Exploring the biochemical and phylogenetic fingerprint of Australian native plants for sustainable use of saline lands

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Abstract

The remarkably rich Australian native vegetation has developed some unique morphological and genetic mechanisms to adapt to severe drought, salinity and water logging. However, the utilisation and significance of Australian native plant bio-resources has been under-exploited, with relatively few dedicated studies, particularly in comparison to crop plants such as rice, wheat or barley. This project investigated the unique gene pool of certain Australian salinity-tolerant plants (three saltbushes- *Atriplex nummularia, A. semibaccata, A. amnicola* and four *Acacia* species- *Acacia victoriae, A. salicina, A. pendula* and *A. stenophylla*). The osmoprotectants glycine betaine (GB), proline and trehalose known to impart salt tolerance were investigated in these plants. Genes encoding the enzymes choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) involved in GB biosynthesis were identified in the three saltbushes. *In-silico* analyses of their cDNA sequences and predicted proteins revealed valuable predictive data pertaining to their extremely conserved functional and structural motifs, subcellular localisation and physico-chemical properties. Gene expression analyses indicated that the saltbush genes for CMO and BADH were differentially expressed in leaves and roots, with significant up-regulation (>1.5 fold change) of *CMO* and/or *BADH* mRNA in the leaf tissues indicating that these genes serve as ideal candidates in transgenic work to enhance salt tolerance in salt sensitive plants. Chemical analyses indicated that *Atriplex semibaccata* and *A. nummularia* produced high quantities of the compound GB in their leaves under salt stress relative to reported low levels in cereals such as barley (2.5 to 10.6 fold change differences). The *Acacia* species, on the other hand, did not produce any detectable levels of GB. Proline production was enhanced by salt in both *Atriplex* (two fold) and *Acacia* species (four fold). HPLC analysis for trehalose detection indicated its absence under salt stress, signifying that trehalose accumulation may not be involved in salt tolerance of these native plants. Another dimension of this study was the use of molecular phylogenetics for assisting in identification of further salinity tolerant *Acacia* species. On the basis of the comparative biology hypothesis that closely related species are most likely to share traits, using DNA markers, a phylogenetic tree of 178 *Acacia* species including four candidate salt tolerant species, i.e., *Acacia pendula*, *A. salicina*, *A. victoriae* and *A. stenophylla* used in the Kamarooka land reclamation project (Australia), was

constructed. Based on phylogenetic relatedness, their historically known potential in agroforestry, and seed availability, 15 species were tested for salt tolerance under controlled laboratory conditions. A method was developed for comprehensive analyses of the datasets using three salt tolerance indices to rank the species. Two new highly tolerant and three moderately salt-tolerant *Acacia* species were thus identified. These will be further used for testing in field conditions (at Kamarooka). In summary, the data on GB analysis, in light of reports from literature on GB-associated health benefits in grazing animals, highlight the potential of saltbushes as sustainable, unseasonal mixed fodder species. Further, the work provides strong rationale for the use of initial phylogenetic screening for large genera, such as *Acacia*, for identifying candidate species for agroforestry and provides an experimental methodology for this purpose.

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How great are your works, O Lord, how profound your thoughts! (Psalm 92:5)

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This thesis is lovingly dedicated to my parents and Richard…

Declaration

I, Shanthi Safrina Maria Monica Joseph, declare that this PhD thesis entitled 'Exploring the biochemical and phylogenetic fingerprint of Australian native plants for sustainable farming on saline lands' is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma, and has not been previously published by another person. Except where otherwise indicated, this thesis is my own work.

Shanthi Safrina Maria Monica Joseph

2014

Publications arising from this work

Refereed journal articles

Joseph S, Murphy DJ, Miller JT and Bhave M (2013) Rapid identification of *Acacia* species with potential salt tolerance using nuclear ribosomal DNA markers. *Sustainable Agriculture Research*, 2 (4): 77-86.

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Joseph S, Murphy DJ, Miller JT and Bhave M (2013). Application of molecular markers for identification of potential salt tolerant plant species for use in agroforestry and saline land reclamation. APCBEE Procedia, 5, 514-519.

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Oral conference presentations

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Joseph S, Murphy DJ and Bhave M (2011) Identification of genes encoding osmoprotectants in Australian native plants. Annual conference of the Australian Society for Biochemistry and Molecular Biology (ComBio 2011) 25-29 September 2011, Cairns, Australia.

Abbreviations

BADH	Betaine aldehyde dehydrogenase
BLAST	Basic Local Alignment Search Tool
BDT	Big Dye Terminator
bp	Base pair (s)
CDH	Choline dehydrogenase
cDNA	Complementary DNA
CDS	Coding sequence
CMO	Choline monooxygenase
COX	Choline oxidase
C-terminal	Carboxyl terminal
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide
dS m^{-1}	Decisiemens per metre
DW	Dry weight
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
ETS	External transcribed spacer
FW	Fresh weight
gDNA	Genomic DNA
GB	Glycine betaine
HKT	Histidine kinase transporter
HSP	Heat shock proteins
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal transcribed spacer
Kb	Kilobase pairs
LB	Luria-Bertani media
LEA	Late embryogenesis abundant
LRWC	Leaf relative water content
matK	Maturase K
mRNA	Messenger RNA
MW	Molecular weight

NCBI	National Centre for Biotechnology Information
N-terminal	Amino terminal
NHX	Na^+/H^+ exchanger
NUFG	Northern united forestry group
OAT	Ornithine δ- aminotransferase
ORF	Open reading frame
P5CR	Pyrroline-5-carboxylate reductase
P5CS	Pyrroline 5-carboxylate synthetase
PCR	Polymerase chain reaction
PDH	Proline dehydrogenase
PSII	Photosystem II
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT	Room temperature
RWC	Relative water content
SOS	Salt overly sensitive
STI	Salt tolerance index
TAE	Tris acetate ethlenediaminetetraacetic acid buffer
TPP	Trehalose-6-phosphate phosphatase
TPS	Trehalose-6-phosphate synthase
UV	ultra-violet
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

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CHAPTER 1

General introduction and literature review

1.1 Introduction

The main aim of this project is to provide an insight into the genetic and biochemical basis of salt tolerance in two Australian native plant genera, *Acacia* and *Atriplex*, for their potential in reclaiming salt-affected lands. Unravelling their biochemical fingerprint can have translational applications, such as production of salinity tolerant plants of agricultural, biodiversity and economic importance. This chapter discusses the causes and impacts of salinity, its effects on plant physiology, a summary of the mechanisms of salt tolerance exhibited by plants, the role of osmoprotectants, followed by a discussion of molecular phylogenetics and its applications in this research area.

1.2 Definition of salinity

Soil salinity is a condition caused by high concentration of soluble salts and is measured in terms of electrical conductivity (ECe) as decisiemens per metre (dS/m). Soil saturation extracts with an ECe of 4 dS/m (equivalent to approximately 40 mM NaCl) or more, and generate an osmotic pressure of 0.2 MPa, are classified as saline (USDA-ARS, 2008). Saline soils are made up of different salts, such as NaCl, Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl, and Na_2CO_3, of which NaCl has been widely investigated as the principal cause of salinity stress to date (Munns and Tester, 2008).

1.2.1 Types and causes of salinity

Salinity can be classified as primary and secondary salinity. Primary salinity refers to the natural occurrence of high salt level over a long period of time, as seen in the salt lakes of Central and Western Australia. Secondary salinity refers to the accelerated rise of a water table, which in turn mobilises the salt in the soil to the surface. As shown in Figure 1.1, native vegetation such as trees with deep roots help in maintaining the water table at a lower level compared to pastures and plants with shallow roots. When these plants are replaced by plants with shallow roots, the water table rises, mobilising the salts to the surface and affecting the land and the water systems, such as rivers and lakes. Secondary salinity is mainly due to the impact of human activity such as irrigation and removal of native vegetation for farming (Pannell, 2001). Furthermore, irrigation of crop land with water containing dissolved salts can leave behind considerable quantities of salt on the soil surface after evaporation.

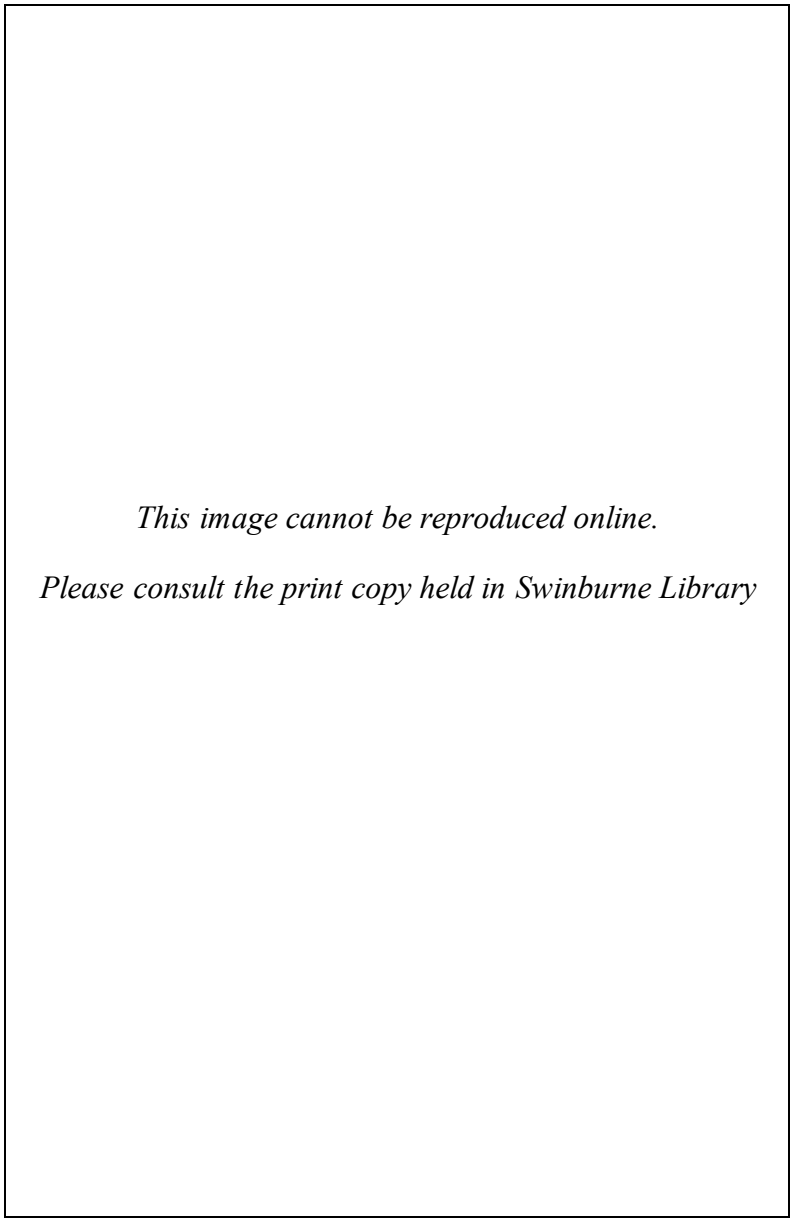

Figure 1.1: Rise in ground water level and salt content due to clearing of native vegetation
(Source: DEPI, 2014)

1.2.2 Salinity: A global problem

Salinity currently affects around 400 million hectares of land worldwide (FAO, 2006) and is a widespread issue affecting at least half the world's countries (Figure 1.2; Corbishley and Pearce, 2007), necessitating immediate action. The current world population of 7.2 billion is expected to rise to 8.1 billion in 2025 and 9.6 billion in 2050, with most of the growth in developing countries (United Nations, Department of Economic and Social Affairs, Population Division, 2013). Crops form an integral component of human consumption and cereals contribute to almost 50% of global food

production (Langridge and Fleury, 2011). In order to meet the demands of the growing population, food production will need to increase by 38% annually (Tester and Langridge, 2010). This trend places a particular emphasis on combatting abiotic stresses such as salinity and drought that directly affect the agriculture industry.

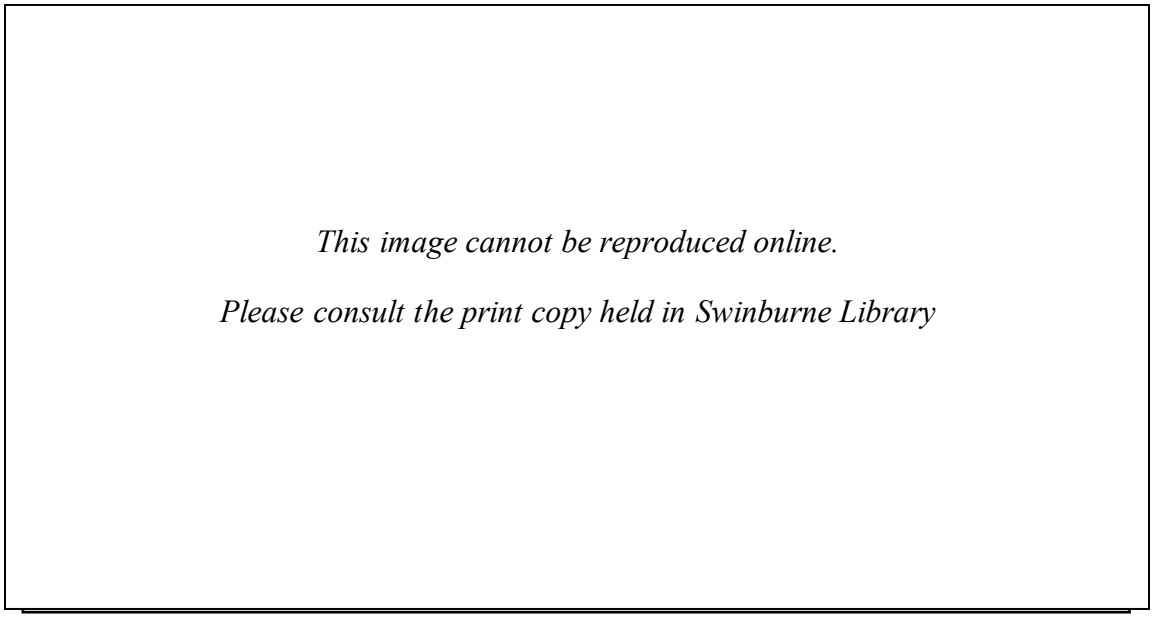

Figure 1.2: Global distribution of salinity affected lands
(Source: Corbishley and Pearce, 2007)

Soil salinity severely affects all aspects of plant physiology, leading to significant losses of crop productivity and sustainability, due to the salt-sensitive nature of most cereal crops. The yields of most crops are significantly reduced at 4 dS m^{-1}. A reduction of up to 25% in yield can occur in crop plants such as wheat, barley, oats, canola and rice when the root zone soil salinity reaches 9.5, 13, 6.3, 11 and 5.1 dS/m respectively (Yiasoumi *et al.,* 2005). An increase in salt concentration to 13.4 dS m^{-1} caused a higher reduction in yield, by 29.6 % for cotton and 35.4% for wheat (Cullu, 2003). In similar conditions, rice would not survive to maturity (Zeng and Shannon, 2000). Barley, one of the most salt tolerant cereals, would not survive after extended periods of salinity above ~ 25 dS m^{-1} NaCl (Munns *et al.,* 2006).

In addition to its severe impacts on crop production and yield, salinity also has detrimental effects on biodiversity and native flora and fauna. In Western Australia, high salinity levels (>10,000 mg/L) lead to a decline in waterbird species inhabiting the wheat belt was observed (Halse *et al.*, 2003). Rahman *et al.* (2011) highlights that

increased salinity in Bangladesh reduced the number of indigenous rice varieties that were cultivated forcing farmers to opt for a limited number of salt tolerant varieties.

1.2.3 The Australian landscape and salinity

The geology of the Australian landscape has vast reserves of salt beneath the surface. Salt also enters the aquatic systems and terrestrial area through natural processes, such as salt particles carried by the wind and rain from nearby seas and weathering of rocks (Neilson *et al.*, 2003). The perennial Australian native vegetation such as the eucalypts, wattles and melaleucas, are well-adapted to these harsh conditions by utilizing the available water, thereby maintaining the water table low (FAO, 2002). However, dramatic changes in the landscape since the European settlement have led to the replacement of deep-rooted perennials with shallow-rooted cereal crop plants and pastures. This has led to the rise in water tables in many regions (Figure 1.1), mobilizing salts to the soil surface and causing toxicity to plants and loss of significant crop land and native vegetation. In Australia, dryland salinity has affected approximately 3.3 million hectares of arable and farming lands, and could further increase up to 5.7 million hectares if no action is taken (van Bueren and Price, 2004). The damage that can arise due to dryland salinity may result in tremendous loss of capital investments, vegetation, agriculture, wildlife and human settlement. The scale of impact on almost one-third of the Australian landscape has prompted serious action by the Australian government to implement programs such as the National Action Plan for Salinity and Water Quality (now, Caring for our Country), through which A$1.4 billion was allocated to fund 1700 projects aimed at addressing salinity related issues (Pannell and Roberts, 2010).

1.3 Effect of salinity on plants

Salinity can be detrimental to plants or render them less productive depending on their ability to tolerate salt stress. The significant factors affected by salinity are growth, water relations, ion concentrations, protein and lipid levels, enzyme activity, cell metabolism and photosynthesis (Figure 1.3; reviewed in Parida and Das, 2005) and have a combined effect on the viability of the plant.

1.3.1 Plant growth, physiology and anatomy

The primary step towards establishing successful plant growth is seed germination. Salinity stress affects seed germination by altering water imbibition (Khan and Weber, 2006), nucleic acid synthesis (Gomes- Filho *et al.,* 2008), protein metabolism (Dantas *et al.,* 2007), hormone balance (Khan and Rizvi, 1994) and utilisation of storage nutrients (Othman *et al.,* 2006). As a result, salinity inhibits germination (*Acacia catechu*; Ramoliya *et al.,* 2004), or delays the onset of germination and seedling emergence (*Solanum lycopersicum*; Kaveh *et al.,* 2011), or decreases seedling vigor (*Zea mays*; Khodarahmpour, 2012). Reduced plant height and biomass is the most significant phenotypic effect of salt stress. *Acacia auriculiformis* (Patel *et al.,* 2010), *Glycine max* (Dolatabadian *et al.,* 2011) and a halophyte *Suaeda salsa* (Guan *et al.,* 2011), have shown reduced shoot and root growth under salinity stress. Additionally, salt-sensitive plants exhibit leaf injury, chlorosis and wilting if the salt load in the leaf far exceeds the plant's ability to compartmentalise salt in to vacuoles (Munns *et al.,* 2006). In addition, salinity stress in plants leads to inhibition of root and hypocotyl growth (cowpea, *Vigna unguiculata*; Pujari and Chanda, 2002), inferior produce quality (lettuce, *Lactuca sativa*; Al-Maskri *et al.,* 2010) and reduced biomass and water uptake (faba bean, *Vicia Faba*; Tavakkoli *et al.,* 2010) (Table 1.1).

Changes in leaf anatomy have also been induced by varying levels of salinity, e.g., increase in thickness of mesophyll, epidermis and leaf succulence. This has been observed in plants such as the salt sensitive bean (*Phaseolus vulgaris*), the moderately tolerant cotton (*Gossypium hirsutum*), and a saltbush *Atriplex patula* (Longstreth and Nobel, 1979). These changes affect vital processes such as transpiration and photosynthesis (Meidner and Mansfield, 1968). Maintenance of a stable chloroplast structure is linked to salt resistance (Xing *et al.,* 2013). However, salinity stress disrupts the chloroplast by dilation of the thylakoid membrane, near-absence of chloroplast grana, larger starch grains and enlarged mesophyll cells (Keiper *et al.,* 1998). Salt-stressed rice chloroplasts showed a significant disorganisation of thylakoid membrane, decreased activity of chlorophyll enzyme and chlorophyll breakdown (Xing *et al.,* 2013).

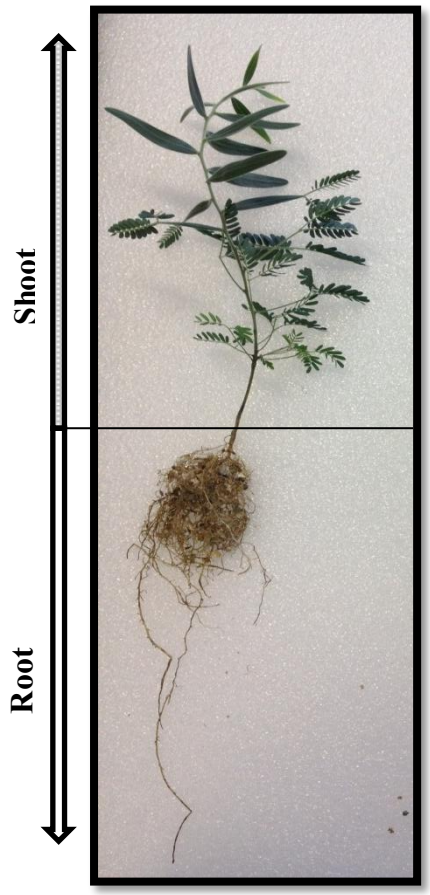

Figure 1.3: Effects of salinity on plant physiology
The figure above shows the effects of salinity on the shoot and root system as demarcated by a line.

1.3.2 Water relations

A positive correlation between water potential (potential energy of water per unit mass of water in the system) and osmotic potential (the potential of water molecules to move from a hypotonic solution to a hypertonic solution across a semi-permeable membrane) in leaves is essential for maintaining stomatal conductance and leaf expansion (Cha-um *et al.,* 2010; Eisa *et al.,* 2012). A negative correlation between water potential and osmotic potential of plants, with increasing salinity, has been reported in several plants such as *Suaeda salsa*, *Chrysanthemum paludosum* and *Aster tripolium* (Lu *et al.,* 2002, Matsumura *et al.,* 1998). The accumulation of NaCl in leaves limits water availability, affects the leaf water potential (Ghoulam *et al.,* 2002) and turgidity of the cell (Katerji *et al.,* 1997) and also leads to the reduction of root hydraulic conductance (Fricke *et al.,* 2013).

Table 1.1: A summary of reported effects of salinity stress on plant growth and other physiological parameters in some *Acacia* species

Plant species[1] and references	Plant age/state at the time of harvest	Level of salinity stress	Effects of salinity on growth parameters				
			Germination/ Seedling emergence	Biomass[2]	Water content (WC)	Plant growth[3]	Other observations
A. auriculiformis (Patel *et al.*, 2010)	Seedling	3.9-11.9 $dS^{-1}m$	Reduction in seed germination ($p< 0.01$) with increasing salt stress.	Decrease ($p<0.01$) in shoot and root DW compared to controls.	Decrease ($p<0.01$) in shoot and root WC compared to controls.	Retardation ($p<0.01$) of shoot and root length.	Root length was remarkably higher than shoot length in control and stressed plants.
A. catechu (Ramoliya *et al.*, 2004)	Seedling	>10.1 $dS^{-1}m$	Seeds did not germinate on soil with salinity greater than 10.1 $dS^{-1}m$.	Reduction in DW (by 50%) of plants at salinity levels of 11.9, 13.7 and 12.5 $dS^{-1}m$.	-	Leaf emergence and expansion were altered. Seedlings grown on soil with salinity of 12.2 $dS^{-1}m$ were very 'weak'.	Root/shoot DW ratio was unaltered in control and stressed plants.
A. longifolia (Morais *et al.*, 2012)	3 months	200 mM	-	Decrease ($p<0.01$) in DW compared to controls.	-	Reduced shoot and root length; roots were long, thin with fewer root hairs compared to control.	-
A. mangium (Nguyen *et al.*, 2004)	45 days	34 mM	-	Decrease ($p<0.05$) in DW compared to controls.	-	-	Growth reduction (as a measure of DW) was less in leaves compared to stems and roots.
A. nilotica (Shirazi *et al.*, 2006)	9 months	15.5-60.9 $dS^{-1}m$	-	-	-	Plants were well established after 9 months of transplantation in saline soil compared to after 3 and 6 months.	Survival of plants became stable after 2-3 months of transplanting in saline soil.
A. senegal (Hardikar and Pandey, 2008)	Seedling	3.9-11.9 $dS^{-1}m$	Reduction in seed germination ($p< 0.01$) with increasing salt stress.	Decrease ($p<0.01$) in shoot and root DW compared to controls.	Decrease ($p<0.01$) in shoot and root WC compared to controls.	Retardation ($p<0.01$) of shoot and root length.	Root length was remarkably higher than shoot length in control and stressed plants.

[1] *Acacia* species; [2] In terms of fresh weight (FW) and /or dry weight (DW); [3] In terms of shoot and root length.

1.3.3 Photosynthesis

The effects of salt stress on photosynthesis, as summarised in Figure 1.4, are primarily manifested as changes in chlorophyll pigments composition, structural disorganisation of chloroplasts, decreased rate of photosynthesis and transpiration, reduction of CO_2 assimilation (Abogadallah, 2011; Biswal *et al.,* 2011; Omoto *et al.,* 2012; Xing *et al.,* 2013). Increases in Na^+ and Cl^- ions in the leaves impair the process of photosynthesis due to reduction of chlorophyll content and leaf area, leading to senescence (Parida and Das, 2005).

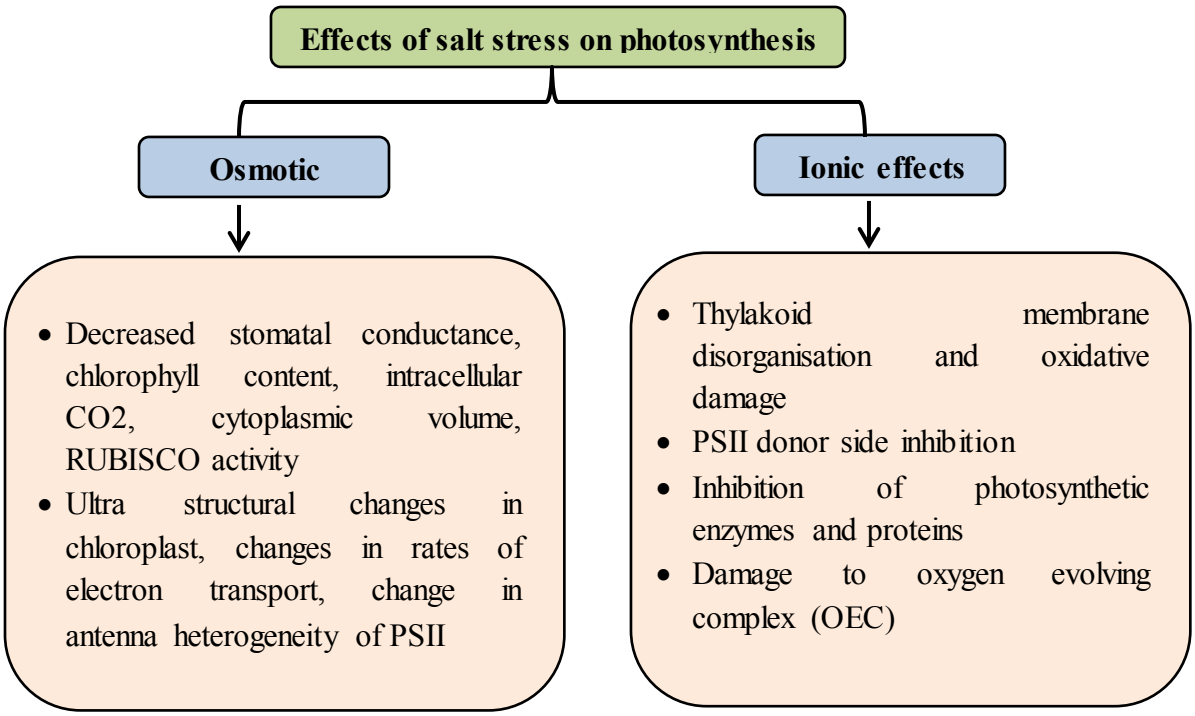

Figure 1.4: Flow diagram showing the effects of salinity on photosynthesis (Adapted from Jajoo, 2013)

1.3.4 Cellular and metabolic activities

The reactive oxygen species (ROS) are normally produced in photosynthesising plants due to the ability of the generated oxygen to accept electrons. However, plants have the natural ability to maintain equilibrium between the ROS and antioxidants (Djanaguiraman and Prasad, 2013), although high levels of salt stress results in oxidative stress due to the formation of ROS, such as, superoxides, hydroxy radicals, singlet oxygen and peroxy radicals. These ROS lead to membrane dysfunction and cellular damage through oxidation of macromolecules, including nucleic acids, proteins and lipids (Parida and Das, 2005).

Nitrogen uptake and metabolism are critical cellular activities also affected by salinity. The antagonistic effect of chloride accumulation impacts nitrogen uptake, as seen in several plants e.g. *Brassica oleracea* (López-Berenguer *et al.,* 2009), *Trigonella foenum-graecum* (Evelin *et al.,* 2012). Nitrogen is a key element required for the synthesis of nucleic acids and amino acids. Salinity has been reported to inhibit nitrogen fixation by decreasing nodulation and nitrogenase activity in *Cicer arietinum* (Soussi *et al.,* 1999); decrease nitrate reductase activity, nitrogen and nitrate uptake in the leaves of *Bruguiera parviflora* (Parida and Das, 2004) and durum wheat (Yousfi *et al.,* 2012).

Lipids are essential for maintaining cell membrane stability and regulating the movement of solutes and essential ions within the cell and its environment (Schuler *et al.,* 1991). Changes in lipid levels of salt-stressed cells, particularly in the level of sterols, phospholipids and fatty acids, have a significant effect on cell membrane fluidity (Surjus and Durand, 1996). An increase in the level of salt accumulation in the chloroplast membranes of leaves of barley seedlings, affected lipid-synthesizing enzymes galactosyl transferase and acylase (Muller and Santarius, 1978). Another deleterious effect is the increase in lipid peroxidation, as reported in tomato (Mittova *et al.,* 2004), wheat (Sairam *et al.,* 2005) and chick peas (Sheokand *et al.,* 2008).

1.3.5 Ion concentration

When salt dissolves in water, dissociation of sodium and chloride ions occur. These ions are then taken up by the roots and translocated to the leaves, which results in impaired uptake of essential nutrient ions, such as K^+, Ca^{2+}, Mg^{2+} and NO^{3-} (Ashraf and Foolad, 2007). Several studies have focussed on the mechanisms that contribute to toxicity by Na^+ and Cl^- accumulation. Accumulation of high levels of Na^+ and Cl^- disrupts several physiological processes, such as, but not limited to, photosynthesis (as discussed above), protein catabolism (Parida and Das, 2005), ion homeostasis (Karimi *et al.,* 2005), stomatal regulation (Redondo-Gómez *et al.,* 2007) and nitrogen uptake (Evelin *et al.,* 2012). Toxic levels of Cl^- also induce chlorotic toxicity symptoms such as chlorosis (yellowing of leaf), due to disconcerted chlorophyll production (Slabu *et al.,* 2009). A significant consequence of Na^+ and Cl^- accumulation is the perturbation in the ratio of Na^+ ions to essential nutrient ions, such as ratios of Na^+/K^+, Na^+/Ca^{2+},

Ca^{2+}/Mg^{2+} and Cl^-/NO^{3-} (Munns and Tester, 2008; Nawaz *et al.,* 2010; Shabala and Munns, 2012).

For some plants Na^+ has been reported to be beneficial at low levels, e.g. in cotton (Ali *et al.,* 2009) and sugarbeet (Wakeel *et al.,* 2009), to maintain the osmotic potential when K^+ supply is limited. However, Na^+ competes with K^+ and has been predominantly reported to be toxic in many plants, such as wheat (*Triticum aestivum*; Munns *et al.,* 2006), faba bean (*Vicia faba*; Slabu *et al.,* 2009) and Mexican redbud (*Cercis canadensis*; Niu *et al.,* 2010). Potassium is an essential cation required for tRNA-ribosome binding in protein biosynthesis, and activates more than 50 enzymes (reviewed in Todorova *et al.,* 2013). Maintenance of high K^+:Na^+ ratio is thus considered an important measure of salt tolerance in brassicas (Ashraf and McNeilly, 2004) and in salinity tolerant genotypes of barley (Shabala *et al.,* 2010). The ratio is crucial for sustaining cellular metabolism, enzyme activity, upholding membrane potential and cell volume regulation (Munns and Tester, 2008).

Under salt stresses, high Na^+ concentrations not only displace K^+ ions but also Ca^{2+} ions. Calcium ions are vital for maintaining the structural rigidity of the cell wall and its associated functional characteristics, such as membrane permeability (Hepler, 2005). Calcium is also an initiator in signal transduction pathways under stress (Tuteja, 2007). Salt stress perturbs the pectin-associated cross-linking of Ca^{2+}, thereby disrupting membrane stability (Essah *et al.,* 2003). This disruption could have major consequences, such as altering the K^+/Na^+ selectivity, increasing Na^+ influx, or the leakage of cytosolic K^+ from the cell, as shown in rice (Wu and Wang, 2012).

1.4 Mechanism of salinity tolerance by plants: A summary

Certain plants have the innate ability of adapting to salt stress. These plants, called halophytes, have developed several physical, biochemical and molecular mechanisms to combat salinity. Many non-halophytes also have similar mechanisms to tolerate salinity to some extent. They can either minimize the entry of salt into the plant or minimize the level of cytoplasmic salt content (Munns, 2002). Figure 1.5 illustrates the tolerance mechanism of plants which undergo a primary abiotic stress e.g. drought, salinity, cold, heat and chemical pollution (Wang *et al.,* 2003). These factors in turn

contribute to secondary levels of damage to plants such as oxidative stress, osmotic stress and ultimately cellular damage. However, the ability of some plants to perceive these stresses at various levels and respond to them, via different mechanisms, helps the plant to survive. Some of these mechanisms are discussed below.

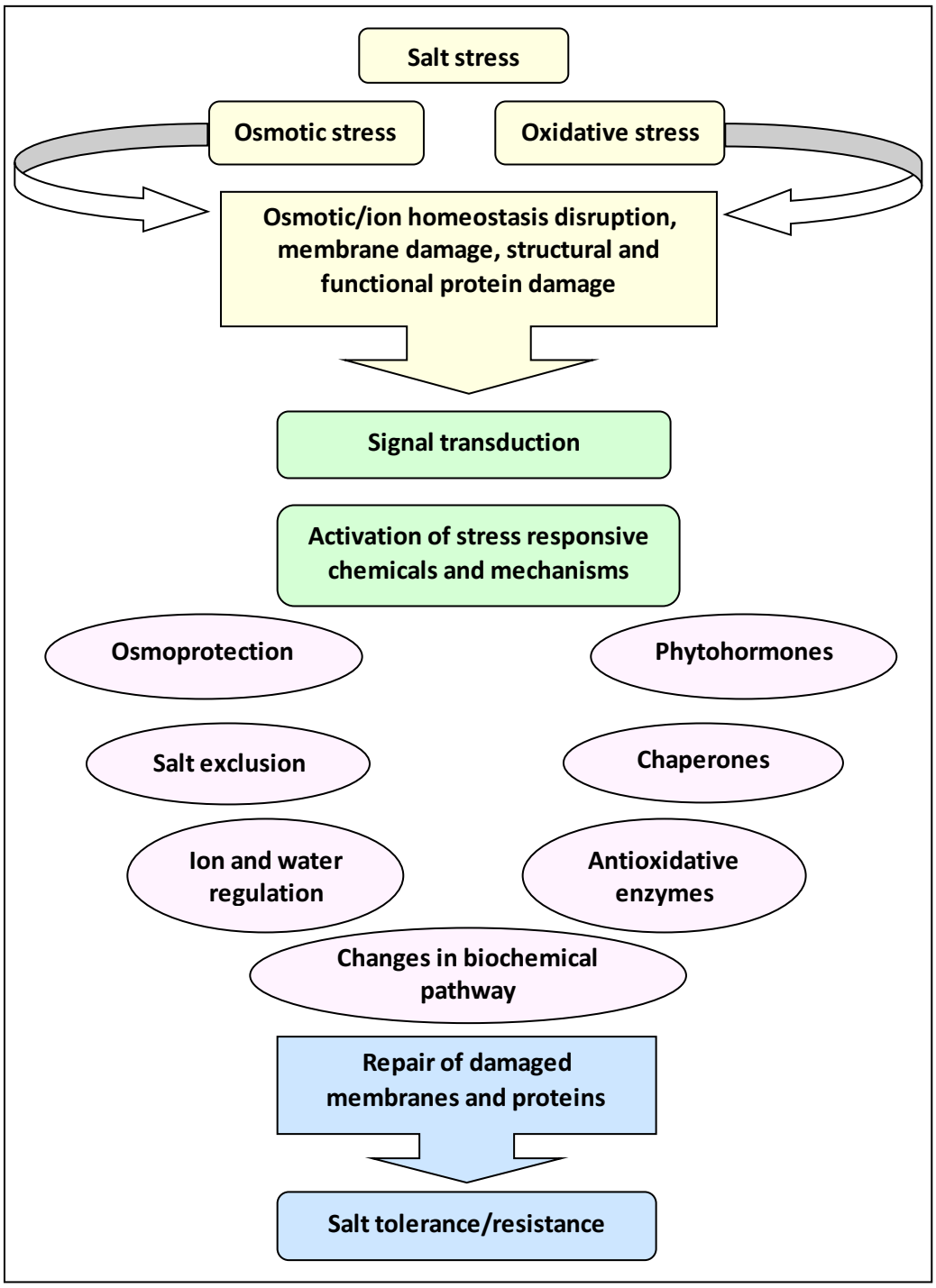

Figure 1.5: Schematic representation of the different tolerance mechanisms triggered at the molecular level by plants in response to salt stress

1.4.1 Salt exclusion

Salt exclusion is a process by which the plant cell reduces the rate of salt accumulation (Munns, 2002). Three mechanisms have been proposed for salt exclusion from leaves: (i) selective salt uptake by root cells based on their cell types (epidermis, exodermis or endodermis); (ii) preferential loading of the xylem with nutrient K^+ ions rather than Na^+ ions, and (iii) exclusion of salt from the upper parts of the root system and parts of the shoot system such as stem, petiole or leaf sheaths (Munns, 2002). Halophytes have the added advantage of excluding excess salts through specialised structures such as salt glands which mainly exclude the salt to the leaf surface and bladder hairs which compartmentalise salts in their central vacuoles (Pessarakli, 1999).

1.4.2 Transport of ions and regulation of ion concentration

Accumulation of Na^+ in the plant affects leaf water potential, requiring Na^+ removal. Several transporter systems that are involved in the uptake, efflux, translocation or compartmentation of ions such as Na^+, K^+ and Cl^- have been studied in various plants (reviewed in Tuteja, 2007; Yamaguchi *et al.,* 2013). Sodium transporters can be involved in the efflux of Na^+ ions or mediate the vacuolar sequestration of Na^+ ions. The main Na^+ transporters, i.e. the Na^+/H^+ exchanger (NHX), salt overly sensitive (SOS) and histidine kinase transporter (HKT) transporters, have gained momentous standing for their roles in salinity tolerance. Overexpression of genes encoding NHX, SOS and HKT has conferred resistance to salinity stress in the model dicot plant, *Arabidopsis thaliana* (Apse *et al.,* 1999; Shi *et al.,* 2003; Horie *et al.,* 2009).

In addition to sodium transporters, three unique proton pumps are involved in establishing proton electrochemical gradients in plants: (i) the plasma membrane P-type ATPase; (ii) vacuolar H^+-ATPase; and (iii) the vacuolar H^+- pyrophosphatase. These pumps establish the proton gradients required for the active transport and efflux of ions and metabolites across membranes (plasma membrane, tonoplast) (Sze *et al.,* 1999; Xiong and Zhu, 2002). Accumulation of excess Na^+ ions in the vacuole regulates ion homeostasis by maintaining the H^+ electrochemical gradient across the vacuolar membrane. In *Arabidopsis*, the P-type ATPase is encoded by 12 genes and determines the direction of ion movement and the extent of ion flow through ion-specific channels

(Sze *et al.,* 1999). It also plays an important role in salt tolerance in plants (reviewed in Bartels and Sunkar, 2005).

1.4.3 Change in photosynthetic pathway

Some halophytic plants alter the mode of photosynthesis in order to maximise the use of available water. Under salinity, *Atriplex lentiformis* shifts the C3 photosynthetic pathway to C4 carbon fixation mode (Meinzer and Zhu, 1999), whereas, *Mesembryanthemum crystallinum* shifts from the C3 mode to the crassulacean acid metabolism (CAM) mode (reviewed in Cushman, 2001). These changes help the plant in reducing transpirational water loss by opening their stomata at night rather than during the day (reviewed in Cushman, 2001). Australia is colonised by both C3 and C4 lineages of Atriplex species (Kadereit *et al*., 2010). Also, Brownell and Bielig (1996) suggest that sodium is small quantities is essential for the functional integrity of mesophyll chloroplasts of C4 plants during conversion of pyruvate to phosphoenol pyruvate.

1.4.4 Induction of stress-responsive proteins

Advances in proteome studies have enabled the analysis of differential responses of plants to various stress treatments. Several proteins involved in processes, such as stress signalling, energy metabolism, lipid metabolism and protein metabolism, are expressed differentially under salt stress. Some of these proteins involved are discussed here.

The 'late embryogenesis abundant' (LEA) proteins are group of proteins expressed at high levels in plant seeds during the post-abscission stages of embryo development (Tunnacliffe and Wise, 2007). Transgenic studies of certain LEA proteins have conferred stress (drought, salinity and cold) tolerance to host plants. Expression of the cDNA clone encoding *Hordeum vulgare* LEA3 protein in rice with a constitutive promoter resulted in high accumulation of the protein and conferred salinity and drought tolerance (Xu *et al.,* 1996). Similar effects of tolerance were observed in transgenic rice with the barley LEA3 encoding gene under the control of a stress inducible promoter, and in transgenic wheat plants under osmotic stress (Rohila *et al.,* 2002, Sivamani *et al.,* 2000). Another barley LEA encoding gene, *HVA1*, conferred

dehydration tolerance in transgenic rice (Chandra Babu *et al.,* 2004). Transgenic *Brassica campestris* with *Brassica napus* group 3 LEA protein gene conferred tolerance to water-deficit and salt-stress (Park *et al.,* 2005).

Another family of genes induced, mainly under heat stress but to a certain extent also by osmotic stress are the 'heat shock' proteins (HSP) that act as molecular chaperones involved in protein folding and protection of cells against denaturation. HSP70 was found to be induced under high salt stress in *Atriplex nummularia* (Zhu *et al.,* 1993). Over expression of a low molecular weight HSP, HSP17, in *A. thaliana* also conferred salt and drought tolerance (Sun *et al.,* 2001).

1.4.5 Induction of antioxidative enzymes

To counteract the effects of ROS and oxidative stress induced by salinity, plants produce enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione-S-transferases (GST) and glutathione peroxidases (GPX) and non-enzymatic antioxidants such as ascorbic acid (AA), flavones, carotenoids, anthocyanins and tocopherols (Djanaguiraman and Prasad, 2013). Meloni *et al.* (2003) showed that an increase in GR, SOD and peroxidase offered better protection against ROS in salt-tolerant cotton plant varieties. Tang and Newton (2005) showed that an increase in polyamines increased the activities of APX, GR, and SOD in salt-stressed Virginia pine plantlets and defended against oxidative damage.

1.4.6 Modulation of phytohormones

Increased levels of plant hormones, such as, auxin, ethylene, cytokinins and absicisic acid, and other substances that function as phytohormones (like jasmonic acid and salicylic acid) play an important role in signalling in plants in response to external stimuli (Staswick and Tiryaki, 2004). Pre-treatment of crops with the phytohormones indole acetic acid, gibberrelic acid and kinetin may help in salinity tolerance, as they increase the efficiency of nitrogen fixation in salt-stressed plants (Chakrabarti and Mukherji, 2003). Although jasmonates function mainly in response to pathogen attack and physical wounding (McConn *et al.,* 1997), a significant increase in the accumulation levels of jasmonic acid in plants under salt and osmotic stress, such as

barley (Lehmann *et al.,* 1995) and tomato (Pedranzani *et al.,* 2003), have also been reported. Exogenous application of salicylic acid on salt-stressed tomato showed an increase in SOD, GPX and CAT that are involved in scavenging ROS (He and Zhu, 2008).

1.4.7 Synthesis and accumulation of compatible solutes

Osmotolerance or osmoregulation is one of the effective mechanisms by which many plants and bacteria overcome salt toxicity. It involves the accumulation of non-toxic organic solutes, commonly known as osmolytes or osmoprotectants. These include polyhydroxylic compounds (sugars and polyols) and zwitterionic alkylamines (quaternary ammonium compounds and aminoacids). Due to the accumulation of salt in the vacuole or apoplast, the ionic and osmotic balance within the cell is altered. By accumulating nontoxic osmolytes in the cytoplasm, the influx of water into the cell is regulated, thereby maintaining cell turgor pressure and water absorption. This is important for sustaining several essential activities such as photosynthesis, stomatal opening, cell expansion and growth. The term 'compatible solute' was introduced by Brown and Simpson (1972) to describe their non-toxic nature and non-inhibitory effect on enzyme activities or metabolic processes of the cell (Wyn-Jones *et al.,* 1977). Le Rudulier *et al.* (1984) demonstrated that exogenous application of osmoprotectants to *E. coli* cells under completely inhibitory levels of osmotic strength were capable of exhibiting significant growth. They also serve to stabilise protein structure and function (Yancey *et al.,* 1982).

Osmoprotectants can be classified into three groups: amino acids (e.g. proline and ectoine); quaternary ammonium compounds and tertiary sulfonium compounds (e.g. glycine betaine (GB), dimethylsulfoniopropionate and choline-O-sulfate); and polyols and sugars (e.g. glycerol, mannitol, sorbitol, sucrose and trehalose) (Rontein *et al.,* 2002). Amino acids are vital in mitigating the effects of salinity by regulating K^+ homeostasis (Cuin and Shabala, 2007). Many proteinogenic amino acids, such as proline, arginine, alanine, glycine, serine, leucine, and valine, as well as some non-proteinogenic amino acids, such as citrulline and ornithine are accumulated under salinity stress (Rabe, 1990). Among these, proline has gained significant momentum, in terms of research and transgenic applications. Proline accumulation occurs plays a

substantial role in osmoprotection, protein and cell membrane stabilization and ROS scavenging (Kavi Kishor *et al.,* 2005). Another widely studied osmoprotectant, GB, also plays a crucial role in ameliorating salinity and osmotic stress tolerance in plants, especially in chenopods (Rhodes and Hanson, 1993). Exogenous application of GB (Lutts *et al.,* 1999; Habib *et al.,* 2012), as well as expression of GB biosynthetic genes transgenically (Bao *et al.*, 2011; Zhang *et al.,* 2011), has shown promising results in imparting stress tolerance to plants. Trehalose is an osmolyte, whose presence and function in plants under stress is ambiguous. It has been predominantly reported in desiccation tolerant plants, such as *Myrothamnus flabellifolia* (Drennan *et al.,* 1993), *Sporobolus stapfianus* (Albini *et al.,* 1994) and *Selaginella lepidophyla* (Vázquez-Ortíz and Valenzuela-Soto, 2004). Several plants have genes that encode enzymes involved in the biosynthesis pathway, but do not exhibit trehalose accumulation. However, transgenic overexpression of trehalose 6-phosphate synthase (TPS) and trehalose 6-phosphate phosphatase (TPP) has conferred salinity tolerance to plants and indicated trehalose accumulation (Garg *et al.,* 2002; Jang *et al.,* 2003). Osmoprotectants of particular interest to this project (Figure 1.6) are described below.

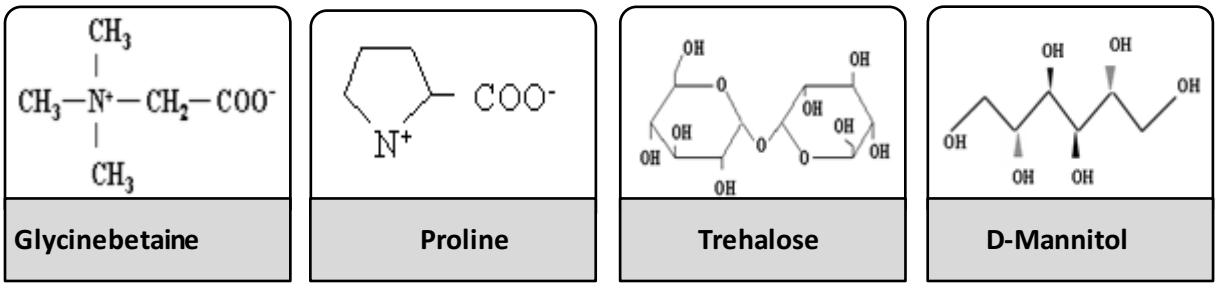

Figure 1.6: Chemical structure of some of the major plant osmoprotectants

1.5 Glycine betaine (GB) (MW: 117.15 g mol^{-1})

Glycine betaine (N, N, N-trimethyl glycine) (GB) is a quaternary amine with a neutral charge over a broad range of physiological pH values (Sakamoto and Murata, 2002). It is termed a "compatible solute" because of its ability to accumulate in stressed plants without interfering or inhibiting their enzymatic or metabolic activities (Wyn-Jones *et al.,* 1977). Accumulation of GB does not occur naturally in all plants under stress (Arabidopsis and Tobacco, for example, do not produce GB naturally under stress), yet they are the most abundant solute found to accumulate in many families of plants, as

shown in Figure 1.7 (Rhodes and Hanson, 1993). Accumulation of GB can confer abiotic stress tolerance by means of osmoregulation/osmoprotection, cell membrane and organelle stabilisation, and induction of antioxidative enzymes and ROS scavengers (reviewed in Wani *et al.,* 2013).

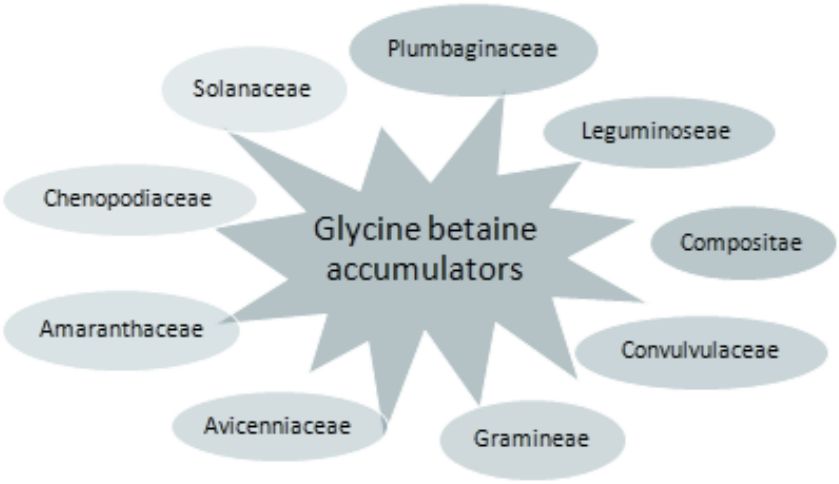

Figure 1.7: Plant families capable of GB accumulation

1.5.1 Biosynthesis

Synthesis of GB occurs from two distinct molecules, choline and glycine (Figure 1.8).

a. *GB biosynthesis from choline:* The first step involves the oxidation of choline to its hydrated form, betaine aldehyde. Although the reaction is conserved in plants and bacteria, it is catalysed by different enzymes. In plants, this reaction is catalyzed by a ferredoxin-dependent enzyme choline monooxygenase (CMO), while in bacteria, such as *Escherichia coli, Pseudomonas aeruginosa* and *Synorhizobium meliloti* it is catalysed by the enzyme choline dehydrogenase (CDH). The second step is the synthesis of GB from betaine aldehyde, by the NAD^+ dependent enzyme betaine aldehyde dehydrogenase (BADH).

b. *GB biosynthesis from glycine:* A novel pathway for the synthesis of GB from glycine was identified in two phylogenetically distant halophytes, *Actinopolyspora halophila* and *Ectothiorhodospira halochloris.* It occurs via a three-step series of methylation catalysed by glycine sarcosine methyltransferase (GSMT) and sarcosine dimethylglycine methyltransferase with partially overlapping substrate specificity (Nyyssölä *et al.,* 2000). This pathway (Figure 1.8) was also identified in a halotolerant

cyanobacterium, *Aphanothece halophytica* (Waditee *et al.,* 2003). The enzyme GSMT catalyses the methylation of glycine to sarcosine, followed by the methylation of sarcosine to dimethyl glycine. The DMT enzyme (sarcosine dimethylglycine methyltransferase) methylates dimethylglycine to betaine.

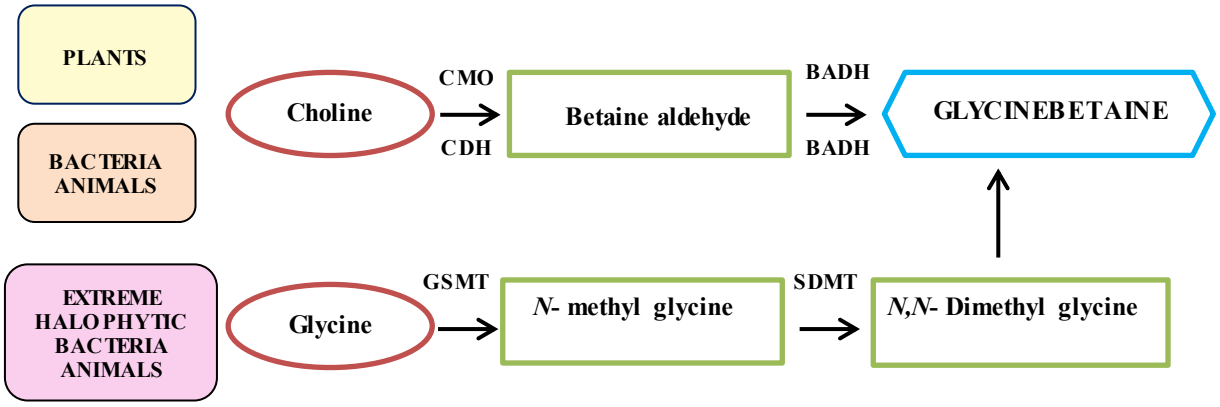

Figure 1.8: Synthesis of glycine betaine from choline and glycine
CMO: choline monooxygenase; CDH: choline dehydrogenase; BADH: betaine aldehyde; GSMT: glycine sarcosine methyltransferase; SDMT: sarcosine dimethylglycine methyltransferase. (Substrates are highlighted in red, the intermediate products in green and the final product in blue)

1.5.2 Major enzymes and genes involved in GB biosynthesis

1.5.2.1 Choline monooxygenase (CMO; EC 1.14.15.7)

CMO is a soluble enzyme that is unique to plants (Rathinasabapathi *et al.,* 1997). It is localised in the chloroplast stroma in many plants (e.g. spinach, barley, and Oldman salt bush). Partially purified CMO from spinach has an optimum pH of 8, and a native molecular mass of 98,000 daltons (Rhodes and Hanson, 1993). CMO is a novel Rieske type iron-sulfur enzyme, i.e. coordinated by two Cys and two His ligands, and catalyses the first step of oxidation of choline to betaine aldehyde. Iron-sulfur (Reiske) clusters are involved in vital processes, such as cell respiration and photosynthesis, due to their ability to transfer electrons, and have other functions, such as centres of catalytic activity, regulators of gene expression (in bacteria), and sensors of iron and oxygen (reviewed in Balk and Lobreaux, 2005). Reiske proteins typically contain two Fe atoms and two acid-labile sulfide groups, and the Reiske motif typically involves coordination of one Fe, by the sulfides of two Cys residues, and the other Fe by the δ-nitrogen atoms in the imidazole rings of two His residues (Fee *et al.,* 1984). However, the Reiske-type cluster (2Fe-2S) in plants has both iron atoms co-ordinated by the two Cys, making

them 'novel' (Kimura *et al.,* 2005), and the Cys ligands instead of Ser are suggested to make them more stable (Cheng *et al.,* 1994). The signature motif for the novel Reiske-type cluster [2Fe-2S] is $CXHX_{15-17}CX_2H$, and for the mononuclear non haeme cluster, it is $G/DX_{3-4}DX_2HX_{4-5}H$ (Hibino *et al.,* 2002). These regions constitute the putative active site for CMO.

The biochemical characterisation of CMO is a difficult task, since purified CMO has low activity and is labile (Burnet *et al.,* 1995). Hence, comparative sequence analysis, based on extant protein data, will provide an insight into protein characteristics with bioinformatics tools such as Protparam (http://web.expasy.org/protparam/). This study intends to characterise GB biosynthetic proteins (using this tool) in order to assess their potential as a genetic resource for osmoprotection, in genetic screening and for future transgenic studies.

1.5.2.2 Betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8)

BADH is a pyridine nucleotide dependent enzyme. It has been cloned from several plants, such as *Spinacia oleracea* (Weretilnyk and Hanson 1990), *Beta vulgaris* (McCue and Hanson 1992), *Atriplex hortensis* (Jia *et al.,* 2002), *Hordeum vulgare* (Ishitani *et al.,* 1995), *Sorghum bicolor* (Wood *et al.,* 1996), *Oryza sativa* (Nakamura *et al.,* 1997), *Amaranthus hypochondriacus* (Legaria *et al.,* 1998), *Avicennia marina* (Hibino *et al.,* 2001), *Atriplex centralasiatica* (Yin *et al.,* 2002), and *Ophiopogon japonicus* (Liu *et al.,* 2010). Although most of these plants have the functional genes for GB synthesis, their level of expression varies. For example, *Oryza sativa* does not exhibit any detectable quantity of GB accumulation (Nakamura *et al.,* 1997, Niu *et al.,* 2007), whereas members of the grass family e.g. maize (Lerma *et al.,* 1991), sorghum (Yang *et al.,* 2003) and barley (Jagendorf and Takabe, 2001), accumulate much less GB, in comparison to spinach and sugar beet, two members of the Chenopodiaceae family (Rhodes and Hanson, 1993).

The enzyme has three major domains: a coenzyme binding domain, a catalytic domain and an oligomerization domain (Johansson *et al.,* 1998), as shown in Figure 1.9. In addition, two functionally important residues characteristic of the Aldehyde Dehydrogenase enzyme family i.e. a glutamic acid (E) and a cysteine residue (C), have

been implicated in the catalytic activity of mammalian aldehyde dehydrogenase and are conserved in all enzymes of this family (http://prosite.expasy.org/PDOC00068). The *consensus pattern of* aldehyde dehydrogenases for the cysteine active site is [FYLVA]-x-{GVEP}s-{DILV}-G-[QE]-{LPYG}-**C**-[LIVMGSTANC]-[AGCN]-{HE}-[GSTADNEKR] (C is the active site residue), and for the glutamic acid active site it is [LIVMFGA]-**E**-[LIMSTAC]-[GS]-G-[KNLM]-[SADN]-[TAPFV] (E is the active site residue). The cysteine residue has been proposed to contribute towards substrate specificity and the catalysis of BADH (Hempel *et al.,* 1993 and Perozich *et al.,* 1999).

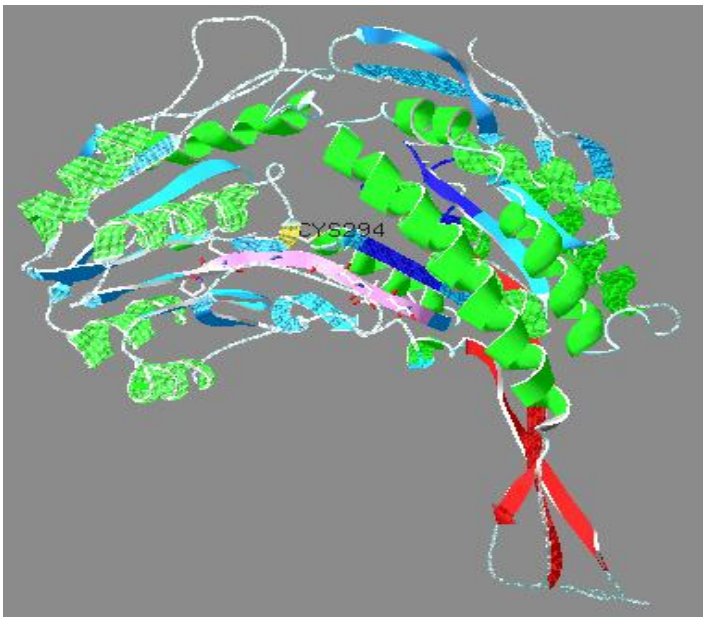

Figure 1.9: 3D model of *Atriplex centralasiatica* BADH monomer showing major functional domains

Atriplex centralasiatica BADH monomer was modelled against *Pisum sativum* AMADH2 (PDB model 3iwj, chain A), which showed sequence identity of 76.5%. The alpha helices are shown in green, the beta sheets in blue, the conserved decapeptide VTLELGGKSP in pink, the oligomerization domain in red, NAD binding domain in dark blue and the active site Cysteine is labelled (modelled using CPHmodels-3.0, Nielsen *et al.,* 2010). The model was assessed using PROCHECK, which determined an overall G-factor of 0.01 and had 86.4% of all residues in the favoured regions.

In plants, the BADH-encoding gene homologue may exist singly or as two isozymes and are reported as BADH1 and BADH2 (reviewed in Fitzgerald *et al.*, 2009). The *BADH2* homologue may not encode functional BADHs capable of reducing betaine aldehyde to glycine betaine, if the *BADH2* transcripts are truncated leading to incorrectly processed pre-proteins (Niu *et al.*, 2007). Although these transcripts may not be contributing to GB synthesis, they are involved in the production of another desirable trait. Buttery *et al.* (1983) reported that the fragrance of particular rice

varieties is due to the accumulation of a chemical called 2-acetyl-1-pyrroline (2AP). Over the last two decades, the genetic cause of the 2AP-based fragrance property in rice was established to be caused by a non-functional allele of the *BADH* homologue, *BADH2* (Bradbury *et al.*, 2008). The proposed involvement of the recessive *BADH2* allele is in the accumulation of the substrate γ-aminobutyraldehyde (GABald) which undergoes spontaneous cyclisation on reaction with an acetyl group to form 2AP (Bradbury *et al.*, 2008).

1.5.2.3 Choline dehydrogenase (CDH; EC 1.1.99.1)

Choline dehydrogenase catalyses the oxidation of choline to betaine aldehyde. Glycine betaine synthesis via CDH has been reported in *Escherichia coli* (Landfald and Strøm, 1986), *Pseudomonas aeruginosa* (Russell and Scopes, 1994), *Sinorhizobium meliloti* (Pocard *et al.,* 1997), oysters (Perrino and Pierce, 2000) and *Halomonas elongata* (Cánovas *et al.,* 2000). In *E. coli,* synthesis of GB requires four genes encoding choline dehydrogenase (*betA*), betaine-aldehyde dehydrogenase (*betB*), a putative regulator (*betI*), and a choline transporter (*betT*); clustered in the *bet* operon (Andresen *et al.,* 1988). Transgenic tobacco plants, with *E. coli betA* and *betB* genes, showed improved resistance towards salinity (Holmström *et al.,* 2000). Although transgenic studies with CDH culminated in GB production, the amount of GB produced was very low (less than 66 nmol g^{-1} fresh weight). This is a delimiting factor for CDH use in plants, since the role of such low GB levels is questionable in terms of stress alleviation and osmoprotection.

1.5.2.4 Choline oxidase (COX; EC 1.1.3.17)

Choline oxidase belongs to the glucose-methanol-choline (GMC) oxidoreductase enzyme superfamily (Fan and Gadda, 2005). The enzyme was first reported in *Arthrobacter globiformis* by Ikuta *et al.* (1977). It has an isoelectric point around pH 4.5, an optimum pH of 7.5 (Ikuta *et al.,* 1977) and a reported monomeric molecular mass of 66 kDa (Ohta-Fukuyama *et al.,* 1980). The enzyme contains covalently bound flavin adenine dinucleotide (FAD) in a 8α-N(1)-histidyl linkage, and catalyzes the four-electron oxidation of choline to GB through betaine aldehyde, with molecular oxygen as primary electron acceptor (Gadda, 2003). Engineering plants with bacterial COX has

the additional advantage of not involving a secondary enzyme or any additional soluble cofactors, and does not interfere with other metabolic pathways (Lilius *et al.,* 1996).

1.5.3 Role of GB in salt stress

1.5.3.1 Effects of exogenous application of GB

GB is a metabolically inert osmolyte, capable of penetrating readily through the leaves and then translocating to the other parts of the plant (Ladyman *et al.,* 1980; Hanson and Wyse, 1982). Exogenous foliar application of GB led to significant reduction in Na^+ and maintenance of K^+ levels in salt-stressed perennial ryegrass (*Lolium perenne*, Hu *et al.,* 2012), enhanced seed oil quality and antioxidant activity under water stress in maize (Ali and Ashraf , 2011), and GB pre-treated pepper seeds showed increased germination under salinity (Korkmaz and Siricki, 2011). The applications of pure GB and sugarbeet extracts to salt-stressed varieties of okra (*Abelmoschus esculentus*) led to alleviation of effects of salinity stress on plant biomass and other parameters, including ion contents, with some parameters showing greater improvement with pure GB compared to sugarbeet extract, and vice versa (Habib *et al.,* 2012). Hence, extraction of GB from natural accumulators and its application on plants under stress can alleviate salt stress may be economically feasible (Ashraf and Foolad, 2007).

1.5.3.2 Transgenic applications of GB biosynthesis genes

Accumulation of GB naturally in stress-responsive plants has been reported in barley, sorghum, spinach, maize and other plants (Table 1.2). Certain plants, such as arabidopsis, rice, mustard and tobacco, do not accumulate GB naturally, but on introducing GB synthesising genes, they exhibited an increase in GB level (Rhodes and Hanson, 1993). Many examples show transgenic overexpression of *CMO* and *BADH* genes causatively leading to salinity tolerance (Tables 1.4 and 1.5). For example, the biomass and seed cotton yield of cotton expressing *CMO* from *Atriplex hortensis* was less affected by salinity compared to controls (Zhang *et al.,* 2009). Transgenic tobacco expressing *Salicornia CMO* was capable of rooting in medium with 300 mM NaCl (Wu *et al.,* 2010), and transgenic potato plants expressing spinach *BADH* were significantly taller and heavier (Zhang *et al.,* 2011). However, the overall level of GB in transgenic plants is comparatively lower than natural accumulators (Tables 1.2 and 1.3). This

justifies the quest to identify more natural accumulators, especially for the purposes of saline land reclamation.

Table 1.2: Levels of GB in some natural GB accumulating plants

Plant species	NaCl treatment	GB content µmol g^{-1} FW	
		Control	Stressed
Amaranthus tricolor	0.3 M	2	10
Atriplex spongiosa	0.8 M	15	45
Gossypium hirsutum L.	0.15 M	3.5	6.7
Hordeum vulgare L.	0.2 M	1.5	5.5
Sorghum bicolor L.	0.1 M	4	9
Spinacia oleracea	0.3 M	5	25
Zea mays	0.15 M	1	2.9

Adapted from Chen and Murata (2011); FW - Fresh weight of leaf tissue

Table 1.3: Levels of GB in transgenic plants overexpressing GB biosynthetic genes (*CMO* and *BADH*)

Plant species	Gene	Gene source	GB level
Arabidopsis thaliana	*CMO* + *BADH* (co-expression)	*Spinacia oleracea*	0.9 µmol g^{-1} FW
Nicotiana tabacum	*CMO*	*Beta vulgaris*	0.25 µmol g^{-1} FW
Oryza sativa	*CMO*	*Spinacia oleracea*	0.45 µmol g^{-1} DW
Nicotiana tabacum	*BADH*	*Atriplex hortensis*	7 µmol g^{-1} DW
Daucus carota	*BADH*	*Spinacia oleracea*	101 µmol g^{-1}DW
Lycopersicon esculentum	*BADH*	*Atriplex hortensis*	0.45 µmol g^{-1} DW
Oryza sativa	*BADH*	*Hordeum vulgare*	56.4 µmol g^{-1} DW

Adapted from Chen and Murata (2011); FW - Fresh weight of leaf tissue
GB levels that are highlighted indicate accumulation in dry weight of tissue.

Table 1.4: Expression of *choline monooxygenase* gene in transgenic species

Transgenic plant	Gene source	Method of transformation	Level of salt stress	Observed effects	Reference
Lolium perenne	*Spinacia oleracea*	Particle bombardment	Enhanced salt stress tolerance	First report of dwarfism (height of transgenic plants was decreased by 63% compared to the control plants) and enhanced salt stress tolerance by coexpression of *SoCMO* and *SoBADH* genes in transgenic *L. perenne*.	Bao *et al.*, 2011
Nicotiana tabacum	*Salicornia europaea*	Agrobacterium-mediated transformation	100-350 mM NaCl for 4 weeks	Transgenic plants were capable of rooting in a medium containing up to 300 mM NaCl, whereas the control plants could not root in medium containing more than 100 mM NaCl.	Wu *et al.,* 2010
Gossypium hirsutum	*Atriplex hortensis*	Agrobacterium-mediated transformation	Field experiment, with the soil containing 420 g kg^{-1} total soluble salts, 0.98% organic matter, 580 mg kg^{-1} total N, 12 mg kg^{-1} available P and 110 mg kg^{-1} available K.	Biological (biomass) and economic (seed cotton) yields of transgenic cotton was less affected by salinity stress than those of non-transgenic cotton.	Zhang *et al.,* 2009

Table 1.5: Expression of *BADH* in transgenic species

Transgenic plant	Gene source	Method of transformation	Level of salt stress	Observed effects	Reference
Solanum tuberosum	*Spinacia oleracea*	Agrobacterium-mediated transformation	50 mM of NaCl initially and increased gradually by 50 mM to a final concentration of 500 mM.	Transgenic plants were 0.4–0.9 cm taller and 17–29% heavier (fresh weight per plant) compared to control plants.	Zhang *et al.,* 2011
Medicago sativa	*Atriplex hortensis*	*Agrobacterium tumefaciens*-mediated transformation	200 mM NaCl solution in an interval of 10 days for four times.	Survival period of transgenic line B203 was 8 months and other transgenic lines for about 4–5 months. Wild type plants did not survive after two weeks of the last salt water irrigation.	Liu *et al.,* 2011
Nicotiana tabacum	*Ophiopogon japonicus*	Particle bombardment	400 mM NaCl for 48 h at room temperature and then returned to normal growth condition.	2–2.5-fold increase of GB content and 60-85% increase in survival.	Liu *et al.,* 2010
Lolium multiflorum	*Zoysia tenuifolia*	Particle bombardment	300 mM NaCl for one month	Transgenic plants had higher root length compared with non transgenic plants with a relative root length of 5.6%	Takahashi *et al.,* 2010
Nicotiana tabacum	*Oryza sativa*	*Agrobacterium tumefaciens*-mediated transformation	0, 171, and 342 mM NaCl was added every 2 days to two week old seedlings upto 4 weeks.	Transgenic seedlings showed a gradual increase in the fresh and dry weights in the first two weeks and then significant increases in the fourth week.	Hasthanasombut *et al.,* 2010
Zea mays	*Suaeda liaotungensis*	Pollen-tube pathway	250 mM NaCl Hoagland solution.	73.9–100% of the transgenic seedlings survived and grew well, unlike the wild type seedlings which had a survival rate of 8.9%.	Wu *et al.,* 2008

1.5.4 Role of GB in other abiotic stresses

Glycine betaine has been implicated in protective roles against other abiotic stresses, such as drought (Wang *et al.,* 2010), heat (Yang *et al.,* 2005), cold (Karabudak *et al.,* 2014), osmotic (Chen and Murata, 2011) and oxidative stress (Park *et al.,* 2007). The photosynthetic machinery (chloroplast ultrastructure) is highly susceptible to abiotic stress as mentioned earlier (section 1.6.2). Glycine betaine plays a pivotal role in protecting the photosynthetic machinery. Transgenic tobacco plants with spinach *BADH* gene enhanced the activity of the enzyme RuBisCo activase (Yang *et al.,* 2005). Under heat stress, RuBisCo activase associates with the thylakoid membranes, interfering with the activity of Rubisco and therefore reduces CO_2 assimilation. But GB accumulation has been shown to prevent RuBisCo activase and thylakoid membrane association (Yang *et al.,* 2005). Wang *et al.* (2010) showed that transgenic wheat (with *Atriplex hortensis BADH*) overexpressing GB appeared had better thylakoid membrane stability and reduced structural damages, in comparison to the chloroplast, when subjected to combined heat and drought stress (compared to control plants).

Arabidopsis engineered with bacterial choline oxidase was capable of GB production, and this conferred protection against freezing temperatures (cold stress), via expedited recovery of photosystem II (PSII) in the leaves (Sakamoto *et al.,* 2000). Murata *et al.* (2007) also suggest that GB is involved in limiting the production of ROS in plants subjected to low temperature. Glycine betaine accumulating transgenic tomato plants were also reported to have enhanced cold tolerance and survival rates (Park *et al.,* 2004). Ahmad *et al.* (2010) engineered GB-synthesizing transgenic potato plants, called SSAC plants which were capable of GB production coupled with SOD and APX expression, and showed decreased ion leakage when subjected to methyl viologen induced oxidative stress (Ahmad *et al.,* 2008; 2010) in comparison to wild type or SOD+APX only expressing plants.

1.5.5 Role of GB in animal health

While the previous sections have clearly outlined the protective properties of GB to plants (natural GB-accumulators and transgenics), the use of GB accumulators in agroforestry related applications is yet to be explored. There are several reports that show GB to play an essential role in maintaining the health of vital organs, such as liver,

heart and kidney in humans (Craig, 2004; Likes *et al.,* 2007). In humans, it has been found to play essential roles as an osmolyte, a lipotrope and a source of methyl groups. Glycine betaine reduces cardiovascular risk factors, and betaine and choline decrease the risk of infant neural tube defects (Raman and Rathinasabapathi 2003). Glycine betaine also plays a vital role in nutrition and health of pigs and poultry that consume GB-rich fodder (Eklund *et al.,* 2005).

1.6 Proline (MW: 115.13 g mol^{-1})

Proline is a highly water soluble amino acid with no net charge at neutral pH. The presence of a secondary amino group and no primary group makes proline an alpha imino acid, rather than an amino acid. Proline is one of the common solutes accumulated in response to salt or drought stress in many bacteria and plants (reviewed in Verbruggen and Hermans, 2008).

1.6.1 Biosynthesis

Proline biosynthesis occurs from L- glutamic acid and involves two enzymes, pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). In plants, proline is synthesised either through the glutamate pathway or arginine/ornithine pathway, though the latter is utilised under higher levels of nitrogen (Delauney and Verma, 1993).

a. Glutamate pathway: Phosphorylation of glutamate, by glutamyl kinase, is an ATP-dependent process that results in the formation of glutamyl phosphate. The enzyme, glutamyl semialdehyde dehydrogenase, reduces glutamyl phosphate to glutamyl semialdehyde (GSA) which is then coupled with glutamyl kinase. This enzyme complex undergoes a spontaneous cyclic reaction to form pyrroline-5-carboxylate (P5C), which on reduction by P5CR yields proline (Yoshiba *et al.,* 1997) (Figure 1.10)

b. Arginine/Ornithine pathway: This pathway involves the enzymatic conversion of arginine to ornithine by arginase, followed by conversion of ornithine to α-keto-δ-aminovalerate by α-aminotransferase (α-OAT); α-keto-δ-aminovalerate then undergoes a spontaneous cyclization to form pyrroline 2-carboxylate (P2C), which is then reduced by P2C reductase to proline. Although this pathway has been established only in bacteria, and not yet discovered in plants, GSA in the glutamate pathway can also be directly obtained by the conversion of ornithine to GSA by ornithine δ-

aminotransferase (δ-OAT), which then follows the glutamate pathway of GSA reduction (Yoshiba *et al.,* 1997).

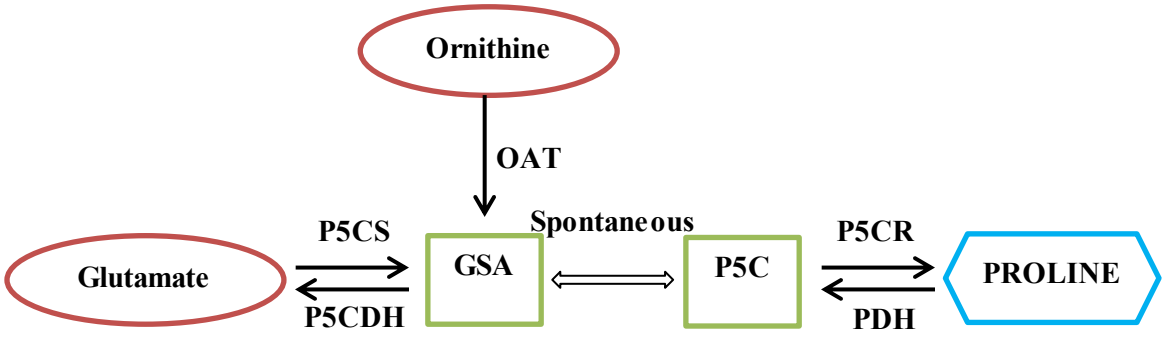

Figure 1.10: Proline metabolism in plants
P5CS: pyrroline 5-carboxylate synthetase; P5CDH: pyrroline 5-carboxylate dehydrogenase; GSA: glutamyl semialdehyde; P5C: pyrroline-5-carboxylate; P5CR: pyrroline-5-carboxylate reductase; PDH: proline dehydrogenase; OAT: Ornithine-δ-aminotransferase. (Substrates are highlighted in red, the intermediate products in green and the final product in blue)

1.6.2 Major enzymes and genes involved in the biosynthetic pathway

1.6.2.1 Pyrroline 5-carboxylate synthetase (P5CS; EC not assigned)

Pyrroline 5-carboxylate synthetase is a bifunctional enzyme involved in the first two steps of proline biosynthesis, through the glutamate pathway in plants (Hu *et al.,* 1992). The bifunctional property of P5CS is attributed to the presence of both γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase activities. However, in bacteria and yeast, there are two separate enzymatic domains, ProB and ProA (in bacteria) and PRO1 and PRO2 (in yeast) encoding γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase (Lehmann *et al.,* 2010). The *P5CS* gene has been isolated from many plants such as *Medicago truncatula* (Strizhov *et al.,* 1997), *Arabidopsis thaliana* (Fujita *et al.,* 1998), *Medicago sativa* (Ginzberg *et al.,* 1998), *Sorghum bicolor* (Su *et al.*, 2011), *Carica papaya* (Zhu *et al.*, 2012), *Nitraria tangutorum* (Zheng *et al.*, 2014); and in many of these species two copies of the gene (*P5CS1* and *P5CS2)* resulting from independent evolutionary duplication events have been reported (Turchetto-Zolet *et al.*, 2009). Although there is high sequence similarity between the two isoforms, they differ substantially in their functionality. The functions of *P5CS1* isoform are confined to being a 'house-keeping' gene (Verdoy *et al.*, 2006) that is expressed is ubiquitously in most organs (Fujita *et al.,* 1998), induced by abiotic stresses such as salt, dehydration, cold and abscissic acid and involved in proline production (Székely *et al.*, 2008). The *P5CS2* isoform, on the other hand, is expressed in dividing cells (Strizhov *et al.*, 1997)

and is required for seed development (Székely *et al.*, 2008). A recent study reports a third *P5CS* gene, *MtP5CS3* from *Medicago truncatula*, which shows sequence homology to both *MtP5CS1* and *MtP5CS2* except for the presence of an extra amino-terminal segment (Kim and Nam, 2013). The *MtP5CS3* isoform plays a regulatory role in salt and drought induced proline accumulation and during nitrogen fixation. Increased P5CS enzyme activity has a positive correlation to increased proline content in many plants such as *Vigna aconitifolia* (Hong *et al.*, 2000), *Gossypium hirsutum* (Parida *et al.*, 2008) and Brassica juncea (Chakraborty *et al.*, 2012) and plays a key role in the rate-limiting step of proline biosynthesis via feedback inhibition of P5CS by proline (Zhang *et al.*, 1995).

1.6.2.2 Pyrroline-5-carboxylate reductase (P5CR or P5R; EC 1.5.1.2)

The enzyme P5CR reduces pyrroline-5-carboxylate (P5C) to yield proline (Yoshiba *et al.,* 1997). The P5CR gene has been isolated from in many plants including soy bean (Delauney and Verma, 1990), *Pisum sativum* (Williamson and Slocum, 1992), *Arabidopsis thaliana* (Verbruggen *et al.,* 1993) and kiwifruit (Walton *et al.,* 1998). A single form of P5CR has been reported in these plants (arabidopsis, pea and soy bean), however, there have been two P5CR isoenzymes reported in spinach (Murahama *et al.,* 2001). But, DNA hybridisation studies of the soybean and pea genome showed the presence of two to three copies of the P5CR gene (Delauney and Verma, 1990; Williamson and Slocum, 1992) and Szoke *et al.* (1992) showed that P5CR activity was detected in cytosol and chloroplast fractions. The spinach P5CR1 was purified from intact chloroplasts. Since the P5CR gene is not localised in the chloroplast genome, the identification of P5CR2 in chloroplasts suggests a transport mechanism from the cytosol to the chloroplast (Murahama *et al.,* 2001). It was also reported that the level of P5CR2 is comparatively higher than P5CR1 under severe salt stress in spinach leaves, and it was more heat stable at 40°C than P5CR1 in spinach (Murahama *et al.,* 2001). P5CR is suggested to be required during embryogenesis in *Arabidopsis* (Meinke *et al.*, 2008) and its activity has been up-regulated in salt-stressed rice (Nounjan *et al.,* 2012).

1.6.2.3 Ornithine δ- aminotransferase (δ-OAT; EC 2.6.1.13)

Proline can be synthesised by transamination of ornithine, through P2C or P5C, followed by reduction to proline. Although reduction of P5C appears to be more

prominent in proline synthesis, proline synthesis from P2C reduction also occurs (Delauney *et al.,* 1993). Delauney *et al.* (1993) studied the expression of P5CS and δ-OAT under varying levels of nitrogen and salt, and found that the mRNA transcripts of P5CS was higher than δ-OAT levels, whereas the effect was reversed under low salt concentration and high nitrogen concentration, suggesting that upregulation of the δ-*OAT* gene and subsequent proline synthesis via the ornithine pathway occurs under high nitrogen concentration. However, it may not be as effective as the glutamate pathway independently. Yang and Kao (1999) demonstrated that rice plants subjected to water stress and gabaculine treatment (a potent OAT inhibitor) showed 75% decrease in OAT activity, but only 20% reduction in proline content, suggesting that the ornithine pathway, in conjunction with the glutamate pathway, may enhance stress alleviation.

1.6.3 Role of Proline in abiotic stress

Accumulation of proline as an adaptive and protective mechanism has been observed in plants subjected to a range of stresses such as drought (*Capsicum annuum*, Anjum *et al.,* 2012), salinity (*Iris lacteal*, Zhang *et al.,* 2012), oxidative stress (*Zea mays*, Yang *et al.,* 2009), temperature stress (*Carica papaya*, Zhu *et al.,* 2012) and heavy metals (*Nicotiana benthamiana*, Ku *et al.,* 2012). In addition, proline may also play an important role in plant development (reviewed in Mattioli *et al.,* 2009) and as a signalling molecule, regulating the transcript levels of stress-related genes (Carvalho *et al.,* 2013).

1.6.3.1 Effects of exogenous application

Exogenous proline application on salt-stressed soybean cell cultures exhibited increased antioxidant enzyme levels and contributed to a significant level of salt tolerance (Yan *et al.,* 2000). Application of proline to salt-stressed tobacco plants showed increased POD and CAT enzyme activities and increased fresh weight (Hoque *et al.,* 2008). Ahmad *et al.* (2010) reported similar observations on application of proline to long term salt-stressed *Olea europaea* plants. Nounjan *et al.* (2012) also found similar benefits from proline application on salt-stressed rice seedlings. Additionally, increased transcript levels of *P5CS* and *P5CR,* and endogenous proline levels were noted (Nounjan *et al.,* 2012). Although exogenous application of proline has been proved to be advantageous in many plants for a long time (Yan *et al.,* 2000, Hoque *et al.,* 2008; Ahmad *et al.*,

2010, Nounjan *et al.,* 2012), some reports show there were no noteworthy benefits. Exogenous application of proline on cucumber, a proline non-accumulating plant, failed to provide stress resistance to the plant (Itai and Paleg, 1982). Foliar application of proline to salt-stressed rice plants did not show any significant reductions in the levels of Na^+ and Cl^- ions (Krishnamurthy and Bhagwat, 1993). De Lacerda *et al.* (2003) suggested that proline accumulation in two salt-stressed sorghum genotypes occurred as a response to salt stress and may not be attributed to salt tolerance.

1.6.3.2 Transgenic applications of proline biosynthetic genes

Transgenic expression of *P5CS* gene has been studied more predominantly compared to *P5CR* and *OAT* genes and the transformants have shown better tolerance to salinity. Some of the reported effects of proline biosynthetic gene expression under salt stress include increased RWC, relatively lower decrease in biomass (fresh weight), greater recovery of transgenic seedlings when transplanted into normal soil conditions and increased proline accumulation (Roosens *et al.,* 2002; Ma *et al.,* 2008; Kumar *et al.,* 2010; Karthikeyan *et al.,* 2011; Surekha *et al.,* 2014) (Table 1.6).

1.6.4 Role of proline in other abiotic stresses

In addition to salt stress, proline accumulation in plants is induced by several other environmental stimuli such as UV irradiation (Salama *et al.,* 2011), heavy metal toxicity (Theriappan *et al.,* 2011), heat stress (Rasheed *et al.,* 2011) and cold stress (Aghaee *et al.,* 2013). Rice plants subjected to drought showed increased proline content in leaves (Hsu *et al.,* 2003), transgenic 'Carrizo' citrange rootstocks over-expressing the *Vigna aconitifolia P5CS* gene displayed a five-fold increase in proline content under drought stress (Molinari *et al.,* 2004). Transgenic 'Swingle' citrumelo plants over-expressing the P5CS gene also showed proline accumulation and maintained a positive leaf pressure potential contributing to drought tolerance (De campos *et al.,* 2011). A critical and advantageous 'after-effect' of stress induced proline accumulation, is the availability of free proline to be metabolised and yield energy molecules such as ATP and NAD(P)H, which are critical in restoring the energy status of the plant during recovery (De Ronde *et al.,* 2004).

Table 1.6: Effects of expressing proline biosynthetic genes (*P5CS, P5CR and OAT)* in transgenic species

Transgenic plant*	Gene	Gene source	Level of salt stress	Observed effects	Reference
Cajanus cajan	*P5CS*	*Vigna aconitifolia*	200 mM NaCl for 7 days	Transgenic plants maintained higher RWC, increased plant height, high proline levels and lower lipid peroxidation compared to NT plants.	Surekha *et al.,* 2014
Oryza sativa (*indica* rice cultivar ADT 43)	*P5CS*	*Vigna aconitifolia*	200 mM NaCl for 4 weeks	Transgenic plants showed lesser reduction in plant height, leaf growth and biomass, and grew to maturity with flowering and seed set while NT plants died within 10 days of salt treatment.	Karthikeyan *et al.,* 2011
Oryza sativa (*indica* rice cultivar Karjat-3)	*P5CS*	*Vigna aconitifolia*	150 mM NaCl for 7 days	Transgenic plants showed lesser reduction in plant height, leaf growth and biomass compared to NT plants. They also expressed four or more times of proline compared to NT plants.	Kumar *et al.,* 2010
Arabidopsis thaliana	*P5CR*	*Triticum aestivum*	Different concentrations of NaCl (0, 50, 100 and 150 mM) for 7 days	Root growth of transgenic plants was less inhibited by NaCl treatment (upto 100 mM NaCl) and produced 2.5–4 times higher levels of proline compared to control plants. Transgenic seedlings also showed greater recovery when transplanted into normal soil conditions.	Ma *et al.,* 2008
Nicotiana plumbaginifolia	*OAT*	*Arabidopsis thaliana*	200 mM NaCl for 6 weeks	Under salt stress, transgenic lines expressing OAT showed 3 fold increase in proline accumulation compared to NT plants and to OAT transgenic lines grown under normal conditions. OAT transgenic lines also showed lesser fresh biomass decrease under 200 mM NaCl stress.	Roosens *et al.,* 2002

*Method of transformation: *Agrobacterium tumefaciens*-mediated transformation; NT: 'non transformed' plants

1.7 Trehalose (MW: 342.31 g mol^{-1})

Trehalose is a non-reducing alpha-D-glucopyranoside disaccharide made of two D-Glucose molecules. It has a more stable structure than sucrose due to the low energy glycosidic bond between the two sugars; hence, it is not easily broken into its constituent hexoses except in the presence of the enzyme trehalase. Trehalose is not accumulated at high levels in most vascular plants. It is mainly accumulated in many bacteria (Shimakata and Minatogawa, 2000), fungi (Nwaka and Holzer, 1998) and a few dessication tolerant higher plants like resurrection plants (Goddijn and van Dun, 1999; Iturriaga *et al.,* 2000) and a few others such as *Botrychium lunarian*, *Echinops persicus*, *Carex brunnescens*, *Fagus silvatica* (Elbein, 1974), *Glycine max* (Müller *et al.,* 1992), *Sporobolus stapfianus*, *Ophioglossum vulgatum* (Müller *et al.,* 1995), *Borya constricta*, *Coleochloa setifera*, *Eragrostiella nardoides*, *Eragrostis nindensis*, *Microchloa kunthii*, *Tripogon jacqemontii*, *Ramonda myconi*, *Sporobolus pyramidalis* (Ghasempour *et al.,* 1998), *Selaginella sartorii* (Iturriaga *et al.,* 2000), *Arabidopsis thaliana* (Müller *et al.,* 2001), *Selaginella lepidophylla* (Müller *et al.,* 2001), *Triticum aestivum* (El-Bashiti *et al.,* 2005), *Phaseolus vulgaris* (García *et al.,* 2005), *Lotus japonicus* and *Medicago truncatula* (Lopéz *et al.,* 2006; 2009). Studies on the effects of validamycin A, a potent trehalase inhibitor, indicated that most angiosperms are capable of trehalose biosynthesis but because of its rapid degradation, it is not accumulated to detectable levels (Goddijn *et al.,* 1997).

1.7.1 Biosynthesis

Synthesis of trehalose can occur by at least five different pathways (Figure 1.11). However, it predominantly occurs through the TPS/TPP pathway from uridine diphosphoglucose and glucose-6-phosphate, with trehalose-6-phosphate formed as an intermediate. This reaction was first studied in 1957 by Cabib and Leloir, who performed partial purification of trehalose phosphate phosphatase and suggested the dephosphorylation of trehalose 6-phosphate to a disaccharide (Cabib and Leloir, 1958).

TPS/TPP pathway: Trehalose 6-phosphate synthase (TPS) catalyses the formation of trehalose phosphate by the transfer of glucose from a glucosyl donor such as UDP-glucose or GDP-glucose to glucose 6-phosphate. Trehalose 6-phosphate phosphatase (TPP) dephosphorylates trehalose 6-phosphate to form trehalose and inorganic phosphate.

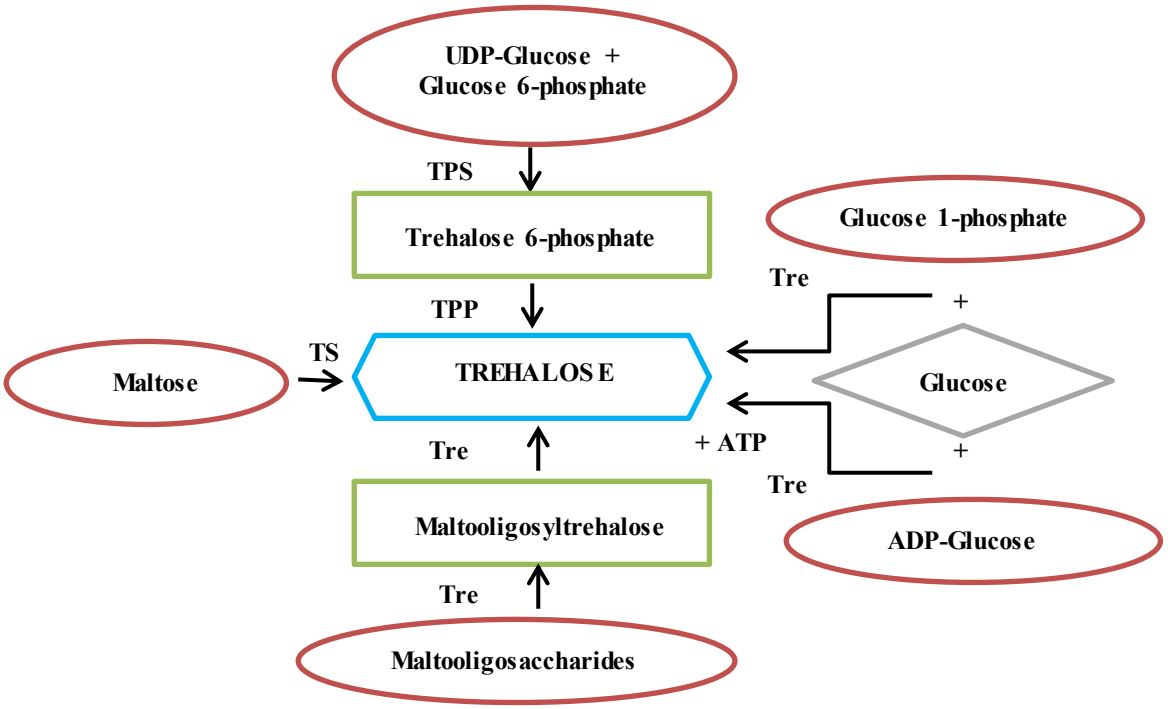

Figure 1.11: Biosynthesis of trehalose via various enzyme pathways

The figure illustrates the various substrates and pathways that are available for trehalose biosynthesis. TPS: Trehalose 6-Phosphate Synthase; TPP: Trehalose 6-Phosphate Phosphatase; TS: Trehalose Synthase; TreY: Maltooligosyl-Trehalose Synthase; TreZ: Trehalohydrolase; TreP: Trehalose Phosphorylase; TreT: Trehalose Glycosyltransferring Synthase. (Substrates are highlighted in red, the intermediate products in green and the final product in blue)

1.7.2 Major enzymes and genes involved in the biosynthetic pathway

1.7.2.1 Trehalose-6-phosphate synthase (TPS; EC 2.4.1.15)

With advances in genome sequencing, the number of TPS encoding genes being identified varied significantly. Initially, Vogel *et al.* (1998) reported nine putative *TPS* genes within the rice genome and then this number was increased to 11 TPS encoding genes by Zang *et al.* (2011). In the case of *A. thaliana,* genome sequencing revealed 11 putative TPS genes within the genome (The Arabidopsis Genome Initiative, 2000; Leymann *et al.*, 2001). Later based on sequence analysis and ignoring TPS-TPP fusion proteins, Avonce *et al.* (2006) reported eight *TPS* genes in *A. thaliana*; since in prokaryotes, the TPS protein is formed by a single phosphatase domain, whereas in most eukaryotes and the microaerophile *Pyrobaculum aerophylum* the TPS proteins are fused to the TPP domain (Avonce *et al.*, 2006). But many studies acknowledge the presence of 11 TPS encoding genes (Vandesteene *et al.*, 2010; Yang *et al.*, 2012). These 11 genes are divided into class I (*AtTPS1-AtTPS4*) and class II (*AtTPS5-AtTPS11*) subfamilies (reviewed in Lunn *et al.*, 2014). The 3D structure of the *E. coli* TPS enzyme showed that the aminoacid residues involved in the binding of substrate

glucose 6-phosphate are Arg9, Trp40, Tyr76, Trp85 and Arg300; while Gly22, Asp130, His154, Arg262, Asp361 and Glu369 are involved in the binding of UDP-glucose (Gibson *et al.*, 2002). These residues are reported to be conserved in organisms exhibiting TPS activity or in organisms known to produce trehalose (Avonce *et al.*, 2006). Of the 11 TPS genes in the Arabidopsis genome, only AtTPS1-AtTPS4 (class I) have the conserved residues for substrate binding of which only AtTPS1 has been reported to have enzyme activity (van Djick *et al.*, 2002; reviewed in Lunn *et al.*, 2014). *AtTPS3* is possibly a pseudogene (Lunn, 2007; Vandesteene *et al.*, 2010) whereas *AtTPS2* and *AtTPS4* genes are expressed in developing seeds suggesting a role in plant development (Schmid *et al.*, 2005). Avonce *et al.* (2006) reported that the rice TPS-encoding gene family did not have these conserved residues but were capable of trehalose accumulation under salt stress (Garcia *et al.*, 1997). The class II (*AtTPS5-AtTPS11*) subfamily have a phosphatase domain (Avonce *et al.*, 2006). However, Harthill *et al.* (2005) reported that no detectable TPP activity was observed in *in vitro* assays of heterologously expressed AtTPS5, AtTPS7 and AtTPS8.

1.7.2.2 Trehalose-6-phosphate phosphatase (TPP; EC 3.1.3.12)

Trehalose-6-phosphate phosphatase is a magnesium-dependent enzyme that belongs to the class of haloacid dehalogenase (HAD) superfamily, characterised by three highly conserved motifs (Avonce *et al.,* 2006). The first motif DXDX(T/V) has two aspartic acid residues which are involved in the formation of a phosphorylated intermediate with the substrate, and the second residue plays a role in catalysis. The second motif (S/T)(G/X) is required for formation of a hydrogen bond with the phosphate group of the substrate. The third motif $KX_{16-30}(G/S)(D/S)X_3(D/N)$ is an important segment of the active site and coordinates the magnesium ion required for catalysis. Two *TPP* genes were initially reported in Arabidopsis (*AtTPPA* and *AtTPPB*) (Vogel *et al.,* 1998) and rice (*OsTPP1* and *OsTPP2*) (Shima *et al.,* 2007). But genome sequencing revealed 10 putative *TPP* genes within the Arabidopsis genome (Leymann *et al.*, 2001) and nine putative *TPP* genes within the rice genome. Although the function of the class II TPS proteins/TPP encoding genes is unclear, rice *TPP*s were induced transiently by salt, cold and drought stress, as well as under exogenous ABA applications (Shima *et al.,* 2007).

1.7.3 Role of trehalose in salt stress

1.7.3.1 Effects of exogenous application of trehalose

External applications of trehalose were beneficial to salt-stressed rice plants based on the concentration of trehalose used. When concentrations of up to 5 mM trehalose were used, plant growth inhibition was reduced; whereas a concentration of 10 mM trehalose stopped chlorophyll loss in leaf blades and conserved root integrity (Garcia *et al.,* 1997). Exogenous application (100 mM) was also shown to induce several other stress-responsive genes e.g. *ATPK19* (a salt and cold stress induced kinase), and increased trehalose-6-phosphate levels and calcium and phosphorylation signalling proteins, based on the microarray data of Schluepmann *et al.* (2004). Zeid (2009) reported that pre-soaking maize grains in 10 mM trehalose rendered the maize seedlings capable of alleviating adverse effects of salinity stress on photosynthesis, nucleic acid concentrations, total soluble sugars and protein as well as increased K^+/Na^+ ratio. Pre-treatment of winter wheat with 1.5 mM trehalose increased endogenous trehalose content by 150% under heat stress, and also protected the chloroplast ultrastructure, thylakoid membranes, preserved cell membrane integrity and abridged ROS accumulation from heat stress (Luo *et al.,* 2010).

1.7.3.2 Transgenic applications of trehalose biosynthetic genes

Transgenic expression of microbial *TPS* and *TPP* genes (Table 1.7) showed increased stress tolerance in many plants. For example, transgenic rice with an *E. coli* TPS–TPP fusion enzyme encoding gene imparted tolerance to drought, salt and cold stress by accumulating trehalose up to 0.1% of the fresh weight no visible growth inhibition (Jang *et al.,* 2003). However, pleiotropic effects such as stunted growth or dwarfism were reported in transgenic potato (Yeo *et al.,* 2000), tomato (Cortina and Culiáñez-Macià 2005) and tobacco (Almeida *et al.,* 2005). Transgenic Arabidopsis plants overexpressing *AtTPS1* showed a small increase in trehalose and trehalose-6-P levels and dehydration tolerance, but displayed delayed flowering (Avonce *et al.,* 2004). One of the contributing factors to such phenotypical changes is in fact the accumulation of trehalose-6-phosphate, an intermediate metabolite, rather than trehalose itself (Almeida *et al.,* 2005). However, transgenic plants such as rice (Garg *et al.,* 2002; Jang *et al.,* 2003) and tomato (Lyu *et al.,* 2013), expressing the TPS–TPP fusion enzyme did not

show any pleiotrophic effects; hence, the TPS–TPP fusion enzyme may be better suited for crop plants where plant biomass is a contributing factor to estimating its yield.

1.7.4 Role of trehalose in other abiotic stresses

Evidence on increased cycling of trehalose and its precursors has been reported in osmotically stressed *E. coli* (Serrano, 1996) and *S.cerevisiae* (Parrou *et al.,* 1997) subjected to mild heat, osmotic and oxidative stress. Transgenic alfalfa plants expressing yeast *TPS1-TPS2* fusion gene were capable of enduring extreme temperatures, drought and salt stress (Suárez *et al.,* 2009). In rice, overexpression of *OsTPP1* enhanced tolerance to salt and cold stress (Ge *et al.,* 2008) and increased trehalose accumulation and contributed to chilling stress (Pramanik and Imai, 2005). Transgenic tomato plants overexpressing yeast *TPS1* gene endured 15 days of drought and recovered well on subsequent rewatering (Cortina and Culiáñez-Macià, 2005). Although these reports show the benefits of expressing trehalose biosynthesis gene(s), research has been limited to a few plant species. Studies on agronomical and environmentally important plants are still in its infancy.

Table 1.7: Effects of trehalose accumulation in transgenic plants

Transgenic plant*	Gene	Gene source	Level of stress	Observed effects	Reference
Lycopersicon esculentum	*TPS1*	Yeast	SS: 100 mM NaCl or 25 mM LiCl	Higher trehalose content; altered phenotypes (dwarfism and lancet shaped leaves); tolerance to drought, salt and oxidative stress	Cortina and Culiáñez-Macià, 2005
Medicago sativa	*TPSP*	Yeast	SS: 50 to 300 mM NaCl for 2 weeks; FS: –5, –10, and –15°C for 6, 12, 24, 48 and 72 h; DS: suspension of irrigation for 5, 10, 20 and 30 days followed by re-watering	Transgenic plants displayed a significant increase in drought, freezing, salt, and heat tolerance.	Suárez *et al.,* 2009
Nicotiana tabacum	*TP*	*Pleurotus sajor-catu*	DS: suspension of irrigation for 10 days	Higher trehalose content; no morphological alteration; tolerance to water deficit	Han *et al.,* 2005
	TPS1	*A thaliana*	OS: varying concentrations of mannitol (0, 0.25, 0.5, 0.75 M) and sodium chloride (0, 0.07, 0.14, 0.20, 0.27 and 0.34 M)	Tolerance to osmotic stress; plants smaller than wild type; absence of lancet-shaped leaves	Almeida *et al.,* 2005
Oryza sativa	*ots A, ots B*	*E. coli*	SS: 100 mM NaCl stress for 4 weeks; DS: periodic withholding of irrigation for 100 hours followed by rewatering for 2 days; CS: 10°C for 72 h	Higher trehalose levels; sustained plant growth, less photo oxidative damage and favorable mineral balance leading to abiotic stress tolerance	Garg *et al.,* 2002
	TPSP	*E. coli*	SS: 100 mm NaCl for 7 days	Increased trehalose levels; absence of phenotypic alterations to growth; tolerance to drought, salt and cold stress	Jang *et al.,* 2003
Solanum lycopersicum	*TPSP*	*E. coli*	SS: 150 mM NaCl; DS: suspension of irrigation for 10 days followed by re-watering	Increased trehalose levels; absence of phenotypic alterations to growth; tolerance to drought and salt stress	Lyu *et al.,* 2013

*Method of transformation: *Agrobacterium tumefaciens*-mediated transformation; SS: salt stress; DS: drought stress; FS: freezing stress; HS: heat stress; CS: cold stress; OS: osmotic stress; TPS: trehalose-6-phosphate synthase; TPSP: a bifunctional fusion (TPSP) of the TPS and TPP genes; TP: trehalose phosphorylase

1.8 Management of dryland salinity through revegetation

Effective land and water resource management will help in reducing the effects of dryland salinity. Reclamation of saline degraded land through revegetation, replacing annual pastures with perennial deep rooted trees, cultivation of salt tolerant plant species such as wattles and salt bushes, and use of native vegetation and saline aquaculture, are some of the potential alternatives (Pannell, 2001). As the water requirements of trees are much higher than those of cereal crops, planting trees in salinity prone areas assists in lowering ground water level and reduce the mobility of free salt (Niknam and McComb, 2000). The amount of reforestation as high as 80% in catchment areas may be required to have a pronounced decrease in water table levels and the incidence of salinity (George *et al.,* 1999). Additional economic incentives related to agroforestry could also be facilitated, e.g. saline degraded lands may be suitable to grow native plants that serve as sources of food, fuel, fodder, fibre, resin, essential oils, and pharmaceutical products and for landscape reintegration.

1.8.1 Revegetation through native trees

From an Australian landscape perspective, native trees are expected to outperform introduced species, as they are acclimated and selective species may be pre-adapted to the available environments (bush fires, aridity and nutrient deficient soils). For example, some of the early tests indicated that some *Eucalyptus* species can survive salt levels of ~1.8% e.g. *E. calophylla*, *E. erythrocorys*, *E. incrassata*, *E. largiflorens*, *E. neglecta* and *E. tereticornis* (Blake, 1981). Van der Moezel *et al.* (1988) reported that some species of *Casuarina* (*C. cristata*, *C. glauca*, and *C. obesa*) are salt tolerant as well as well-adapted to grow in waterlogged conditions. A number of other Australian native species have also been tested for their ability to tolerate different levels of salinity. In particular, species of *Acacia* (*A. cyclops, A. stenophylla, A. ampliceps*), *Atriplex* (*A rhagodioides, A. vesicaria, A. paludosa, A. amnicola, A. bunburyana, A. cinerea, A. lentiformis, A. muelleri, A. nummularia, A. semibaccata, A. undulata*), *Casuarina* (*C. glauca, C. obesa*), *Frankenia* (*F. ambita, F. brachyphylla, F. fecunda*), *Melaleuca* and *Puccinellia (P. ciliata*), have been reported to grow in extreme saline soil conditions with ECe >16 dS/m, while several species of *Eucalyptus* have been reported to grow on moderately saline (ECe 4-8 dS/m) soils (Department of Agriculture, Western Australia, 2002). Recent reports on salinity tolerance of eight other species by

Xianzhao *et al.* (2013) concluded that *Tamarix chinensis* had the highest level of tolerance among the species tested, followed by *Suaeda salsa*. *Sesbania cannabina* exhibited the lowest level of salt tolerance comparatively. The results were based on seedling growth yield at seven NaCl concentrations (0, 50, 100, 150, 200, 250, and 300 mM) (Xianzhao *et al.*, 2013). Australian based research project, Enrich, was initiated by the CRC for Plant-based Management of Dryland Salinity, RIRDC Joint Venture Agroforestry Program, Meat and Livestock Australia and Australian Wool Innovation and screened more than 60 native shrub species (including *Atriplex, Rhagodia, Maireana*, *Acacia, Medicago, Drosophila* and *Kennedia*) for their suitability in forage applications (Future Farm Industries Co-operative Research Centre Limited and Enrich project, 2011). Revell *et al.* (2013) substantiated the latent prospects of the above mentioned species as feed additives based on nutritive values and digestibility.

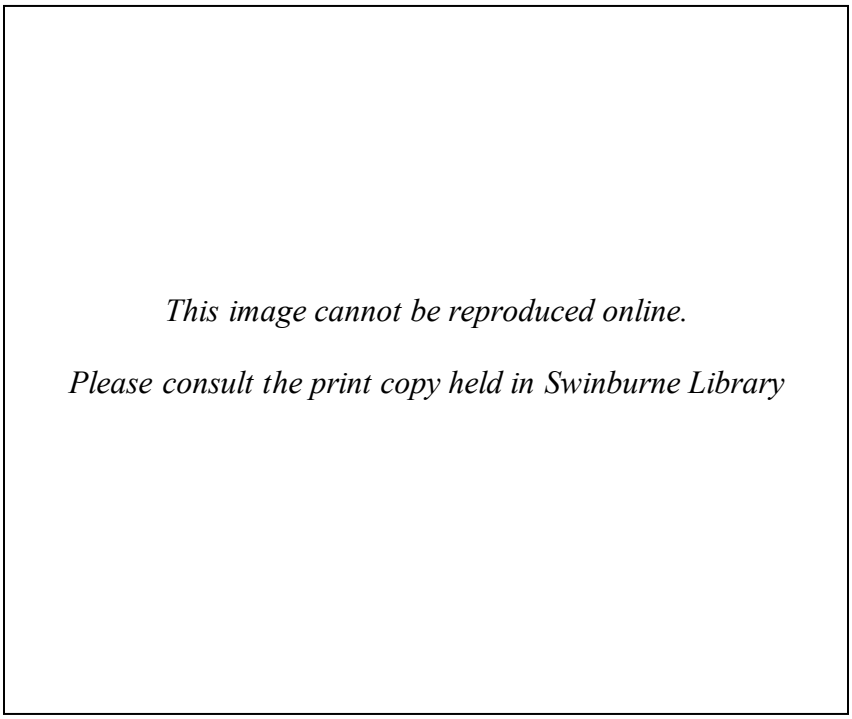

Figure 1.12: Extent of utilisation of native species for forage applications
The figure illustrates that although there are more than 20,000 Australian plant species, current knowledge and laboratory testing is limited only to 101 species.
(Source: Future Farm Industries Co-operative Research Centre Limited and Enrich project, 2011)

However, despite such efforts, native species are still under-investigated and underutilized considering the number of species available (as shown in Figure 1.12), and information on salinity tolerant species with agroforestry potential is especially limited. Also, there is no exhaustive data that demonstrates their potentials and

distinguish the upper and lower limits of tolerance. Testing these parameters would provide invaluable information when combined with other data on aspects of economic importance, weediness, phytochemical composition, forage and other characteristics.

1.8.2 The Kamarooka project

Kamarooka is located approximately 20 kilometres north of Bendigo, in Victoria, Australia. Dryland salinity first became evident at Kamarooka in the 1950s. The level of sub-soil salinity measured varied from about 2-4 dS/m (NUFG, 2013). The Northern United Forestry Group (NUFG), first established in the 1990s, comprised of a group of people working towards sustainable farming systems and establishing low-rainfall farm forestry (NUFG, 2013). The Kamarooka project was a successful land reclamation program that established and managed halophytic vegetation (largely saltbushes) on the most degraded salt-affected land, along with salt-tolerant native trees like acacias, shrubs and grasses in adjacent land moderately affected by salinity (Figure 1.13) (NUFG, 2013). The work of the NUFG highlighted the importance of native plants in reclaiming salt-affected lands, and paved the way for this study to explore what makes these plants salt-tolerant and devise a rapid method to identify new candidate species for planting on saline lands. Of interest to this project are some of the species of the genus *Acacia* and *Atriplex* used in the Kamarooka project and these are described in the following section.

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Figure 1.13: Transformation of Kamarooka after saltbush and *Acacia* planting
(Source: NUFG, 2013)

1.9 The genus *Atriplex* (Common name: Salt bush)

1.9.1 Physical description and distribution

Salt bush is the common name given to plants of genus *Atriplex* (Chenopodiaceae family). Salt bushes are deep-rooted perennial shrubs that can grow to three metres. The leaves have a scaly coating that often gives species a silver grey colour. The leaves in this genus are typically 1-3cm long; however, there is considerable variation in leaf shape, from elliptical to orbicular (ANBG, 2013). Salt bush flowers are usually small, green, and terminal, and produce triangular, laterally compressed fruits (Aganga *et al.,* 2003).

The genus *Atriplex* has more than 250 species mainly found in sub-tropical and temperate regions of the world. Australia is home to about 61 species (Figure 1.14). Saltbushes are generally well adapted to areas with an annual rainfall of 250-600 mm and grow in slightly acidic conditions and at alkaline soils. The most widespread species in the inland semi-arid and arid regions of mainland Australia is *Atriplex nummularia,* the Oldman saltbush.

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Figure 1.14: Distribution of saltbushes in Australia
(Source: ANBG, 2013)

1.9.2 Potential for revegetation and other applications

Halophytes have inherent physical, biochemical and/or molecular mechanisms (as mentioned in Section 1.4) to combat salinity and can be used as a source of food, fodder, forage, ornamentals and chemicals (Lokhande and Suprasanna, 2012). But these groups of plants are seemingly under-utilised for these purposes. Only now saltbushes are being considered as new economic opportunities, not only for perennial revegetation but also as pastures (NUFG, 2013; Saltland Pastures Association). The

Land and Water Australia 'Options for the Productive Use of Saline Land' (OPUS) promotes 'Living with salt', and the benefits of saltbush include availability of feed in autumn/winter, in addition to environmental benefits such as reductions in erosion, surface salinity and groundwater recharge (salt loading) (LWA, 2004). Saltbushes are among the Australian native vegetation most tolerant to drought and salt extremes (Department of the Environment and Water Resources, 2007) and form salt-tolerant forage, e.g., Oldman saltbush (*Atriplex nummularia*) and River saltbush (*A. amnicola*). Many species such as *A. semibaccata* and *A. prostrata,* have been classified as highly salt tolerant; while others such as *A. cinerea* and *A. paludosa* are classified as extremely salt tolerant i.e. capable of growth in saline soils with an ECe range > 16 dS/m (VRO, 2012). Research shows that at high salt concentration, the germination ability of some species, such as *A. centralasiatica* (Liu *et al.,* 2006), *A. prostrata* and *A. patula* (Katembe *et al.,* 1998), was not permanently inhibited, confirming their inherent ability to withstand harsh environments. Furthermore, saltbushes such as *A. nummularia* can live for up to 50 years (DEPI, 2009), making them an excellent candidate for revegetation and recovery of saline soils. They also have the ability to recover well after intense defoliation, making them very suitable for pruning. Saltbushes have been used to reduce soil erosion, as they bind the topsoil and reduce winds, which enable the protection of other plants and animals. Additionally, they contain high levels of nitrogen and phosphorous and do not alter the organoleptic properties (taste, colour, odour, aesthetic appearance) of meat produced from cattle fed on saltbushes (Aganga *et al.,* 2003), making these plants an ideal feed crop. Further, grazing trials show that saltbushes make a good mixed-fodder species (NUFG, 2013; SPA: Nutritional Value of Plants growing On Saline Land; Saltland Pastures can sustain sheep during autumn; opportunities and constraints to grazing saline pastures) as shown in many large animal grazing trials in Africa and Australia (Ben-Salem *et al.,* 2010). For example, sheep fed on a mixed diet containing saltbush and hay showed increase live weight (Aganga *et al.,* 2003).

Another saltbush species under evaluation as drought fodder is *Atriplex semibaccata* (Palmer and Ainslie, 2002, Harris *et al.,* 2009). In the United States, it was introduced as a supplementary forage crop and soil binder as early as 1888 (Tull, 1999). *Atriplex semibaccata* has excellent potential as an animal feed with the ability to improve animal

health and wool growth. This is due to high levels of sulphur, nitrogen, salt and vitamin E in the foliage, and the potential to control internal parasites (Fancote *et al.*, 2013). The basis of its food functionality is not yet known; but it is noteworthy that most chenopods produce betaines (CAS number 107-43-7) and several health benefits of betaine and choline are now known (section 1.5.5; Likes *et al.,* 2007).

Oldman saltbush (*Atriplex nummularia*), River saltbush (*A. amnicola*), and Creeping saltbush (*A. semibaccata*) are of interest to this project due to the benefits detailed above. Despite their numerous environmental and animal health benefits, little is known as to whether the saltbushes have the genetic ability to synthesise GB, and if so, whether it is in quantities superior to other fodder species. Addressing this gap is essential if these species are to be fully exploited for environmental and economic sustainability on saline and/or drought-prone land.

1.10 The genus *Acacia* (Common name: Wattle)

1.10.1 Physical description and distribution

The genus *Acacia*, commonly known as Wattle (also acacia, when written with a lower case initial letter and not italicised), was first described in 1754 by Philip Miller. The genus is a legume (family Leguminosae), in the mimosoid legume group, which is variously treated as a family, Mimosaceae, or subfamily, Mimosoideae. In 1842, George Bentham restricted the genus name *Acacia* to mimosoid legumes that have numerous free stamens. The majority of Australian *Acacia* spp. are defined by the presence of phyllode, except a small number placed in sections *Botrycephalae* and *Pulchellae,* which have compound leaves. Phyllodes function like a leaf and are capable of photosynthesis, but are thought to derive from expanded and flattened leaf petiole tissue. Acacias in the broad sense are widespread, found mainly in dry and semi dry regions of Australia, Africa, Asia and America (Figure 1.15). It is the largest genus of vascular plants found in Australia. There are some 1350 species of *Acacia* found throughout the world and close to 1000 found in Australia (reviewed in Murphy, 2008).

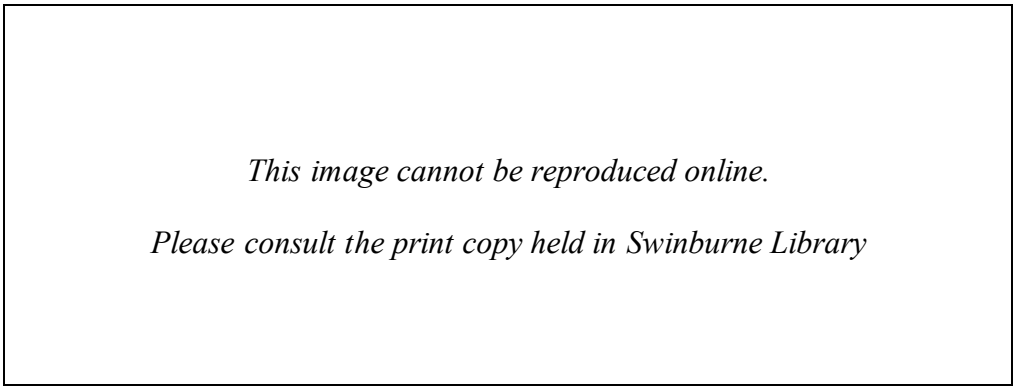


Figure 1.15: Distribution of *Acacia* worldwide
Dark grey represents the areas where acacias are mainly grown.
(Source: World Wide Wattle, 2013)

1.10.2 Potential for revegetation and other applications

Australian *Acacia* can thrive in a diverse range of habitats and environments, and many species can tolerate high pH and waterlogged soils (Niknam and McComb, 2000). Several salt-tolerant *Acacia* species, such as *A. saligna, A. stenophylla, A. salicina* and *A. ampliceps,* have the potential to provide forage and fodder (Vercoe, 1987). *Acacia* species harbor nitrogen-fixing rhizobia that can improve soil fertility (Hoque *et al.,* 2011). They provide edible fruits and seeds, gum arabic and timber for fuel, construction and fencing (reviewed in Lokhande and Suprasanna, 2012). Species such as *A. dealbata* are used in the production of base oils for perfumes (Panda, 2003).

Craig *et al.* (1990) found that a range of acacias from naturally saline lands were moderately tolerant of waterlogged and saline conditions in controlled greenhouse trials. Species with good tolerance to waterlogging and salinity included *Acacia* aff. *lineolata* and *A. mutabilis* subsp. *stipulifera*. *Acacia stenophylla* is also a very tolerant species and *A. auriculiformis*, *A. cyclops*, *A. ligulata*, *A. maconochieana* and *A. sclerosperma* are moderately tolerant for use in the revegetation of damaged agricultural catchments (McComb *et al.*, 1989; Sun and Dickinson 1995). The species of interest to this study are Weeping myall/boree (*Acacia pendula),* Willow wattle (*Acacia salicina*), River Cooba (*Acacia stenophylla*) and Bramble/Prickly wattle (*Acacia victoriae*) that were utilised in the Kamarooka project.

1.11 Molecular phylogenetics in identification of salt tolerant species

Another focus of this study is to identify *Acacia* species for sustainable agroforestry applications in salinity prone areas. It is highly desirable to identify which species would carry a favourable gene pool to combat salinity. The use of native salt tolerant varieties could provide an ideal solution; as, such species may have the trait of salinity tolerance in addition to having economic and agronomic benefits. The selection of species, however, needs to consider key factors such as substantial investment, both economically and time wise.

Comparative biology is based on the expectation that closely related organisms share traits, such as salinity tolerance, that are less common in more distantly related organisms (Cracraft, 2002). Therefore, clarifying molecular phylogenetic relationships can aid in selecting candidate species for a particular trait. Miller *et al.* (2011) used plastid and nuclear rDNA data to test whether invasiveness of species had a phylogenetic component, across a broad phylogenetic framework of 110 *Acacia* species. Although the invasive species did not form a monophyletic group, some evidence for phylogenetic grouping of invasive *Acacia* species was found. The study also identified sister species of known invasive species that may have increased potential for invasiveness. The need for phylogenetic data on the species of interest in the present study is to establish genetic relationships or interspecies similarities of the *Acacia* species, as well as identify close relatives to the four species utilised in Kamarooka mentioned earlier. This information can subsequently be used to generate lists of species that can be tested for salinity tolerance traits. In a very large genus, like *Acacia*, this may reduce unnecessary field trials, prevent further expansion of salinity, and assist in biodiversity conservation and sustainability.

Molecular phylogeny is a potential tool for comparative genomics and phylogenetic classification. It is based on the principle that nucleotide sequences obtained from the nuclear or chloroplast genomes are highly conserved in individuals within a species, but differs among different species (Shneyer, 2009). These differences provide information about evolutionary relationships and by inference the potential sharing of traits between taxa. The identification of DNA regions for phylogenetics can also be used for species identification, by comparison of the target sequences against a known reference

database - a technique known as DNA barcoding. Ideally, the DNA barcode region should match certain selection criteria, which include (i) the barcode region should be conserved in all species of a major taxon, (ii) be of short length (not exceeding 700-800 bp) for efficient isolation from damaged samples, (iii) be sufficiently divergent in different and closely related species, (iv) exhibit high similarity in individuals within a species, (v) be flanked by evolutionary conserved region for ease of primer design and sequence amplification, and (vi) presence of indel sequences (insertions and deletions) for ease of alignment (Shneyer, 2009). In animals, a 650 bp 5' section of the mitochondrial gene coding for cytochrome *c* oxidase 1 (CO1 or cox1) serves as a barcode region. However, in plants mitochondrial DNA has been found to be unsuitable for DNA barcoding (Erpenbeck *et al.,* 2006, Hellberg, 2006), and hence the search for a suitable barcode region was confined to the nuclear and chloroplast genomes. Several sections of the chloroplast genome, such as *trnH-psbA, matK, rpoC, rpoB, rbcL* are now the preferred choice of chloroplast markers for phylogenetic studies of closely related plant species. These are often combined with the internal transcribed spacer (ITS) of 18S-26S rDNA and external transcribed spacer (ETS) of 18S-26S rDNA in the nuclear regions.

1.11.1 Nuclear ribosomal DNA spacers as molecular phylogenetic markers

Apart from DNA barcoding, the ITS1 and ITS2 regions of plant rRNA genes are used most frequently to study phylogeny at genus and species levels due to advantages such as high sequence variability, high copy number and ease of amplification (Kay *et al.,* 2006; Mort *et al.,* 2007). The ITS is adjacent to the conserved 5.8S rRNA gene region and is flanked by the conserved 18S and 26S rRNA genes, and this entire region is tandemly repeated thousands of times to make up the rDNA cistron. The ETS region of 18S-26S rDNA belongs to the same transcriptional unit. It may have evolved under similar functional constraints and complements the ITS data to yield more characters for significant phylogenetic inferences in angiosperms (Baldwin and Markos, 1998). The ITS and ETS regions are currently the most commonly sequenced published loci for a wide range of *Acacia* species and therefore provide the best available comparative dataset.

1.11.2 Role of chloroplast markers in molecular phylogenetics

Chloroplast markers (noncoding sequences: introns and spacers) based on chloroplast genes are easy to isolate and abundant in the cell. However, a single gene may not have sufficient variation, or may have low sequence divergence in closely related species, and hence phylogenetic resolution at lower taxonomic levels may be unfavourable. A more efficient way to apply these regions for phylogenetic analysis is to use a combination of chloroplast DNA markers. Two combinations of chloroplast loci were proposed as potential "official" plant DNA barcodes: *rpoC1+rpoB+matK* and *rpoC1+matK+psbA-trnH* (Chase *et al.,* 2007). Of these *rpoC1, rpoB* and *matK* are coding sequences and *psbA-trnH* is non-coding. The markers were tested for degree of variability and phylogenetic usability and were determined to be of potential use (Chase *et al.,* 2007). Several other regions and combinations were also tested such as *rbcL, rpoB, rpoC, matK, accD, trnH-psbA* and the universal plastid amplicon (UPA) (Newmaster *et al.,* 2008), (chloroplast *rpoB, rpoC1, matK, accD, ycf5, ndhJ, trnH-psbA, rbcL* and nuclear ITS) (Kress and Erickson, 2007*), matK-trnK*, *psbA-trnH*, *trnL-rpl32*, *trnL-trnF* (Miller *et al.,* 2003). The recent availability of many chloroplast genomes has facilitated the identification of potential marker regions that have high evolutionary rates which is critical to resolution in molecular phylogenetics study.

1.12 Summary and research aims

The above review of literature shows that salinity is a serious global issue that has damaging consequences to agriculture, food security, land management and biodiversity. While the effect of salinity on plants can be deleterious, some plants are capable of tolerating salinity and exhibit various sensing, signalling and regulatory pathways. There is need for better crops capable of sustaining a growing human population via high productivity, which requires the exploration of more genetic resources. There is a lot of emphasis on improving salinity tolerance in crop plants by transgenic technologies.

Much of the work on genetics of salt tolerance has been conducted in model plants and crop species, but data related to native plants are very limited. Some possible candidates for investigation are identified here and include the saltbushes and wattles. Australian native flora, such as the saltbushes (*Atriplex* spp.) and wattles (*Acacia* spp.),

are rich genetic and environmental resources yet to be utilised to the fullest. The following chapters aim to explore the biochemical basis of salt tolerance of these plants; and utilise phylogenetics as a molecular tool to identify salt tolerant *Acacia* genotypes.

1.13 Specific aims

- To determine the presence of select osmoprotectant genes in the saltbushes (*Atriplex nummularia, Atriplex semibaccata* and *Atriplex amnicola*) and wattles (*Acacia salicina, Acacia pendula* and *Acacia victoriae*) by means of gene isolation, amplification, cloning and sequencing and characterise the genomic and protein sequences using bioinformatics tools.
- To investigate if the saltbushes have the genetic and biochemical ability to synthesise glycine betaine
- To investigate other osmoprotectants (proline and trehalose) in salt treated against control plant seedlings by biochemical and enzyme expression analysis.
- To identify potential salt tolerant *Acacia* species using Bayesian and maximum parsimony inference in phylogenetics as a predictive molecular tool.
- To test growth and physiology parameters indicative of salt tolerance in putative salt tolerant *Acacia* species.

CHAPTER 2

Materials and Methods

2.1 Equipment

Table 2.1: Equipment used in this study

Manufacturer	Equipment	Purpose
Bio-Rad, California, USA	MyCycler™	PCR
	Chemidoc XRS documentation station	Visualisation of agarose gels under UV light
BMG LABTECH Pty. Ltd, Offenburg, Germany	Microplate reader	For reading absorbance of reaction mixtures used in enzyme assays
Eppendorf, Hamburg, Germany	Mini spin plus	Centrifugation
General Electric (GE) Healthcare, Buckinghamshire, UK	Electrophoresis power supply-EPS301 Minnie Gel Unit, Gel tank	Agarose gel electrophoresis
	GeneQuant™ Pro UV/Vis Spectrophotometer	Quantification of nucleic acid concentration, measuring absorbance of microbial cultures
Grace Discovery Sciences, Illinois, USA	Prevail™ Carbohydrate ES column (250x4.6 mm)	HPLC
	Prevail™ All-Guard™ Carbohydrate ES 5µm guard column (4.6x7.5 mm)	HPLC
Thermo Scientific, Madison, USA	Finnpipettes (1–10, 10–100, 100–1000 µL)	Dispensing liquids
	Sorvall RC6	Centrifugation
Olympus, Tokyo, Japan	C3040 digital camera	Taking images of UV exposed gels
Ratek, Victoria, Australia	Orbital shaker/incubator	Bacterial growth
Shimadzu, Kyoto, Japan	SCL-10A VP system controller, FCV-10AL VP pump, DGU-14A degasser, SIL-10 VP sample auto injector, UV-VIS detector SPD-10AD VP and a CTO-10AC VP column oven	High performance liquid chromatography (HPLC)
Thermoline, Victoria, Australia	Plant growth cabinet (with light, temperature and humidity control)	Propagation of plants
Varian, Victoria, Australia	Varian Spectra AA220 atomic absorption spectrophotometer	Quantitative analysis of cations

2.2 Commercial kits, materials, reagents, solutions

Table 2.2: Commercial kits and materials used in this study

Manufacturer	Kit/material/reagents/solution	Purpose
Applied Biosystems, Australia	BDT (Big Dye Terminator) v3.1 Ready Mix	DNA sequencing
Bioline, Alexandria, Australia	Biomix™ (2×)	PCR
	BioScript™ MMLV RT(Moloney Murine leukaemia virus reverse transcriptase)	Reverse transcription
	dNTP set (4×25 µmol)	dNTPs for reverse transcription
	Hyperladder1™	DNA Molecular weight marker for agarose gel electrophoresis
	RNase Inhibitor	Inhibition of RNase activity
	TRIsure™	RNA isolation reagent
Eppendorf, Hamburg, Germany	Perfectprep® Gel Cleanup Kit	Purification of DNA from gels
Merck, NJ, USA	Acetonitrile	Solvent for HPLC
Promega, Madison, USA	Restriction endonucleases	DNA digestion
	Wizard® SV Plasmid DNA Miniprep Kit	Plasmid DNA isolation
	RNase-free DNaseI	Digestion of genomic DNA from RNA samples
	T4 DNA ligase	Ligation of DNA
	2× Rapid Ligation Buffer	Ligation buffer
	pGEM®T Easy Vector System 1	Gene cloning
	RNase-free DNaseI	DNA digestion during RNA preparation
Qiagen, Victoria, Australia	DNeasy Plant Mini kit	DNA extraction
Sigma, Victoria, Australia	Ethidium bromide (10 mg/mL)	Agarose gel electrophoresis

2.3 Preparation of solutions

2.3.1 Sterilisation

Solutions were sterilised by autoclaving (121°C for 20 min), or filter sterilised through a 0.22 µm syringe filter (Millipore, Germany). All glassware and disposable plastic ware were also autoclaved as above. The sterilisation methods used in the preparation of buffers, media and solutions listed below are indicated with * (autoclaved) or ** (filter sterilised).

2.3.2 Buffers and Solutions

All buffers and solutions were prepared using autoclaved MilliQ water (Millipore, Germany). The general use buffers and solutions listed below were prepared according to the instructions in Sambrook and Russell (2001).

*TAE buffer, 50X: 2.0 M Tris base, 6.5 M EDTA disodium salt, pH 8.0

*Agarose gel electrophoresis loading dye, 6X: 0.25% (w/v) xylene cyanol, 0.25% bromophenol blue, 30% (v/v) glycerol

**TB Buffer: 10 mM Hepes, 15 mM CaCl2, 250 mM KCl, pH 6.7, then add $MnCl_2$ to a final concentration of 55 mM

The following solutions used for DNA sequencing were prepared according to instructions by AGRF (Australian Genome Research Facility Ltd, Melbourne, Australia).

*BDT dilution buffer, 5X: 400 mM Tris pH 9.0, 10 mM $MgCl_2$

Sequencing clean-up solution: 0.2 mM $MgSO_4$ in 70% ethanol

The following solution used for plant growth was prepared according to Hoagland and Arnon (1950).

*Hoagland's solution: 7 mM $Ca(NO_3)_2.4H_2O$, 5 mM KNO_3, 2 mM KH_2PO_4, 2 mM $MgSO_4.7H_2O$, 45 µM H_3BO_3, 9 µM $MnCl_2.4H_2O$, 0.7 µM $ZnSO_4.7H_2O$, 0.32 µM $CuSO_4.5H_2O$, 0.12 µM $NaMoO_4$, 28 µM FeEDTA in 1 M KOH.

2.3.3 Media and Solutions for Microbial Growth

The media and solutions used for culturing bacteria were prepared according to Sambrook and Russell (2001).

**Ampicillin: 20 mg/mL in sterile MilliQ water

*Luria-Betani (LB) medium: 10% (w/v) tryptone, 5% (w/v) yeast extract, 5% (w/v) NaCl, 15% (w/v) agar (added for plates only)

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside): 5% (w/v) in dimethylformamide

**IPTG (isopropyl β-D-1-thiogalactophyranoside): 0.1 M in sterile MilliQ water

*2xYT: 16% tryptone, 10% yeast extract, 5% NaCl, pH 7.0

SOB: 0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$*

2.3.4 Microbial Strains

Escherichia coli JM109 (Promega, USA), was used for general molecular cloning procedures. The 'competent' cells were used for transformation of recombinant DNA.

2.3.5 Plant propagation

Seeds of *Atriplex nummularia*, *Atriplex semibaccata*, *Atriplex amnicola*, *Acacia pendula*, *Acacia salicina* and *Acacia victoriae* were purchased from AustraHort Pty Ltd. (Queensland, Australia) and/or Nindethana Seed Services (Western Australia, Australia) and germinated for 4-5 days on sterile filter paper moistened with sterile water. Seedlings were then grown on vermiculite:perlite mixture (3:2) under controlled conditions (25°C, 70% humidity, 16 h photoperiod) in a plant growth cabinet, irrigated with Hoagland's nutrient solution every alternate day. Leaves of individual plants were snap-frozen in liquid nitrogen and stored at -80 ºC and used for RNA extraction for cDNA synthesis.

2.3.5.1 Plant propagation for gene expression and biochemical analyses

For differential gene expression and biochemical analyses, six seedlings of uniform growth were transferred to two pots (three plants in each) and grown as above for 8 weeks. Three of these were then exposed to salt stress in increments of 50 mM NaCl in Hoagland's solution every alternate day, to the final concentration of 300 mM NaCl (for a total period of 13 days), while three other seedlings continued to be irrigated with Hoagland's solution (as controls). Salt tolerant plants favour low amounts of salt (up to 200 mM) and may need it for growth, hence a higher concentration is needed to evaluate the effects of salt stress. Hence the salt concentration to be used was based on several studies that utilised 300 mM NaCl to study effects of salinity stress in saltbushes; e.g., *Atriplex prostrata* (Khan *et al.,* 2003), *Atriplex nummularia* (de Araujo *et al.,* 2006) and *Atriplex halimus* (Ahmad *et al.,* 2008; Bouchenak, 2012). The gradual increase in salt is critical to avoid physiological shock that can result in the loss of differential response (Peel *et al.*, 2004). The leaves and roots of individual salt-stressed plants were harvested after 48 h at 300 mM NaCl and snap-frozen and individual control plants harvested simultaneously. One hundred mg of leaf and root tissue of each plant was used for RNA extraction.

2.4 Methods specific to Chapter 3

2.4.1 Total RNA extraction

Total RNA was extracted using TRIsure reagent according to the manufacturer's protocol (Bioline, Australia). About 100 mg of plant leaf tissue was ground to a fine

powder under liquid nitrogen and mixed with 1 mL of TRIsure (Bioline, Australia) and incubated at room temperature (RT) for 5 minutes. To this mixture, 200 µL of chloroform: isoamyl alcohol (24:1) was added and shaken vigorously for 15 seconds, then incubated at RT for 3 minutes. The mixture was centrifuged at 12,000 rpm for 15 minutes at 4 °C. The upper, clear, aqueous phase was removed, mixed with 500 µL isopropanol, incubated at RT for 10 minutes and centrifuged as above for 10 minutes. The RNA pellet was washed with 1 mL of 75% ethanol made in diethylpyrocarbonate (DEPC)-treated water and centrifuged at 7,500 rpm for 5 minutes at 4 °C. The pellet was air-dried, re-suspended in 40 µL DEPC-treated water and incubated for 10 minutes at 60°C. The quality of the total RNA extracted was assessed by running 4 µL of RNA on an agarose gel.

2.4.2 DNase treatment of total RNA

To ensure there was no contamination with genomic DNA, the RNA preparations were treated with DNase. Total RNA was treated with RNase-free DNaseI (Promega, USA) according to the supplier's instructions. Total RNA (50 µL) was incubated with 10 U of DNase, 2 U RNase inhibitor and 10 µL of 10× reaction buffer in a total volume of 100 µL (made up with sterile DEPC-treated MilliQ water) for 1 hour at 37 ºC. RNA was precipitated by addition of 5.0 µL 3 M sodium acetate and 250 µL absolute ethanol and incubated for 10 minutes at -80 ºC, followed by centrifugation at 14000 x g for 10 minutes. The pellet was washed in 70% ethanol; air dried and resuspended in 20 µL sterile DEPC-treated MilliQ water. Concentration of purified RNA was assessed spectrophotometrically (GeneQuant™ pro, GE Healthcare Biosciences) and stored at -80 ºC for further analysis.

2.4.3 Spectrophotometric quantification of RNA

Purified RNA was diluted 1:50 (RNA: DEPC-treated MilliQ water) and the absorbance readings at 230 nm, 260 nm and 280 nm were recorded on GeneQuant™ Pro Spectrophotometer (GE Healthcare, UK). The RNA concentrations were determined based on 1A260 = 40 µg/mL of single-stranded RNA. An RNA to protein absorbance ratio (A260/A280) between 1.8 and 2.0 was used as an indication of high purity (Sambrook and Russell, 2001).

2.4.4 cDNA synthesis

First strand complementary DNA (cDNA) was synthesised using the Bioscript MMLV reverse transcriptase (Bioline, Australia) system. For each reaction 2 µg of each RNA (made upto 11 µL with sterile DEPC-treated MilliQ water) was incubated with 1 µL of 0.5 µg/µL oligo d(T)18 primer (Invitrogen) at 70 ºC for 5 minutes and then chilled on ice. To this reaction mixture, 1 µL dNTPs (10 mM each), 10 U RNase inhibitor (Bioline, Australia), 4.0 µL 0f 5× reaction buffer (Bioline, Australia), 2.5 µL of DEPC-treated water and 50 U Bioscript (Bioline, Australia) were added and incubated at 37 ºC for 1 hour. The reaction was stopped by incubating the mixture at 70 ºC for 10 minutes. The quality of cDNA was assessed by PCR using 1 µL of the cDNA preparations and the CMO F2-R2 or BADH F9-R3 intron-spanning primers (Figures 2.1 and 2.2, Table 2.3), using conditions of semi-quantitative reverse transcriptase PCR (sqRT-PCR) and comparing the sizes of PCR products to those from gDNA.

2.4.5 Cloning of full-length cDNAs

Multiple sequence alignments of all available plant CMO and BADH cDNAs from GenBank were conducted using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/) and BioEdit v7.0.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The alignments were used to design exon-based primers (Table 2.3). One µL of first-strand cDNA was then amplified with CMO F1-R1 and BADH F7-R1 to obtain full-length cDNAs (from start to stop codons). The PCR conditions were: initial denaturation (94°C, 5 minutes), then 30 cycles of denaturation (94°C, 45 sec), annealing (temperature various, see Table 2.3; 45 sec), extension (72°C, 90 sec) and a final extension (72°C, 10 minutes). The PCR products of interest were purified and cloned into pGEM-T Easy vectors (Promega, USA) following the manufacturer's instructions. For each reaction 2.0 µL PCR product was combined with 1.0 µL vector DNA, 5.0 µL 2× Rapid Ligation buffer (Promega, USA) and 1.0 µL T4 DNA ligase (Promega, USA). Ligation reactions were incubated at room temperature for one hour and then at 4 ºC overnight. The ligation mixture (5.0 µL) was then mixed with 100 µL competent JM109 *E. coli* cells, incubated on ice for 20 minutes, heat-shocked at 42 ºC for 50 seconds and incubated on ice for 2 minutes. This reaction mixture was added to 1 mL of Luria Bertani (LB) broth and incubated at 37 ºC for 1 - 1.5 hours with shaking at 200 rpm. After incubation, the transformation mixture was centrifuged at 1,000 ×g for 3 minutes and 800 µL of the supernatant was discarded.

An aliquot (50 µL) of the remaining transformation mixture was plated onto LB agar containing ampicillin, IPTG and X-gal for identification of recombinant colonies through blue/white screening. Colony PCR was conducted using vector-based primers T7 and SP6 to identify colonies with recombinant plasmids. Six to ten such colonies per ligation were cultured in Luria Bertani broth and used for plasmid extractions using Wizard® Plus SV Minipreps system (Promega, USA). Plasmids were sequenced using the BigDye® Terminator v3.1 (Applied Biosystems, USA) as per instructions of the Australian Genomic Research Facility (Melbourne) (http://www.agrf.org.au/) and analysed on a 3730xl DNA Analyser at AGRF.

2.4.6 Extraction of genomic DNA

Genomic DNA was extracted from frozen leaf tissues using DNeasy Plant Mini Kit (Qiagen, Australia) according to the supplier's protocol. About 100 mg of fresh leaf tissue was pulverised into a fine powder under liquid nitrogen to which Buffer AP1 (400 µL) and RNase A (4 µL) were added. The mixture was vortexed briefly then incubated at 65°C in a water bath for 10 minutes. Buffer AP2 (130 µL) was added to the reaction components, mixed well and incubated on ice for 5 minutes. The lysate was centrifuged for 5 minutes at 20,000 x g. The supernatant was transferred into a QIAshredder Mini spin column in a 2 mL collection tube and centrifuged for 2 minutes at 20,000 x g. The eluent in the collection tube was mixed with Buffer AP3/E (1.5 times the eluent volume). The solution (maximum of 650 µL) was loaded onto a DNeasy mini spin column in a 2 mL collection tube and centrifuged at 6000 x g for 1 minute. This step was repeated if there was any remaining sample. The column was washed with Buffer AW by centrifugation at 20,000 x g for 2 minutes followed by DNA elution in 50 µL of Buffer AE.

2.4.7 Amplification, cloning and sequencing of genomic copies of *CMO* and *BADH* genes

Alignments of all putative plant CMO and BADH cDNAs and putative proteins available in GenBank were performed using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/) and edited using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Based on these, several exon-based degenerate primers were designed to amplify various overlapping sections of the genes

from gDNAs (Figures 2.1 and 2.2, Table 2.3). Since the BADH genes typically contain many and large introns (Yin *et al*., 2002), a nested PCR approach was applied using primer pairs F7-R6, F8-R6, F1-R3, F4-R4, F5-R4 and F5-R1; for CMO, the primer pairs F3-R3, F4-R4, F6-R5, F7-R4, F7-R5 and F8-R3 were used. The reactions were conducted in 50 µL volumes containing 25 µL Biomix (Bioline, Australia) (which contains dNTPs and Taq polymerase), 100 ng of each primer and 100 ng of gDNA templates. The cycling conditions used for PCR amplifications were initial denaturation (94 °C, 5 minutes), followed by 35 cycles of denaturation (94°C, 45 sec), annealing (at 5°C lower than the lowest Tm of the primer in a pair, 45 sec) and extension (72°C, 1 minute), then a final extension (72°C, 10 minutes). The products of expected size (or larger, possibly due to presence of introns) were purified from gels using Wizard® SV Gel or PCR Clean-Up Systems (Promega, USA and cloned into the pGEM-T Easy vectors (Promega, USA). To test for the presence of inserts, colony PCR was conducted using the vector-based primers T7 and SP6 (Table 2.3), except for annealing temperatures of 45°C. At least 6-10 recombinant (white) colonies per ligation were cultured individually overnight in Luria Bertani (LB) broth and used for plasmid DNA extraction using Wizard® Plus SV Minipreps DNA Purification System (Promega, USA). Plasmids were sequenced using the T7 and SP6 primers and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), using the protocol provided by the Australian Genomic Research Facility (AGRF), Melbourne (http://www.agrf.org.au/).

Table 2.3: Primers used for gene cloning and expression analyses

Primer name[1]	Sequence (5'-3')[2]	Primer location, region in putative protein[3]	Tm (˚C)	Expected amplicon size (bp)[3]	Ta (˚C)
Primers used for amplification and cloning of full-length cDNAs					
*CMOF1	ATG GCA GCA AGT GCA ACA AC	Exon 1, MAASATT	47	1269	45
*CMOR1	TCA CTT CAA WAC TTG GTG TAA CC	Exon 10 CWLHQVLK	47		
BADHF7	ARA ATG GCG TTY CCW ATK YC	Exon 1, KMAFPMP	61	1503	57
BADHR1	GGA GAC TTG TAC CRK CCC CTG A	Exon 15, GWYKSP	59		
Additional primers used for amplification and cloning of GB genes from *Acacia* gDNA and cDNA					
CMOF3	CAT GCW TTT CAC AAT GTT TGY	Exon 3, HAFHNVC	59	1062	52
CMOR3	GTA KYC ATC CTA GKK CCA TAC C	Exon 8, RYGPWMDT	56		
CMOF4	CTT GGT AYA CYG AAC CTG C	Exon 1, WYTD/EP	55	1783	50
CMOR4	CAG TGG AAR TGG TGR ATT CC	Exon 10, PMHHFH	59		
CMOF6	GTT TYG TNT GCC CTT AYC	Exon 3, CFVCPY	52	1048	47
CMOR5	ART AGT CCA CCA CTA RTT TGC	Exon 8, CKVVFD	54		
CMOF7	SNT AYC ATG TTC CTT ATG C	Exon 6, YHYPYYA	50	1394	50
CMOR4	CAG TGG AAR TGG TGR ATT CC	Exon 10, PMHHFH	59		
CMOF7	SNT AYC ATG TTC CTT ATG C	Exon 6, YHYPYYA	50	455	45
CMOR5	ART AGT CCA CCA CTA RTT TGC	Exon 8, CKVVFD	54		
CMOF8	CAA GYG CWA CMA CMA TGT TGC	Exon 1, SATTML	58	1645	54
CMOR3	GTA KYC ATC CTA GKK CCA TAC C	Exon 8, RYGPWMDT	56		
BADHF1	ACT GGA AAC CCT TGA TTC TGG A	Exon 3, LETLDSG	57	1498	50
BADHR3	GCA GCA GAA GCC ATA ATC	Exon 7, KIMASA	54		
BADHF4	AGT KTG TAA HGA AGT GGG AC	Exon 6, VCNEVG	52	2195	49
BADHR4	CCA RAC ARY TCC AAC TTC	Exon 14, EVGAVW	51		
BADHF5	CGR CTT GGT CCT GTT ATC	Exon 10, RLGPVI	54	1298	46
BADHR4	CCA RAC ARY TCC AAC TTC	Exon 14, EVGAVW	51		
BADHF5	CGR CTT GGT CCT GTT ATC	Exon 10, RLGPVI	54	1076	52
BADHR1	GGC TTC ATC TTC AGT WYT A	Exon 12, S/KTEDE	57		
BADHF7	ARA ATG GCG TTY CCW ATK YC	Exon 1, KMAFPMP	61	2119	56
BADHR6	TCC AGA ATC AAG GGT TTC CAG	Exon 3, LETLDSG	61		
BADHF8	GCA ACT GCA GAG GAT GTR G	Exon 2, ATAEDV	55	1727	52
BADHR6	TCC AGA ATC AAG GGT TTC CAG	Exon 3, LETLDSG	61		
Primers used for gene expression studies					
*CMOF2	ATG CCT TTC ACA ATG TTT GC	Exon 3, HAFHNVC	59	506	55
*CMOR2	ACC ATT GTT TGA AGT CCC AG	Exon 7, GTSNNG	57		
BADHF9	GCG TGC TAT TGC TGC TAA G	Exon 2, RAIAAK	57	539	51
BADHR3	GCA GCA GAA GCC ATA ATC	Exon 7, KIMASA	54		
ActinF	ATG GTS AAG GCT GGD TTT GC	NA	47	900	52
ActinR	GGG AAC ATR GTK GAH CCA CCA C	NA	52		
Primers used for amplification of inserts from clones					
T7	GTA ATA CGA CTC ACT ATA GGG C	pGEM-T Easy vector-specific primers	51	Insert-specific	45
SP6	ATT TAG GTG ACA CTA TAG		35		

[1]F: forward primer, R: reverse primer.
[2]R=A+G; Y=C+T; M=A+C; K=G+T; S=G+C; W=A+T; H=A+T+C; B=G+T+C; D=G+A+T; V=G+A+C; N=A+C+G+T.
[3]Estimates based on the *Arabidopsis thaliana CMO* and *Atriplex centralasiatica BADH* gene structures (Figures 2.1 and 2.2).
*Estimates and primer sequence based on *Atriplex nummularia CMO* cDNA (AB112481.1). NA: not applicable.
Ta: Annealing temperature (˚C) used

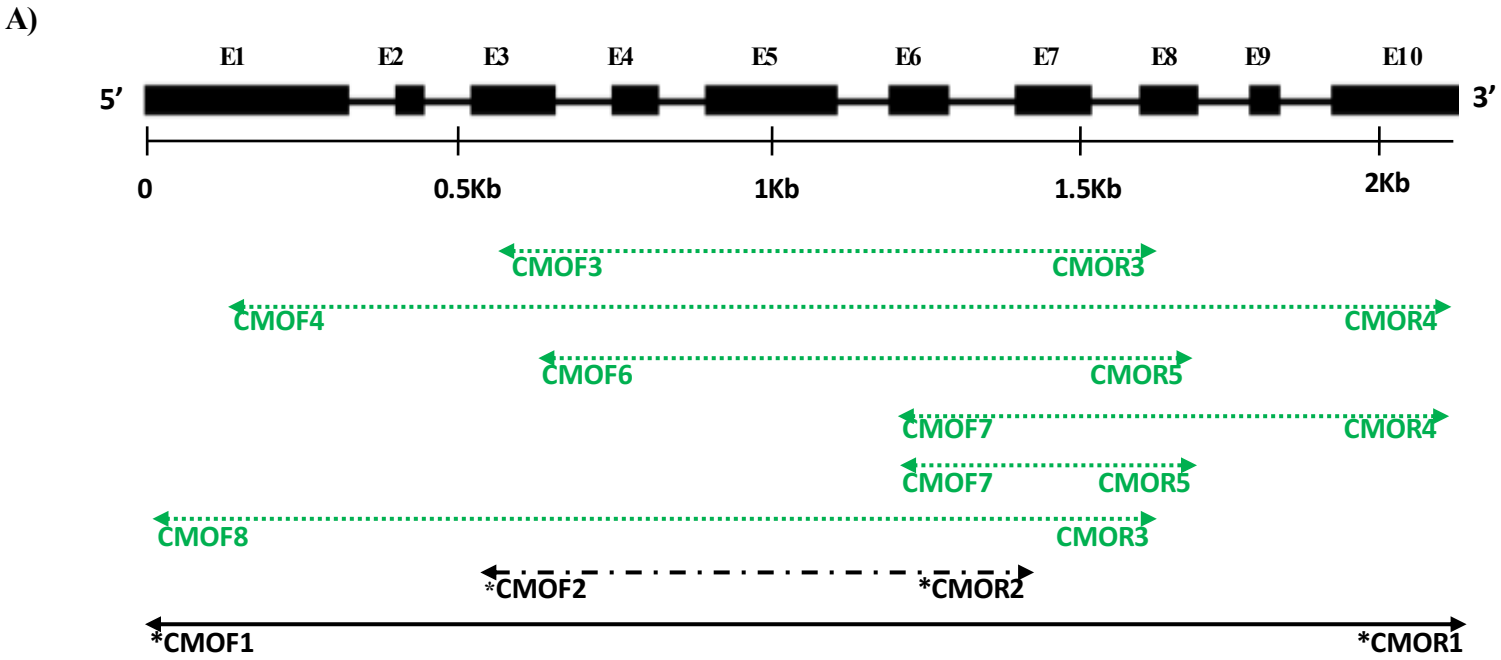


Figure 2.1: Primer design for cloning of cDNAs and differential gene expression analyses of *CMO* gene

A) *CMO* gene structure deduced from *Arabidopsis thaliana* genomic copy (AT4G29890.1) and CDS (NM_119135.4).*Positions and sequences of primers are based on *Atriplex nummularia* CMO cDNA (AB112481.1). Boxes labelled E represent exons, lines between represent introns, 'F' denotes forward primers and 'R' denotes reverse primers. Dash-and-dot arrows indicate PCR products analysed for differential gene expression of saltbush cDNA; plain arrow indicates PCR for obtaining full length saltbush cDNA (excluding introns). Green dotted lines indicate additional primer pairs attempted for *CMO* amplification from gDNA and cDNA of *Acacia* species. Lines drawn to scale.

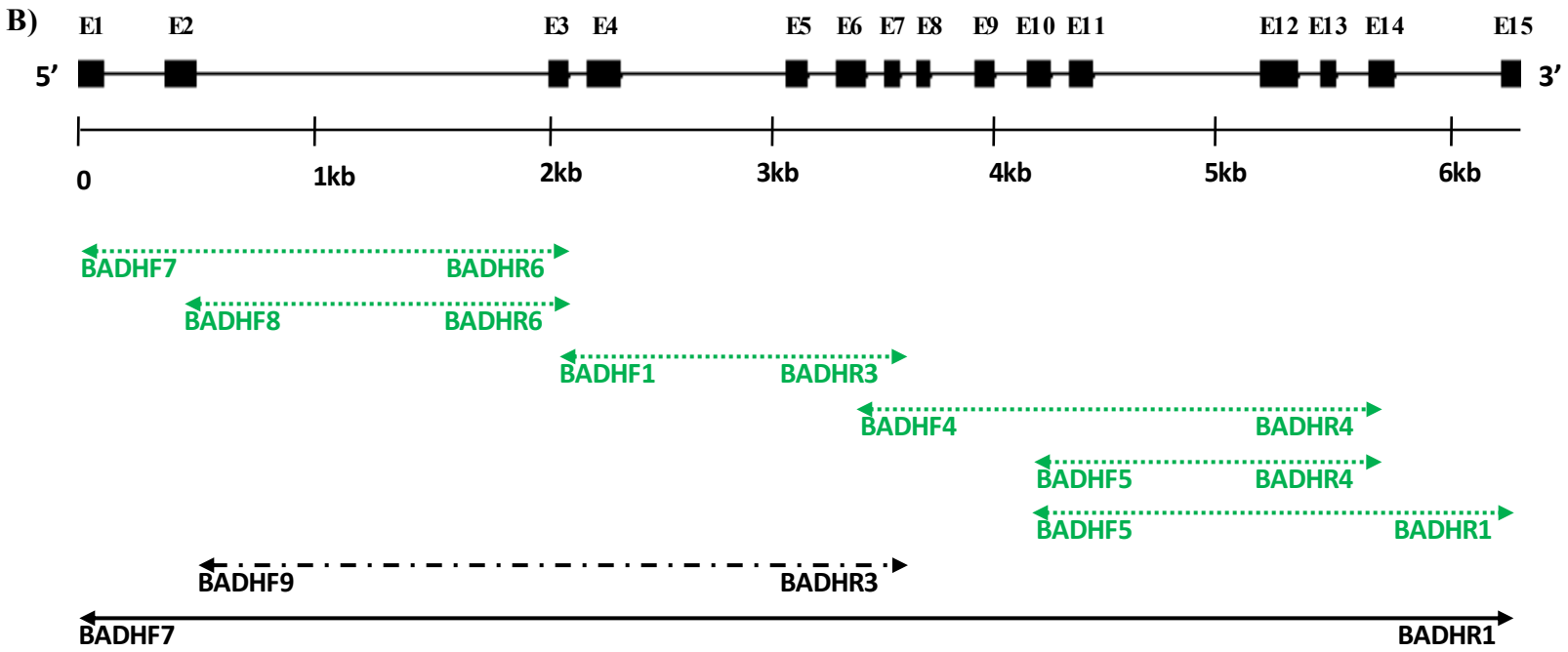


Figure 2.2: Primer design for cloning of cDNAs and differential gene expression analyses of *BADH* gene

BADH gene structure deduced *Atriplex centralasiatica* genomic copy (AY093684.1) and CDS (AY093682.1). Boxes labelled E represent exons, lines between represent introns, 'F' denotes forward primers and 'R' denotes reverse primers. Dash-and-dot arrows indicate PCR products analysed for differential gene expression; plain arrow indicates PCR for obtaining full length cDNA (excluding introns). Green dotted lines indicate additional primer pairs used for *BADH* amplification from gDNA and cDNA of *Acacia* species. Lines drawn to scale.

2.4.8 Sequence analyses, alignments and phylogenetic trees

The cDNA sequences were subjected to BLASTN (nr database) (http://www.ncbi.nlm.nih.gov/Blast.cgi), and the cDNAs and deduced amino acid sequences also compared to those of *A. thaliana* (for CMO) and *S. oleracea* and *Atriplex centralasiatica* (for BADH). Percent identity with all putative plant CMO and BADH proteins in Genbank was calculated using Sequence Identity Matrix in BioEdit v7.0.0. Phylogenetic trees were produced based on amino acid alignments using the Neighbor-Joining algorithm in MEGA4 (http://www.megasoftware.net/mega4/mega.html) with a bootstrap replication value of 1000. The following bioinformatics tools were also used: SIM4 (http://pbil.univ-lyon1.fr/members/duret/cours/inserm210604/exercise4/sim4.html) to align cDNA and genomic sequences and predict splice junctions; GSDS (gene structure display server) (http://gsds.cbi.pku.edu.cn/) for drawing gene structure schematics; ProtParam (http://web.expasy.org/protparam/) for analysing the putative mature proteins for biochemical parameters (molecular weights, pI, amino acid composition, instability index, aliphatic index, and GRAVY (GRand AVerage of hYdropathicity)); Conserved Domain Database Search (CDD-Search), (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi); WoLF PSORT (http://wolfpsort.org/) and TargetP (http://www.cbs.dtu.dk/services/TargetP) to predict subcellular locations and ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) to predict chloroplast transit peptides (cTP) and potential cleavage sites.

2.5 Methods specific to Chapter 4

2.5.1 Glycine betaine quantitation by HPLC

2.5.1.1 Preparation of solutions

Betaine anhydrous was purchased from Sigma, absolute ethanol from Ajax Finechem (Rowe Scientific Pty Ltd, Australia) and acetonitrile from Merck (Australia). All solvents were of HPLC grade and all other chemicals were of analytical grade. Acetonitrile and MilliQ water were vacuum-filtered using a 0.45 µm membrane filter and sonicated for 20 minutes before use. A standard (stock) solution of 5 mg/mL Betaine was prepared in 50% ethanol and diluted to six standard GB solutions ranging in concentrations from 50 µg/mL to 1000 µg/mL.

2.5.1.2 Sample preparation

Plant extracts for betaine analysis were prepared based on a modified protocol by Ahmad *et al.,* 2008. Extracts were prepared in triplicates for each condition- three control plants (grown in no salt condition) and three salt-stressed plants (grown in 300 mM NaCl condition). Frozen powdered leaf tissues weighing 50 to 80 mg was mixed with the solvent- methanol: chloroform: water (60:25:15) at a ratio of 2 µL of solvent per 1 mg sample. The mixture was vortexed for 30 seconds. An equal volume of sterile Milli-Q water was added and the resultant homogenate was shaken gently for 20 minutes. The samples were then centrifuged at 570 x g for 10 minutes at room temperature. The upper clear methanol-water phase was obtained and freeze dried. The concentrated sample was dissolved in 1 mL of 50% ethanol and filtered through a 0.45 µm membrane filter. The filtered extracts were transferred to HPLC vials for GB quantitation. An injection volume of 10 µL per extract was used for each run of 15 minutes. Each sample and standard was injected three times and the average peak area was used for GB estimation.

2.5.1.3 HPLC instrumentation and chromatographic conditions

A Shimadzu model HPLC system equipped with SCL-10A VP system controller, LC-10AT VP liquid chromatograph, FCV-10AL VP pump, DGU-14A degasser, SIL-10 VP sample auto injector, UV-VIS detector SPD-10AD VP and a CTO-10AC VP column oven was used. The chromatographic column used was Prevail™ Carbohydrate ES column (250 x 4.6 mm) along with a Prevail™ All-Guard™ Carbohydrate ES 5 µm (4.6 x 7.5 mm) guard column. The typical conditions used for chromatography were- temperature: 30°C, flow rate: 1 mL/minute, injection volume: 10 µL, pressure (P_{max}) – 2200 psi, UV wavelength: 190 nm and a run time of 15 minutes. The mobile phase used for separation was a mixture of Acetonitrile and MilliQ water in the ratio 75:25. Peak area was calculated using Shimadzu's CLASS VP chromatography analysis software.

2.5.1.4 HPLC method optimisation and validation

Based on the standard protocol provided with the column, various adjustments using betaine standard were made in order to optimise the elution of GB. Some of the parameters that were optimised include solvent concentration (ratio of acetonitrile:

water; 80:20, 75:25), percentage of ethanol for preparation of extracts (40%, 50%, 60%, 70%), time of elution (20 minutes, 15 minutes), and UV wavelength for detection (190 nm, 200 nm) (results not detailed). The HPLC method developed for the quantitation of GB was validated based on five of the parameters detailed by the International Conference on Harmonisation (ICH) guidelines for validation of analytical procedures. These include linearity, intraday and inter day precision as percent relative standard deviation (% RSD), limit of detection (LOD) and limit of quantitation (LOQ).

Linearity was determined based on linear regression (R^2) of six standard GB solutions ranging from 50 µg/mL to 1000 µg/mL. To establish precision of method, 10 µL of three standard GB solutions each (50, 300 and 500 µg/mL) were injected into the HPLC system three times on a single day (intraday precision) and on three different days (inter day precision). Percent relative standard deviation (RSD) was calculated for the three standard GB solutions as % RSD= 100*(σ/average peak area), where σ denotes standard deviation. The following definitions are stipulated by the ICH for LOD and LOQ: (i) limit of detection can be defined as 'the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value' (ICH, 1996); (ii) limit of quantitation can be defined as 'the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy' (ICH, 1996); (iii) limit of detection and limit of quantitation was calculated based on the standard deviation (σ) of response (average peak area). LOD= 3.3*(σ/S) and LOQ= 10*(σ/S) where S is the slope obtained from the standard curve.

2.5.1.5 GB quantitation

Identification of GB in the leaf extracts was based on the retention time of standard GB. The amount of GB in the leaf extracts of control and salt-stressed *Atriplex* and *Acacia* samples was determined from the slope and intercept values projected in the standard curve of glycine betaine. The amounts were expressed as µg of GB per mg FW of leaf tissue.

2.5.2 Proline quantitation by Ninhydrin Assay

2.5.2.1 Preparation of standards

Quantitation of proline from standards as well as plant samples was optimised by modifications of a protocol by Bates *et al.* (1973). Proline (Sigma Aldrich, Australia) was used to prepare a 1 M stock solution in 3% sulphosalisylic acid (Sigma Aldrich, Australia) and standards (100 - 400 μM) were prepared from it. Toluene was used as a blank (Bates *et al.*, 1973).

2.5.2.2 Sample preparation and proline quantitation

Fresh tissue (around 50 mg) was powdered in liquid nitrogen and homogenized in 1.5 mL 3% sulfosalicylic acid and centrifuged at 12000 × g for 7 minutes. Then, 2 mL 1% ninhydrin in 60% acetic acid and 500 μL distilled water was added to the 500 μL of supernatant. The solution was incubated in boiling water bath (100°C) for an hour and the reactions stopped by placing the tubes on ice. Two mL toluene was added to each sample and the solutions mixed well. The absorbance of the toluene phase was determined in 518 nm and proline content was estimated as nmol mg^{-1} FW. Standards were processed in the same way as the plant samples. Toluene was used as a blank. A standard curve with absorbance (AU) plotted against concentration (nanomoles per mL) was used to estimate the quantity of proline in the sample. Any dilution used was considered in determining the proline quantity.

2.5.3 Trehalose quantitation by HPLC

2.5.3.1 Preparation of solutions

Trehalose dihydrate (α,α-Trehalose) was purchased from Sigma, absolute ethanol from Ajax Finechem and acetonitrile from Merck. All chemicals used were of analytical grade and solvents of HPLC grade. Acetonitrile and MilliQ water used for HPLC were vacuum filtered using a 0.45 μm membrane filter and sonicated for 20 minutes before use. A standard solution of 5 mg/mL trehalose was prepared in 50% ethanol. The stock solution was diluted to standard trehalose solutions with concentrations ranging from 1 mg/mL to 5 mg/mL.

2.5.3.2 Sample preparation

Samples prepared for GB determination were also used for Trehalose analysis with some modifications. An aliquot of the GB extracts together with an equal volume of 10 mM sulphuric acid were boiled for 1 hour at 100°C in order to break down sucrose, as typically carried out for estimation of trehalose in plant tissues (El-Bashiti *et al.,* 2005; Ahmed *et al.,* 2013; Li *et al.,* 2014), due to sucrose having the same retention time as trehalose on a HPLC column.

2.5.3.3 HPLC instrumentation and chromatographic conditions

A Shimadzu model HPLC system equipped with SCL-10A VP system controller, LC-10AT VP liquid chromatograph, FCV-10AL VP pump, DGU-14A degasser, SIL-10 VP sample auto injector, RID-10A Refractive index detector, and a CTO-10AC VP column oven was used. The chromatographic column used was Prevail™ Carbohydrate ES column (250 x 4.6mm) along with a Prevail™ All-Guard™ Carbohydrate ES 5 µm (4.6 x 7.5mm) guard column. The typical conditions used for chromatography were- temperature: 30°C, flow rate: 1 mL/minute, injection volume: 20 µL, pressure (P_{max}) – 2200 psi, RID mode: analytical, polarity: + and a run time of 15 minutes. The mobile phase used for separation was a mixture of filtered Acetonitrile and MilliQ water in the ratio 75:25. Peak area was calculated using Shimadzu's CLASS VP chromatography analysis software.

2.5.3.4 HPLC method optimisation and validation

Based on the standardised protocol provided with the column, the method for HPLC analysis using standard trehalose was optimised. The HPLC method developed for the quantitation of trehalose was validated based on five of the parameters mentioned by the International Conference on Harmonisation (ICH, 1996) guidelines for validation of analytical procedures. The parameters include linearity, intraday and inter day precision as percent relative standard deviation (%RSD), limit of detection (LOD) and limit of quantitation (LOQ) as described under GB quantitation using HPLC. Precision of method, percent relative standard deviation (RSD), limit of detection, limit of quantitation and linearity was determined using three standard trehalose solutions ranging from 1, 3 and 5 mg/mL as described for GB estimation.

2.5.3.5 Trehalose quantitation

Identification of trehalose in leaf extracts was based on the retention time of standard trehalose. The amount of trehalose in the leaf extracts of control and salt-stressed *Atriplex* and *Acacia* samples was determined from the slope and intercept values projected in the standard curve of trehalose. The amounts were expressed as µg of trehalose per mg of leaf tissue.

2.5.4 Enzyme assays

2.5.4.1 Total protein extraction for assay of Betaine aldehyde dehydrogenase activity (BADH) enzyme activity

Total protein from leaf tissues of three control plants and three salt-stressed plants were extracted according to the method described in Zhang *et al.* (2008). About 100 mg of freeze dried leaf material was homogenised in 200 µL of protein extraction buffer (50 mM HEPES-KOH (pH 8.0), 1 mM EDTA, and 5 mM DTT). The mixture was centrifuged at 10,000 x g for 10 minutes at 4°C in a cold room. The supernatant was used for estimation of total protein concentration using Bradford assay.

2.5.4.2 Total protein extraction for assay of proline biosynthetic enzymes

Leaf extracts of three control and three salt-stressed plants were prepared from snap-frozen powdered leaf tissue by extracting with a buffer containing 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM β-mercaptoethanol, 4 mM DTT, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 2% polyvinylpolypyrrolidone (PVPP) (Chilson *et al.,* 1992). Briefly, 200 µL of buffer was added to the leaf material and homogenised. The extracts were centrifuged at 10,000 x g for 20 minutes. The supernatants were transferred to pre-chilled microcentrifuge tubes and further clarified by centrifugation at 10,000 x g for 20 minutes at 4°C in a cold room. The supernatant was used for estimation of total protein concentration by Bradford assay.

2.5.4.3 Quantitation of total protein content by Bradford Assay

Protein estimation using Bradford assay was done on 96-well microtitre plates according to the supplied protocol (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf). The Bradford reagent was

brought to room temperature and mixed well before use. Suitable dilutions of protein standards were prepared as shown in Table 2.4.

Table 2.4: Estimation of protein quantity based on serial dilutions of standard BSA protein with 2000 µg/mL concentration

Tube no.	BSA Standard (µL)	Diluent buffer* (µL)	Final Concentration (µg/mL)	Total volume (µL)	Available volume (µL)
1	15	5	1500	20	10
2	10	10	1000	20	10
3	10 (from tube 2)	10	750	20	10
4	10 (from tube 3)	10	500	20	10
5	10 (from tube 5)	10	250	20	10
6	10 (from tube 6)	10	125	20	20
7	- (Blank)	5	0	5	5

*Diluent buffer refers to the buffer used for total protein extraction.

250 µL of Bradford reagent was added to 5 µL each of the above standards and plant extracts. The mixture was incubated for 5 minutes at room temperature and the absorbance measured at 595 nm using a plate reader (protein dye complex is stable for 60 minutes). The net absorbance against the protein concentration of each standard was plotted. The protein concentrations of the plant extracts were determined using the standard curve.

2.5.4.4 Assay for BADH activity

The reaction mixture for assaying BADH activity was prepared according to Zhang *et al.,* 2008. To 100 µL of reaction mixture (20 mM Tris-HCl, 0.5 mM NAD^+, 5 mM DTT) in a 96 well microplate, 20 µL of enzyme extract prepared for BADH assay (prepared as described above) was added. The reaction was initiated on addition of the substrate betaine aldehyde (1 mM). The absorbance of the mixture was read at 340 nm at 0 minute and then after 25 minutes at 25°C. The assay was repeated twice under identical conditions. One unit of BADH activity can be defined as 1 µmol/minute of NAD^+ consumed per mg protein (Zhang *et al.,* 2008).

2.5.4.5 Assay for P5CS activity

A 100 µL reaction mixture (Zhuang *et al.,* 2011) containing 100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, 75 mM Na-glutamate, 5 mM ATP and 0.4 mM NADPH was used for assaying the activity of P5CS in 20 µL of plant extract (prepared for proline

biosynthetic enzymes, see above). The reaction was initiated by addition of NADPH. The assay was conducted in a 96 well microplate and monitored on a microplate reader at A_{340} at 37°C immediately (T_0) and thereafter at 15 minutes (T_{15}), the consumption of NADPH measured as a decrease in absorbance at A_{340}. One unit of P5CS activity can be defined as 1 µmole of NADPH oxidised per minute per mg protein (Zhuang *et al.,* 2011).

2.5.4.6 Assay for PDH activity

To 100 µL of reaction mixture (Rucińska-Sobkowiak *et al.,* 2013) (0.15 M Na_2CO_3–HCl buffer (pH 10.3), 1.5 mM NAD^+, 15 mM L-proline) in a 96 well microplate, 20 µL of plant extract (prepared for proline biosynthetic enzymes, see above) was added. The reduction of NAD^+ to NADH was monitored at 340 nm at 37°C immediately (T_0) and then after 30 minutes (T_{30}). The PDH activity was expressed as nanomoles of NAD consumed per minute per mg of protein. The extinction coefficient of NAD^+ was 6.2 mM/cm. The reaction mixture (without NAD^+) and enzyme extract was used as a blank.

2.5.4.7 Data analysis

Data obtained for the amount of GB and proline in control and stressed plants was analysed using one-way analysis of variance (ANOVA) in SPSS v. 20 (Statistical Package for the Social Sciences). The Least Significant Difference (LSD) and Tukey's-b post-hoc tests was carried out to compare group means with the significance level set at 0.05. Mean differences were significant if $p<0.05$ and not significant if $p>0.05$. For assaying the activity of BADH, P5CS and PDH, specific activity of enzyme was calculated per mg protein in the frozen tissue sample and subjected to the same statistical analyses as above.

2.6 Methods specific to Chapter 5

2.6.1 Plant Tissue Sampling for Genomic DNA Extraction

The thirty species to be analysed were initially selected based on species groups morphologically related to the salt tolerant species (*A. pendula, A. salicina, A. stenophylla* and *A. victoriae*), as described in Flora of Australia (Orchard and Wilson, 2001a; b). The phyllode or leaf tissue (20 mg) was removed from herbarium sheets held at Royal Botanic Gardens, Melbourne (herbarium voucher numbers given in Table

2.5) and used for genomic DNA extraction using the DNeasy Plant Mini Kit (Qiagen Australia).

2.6.2 Amplification and Sequencing of ITS and ETS Markers

A dataset of ITS and ETS sequences was constructed using data from Brown, *et al.* (2012). *Paraserianthes lophantha* (voucher MEL2057862; GenBank accessions: ITS: EF638203; ETS: EF638105.1) was used as the out-group, based on Brown *et al.,* (2008) who concluded that it is sister to *Acacia.* The ITS region was amplified from the genomic DNAs using the primer pair S3 (5'-AACCTGCGGAAGGATCATTG-3') and 26SE (5'-TAGAATCCCCGGTTCGCTCGCCGT-3') (Murphy *et al.,* 2003). The ETS region was amplified using the primer pair 18S-IGS (5'-CACATGCATGGCTTAATCTTTG-3') and AcR2 (5'-GGGCGTGTGAGTGGTGTTTGG-3') (Murphy *et al.,* 2010) (Figure 2.3). The 50 µL reaction mixes contained 2mM dNTPs, 25 pmol of each primer, 10-50 ng of DNA template, 1U *Taq* DNA polymerase and 5 µL 10 X reaction buffer (New England Biolabs, USA). The reaction cycle for amplifying the ITS region was an initial denaturation at 95°C for 15 minutes followed by 30 cycles of 94°C for 30 seconds, 63.8°C for 30 seconds and 72°C for 20 seconds and then a final holding temperature of 72°C for 5 minutes. The reaction cycle for amplifying the ETS region was an initial denaturation at 94°C for 3 minutes followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes and then a final extension temperature of 72°C for 7 minutes. The PCR products were purified using QIAquick PCR kit (Qiagen) and used for DNA sequencing in both directions for each sample, using the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit or Prism Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Australia) and the supplier's protocols.

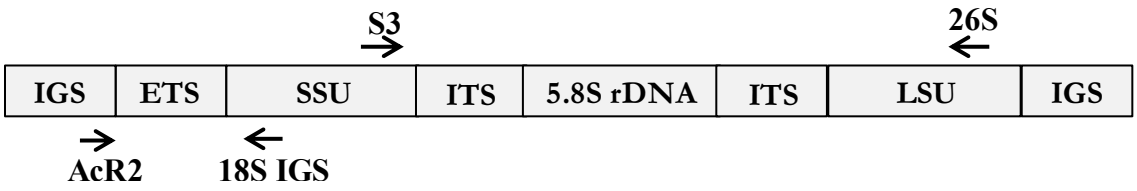


Figure 2.3: Primer design and structure of the ribosomal DNA cistron
S3 denotes the forward primer and 26SE denotes the reverse primer used to amplify the ITS region. AcR2 denotes the forward primer and 18S-IGS denotes the reverse primer used to amplify the ETS region (figure not drawn to scale).

Table 2.5: Identification of species morphologically related to *A. pendula, A. salicina, A. stenophylla* and *A. victoriae*

Morphologically related species	Key indicator(s) of plausible relationship	References	Voucher number
Species of interest: *A. stenophylla*			MEL 2312528
A. coriacea	Long phyllodes and growth habit resembling *A. stenophylla.*	2	MEL 2313071
A. sibilans	Closely related to *A. coriacea* in phyllode morphology.	2	MEL2327552
A. calcicola	*A. coriacea* sometimes confused with *A. calcicola.*	2	MEL 2233895
Species of interest: *A. salicina*			MEL 2286869
A. bivenosa	*A. salicina* belongs to the *A. bivenosa* group.	3	MEL 2306646
A. ligulata	*A. salicina* was often confused with *A. ligulata* and *A. ampliceps*.	1, 3	MEL 2326226
A. ampilceps	*A. salicina* was often confused with *A. ligulata* and *A. ampliceps*.	1, 3	MEL 2306643
A. cupularis	Belongs to the *A. bivenosa* group.	3	MEL 2278496
A. didyma	Belongs to the *A. bivenosa* group.	3	MEL 2283488
A. rostellifera	Belongs to the *A. bivenosa* group.	3	MEL 2319042
A. sclerosperma	Belongs to the *A. bivenosa* group.	3	MEL 2042807
A. startii	Belongs to the *A. bivenosa* group.	3	MEL 2297013
A. telmica	Belongs to the *A. bivenosa* group.	3	MEL 710783
A. tysonii	Belongs to the *A. bivenosa* group.	3	MEL 2137099
A. xanthina	Belongs to the *A. bivenosa* group.	3	MEL 2327555
Species of interest: *A. pendula*			MEL 2233883
A. omalophylla	*A. pendula* closely related to *A. omalophylla.*	2	MEL 2328341
*A. melvillei**	Very closely related to *A. omalophylla*, and in Queensland, both are known as Yarran.	2	MEL 2034608
Species of interest: *A. victoriae**			MELU SRA 260
*A. alexandri**	*A. victoriae* with long and linear phyllodes may be confused with *A. alexandri.*	2, 4	MELU SRA 148
*A. aphanoclada**	Belongs to the *A. victoriae* group.	2, 4	MELU SRA 224
A. chartacea	Belongs to the *A. victoriae* group.	2, 4	MEL 721448
*A. cuspidifolia**	Belongs to the *A. victoriae* group.	2, 4	MEL SRA 115
*A. dempsteri**	Belongs to the *A. victoriae* group.	2, 4	MEL 2096892
A. pickardii	Belongs to the *A. victoriae* group.	2, 4	MEL 2067966
A. ryaniana	Belongs to the *A. victoriae* group.	2, 4	MEL 721629
A. synchronicia	In the absence of flowers, *A. victoriae* may be confused with *A. synchronicia.*	2, 4	MEL 2252506
A. marramamba	Belongs to the *A. pyrifolia* group.	2, 4	MEL 2313077
A. strongylophylla	Presence of spinose stipules.	2, 4	MEL 2287670

*Sequences available in GenBank: *A. melvillei*: FJ868397.1; FJ868438.1; *A. victoriae*: DQ029275.1, DQ029316.1; *A. alexandri*: DQ029264.1, DQ029306.1; *A. cuspidifolia:* DQ029261.1, DQ029302.1; *A. dempsteri*: DQ029259.1, DQ029300.1. References: 1: Maslin (2001); 2: Orchard and Wilson (2001b); 3: Chapman and Maslin (1992); 4: Ariati *et al.,* (2006).

2.6.3 Phylogenetic Analyses

The ITS and ETS sequence datasets generated in this study were edited using Sequencher v3.0 (Gene Codes Corporation), concatenated manually using BioEdit v7.0.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and then aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and manually adjusted where necessary. The combined dataset was analysed using Maximum Parsimony (MP) via the software Molecular Evolutionary Genetics Analysis (MEGA) v4.0 (Tamura *et al.*, 2007), and Bayesian methods using MrBayes v3.2.1 (Ronquist and Huelsenbeck, 2003). For the MP analysis, the ClustalW alignment file was converted into a .meg file on MEGA 4. The phylogenetic trees were generated using the Maximum Parsimony criterion. Bootstrap support statistics were calculated using 1000 replicates. The 'Complete Deletion' option, to eliminate all positions containing gaps or missing data from the dataset, was used. Insertion/deletion (indel) events were scored as multistate characters. Of the total 782 positions in the final dataset, 118 were parsimony-informative. These datasets, analysed using MEGA, will be henceforth referred to as 'parsimony' results.

For the Bayesian analysis, the sequence dataset obtained for the 19 species was combined with previous data (Brown *et al.,* 2012) and subjected to Bayesian analysis using MrBayes v3.2.1. Insertion/deletion (indel) events were scored as multistate characters. The combined ITS and ETS data were divided into six partitions: ITS1, 5.8S, ITS2, LSU, SSU and ETS. An evolutionary model, GTR (Generalised Time Reversible) substitution model with gamma-distributed rate variation, was applied to each partition. A Markov Chain Monte Carlo (MCMC) search was run for 8 million generations, with trees sampled every 100 generations. Starting from different random trees, the analyses were performed twice simultaneously (Nruns = 2) with four Markov chains (N chains = 4) for each tree. Burn-in was set to 25001 (i.e. the first 25001 trees were discarded from each run). A Bayesian consensus phylogram was generated and for each node posterior probability (PP) values were calculated. The phylogenetic tree was visualised and coded for display using FigTree (Rambaut and Drummond, 2008).

2.7 Methods specific to Chapter 6

2.7.1 Species selection for salt tolerance evaluation

The selection of species was primarily based on molecular phylogenetic trees developed earlier based on ribosomal DNA markers (Chapter 6), to determine the species closely related to four species (*A. pendula, A. salicina, A. stenophylla, A. victoriae),* found to be able to grow in salinity affected land (section 1.8.3). The list of 20 *Acacia* species found to be closely related to the four salt tolerant species of interest was reduced to 15 based on any reported unique properties and/or economic value, and seed availability of required quality and quantity for replicate studies of various plant physiological parameters. The 15 species thus selected were: *A. papyrocarpa, A. enterocarpa, A. eriopoda, A. rigens, A. sclerophylla* (related to *A. pendula*), *A. oswaldii* (related to *A. stenophylla*), *A. cupularis, A. rostellifera, A. ligulata, A. xanthina* (related to *A. salicina*), and *A. synchronicia* (related to *A. victoriae*) (Chapter 6). Seeds of species of interest were purchased from AustraHort Pty Ltd and Nindethana Seed Services.

2.7.2 *Acacia* seed pre-treatment, plant culture and application of salt stress (specific to Chapter 6)

Selected *Acacia* seeds were surface sterilised using 5% sodium hypochlorite solution (commercially available bleach). Briefly, seeds were soaked in 95% ethanol for 10 seconds to remove any waxy substances present. The ethanol was then drained off and 5% sodium hypochlorite solution was added enough to submerge the seeds, swirled and left as such for 15 minutes. The solution was then drained off and the seeds were washed six times with sterile distilled water. In addition to surface sterilisation, *Acacia* seeds require pre-treatment to overcome natural inhibitors of germination. The presence of a thick seed coat makes it impermeable to water preventing imbibition. The most common pre-treatment methods are the use of boiling water, smoke water or abrasion (http://asgap.org.au/seed.html). In this study, boiling water was added to surface sterilised seeds and allowed to cool down naturally. The seeds were left in the boiling water overnight. Seeds that were swollen and appeared larger than the initial size were sown on vermiculite. Seeds that floated (infertile seeds) were discarded and those that did not swell were re-treated with hot water. The sterilised viable seeds were sown on a bed of vermiculite:perlite mixture (3:2) under controlled conditions (25°C, 70%

humidity, 16 h photoperiod) in a plant growth cabinet. After the seedlings emerged, they were transferred to pots as mentioned in section 2.3.5.1 for salinity testing.

2.7.3 Determination of effects of salt stress on plant physiological parameters

Shoot and total root lengths were measured in centimetres. Each plant was separated into shoot and root and the fresh weight (FW) of each component was measured on a digital scale. The root and shoot samples were then oven-dried at 70°C for three days and re-weighed to determine the dry weight (DW). The tissue water content (TWC) was calculated as per Jha *et al.* (2010), as TWC (%) = (FW-DW) / FW X 100. For the determination of leaf relative water content (LRWC) (Yamasaki and Dillenburg, 1999), ten leaves were excised and their fresh weight (FW) noted. They were then submerged in distilled water in a petri dish, covered with a filter paper and the lid closed, and incubated at 22°C for 24 h in order to reach full saturation. The RWC was determined after 24 h to avoid variations in the values (Yamasaki and Dillenburg, 1999). The leaves were then blotted dry and weighed on a digital balance to determine turgid weight (TW). The turgid leaves were dried in an oven at 70°C for three days and re-weighed to determine their dry weight (DW). The leaf relative water content (LRWC) was calculated as per the formula of Yamasaki and Dillenburg (1999) as LRWC (%) = $(FW\text{-}DW)_{Leaf}$ / $(TW\text{-}DW)_{Leaf}$ X 100. The root water content (RWC) was determined as per Vysotskaya *et al.* (2010), as RWC (%) = $(FW\text{-}DW)_{Root}$ / DW_{Root} X 100.

2.7.4 Physiology indices

The data obtained to evaluate the effects of salinity on plant physiological parameters were used to ascertain a 'physiology index'. The index for each parameter reflects the effect of salinity stress on the plants. The following indices were calculated (Kausar *et al.*, 2012):

(i) Salt tolerance index $_{Total\ Growth}$ (STI_G): Height of control plant $_{(shoot+root)}$/ Height of stressed plant $_{(shoot+root)}$;

(ii) Salt tolerance index $_{Total\ Fresh\ Weight}$ (STI_{FW}): Fresh weight of control plant $_{(shoot+root)}$/ Fresh weight of stressed plant $_{(shoot+root)}$

(iii) Salt tolerance index $_{Total\ Dry\ Weight}$ (STI_{DW}): Dry weight of control plant $_{(shoot+root)}$/ Dry weight of stressed plant $_{(shoot+root)}$

(iv) Salt tolerance index $_{\text{Root Water Content}}$ (TI_{RWC}): Root Water $Content_{(stressed)}$/Root Water $Content_{(control)}$

2.7.5 Element analysis using Atomic Absorption Spectrometry (AAS)

2.7.5.1 Preparation of standards

Sodium: 0.051 g of NaCl was dissolved in 200 mL 0.5 N nitric acid containing 2 mg/mL potassium (from KNO_3) to give a stock concentration of 100 µg/mL sodium. Sodium standards from this stock (with concentrations ranging from 0-1 µg/mL sodium) and tissue samples were prepared in a solution containing 2000 µg/mL potassium (from KNO_3) to suppress the ionization of sodium in the air-acetylene flame.

Potassium: 0.038 g of KCl was dissolved in 200 mL 0.5 N nitric acid containing 1 mg/mL cesium (from CsCl) to give a stock concentration of 100 µg/mL potassium. Standards ranging from 0-1 µg/mL potassium and tissue samples were prepared in a solution containing 1 mg/mL cesium (from CsCl) to suppress the ionization of potassium in the air-acetylene flame.

Magnesium: 0.203 g of $Mg(NO_3)_2$ was dissolved in 200 mL of 0.5 N nitric acid to give a stock of 100 µg/mL magnesium. Standards from this stock (with concentrations ranging from 0-1 µg/mL sodium) and tissue samples were prepared in 0.5 N nitric acid. Nitrous oxide-acetylene flame was used in order to suppress the ionization of magnesium.

Calcium: 0.05 g of $CaCO_3$ was dissolved in 200 mL 0.5 N nitric acid containing 2 mg/mL potassium (from KNO_3) to give a stock of 100 µg/mL calcium. Standards (0-1 µg/mL calcium) and tissue samples were prepared in a solution containing 2000 µg/mL potassium (from KNO_3) to suppress the ionization of calcium in the nitrous oxide-acetylene flame.

2.7.5.2 Preparation of plant samples

The plant material used for physiological parameters study was also used for cation analysis. After recording the dry weights, the dried shoot and root tissues were separately digested with 0.5 N nitric acid (1 mL of acid/10 mg of tissue) held in a water bath at 80°C for 3 days. The tubes were centrifuged at 14000 x g for 10 minutes. A dilution of either 10 µL or 100 µL aliquot of the supernatant was used for analysis.

Samples were prepared similar to the corresponding ion standards (see above) under investigation.

2.7.5.3 Cation determination

A suitable method with parameters shown in Table 2.6 was set on a Varian AAS instrument. Standards were injected at least three times and the readings plotted as a calibration graph. The biological triplicates of control and salt-stressed shoot and root samples were each injected three times. Samples were diluted suitably when the absorbance values were 'over range' i.e., exceeded the detection range and the dilution factors (DF) considered when calculating the concentration of each element using the following equation:

$$\text{mg/g DW of tissue} = \frac{\text{Concentration (mg/L)} \times \text{DF} \times \text{Sample volume (L)}}{\text{DW of tissue (g)}}$$

In order to express the values as millimole/gram of dry weight of tissue, for enabling comparisons with the reported levels of other plants, the molecular weight of each ion (Table 2.6) was used in the formula:

$$\text{mmole/g DW of tissue} = \frac{\text{Concentration (mg/L)} \times \text{DF} \times \text{Sample volume (L)}}{\text{DW of tissue (g)} \times \text{Molecular weight of ion}}$$

Table 2.6: Recommended instrument parameters used for AAS analysis of plant tissues

Ion	Molecular weight of ion (amu*)	Standard material	Lamp Current	Fuel	Support	Amount of Suppressant added	Wavelength (nm)	Slit width (nm)	Optimum working range (µg/mL)
Na^+	22.989	Sodium chloride	5 mA	Acetylene	Air	2000 µg/mL potassium	589.0	0.2	0.15-0.6
K^+	39.098	Potassium chloride	5 mA	Acetylene	Air	1000 µg/mL cesium	766.5	0.5	0.5-2.0
Mg^{2+}	40.077	Magnesium nitrate	3 mA	Acetylene	Nitrous oxide	none	285.2	0.5	0.1-0.4
Ca^{2+}	24.305	Calcium Carbonate	3 mA	Acetylene	Nitrous oxide	2000 µg/mL potassium	422.7	0.5	0.1-3

*amu- Atomic mass units.

2.7.6 Data analysis

Data were analysed using one-way analysis of variance (ANOVA) in SPSS v.21 (IBM SPSS Statistics for Windows, v21.0). The Least Significant Difference (LSD) post-hoc test was applied to compare group means (where $p < 0.05$ means the group means are significantly different from each and $p > 0.05$ means not different). All graphical representations were created using Microsoft Excel.

CHAPTER 3

Genetic analyses of glycine betaine biosynthetic genes in Australian native plants

3.1 Abstract

This chapter details the identification and in-depth characterisation of genes encoding the enzymes choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) involved in the biosynthesis of glycine betaine (GB) in three native Australian saltbushes- *Atriplex nummularia, Atriplex semibaccata* and *Atriplex amnicola*; and three Australian wattles- *Acacia pendula, Acacia salicina* and *Acacia victoriae.* All three saltbush *CMO* cDNAs were 1317 bp long, encoding full-length proteins of 438 aa predicted to be localised in the chloroplast. The sequences exhibited the Reiske-type motif ($CXHX_{16}CX_2H$) and mononuclear non-heme Fe motif ($DX_3DX_3HX_4H$) consistent with all GB accumulating plant CMOs. The putative saltbush *BADH* cDNAs were 1503 bp long, encoding full-length proteins of 500 aa with a predicted cytoplasmic localisation. The putative BADH proteins showed conservation of active site residues with functional and structural roles (e.g. the ALDH Cys-active site, Glu-active site, NAD-binding sites). The saltbush *CMO* genes showed notable up-regulation relative to *Actin* in salt-stressed leaf and root tissues compared to control plants, whereas the *BADH* genes were up-regulated in the leaf tissues only. On the other hand, *CMO* and *BADH* cDNA isolations of all three *Acacia* species were unsuccessful, hence in order to determine if the genes are actually present in the selected *Acacia* spp., gene isolation from genomic DNA was performed. Amplification of partial *CMO* gene from *Acacia pendula* and *Acacia victoriae* was successful, but no amplifications were obtained for *Acacia salicina CMO* and all three *Acacia* spp. *BADH.* The predicted open reading frames of *Acacia victoriae* CMO was closely related to CMOs of typical GB non-accumulators whereas *Acacia pendula* CMO was related to CMOs GB accumulators. The high degree of conservation in key amino acid residues of saltbushes CMO and BADH indicate their potential to breed salt tolerant plants via transgenic technologies.

3.2 Introduction

Glycine betaine (GB) is one of the most abundant solutes found to accumulate in many genera of plants. Rhodes and Hanson (1993) classified plants into two groups - GB accumulators and GB non-accumulators based on their ability to accumulate GB. Families outlined as GB accumulators are shown in Figure 1.7. However, not all genera in these families are accumulators e.g. among the genera listed in the family Leguminoseae - *Lycium*, *Medicago* and Trifolium are GB accumulators whereas *Aegialitis*, *Armeria*, *Lycopersicon*, *Nicotiana*, *Acacia*, *Goodia*, *Kennedia*, *Lotus* and *Pultanea* are listed as non-accumulators. Blunden *et al.* (2005) determined the widespread occurence of betaines (glycine betaine, trigonelline, proline betaine, *trans*-4-hydroxyproline and pipecolatebetaine) in 143 species of angiosperms, of which 123 species contained at least one type of betaine (Blunden *et al.,* 2005). In spite of its widespread occurrence, some plants that have the genetic and biochemical pathway do not accumulate GB.

As mentioned in sections 1.5.3 and 1.5.4, GB, is proven to aid in salt and drought tolerance in plants, and, interestingly, other independent reports showed livestock fed on GB supplemented diet displayed health benefits such as reduced cardiovascular risks (Raman and Rathinasabapathi 2003; Eklund *et al.,* 2005). However, the missing link is to find a plant that can have both functions – salt/drought tolerance and function as a nutritious feed crop. The Australian acacias and saltbushes have both adapted to similar edaphic conditions in Australia. It would be advantageous to investigate species that have been successfully trialled in land management programs (like the species used in this study previously trialled at Kamarooka). Identification of GB genes will provide conclusive results and validate native species cultivation for revegetation and as perennial fodder in salinity and drought-affected areas.

3.3 Experimental design

Few studies have been reported as yet that have systematically tested for agronomical candidate species for their genetic predisposition to stress tolerance. To this end, this study investigated the Australian native saltbushes, i.e., *Atriplex* species, which are historically known for extreme salt tolerance and longevity and recently shown to impart health benefits to grazing animals (Raman and Rathinasabapathi 2003; Eklund *et*

al., 2005) and native wattles (*Acacia* species). This study investigated whether the three selected native saltbushes and wattles (i) have the genes for GB biosynthesis; if so, ii) are they expressed; (iii) are they functional, i.e., is the final product, GB, made? This chapter provides insights into answering the first two questions- Do the native Australian plants under investigation encode glycine betaine biosynthetic genes and are they transcriptionally active. Gene identification was done using complementary DNA (cDNA) from normal plants (unstressed) followed by gene expression studies under control and salt conditions. But when amplifications failed to occur, they were tried from cDNA of salt-stressed leaf tissues and genomic DNA of normal plants.

3.4 Results – Section A (Gene identification from saltbushes)

3.4.1. Cloning of CMO and BADH from saltbushes

Multiple sequence alignments of the plant CMO and BADH cDNAs and putative proteins available in GenBank were conducted using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/) and edited using BioEdit v7.0.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Based on these, several exon-based degenerate primers were designed to amplify the *CMO* and *BADH* genes from cDNAs (Figure 2.1, Table 2.3). Size of the PCR products were predicted based on the alignment of *Atriplex nummularia CMO* cDNA (AB112481.1) and *Atriplex centralasiatica BADH* cDNA (AY093682.1) sequences. All amplified PCR products that showed a single band on agarose gel, possibly denoting a single product, were directly purified and cloned (Figure 3.1). When a primer pair yielded more than one product close to the predicted size, PCR products close to the expected size were purified from select bands excised from agarose gels and cloned. The amplification of products smaller than expected suggests non-specific binding, possibly because of the degenerate nature of the primers used. The recombinant clones were screened for inserts by colony PCR with T7 and SP6 primers. Positive clones with the expected product were sequenced.

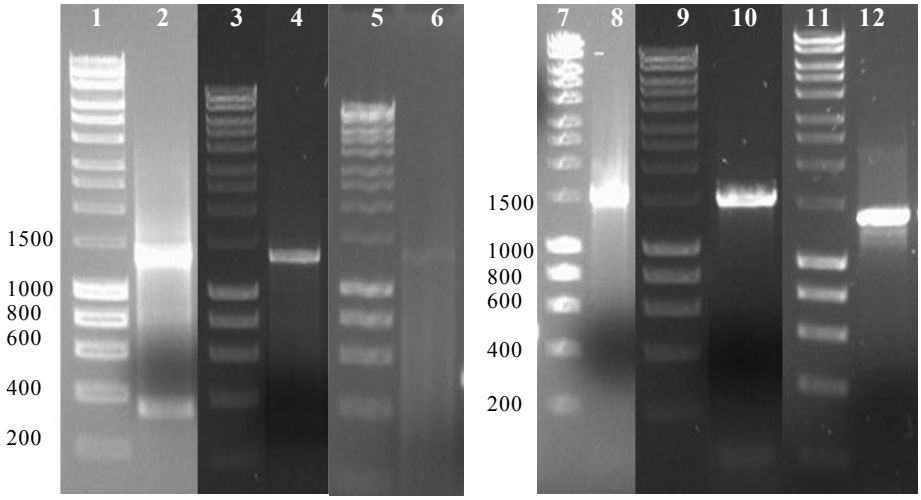


Figure 3.1: *CMO* and *BADH* PCR products (from cDNA) for cloning and sequencing
Lanes 1, 3, 5, 7, 9, 11: DNA Hyperladder 1 marker; Lanes 2, 4 and 6: *CMO* cDNA from *Atriplex nummularia*, *Atriplex semibaccata* and *Atriplex amnicola*; Lanes 8, 10 and 12: *BADH* cDNA from *Atriplex nummularia*, *Atriplex semibaccata* and *Atriplex amnicola*.

3.4.2 Sequence characteristics of the putative CMO enzymes of saltbushes

The sequencing of clones indicated that all three cDNAs were 1317 bp long (GenBank accession- *AsCMO*: JX486549; *AnCMO*: KC785451; *AaCMO*: KC785452). BLAST results showed the *AnCMO*, *AaCMO* and *AsCMO* cDNAs had highest identity to the Genbank sequences of *Atriplex nummularia* (AB112481.1; 97%, 99% and 99% identity for *AnCMO*, *AsCMO* and *AaCMO* respectively), *Atriplex hortensis* (AF270651.1; 97%, 97%, 97%) and *Atriplex prostrata* (AY082068.1; 86%, 96%, 96%). The saltbush cDNAs also had a high identity (84%, 84%, 85%) to *CMO* cDNA (EF362838.1) of *Spinacia oleracea* (spinach), a known GB accumulator (Rhodes and Hanson 1993). The cDNAs encoded full-length proteins of 438 amino acids and mature proteins of 380 amino acids (Figure 3.3; Table 3.2). The phylogenetic tree (Figure 3.2) constructed using the alignment of all the plant putative CMOs available in Genbank (Appendix II) grouped AnCMO, AsCMO and AaCMO with other members of Amaranthaceae such as spinach, sugar beet (*Beta vulgaris*), *Suaeda spp, Amaranthus tricolor* and *Haloxylon ammodendron,* reported to be GB accumulators (Rhodes and Hanson 1993). The only monocot related to this group was the turf grass *Ophiopogon japonicus*, its CMO was previously reported to be phylogenetically related to *S. oleracea* (Wu *et al.,* 2010). The low or non-accumulators formed a distinct clade, comprising all other monocots. Appendix IV also indicates that putative CMO proteins from specific genera, such as *Atriplex* and *Suaeda* have the highest percentage of sequence identity (>90%) within that group followed by broader relationship among their respective families,

Chenopodiaceae (*Atriplex spp, Beta vulgaris, Salicornia europaea, Spinacia oleracea)* and Amaranthaceae (*Suaeda spp, Amaranthus tricolor, Haloxylon ammodendron)*. Likewise, members of the Poaceae family (*Hordeum vulgare*, *Oryza sativa* and *Zea mays*) are closely related based on their sequence identities. These results suggest few evolutionary changes in CMO among species within a genus and the possibility of members of an entire genus having a functional/non-functional CMO.

The CDD search revealed the two signature motifs of plant CMOs, i.e., the novel Rieske-type iron-sulfur cluster and the mononuclear non-heme Fe cluster. The consensus for the Rieske motif in AnCMO, AsCMO and AaCMO was CTHRASILACGSGKKSCFVCPYH (i.e., $CXHX_{16}CX_2H$), which is identical to spinach and sugar beet motifs and highly conserved to the plant consensus ($CXHX_{15-17}CX_2H$) (Figure 3.3; Table 3.2). The mononuclear non-heme Fe cluster in the salt bushes CMO exhibited DNYLDSSYHVPYAH, i.e., $DX_3DX_3HX_4H$, consistent with all plant CMOs ($G/DX_{3-4}DX_2HX_{4-5}H$). The sequences also exhibited a number of other functionally important sites/motifs proposed by CDD search i.e., an active site, a substrate-binding site, a Fe-binding site and an α-subunit interface (or polypeptide binding site) (Table 3.2). Prediction of subcellular localisation by WoLF PSORT suggested that AnCMO, AsCMO and AaCMO are localised in the chloroplast, and is supported by the putative N-terminal chloroplast targeting peptide (cTP) of 58 residues seen in the protein sequence alignment on comparison with spinach CMO (Figure 3.3). TargetP indicated a chloroplast transit peptide with a reliability class of 1 for AnCMO and AsCMO, and 2 for AaCMO. ChloroP predicted the cTP to be 72 residues for AnCMO, 91 residues for AsCMO and 67 for AaCMO. Other biochemical properties of the putative mature AnCMO, AsCMO and AaCMO were construed using ProtParam and compared with the CMOs from spinach, a dicot GB accumulator, *A. thaliana,* a dicot non-accumulator, and a monocot, *Ophiopogon japonicus* (Table 3.2). The results indicated similarity in predicted molecular weights, theoretical pI and aliphatic index. However, the instability values, varied, suggestive of non-monomeric functionality for proteins and the need for additional amino acids, such as, a transit peptide, to prevent premature post transcriptional processing for proteins with the instability values >40. A difference noted in the transit peptide length of *A. thaliana* compared to the other CMOs (Appendix II) may also be indicative of its non-functional CMO.

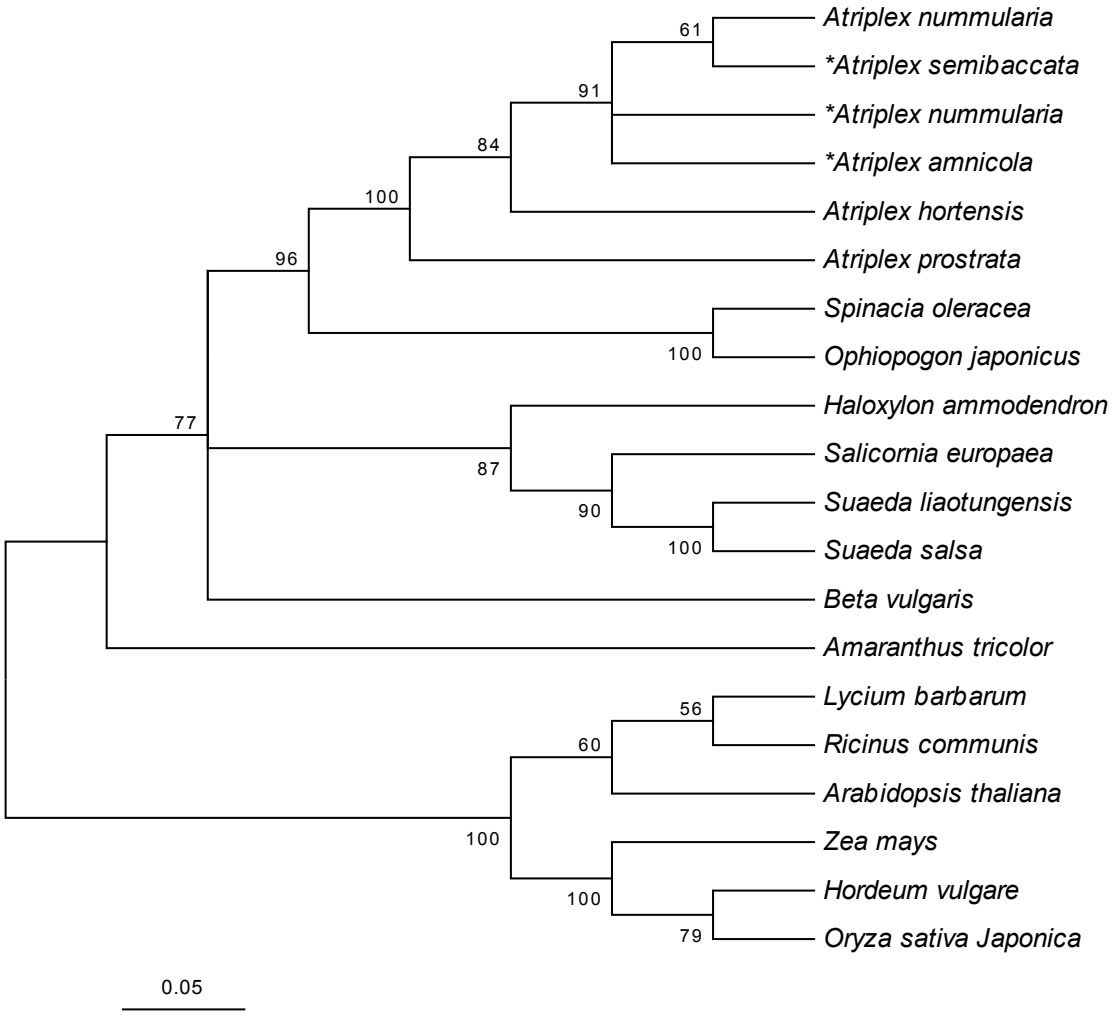
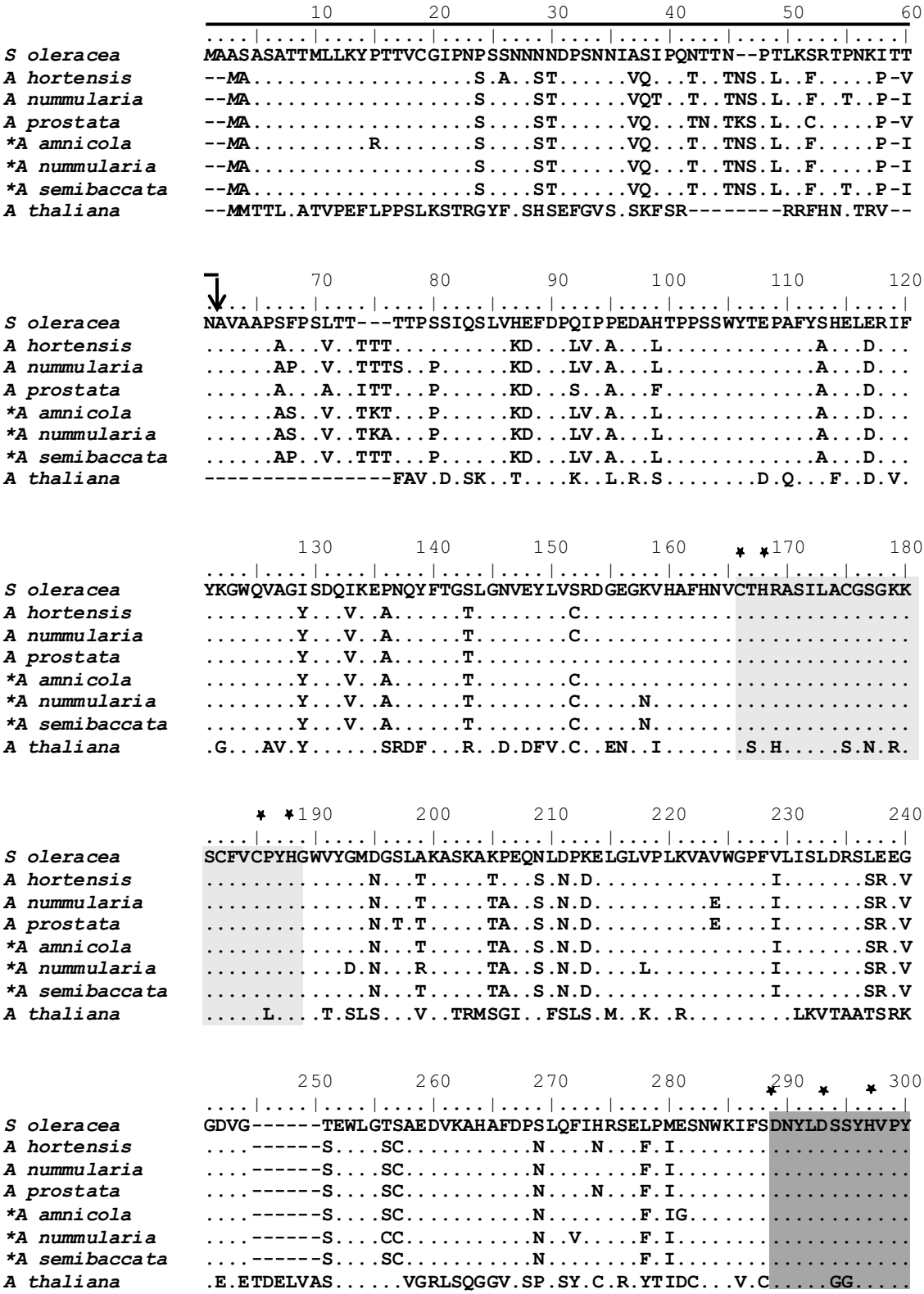


Figure 3.2: Phylogenetic relationships of saltbush CMOs to other full length plant CMO proteins

The phylogenetic tree was constructed using the alignment from Appendix II in MEGA4 with a boot strap replication value of 1000. Numbers at each branching node represents the bootstrapping support value. The branch distance of 0.05 is indicative of the proportion of amino acid changes (a distance of 0.05 = 5 changes per 100 amino acids). *Indicates predicted protein from experimentally obtained cDNA sequences in this study. All other full length protein sequences were obtained from GenBank. *Spinacia oleracea,* EF362838.1; *Arabidopsis thaliana,* BAC21260.1; *Amaranthus tricolor,* AB303389.1; *Atriplex hortensis,* AF270651.1; *Atriplex nummularia,* AB112481.1; *Atriplex prostrata,* AY082068.1, *Beta vulgaris,* AF023132.1; *Haloxylon ammodendron,* GQ379204.1; *Hordeum vulgare,* AB434467.1; *Lycium barbarum,* FJ514800.1, *Ophiopogon japonicus,* DQ645889.1; *Oryza sativa* Japonica, AJ578494.1; *Ricinus communis,* XM_00251821; *Salicornia europaea,* AY849925.1; *Suaeda liaotungensis,* AF354442.1; *Suaeda salsa,* DQ656523.1; *Zea mays,* DQ864498.1).


10 20 30 40 50 60
S oleracea MAASASATTMLLKYPTTVCGIPNPSSNNNNDPSNNIASIPQNTTN--PTLKSRTPNKITT
A hortensis --MA.....S.A..ST.....VQ...T..TNS.L..F.....P-V
A nummularia --MA.....S....ST.....VQT..T..TNS.L..F..T..P-I
A prostata --MA.....S....ST.....VQ...TN.TKS.L..C.....P-V
*A amnicola --MA.....R.....S....ST.....VQ...T..TNS.L..F.....P-I
*A nummularia --MA.....S....ST.....VQ...T..TNS.L..F.....P-I
*A semibaccata --MA.....S....ST.....VQ...T..TNS.L..F..T..P-I
A thaliana --MMTTL.ATVPEFLPPSLKSTRGYF.SHSEFGVS.SKFSR--------RRFHN.TRV--
70 80 90 100 110 120
S oleracea NAVAAPSFPSLTT---TTPSSIQSLVHEFDPQIPPEDAHTPPSSWYTEPAFYSHELERIF
A hortensis .....A...V..TTT.....KD...LV.A...L.....A...D...
A nummularia .....AP..V..TTTS..P.....KD...LV.A...L.....A...D...
A prostata .....A...A..ITT...P.....KD...S..A...F.....A...D...
*A amnicola .....AS..V..TKT...P.....KD...LV.A...L.....A...D...
*A nummularia .....AS..V..TKA...P.....KD...LV.A...L.....A...D...
*A semibaccata .....AP..V..TTT...P.....KD...LV.A...L.....A...D...
A thaliana ----------FAV.D.SK..T....K..L.R.S.....D.Q...F..D.V.
130 140 150 160 170 180
S oleracea YKGWQVAGISDQIKEPNQYFTGSLGNVEYLVSRDGEGKVHAFHNVCTHRASILACGSGKK
A hortensis .....Y...V..A.....T.....C.....
A nummularia .....Y...V..A.....T.....C.....
A prostata .....Y...V..A.....T.....
*A amnicola .....Y...V..A.....T.....C.....
*A nummularia .....Y...V..A.....T.....C.....N.....
*A semibaccata .....Y...V..A.....T.....C.....N.....
A thaliana .G...AV.Y.....SRDF...R..D.DFV.C..EN..I.....S.H.....S.N.R.
190 200 210 220 230 240
S oleracea SCFVCPYHGWVYGMDGSLAKASKAKPEQNLDPKELGLVPLKVAVWGPFVLISLDRSLEEG
A hortensis .....N...T.....T...S.N.D.....I.....SR.V
A nummularia .....N...T.....TA..S.N.D.....E....I.....SR.V
A prostata .....N.T.T.....TA..S.N.D.....E....I.....SR.V
*A amnicola .....N...T.....TA..S.N.D.....I.....SR.V
*A nummularia .....D.N...R.....TA..S.N.D....L.....I.....SR.V
*A semibaccata .....N...T.....TA..S.N.D.....I.....SR.V
A thaliana .....L...T.SLS...V..TRMSGI..FSLS.M..K..R.....LKVTAATSRK
250 260 270 280 290 300
S oleracea GDVG------TEWLGTSAEDVKAHAFDPSLQFIHRSELPMESNWKIFSDNYLDSSYHVPY
A hortensis ....------S....SC.....N....N...F.I.....
A nummularia ....------S....SC.....N.....F.I.....
A prostata ....------S....SC.....N....N...F.I.....
*A amnicola ....------S....SC.....N.....F.IG.....
*A nummularia ....------S....CC.....N..V.....F.I.....
*A semibaccata ....------S....SC.....N.....F.I.....
A thaliana .E.ETDELVAS.....VGRLSQGGV.SP.SY.C.R.YTIDC...V.C.....GG.....

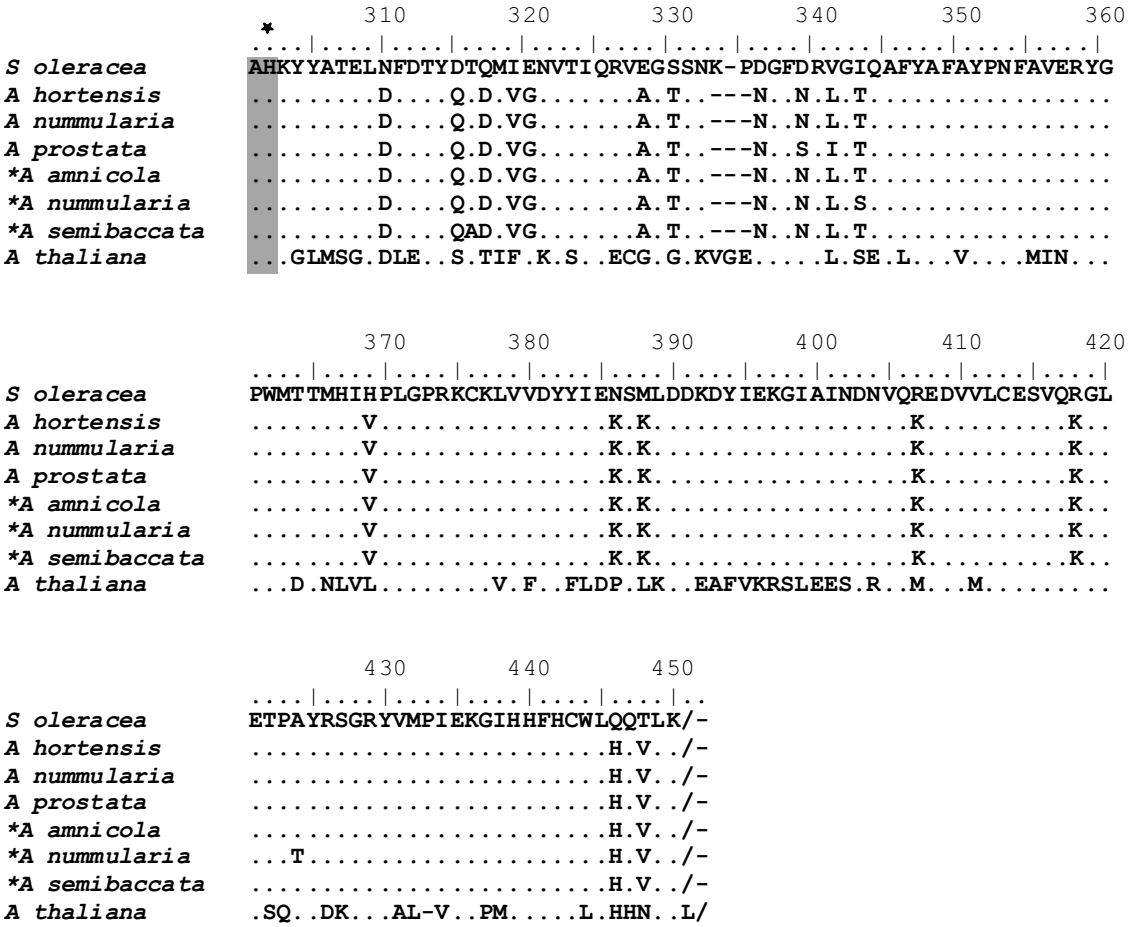


Figure 3.3: Alignment of the deduced amino acid sequences of saltbush CMOs

Amino acid sequence alignment of the CMO proteins deduced from cDNAs of *Atriplex nummularia*, *Atriplex semibaccata and Atriplex amnicola* isolated in this study (indicated by asterisk) with the reported sequences of *Spinacia oleracea* (GenBank EF362838.1), *Arabidopsis thaliana* (AEE85689.1), *Atriplex hortensis* (AF270651.1), *Atriplex nummularia* (AB112481.1) and *Atriplex prostrata* (AY082068.1). The alignments were created in ClustalW in BioEdit. A dot (.) indicates a conserved residue when aligned with *S. oleracea* CMO. A dash (-) indicates a gap introduced to optimally align the sequences, or a missing residue. The over-line at N-terminal denotes the predicted chloroplast transit peptide and the downward arrow indicates the predicted start of mature CMO corresponding to that of mature spinach (*Spinacia oleracea*) CMO (from A69). The light-grey shaded box indicates the Rieske iron-sulfur center [2Fe-2S] ($CXHX_{16}CX_2H$), with the key cysteine (positions C173, C192) and histidine (H175, H195) residues, marked with asterisks. The dark-grey shaded box illustrates the conserved mononuclear non-heme Fe binding motif ($DX_3DX_3HX_4H$), with the key aspartic acid (D296, D300) and histidine (H304, H309) residues, marked with asterisks. The **bold** and *italicized* "***M***" at the start of the sequence denotes the start codon. The "/" symbol denotes the stop codon at the end of the sequence.

Table 3.1: Results of Conserved Domain Database (CDD) search for the putative CMOs and BADHs isolated from saltbushes

Residues involved	Conserved feature	NCBI-CDD search source domain
AnCMO		
N281, Y282, S285, S286, H288, V289, H293, Q332, A333, Y335, A343, E345, T352, M354, I383, G386, V393, Q394, D397	Putative active site	176892
N281,Y282, S285, S286, H288,V289, H293, Q332, A333, Y335, A343, E345, T352, M354, I383, G386, V393, Q394	Putative substrate binding site	176892
H288, H293, D397	Specific Fe binding site	176892
E272, S273, I277, D280, N281, S285, Y287, H288, A292, R362, K363, V399, L400, C401, S403, V404, Q405, K406, G407, Y418, V419, M420, P421, I426, F429, L433	Putative alpha subunit interface	176892
*CTHRASILACGSGKKSCFVCPYH	Reiske-type cluster	-
*DNYLDSSYHVPYAH	Mononuclear non-heme cluster	-
AsCMO		
N281, Y282, S285, S286, H288, V289, H293, Q332, A333, Y335, A343, E345, T352, M354, I383, G386, V393, Q394, D397	Putative active site	176892
N281, Y282, S285, S286, H288, V289, H293, Q332, A333, Y335, A343, E345, T352, M354, I383, G386, V393, Q394	Putative substrate binding site	176892
H288, H293, D397	Specific Fe binding site	176892
E272, S273, I277, D280, N281, S285, Y287, H288, A292, R362, K363, V399, L400, C401, S403, V404, Q405, K406, G407, Y418, V419, M420, P421, I426, F429, L433	Putative alpha subunit interface	176892
*CTHRASILACGSGKKSCFVCPYH	Reiske-type cluster	-
*DNYLDSSYHVPYAH	Mononuclear non-heme cluster	-
AaCMO		
N281,Y282,S285,S286,H288,V289,H293,Q332,A333,Y335,A343,E345,T352, M354,I383,G386,V393,Q394,D397	Putative active site	176892
N281,Y282,S285,S286,H288,V289,H293,Q332,A333,Y335,A343,E345,T352, M354,I383,G386,V393,Q394	Putative substrate binding site	176892
H288,H293,D397	Specific Fe binding site	176892
G272,S273,I277,D280,N281,S285,Y287,H288,A292,R362,K363,V399,L400,C 401,S403,V404,Q405,K406,G407,Y418,V419,M420,P421,I426,F429,L433	Putative alpha subunit interface	176892
*CTHRASILACGSGKKSCFVCPYH	Reiske-type cluster	-
*DNYLDSSYHVPYAH	Mononuclear non-heme cluster	-
AnBADH		
N162, E260, G291, C294	Specific catalytic residues	143428
I158, S159, P160, W161, N162, W170, K185, S187, E188, F236, T237, G238, S239, T242, K245, I246, E260, L261, G262, C294, E393, F395, L421, W459	Specific NADP-binding site	143428
*FWTNGQICSATS	Aldehyde dehydrogenases cysteine active site	143428
*VTLELGGKSP	Aldehyde dehydrogenases glutamic acid active site	143428
AsBADH		
N162, E260, G291, C294	Specific catalytic residues	143428
I158, S159, P160, W161, N162, W170, K185, S187, E188, F236, T237, G238, S239, T242, K245, I246, E260, L261, G262, C294, E393, F395, L421, W459	Specific NAD(P) binding site	143428
*FWTNGQICSATS	Aldehyde dehydrogenases cysteine active site	143428
*VTLELGGKSP	Aldehyde dehydrogenases glutamic acid active site	143428
AaBADH		
N162,E260,G291,C294	Specific catalytic residues	143428
I158,S159,P160,W161,N162,W170,K185,S187,E188,F236,T237,G238,S239, T242,K245,I246,E260,L261,G262,C294,E393,F395,L421,W459	Specific NAD(P) binding site	143428
*FWTNGQICSATS	Aldehyde dehydrogenases cysteine active site	143428
*VTLELGGKSP	Aldehyde dehydrogenases glutamic acid active site	143428

*Conserved feature identified based on multiple sequence alignments of available plant CMOs and BADHs (Appendices 2 and 3)

Table 3.2: Physicochemical characteristics of the putative mature CMO and full length BADH proteins of saltbushes

Parameter*	AnCMO	AsCMO	AaCMO	SoCMO	OjCMO	AtCMO	AnBADH	AsBADH	AaBADH	SoBADH	OjBADH	AtBADH
Number of amino acids	380	380	380	379	379	375	500	500	500	497	500	501
Molecular weight (kDa)	42.67	42.54	42.53	42.88	42.96	42.32	54.65	54.56	54.74	54.27	54.63	54.43
Theoretical pI	5.87	5.77	6.06	5.49	5.49	5.76	5.29	5.24	5.30	5.29	5.45	5.18
Transit peptide length[#]	58[#]	58[#]	58[#]	60[#]	59[#]	47[#]	-	-	-	-	-	-
Instability index	45.15	43.87	42.35	47.15	47.15	31.38	31.75	31.97	32.93	31.41	32.85	31.96
Aliphatic index	76.18	75.42	75.16	72.27	71.24	78.43	91.12	91.12	89.56	91.07	90.54	90.18
Grand average of hydropathicity (GRAVY)	-0.308	-0.288	-0.294	-0.405	-0.417	-0.233	-0.019	-0.023	-0.045	-0.023	-0.052	-0.025

*Deduced using ProtParam (http://web.expasy.org/protparam/)

[#]Transit peptide length for CMO was deduced based on sequence homology with experimentally reported chloroplast transit peptide (Rathinasabapathi *et al.*, 1997). As the reported AVA start site for the mature CMO polypeptide was absent in *A. thaliana* (At) CMO, the cTP length was obtained from UniProt database (http://www.uniprot.org/uniprot/Q9SZR0). Transit peptide length for BADHs could not be determined, as plant BADHs lack a typical N terminal transit peptide (Nakamura *et al.,* 2001) and no experimental evidence is available. The only reported signal peptide is the 'SKL' at the C terminal which targets the peroxisomes (Nakamura *et al.,* 2001); this motif was not found in AnBADH, AsBADH, AaBADH, SoBADH, OjBADH or AtBADH. Genbank Accession numbers: AAB52509.1 (SoCMO), ABG34274.1 (OjCMO), AEE85689.1 (AtCMO), AAA34025.1 (SoBADH), ABG34273.1 (OjBADH), AAM64944.1 (AtBADH).

3.4.3 Sequence characteristics of the putative BADH enzymes of saltbushes

The *BADH* cDNAs isolated from *Atriplex nummularia, Atriplex semibaccata and Atriplex amnicola* were 1503 bp long (*AnBADH*: KC785453; *AsBADH*: KC785454; *AaBADH*: KC785454). The BLAST search results showed 96-99% identity with cDNAs from other saltbushes and also *Ophiopogon japonicus* (DQ645888.1; 96%), but much more limited identity (90%, 89%, 90%) with spinach *BADH* (M31480.1). The cDNAs encoded full-length proteins of 500 amino acids (Figure 3.5; Table 3.2). Plant BADHs lack a typical N-terminal transit peptide (Nakamura *et al.,* 2001) and no experimental evidence for such a signal is available. Alignment of all available putative full-length plant BADH sequences from Genbank (Appendix III) was used to construct a phylogram, which showed close evolutionary relationship of AnBADH, AsBADH and *Aa*BADH with other members of Chenopodiaceae and Amaranthaceae (Figure 3.4). However, *O. japonicus* BADH, like its CMO, resolves in this *Atriplex* clade, suggesting the grouping was based more on similarity pertaining to BADH function and sequence similarity, rather than a separation of dicots/monocots. This observation is further supported by the percentage sequence identity between *O. japonicus* BADH and BADHs from *Atriplex* species (>90%; Appendix V). Unlike the CMOs which have variations at the N-terminal region, the BADHs are much more conserved (Appendix III). Nonetheless, based on identities, the *Atriplex* BADHs do form a highly conserved group, like their CMOs.

The saltbush cDNAs encoded putative proteins of 54.65 kDa (*An*BADH), 54.56 kDa (*As*BADH) and 54.74 kDa (*Aa*BADH) (Table 3.2) similar to sizes of other BADHs determined experimentally, e.g., *Zoysia tenuifolia* (55.5 kDa; Oishi & Ebina, 2005), and rice (55 kDa; Mitsuya *et al.,* 2009). The CDD-search showed the three putative proteins belonged to the aldehyde dehydrogenase (ALDH) family and exhibited several key features, e.g., residues involved in catalytic activity, the NADP-binding site, ALDH Glu-active site and ALDH Cys-active site (Table 3.2). Additionally, the ten most conserved motifs in ALDHs proposed by Perozich *et al.,* (1999) were also identified. Their roles in maintaining the structure and functionality of BADH enzymes are summarised in Table 3.3 and the relative positions in the multiple alignment are displayed in Figure 3.5.

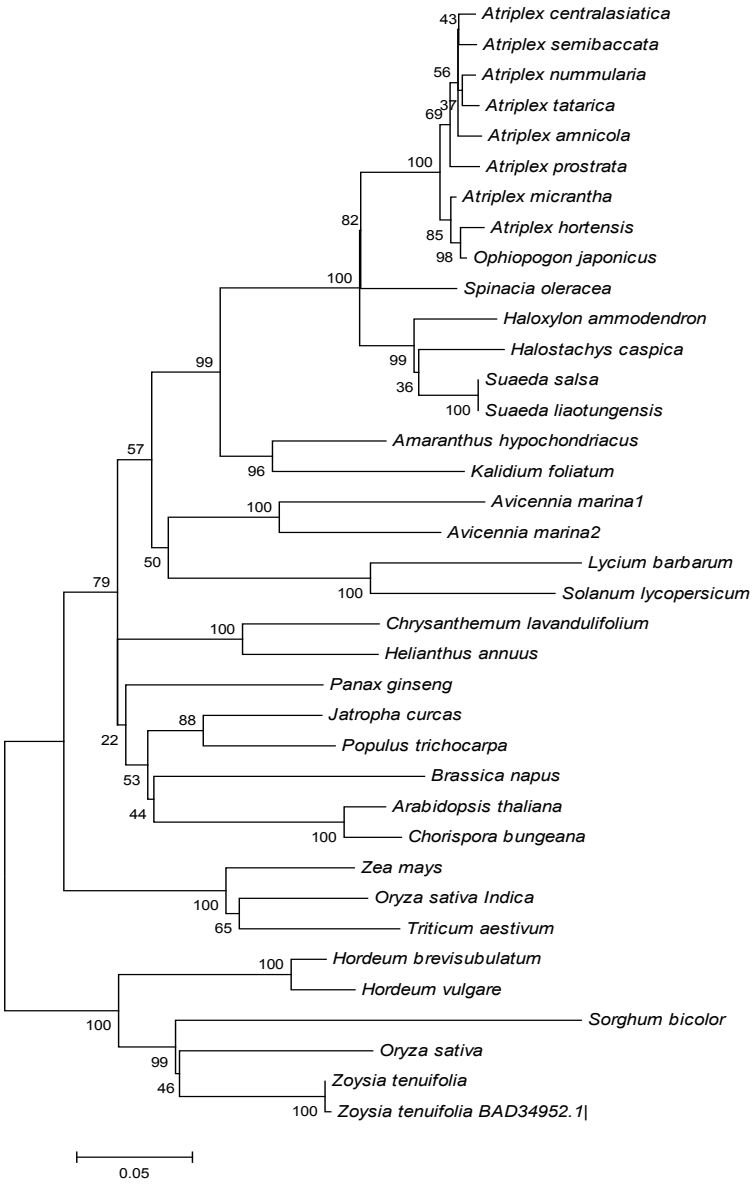


Figure 3.4: Phylogenetic relationships of saltbush BADHs to other full length plant BADH proteins

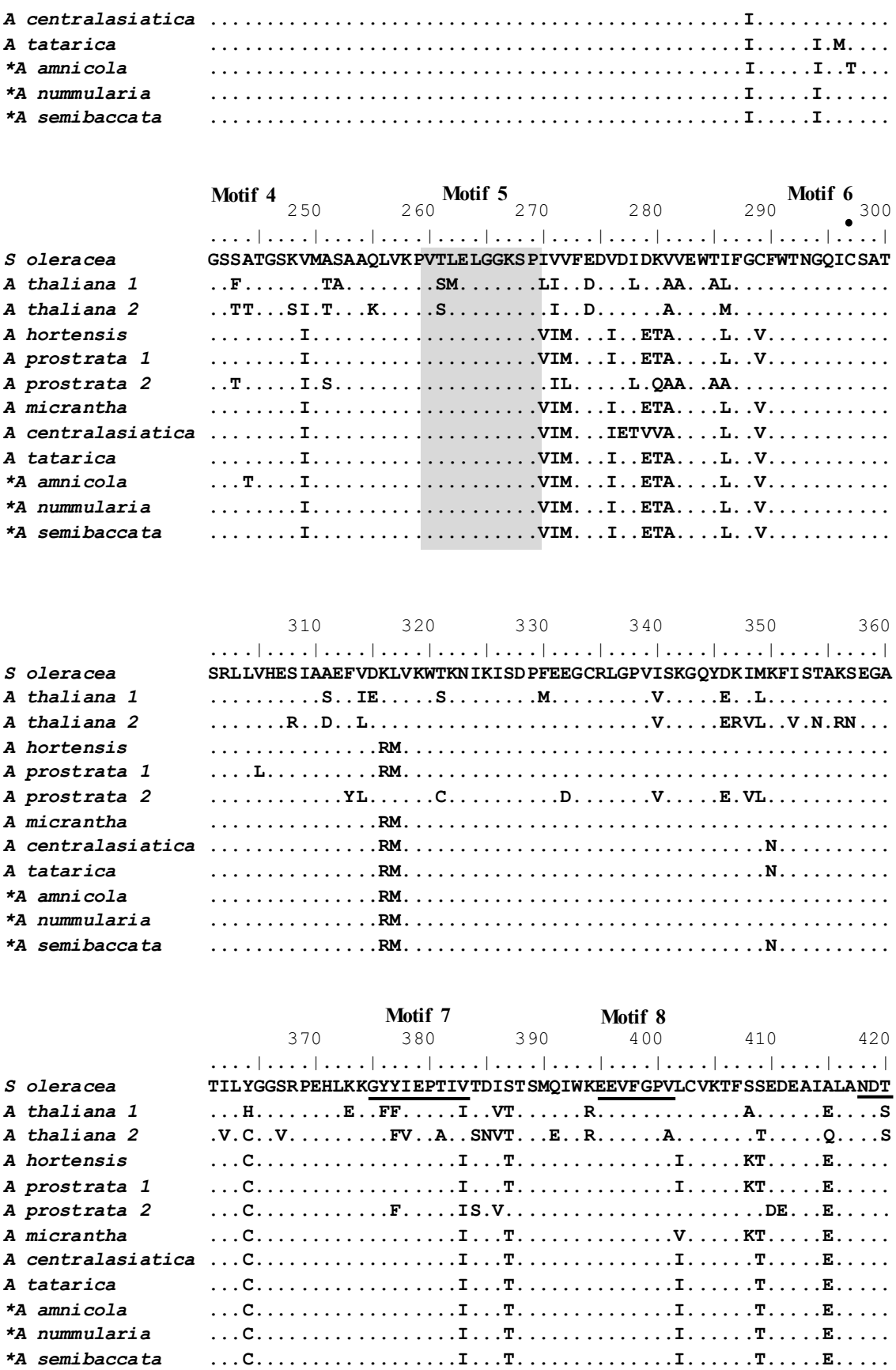
The phylogenetic tree was constructed using data from Appendix III in MEGA4 with a boot strap replication value of 1000. Numbers at each branching node represents the confidence value of bootstrapping. The branch distance of 0.02 is indicative of the proportion of amino acid changes (a distance of 0.05 = 5 changes per 100 amino acids). An asterisk '*' indicates predicted protein from experimentally obtained cDNA sequences in this study. All other full length protein sequences were obtained from GenBank. *Amaranthus hypochondriacus,* AAB58165.1; *Arabidopsis thaliana,* AAM64944.1; *Atriplex centralasiatica,* AAM19159.1; *Atriplex hortensis,* CAA49425.1; *Atriplex micrantha,* ABM97658.1; *Atriplex prostrata,* AAP13999.1; *Atriplex tatarica,* ABQ18317.1; *Avicennia marina* 1, AF170094.1; *Avicennia marina* 2, BAB18544.1; *Brassica napus,* AAQ55493.1; *Chorispora bungeana,* AAV67891.2; *Chrysanthemum lavandulifolium,* AAY33872.1; *Halostachys caspica,* ABO45931.1; *Haloxylan ammodendron,* ACS96437.1; *Helianthus annus,* ACU65243.1; *Hordeum brevisubulatum,* AAS66641.1; *Hordeum vulgare*, BAA05466.1; *Jatropha curcas,* ABO69575.1; *Kalidium foliatum,* ABI95806.1; *Lycium barbarum,* ACQ99195.1; *Ophiopogon japonicas,* ABG34273.1; *Oryza sativa,* ABB83473.1; *Oryza sativa* Indica, ACF06149.1; *Panax ginseng,* AAQ76705.1; *Populus trichocharpa,* XP_002322147.1; *Solanum lycopersicum,* ACI43573.1; *Sorghum bicolour,* AAC49268.1; *Spinacia oleracea,* AAA34025.1; *Suaeda liaotungensis,* AAL33906.1; *Suaeda salsa,* ABG23669.1; *Triticum aestivum,* AAL05264.1; *Zea mays,* AAT70230.1; *Zoysia tenuifolia,* BAD34957.1.

```
                          10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
S oleracea          MAFPIPARQLFIDGEWREPIKKNRIPVINPSTEEIIGDIPAATAEDVEVAVVAARRAFRR
A thaliana 1        ..I.M.T.............L.K...IV..A...V........T...D...N.....LS.
A thaliana 2        ..ITV.R.....G.Q.T..VLRKTL..V..A..D...Y.....S....L..E...K..T.
A hortensis         ...................LL.....I.............................K..K.
A prostrata 1       ......V............LL.....I.............................K..K.
A prostrata 2       ------------------------------------------------------------
A micrantha         ...................LL.....I.............................K..K.
A centralasiatica   ....M.V............LL.....I.............................K..K.
A tatarica          .....SV............LL.....I.............................K.
*A amnicola         ....M.V............LL.....I.............................K..K.
*A nummularia       ....MSVH...........LL.....I.............................K..K.
*A semibaccata      ....M.V............LL.....I.............................K..K.

                          70        80        90       100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
S oleracea          N---NWSATSGAHRATYLRAIAAKITEKKDHFVKLETIDSGKPFDEAVLDIDDVASCFEY
A thaliana 1        .KGKD.AKAP..V..K........VN.R.TDLA...AL.C...L....W.M....G...F
A thaliana 2        .NGKD.ARAT..V..K........VI.R.SELAN..A..C...L...AW.M....G....
A hortensis         .KGRD.A.LW-S...K.....................L.....R...........T....
A prostrata 1       .KGRD.A........K...........R.........L.....................
A prostrata 2       ----------------...........R.........L.........L.......G....
A micrantha         .KGRD.A........K.....................L.....R...........T....
A centralasiatica   .KGRD.A........R...........R.........L.....L...........T....
A tatarica          .KGRD.A........R...........R.........L.....L...........T....
*A amnicola         .KGRD.A........R...........R.........L.....L...........T..D.
*A nummularia       .KGRD.A........R.....................L.....L...........T....
*A semibaccata      .KGRD.A........R...........R.........L.....L...........T....

                                                  Motif 1
                          130       140       150       160       170       180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
S oleracea          ---FAGQAEALDGKQKAPVTLPMERFKSHVLRQPLGVVGLISPWNYPLLMATWKIAPALA
A thaliana 1        ---Y.DL..G..A......S....S...Y..K.........T.........V..V..S..
A thaliana 2        ---Y.DL..G..A...T.LS...DT..GYI.KE.I....M.T.........V..V..S..
A hortensis         FEY.........A.....................I..............D.........
A prostrata 1       ---.........A..........D..........I.....................
A prostrata 2       ---Y.D......A.....IA...DT.........I...................V.....
A micrantha         ---.........A.....................I.....................
A centralasiatica   ---.........A..........D..........I.....................
A tatarica          ---.........A..........D..........I.....................
*A amnicola         ~~~.........A..........D..........I.....................
*A nummularia       ---.........A..........D..........I.....................
*A semibaccata      ---.........A..........DG.........I.....................

                  Motif 2                                 Motif 3
                          190       200       210       220       230       240
                    ....|....|....|....|....|....|....|....|....|....|....|....|
S oleracea          AGCTAVLKPSELASVTCLEFGEVCNEVGLPPGVLNILTGLGPDAGAPLVSHPDVDKIAFT
A thaliana 1        .....I.............LADI.R..........V...F.SE.....A...G.......
A thaliana 2        .....I........L....LADI.R................TE.....A...H....V..
A hortensis         ....T.....................................I.....I..V...
A prostrata 1       .....................................I.....I......
A prostrata 2       ...A.I.....M.......LAD..K............S.Y..E..G..A.......V...
A micrantha         .....................................I.....
```


A centralasiatica
A tatarica
*A amnicola
*A nummularia
*A semibaccata
Motif 4
Motif 5
Motif 6
250
260
270
280
290
300
S oleracea
A thaliana 1
A thaliana 2
A hortensis
A prostrata 1
A prostrata 2
A micrantha
A centralasiatica
A tatarica
*A amnicola
*A nummularia
*A semibaccata
GSSATGSKVMASAAQLVKPVTLELGGKSPIVVFEDVDIDKVVEWTIFGCFWTNGQICSAT
310
320
330
340
350
360
SRLLVHESIAAEFVDKLVKWTKNIKISDPFEEGCRLGPVISKGQYDKIMKFISTAKSEGA
Motif 7
Motif 8
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380
390
400
410
420
TILYGGSRPEHLKKGYYIEPTIVTDISTSMQIWKEEVFGPVLCVKTFSSEDEAIALANDT

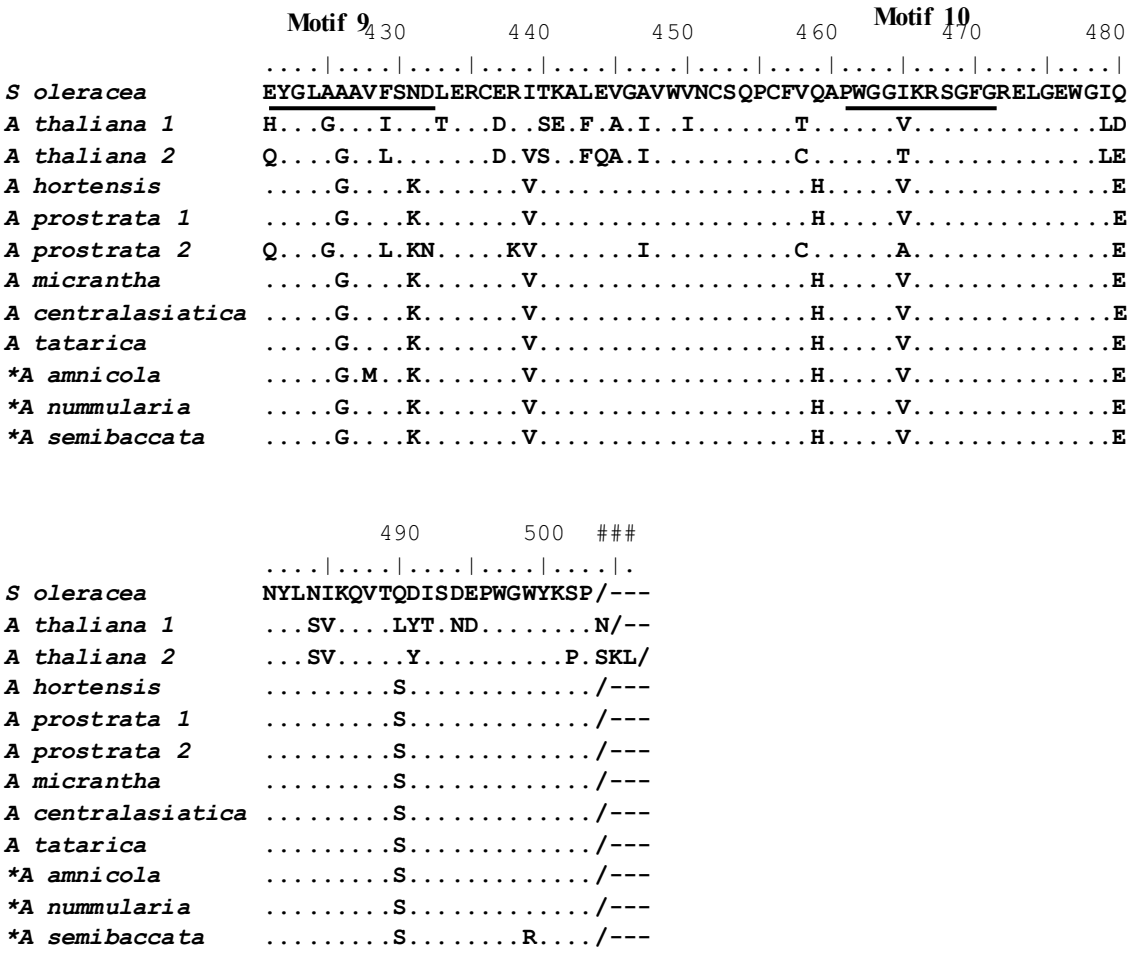


Figure 3.5: Alignment of the deduced amino acid sequences of saltbush BADHs

Sequence alignment of putative saltbushes BADH protein deduced from cDNA (indicated by underline) with reported Genbank BADH protein sequences of *Spinacia oleracea* (AAA34025.1), *Atriplex hortensis* (CAA49425.1), *Atriplex prostrata* BADH1 (AAM08913.1), *Atriplex prostrata* BADH 2 (AAM08914.1), *Atriplex micrantha* (ABM97658.1), *Atriplex centralasiatica* (AAM19159.1), *Atriplex tatarica* (ABQ18317.1), *Arabidopsis thaliana* BADH 1 (AEE35649.1) and *Arabidopsis thaliana* BADH2 (AEE78376.1). An asterisk '*' indicates predicted protein from experimentally obtained cDNA sequences in this study. The alignments were created in ClustalW in BioEdit. A dot (.) indicates the presence of conserved amino acid at the position aligned with *Spinacia oleracea* BADH protein. A dash (-) indicates a gap introduced to align sequences or represent a missing amino acid. The F marked with an asterisk at position 146 denotes the conserved Phe residue implicated in the binding of substrate betaine aldehyde (Hibino, 2001). The grey region shows the decapeptide VTLELGGKSP that is found conserved among plant aldehyde dehydrogenases (Weretilnyk, 1990) and contains the catalytic Glu (E) residue. A circle '•' at position 297 indicates the highly conserved catalytic Cys residue. The ten motifs found to be conserved across all aldehyde dehydrogenases are underlined and numbered. The three hash symbols (###) over the terminal tripeptide 'SKL' represent the peroxisomal targeting peptide. The **bold** and *italicized* "***M***" at the start of the sequence denotes the start codon. The "/" symbol denotes the stop codon at the end of the sequence.

For ALDHs, the consensus for the Glu-active site is [LIVMFGA]-E-[LIMSTAC]-[GS]-G-[KNLM]-[SADN]-[TAPFV], and that for the Cys-active site as [FYLVA]-x-{GVEP}-{DILV}-G-[QE]-{LPYG}-C-[LIVMGSTANC]-[AGCN]-{HE}-[GSTADNEKR], based on alignment of 145 ALDHs from diverse organisms including mammals, plants, fungi and bacteria (Perozich *et al.,* 1999). All three saltbush BADHs exhibited residues that contributed to these domains. The Glu-active site domain in AnBADH, AsBADH and AaBADH comprised of L-**E**-L-G-G-K-S-P (Motif 5) consistent with the consensus previously identified in other plants (Weretilnyk and Hanson, 1990). In plants, this domain is part of the conserved decapeptide V-T-L-**E**-L-G-G-K-S-P (Weretilnyk and Hanson 1990). The ALDH Cys active site in the saltbush BADHs (Motif 6) was F-W-T-N-G-Q-I-**C**-S-A-T-S, exhibiting a few changes compared to the consensus (given above). The third residue {G/V/E/P} was replaced by T, the fourth {D/I/L/V} by N, the seventh {L/P/Y/G} by I and the eleventh {H/E} by T. However, these changes were also noticed in spinach and other GB accumulators (Appendix III), suggesting evolutionary variations between mammalian and plant ALDHs. The Cys of the ALDH Cys-active site in AnBADH, AsBADH and AaBADH was at 28 residues from the decapeptide, as in the other GB accumulator plants (Figure 3.5; Appendix III). Motif 4 in AnBADH, AsBADH and AaBADH was GSSATG, very similar to the signature sequence GxGxxG suggested to be involved in the NAD-binding turn of the Rossmann fold (Hempel *et al.,* 1993), the S instead of the second Gly falling within the consensus [Vil]-[Astvl]-[Fl]-[Tl]-**G**-S-[std**g**vfty]-[Atpe]-T-**G** (Table 3.3). Motif 8 was also highly conserved in AnBADH, AsBADH and AaBADH as per the consensus [Ed]-E-V-**F**-G-P-V. The fifth Gly in Motif 4 along with the invariant Phe in Motif 8 are integral for binding the nicotinamide ring of NAD (Hempel *et al.,* 1993). Absence of the C-terminal SKL, the peroxisomal signal characteristic of monocot BADHs translocating to microbodies, suggests the saltbush BADHs may not be translocated to peroxisomes. Supporting this is the prediction of cytoplasmic localisation by WoLF PSORT. However, it is important to investigate the localisation experimentally.

Table 3.3: Conserved sequence motifs present in plant BADHs and their proposed roles

Motif no.	Motif length	Motif pattern in ALDHs[1]	Corresponding motif pattern in plant BADHs[2]	Proposed role of motif[3]	Relative position[2]
1	5	[Past]-[WFy]-[**N**e]-[FYgalv]-[Ptl]	**PWNYP**	Most conserved motif. Contains an asparagine (N) nearest to the catalytic thiol and is proposed to stabilize the carbonyl oxygen of the aldehyde substrate during catalysis.	163-167
2	14	[Apnci]-[Liamv]-[Avslcimg]-[ACtlmvgf]-**G**-[Ncdi]-[Tavcspg]-[Vaimfcltgy]-[Vil]-[Lvmiwafhcy]-[**K**h]-[Ptvghms]-[ASdhp]-[Epsadqgilt]	[Asv]-**L**-**A**-[Asv]-[Gs]-**C**-[TAs]-[At]-[Vl]-**L**-[Ke]-**P**-**S**-[Eq]	PF	178-191
3	10	[Grkpwhsay]-[FLeivqnarmhk]-[Pg]-[Plakdievsrf]-[Gnde]-[Vliat]-[VLifyac]-[Nglqshat]-[Vllyaqgfst]-[IVlms]	**G**-**L**-**P**-[Pas]-**G**-[Vai]-[Lf]-[NS]-[Iv]-[Lvi]	PF	208-217
4	10	[IVlgfy]-[SAtmnlfhq]-[Fyla]-[Tvil]-**G**-[Sgen]-[Tsvrindepaqk]-[EAprqgktvnldh]-[VTiasgm]-[Gafi]	[Vil]-[Astvl]-[Fl]-[Tl]-**G**-**S**-[stdgvfty]-[Atpe]-**T**-**G**	Involved in NAD-binding turn of the Rossmann fold. The fifth glycine G) along with the invariant phenyl alanine (F) in motif 8 is integral for binding the nicotinamide ring of NAD.	237-246
5	16	[Lamfgs]-[Enlqf]-[Ltmcagi]-[**G**s]-[Ga]-[Knlmqshiv]-[SNadc]-[Pahftswv]-[cnlfmgivahst]-[Ivlyfa]-[Viamt]-[Fdlmhcanyv]-[Daeskprnt]-[Dsntaev]-[Acvistey]-[Dnlera]	[Lm]-**E**-**L**-**G**-**G**-**K**-**S**-**P**-[LIV]-[IV]-[Vmit]-[Fs]-[Ed]-[De]-[vni]-[vhdra]-[Denk]	General base for catalytic reaction.	260-269
6	8	[Fyvlma]-[Fgylrmdaqetwsvikp]-[Nhstyfaci]-[QAsnhtcmg]-**G**-[Qe]-[crvitksand]-[**C**r]	[Fl]-[Wsapf]-[Tn]-[Nag]-**G**-[Qr]-[Iv]-**C**	The terminal invariant residue Cysteine (C) acts as the catalytic nucleophile, highly conserved in all sequences with catalytic activity.	290-297
7	9	[Gdtskac]-[Yfnarthclswv]-[FYlwvis]-[IVlfym]-[Qeapkgrmynhlswyv]-[Pa]-[Tachlmy]-[Vll]-[FLiwn]	**G**-[FY]-[Fy]-[Ivmsl]-[Eq]-**P**-[Ta]-**I**-[Ivn]	PF	375-383
8	7	[Ektdrqgs]-**E**-[Ivtlnfsp]-**F**-[Ga]-[Ps]-[Vilcf]	[Ed]-**E**-**V**-**F**-**G**-**P**-**V**	The second invariant glutamic acid (E) is involved in binding NAD.	395-401
9	15	[Nrst]-[Dnaseqtkrcgi]-[TSrvnalcqgik]-[Epdtgqikvrfshyncl]-[Yfkqvm]-[Gpa]-[Lnmv]-[Astgvqcf]-[Agsltfc]-[AGysct]-[Vllfams]-[Fhwyivlem]-[TSag]-[KRnsqteahdp]-[DNsileakt]	**N**-**D**-[Tsp]-[Eqhkr]-[Yf]-**G**-[Lw]-[AG]-[AGs]-[AG]-[Vi]-[FLim]-**S**-[Knqgsd]-[Dn]	PF	418-432
10	12	[Pasw]-[Fwyahv]-[Gtqs]-**G**-[Fvyesnimtawrq]-[Kgrn]-[mqarelnskghdpt]-[Stm]-[Gfls]-[Ifntlmygshrvq]-[Gdnhrsy]-[Rdpsagkte]	**P**-**W**-**G**-**G**-[Vitskln]-**K**-**R**-[**S**-]-[Gm]-**FGR**	PF	461-472

[1]The ten most conserved sequence motifs proposed by Perozich *et al.,* (1999), based on the alignment of the consensus sequences of ALDH family in diverse organisms including fungal, bacterial, plant and human ALDHs. [2]As per the multiple sequence alignment of putative plant BADH proteins. [3]Roles of the motifs proposed by Hempel *et al.,* (1993) and Perozich *et al.,* (1999)Residues in bold in column 3 indicate highly conserved, invariant residues in at least 95% of known ALDHs, whereas in column 4, they indicate the single most conserved residue in plants. Capitalized letters represent residues that are predominant while less conserved alternative residues are in small letters within at a position are shown in square brackets. 'PF' indicates no specific role has been assigned to the motif other than their involvement in protein folding.

3.4.4 Optimisation of *CMO* and *BADH* gene expression using semi quantitative reverse transcription PCR (SQ-PCR)

In order to study the differential expression of *CMO* and *BADH* genes, PCR cycles were optimised to avoid saturation of mRNA transcripts which might interfere in the intensity values (Figure 3.6). Three µL of the PCR product of *actin*, 3 µL of *CMO* product and 3 µL of *BADH* product from *Atriplex nummularia* cDNA (control and salt-stressed) were used. Higher volume of PCR product showed early saturation on the gel (images not shown), preventing any noticeable changes in expression levels to be observed. The results of optimisation of semi-quantitative RT-PCR for different cycle numbers indicated *CMO* and *BADH* products could be analysed optimally at 20 and 25 cycles, respectively (Figure 3.6; Table 3.4). Gene optimisation was performed with leaf cDNA from one control and one salt-stressed plant.

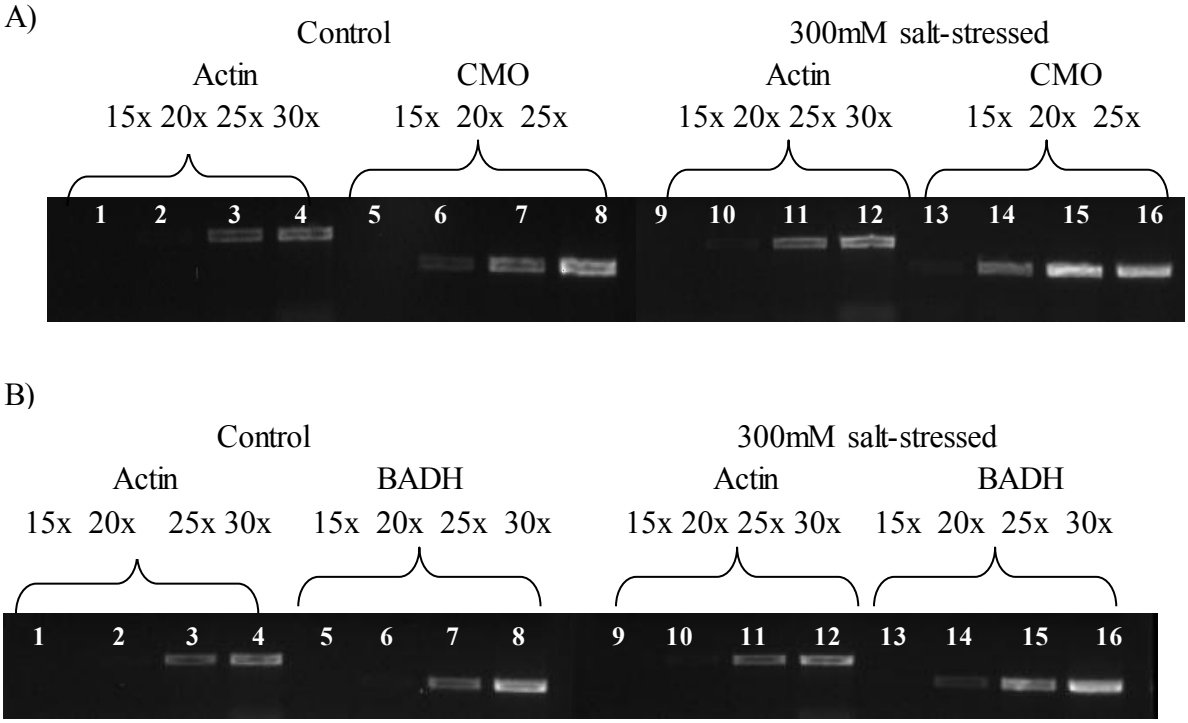


Figure 3.6: Optimisation of cycle numbers for semi-quantitative RT-PCR for *CMO* and *BADH* gene expression study

Table 3.4: Optimisation of cycle numbers for semi-quantitative RT-PCR for *Actin*, *CMO* and *BADH* gene expression

Salt condition	Lane Number	Number of cycles	CMO optimisation		BADH optimisation	
			Band description	Trace Int x mm*	Band description	Trace Int x mm*
Control	1	15	Actin	-	Actin	-
	2	20		433.851		532.309
	3	25		1210.612		1372.688
	4	30		1831.782		2293.340
	5	15	CMO	-	BADH	-
	6	20		855.485		890.446
	7	25		2186.262		1647.669
	8	30		2231.523		3681.175
300 mM NaCl	9	15	Actin	-	Actin	-
	10	20		405.309		624.120
	11	25		1127.496		1450.328
	12	30		1893.265		2403.719
	13	15	CMO	407.962	BADH	-
	14	20		1342.886		1016.316
	15	25		2608.724		3058.207
	16	30		2610.206		4359.761

Lane number indicates the sample loading well in Figure 3.6; * indicates the intensity of the band as calculated by BioRad quantity one software.

3.4.5 Expression of *CMO* and *BADH* genes of saltbushes is up-regulated under salinity stress

The *CMO* and *BADH* genes showed notable up-regulation relative to *Actin* in salt-stressed leaf tissues compared to control plants (Figure 3.7; Appendix VI). The *CMO* expression in leaves was 1.7 fold higher in *A. nummularia,* 1.5 fold higher in *A. semibaccata* and 1.3 fold higher in *A. amnicola*; and even higher in the roots (fold change (FC) of 2.2, 2.9 and 1.9 respectively). The *BADH* genes were also up-regulated in the leaf (FC of 2 in *A. nummularia*, 1.7 in *A. semibaccata* and 2.1 in *A. amnicola*), but exhibited little change in roots (FC of 1.1 in *A. nummularia* , 1.1 in *A. semibaccata* and 1.0 in *A. amnicola*)

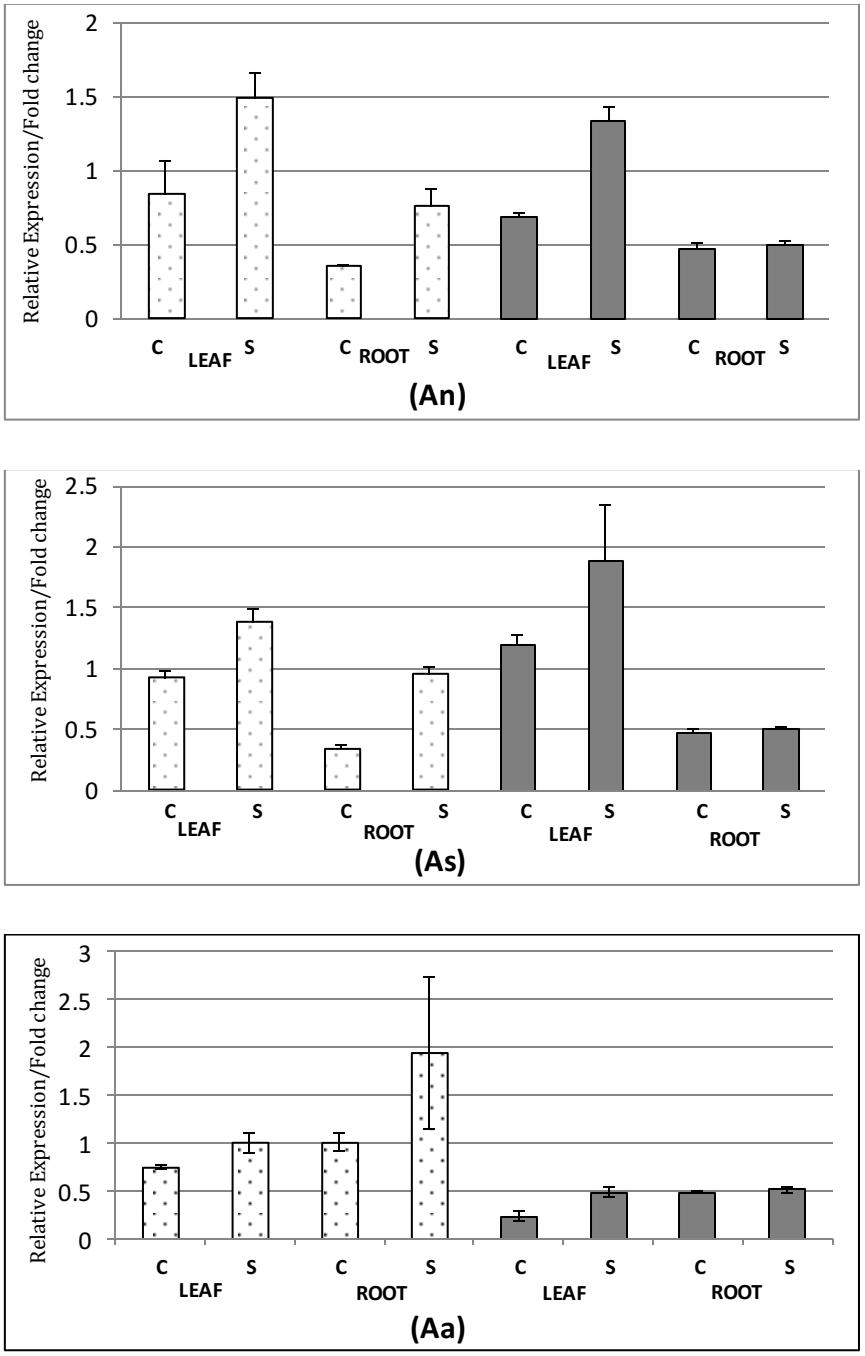


Figure 3.7: Relative expression levels of *CMO* and *BADH* genes in control and salt-stressed tissues of saltbushes.

The mRNA expression levels of *CMO* (white bars) and *BADH* (grey bars) in *A. nummularia* (An), *A. semibaccata* (As) and *A. amnicola* (Aa). Data from leaf and root tissues of control (C) and 300 mM salt-stressed (S) tissues, shown as normalized expression relative to actin. Each bar represents the average of three biological replicates (n=3). Data were analysed using one-way analysis of variance (ANOVA) in SPSS v.21 (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). The Least Significant Difference (LSD) and Tukey's-b post-hoc test was carried out to compare group means (where $p<0.05$ indicates significantly different, $p>0.05$ indicates not significantly different). Difference in expression between control and stressed leaves for *CMO* and *BADH*, and control and stressed roots for *CMO* in both plants were significant ($p=0.003$). Difference in expression between control and salt-stressed roots for *BADH* in all three plants were insignificant.

3.5 Results – Section B (Gene identification from wattles)

3.5.1 Cloning of *CMO* and *BADH* from cDNAs of *Acacia* leaf tissues

Although good quality RNA was extracted and the house keeping gene actin was amplified, *CMO* and *BADH* genes were not amplified from cDNAs of non-treated *Acacia pendula* (Figure 3.8), *Acacia victoriae* and *Acacia salicina* (gels not shown) after repeated attempts. On the other hand, cDNA from *Atriplex nummularia* used as a positive control lead to successful amplification of *Actin*, *CMO* and *BADH* (Figure 3.8). The reactions were also tried with cDNA from salt-stressed tissues but without any success. This prompted the question, if the genomic copies of *CMO* and *BADH* in leaf tissues of *Acacia pendula, Acacia victoriae* and *Acacia salicina* are actually present. Hence, genomic DNA was extracted and used for *CMO* and *BADH* gene amplification.

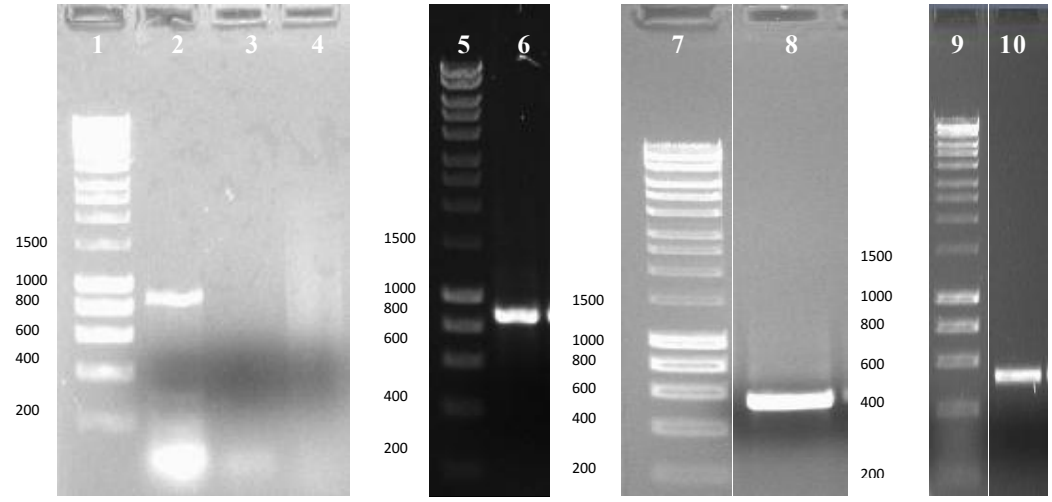


Figure 3.8: Amplification of *Acacia pendula CMO*, *BADH* and *Actin* gene from cDNA

Lanes 1, 5, 7 and 9: Hyperladder 1 marker; Lane 2: Amplification of *Actin* from *Acacia pendula* cDNA, lanes 3 and 4 show absence of *CMO* and *BADH* amplicons from the same cDNA; lane 6: amplification of partial *Actin* gene, lane 8: partial *CMO* gene and, lane 10: partial *BADH* gene from *Atriplex nummularia* cDNA.

3.5.2 Cloning of partial fragment of *CMO* gene from *Acacia* species

The use of Qiagen DNeasy Plant minikit facilitated the extraction of good quality genomic DNA which was used to amplify the *CMO* and *BADH* genes. After several attempts to amplify *BADH* from the *Acacia* species gDNA, it was inconclusive whether the *BADH* gene was absent or the degenerate primers being used were not as efficient as in amplifying *BADH* from saltbush gDNA. Several primer combinations were trialled to amplify the *CMO* gene from the genomic DNAs of *Acacia pendula*, *Acacia salicina* and *Acacia victoriae*. However, only one primer pair CMO F3-R3 yielded products

close to the expected size of 1061 bp based on *A. thaliana CMO* (Figure 3.9). As a positive control, *Atriplex semibaccata* gDNA was used to amplify partial *CMO* gene (close to 1500 bp) which was verified by cloning the product and sequencing (PCR data not shown, sequence data shown in Appendix IX). *Acacia pendula* and *Acacia salicina* produced multiple faint bands whereas *Acacia victoriae* showed one very bright band. Since multiple bands were obtained for *Acacia pendula* and *Acacia salicina,* a gradient PCR (Figure 3.9) at five different primer annealing temperatures (Ta) (48, 49.7, 50.9, 51.9 and 53°C) close to the estimated Ta (51°C) was tried to eliminate products that may have formed due to nonspecific binding of primers. Bands of the expected size and those close to it were gel-purified (Figure 3.9) and cloned into plasmids. At least 11-15 white clones (clones containing recombinant plasmids) were considered positive based on blue-white colony screening. To check if the positive white colonies contained recombinant plasmids with the desired insert size, a colony PCR was performed with vector specific T7 and SP6 primers (Figure 3.10). Initially, three clones from each plant species (with different insert size) were sequenced (*Acacia pendula*- clones 7, 8, 9; *Acacia salicina*- clones 2, 6, 11; and *Acacia victoriae*- clones 2, 3, 4). After the expected product was identified from the BLAST results of the sequence, two more clones with similar insert size were sequenced.

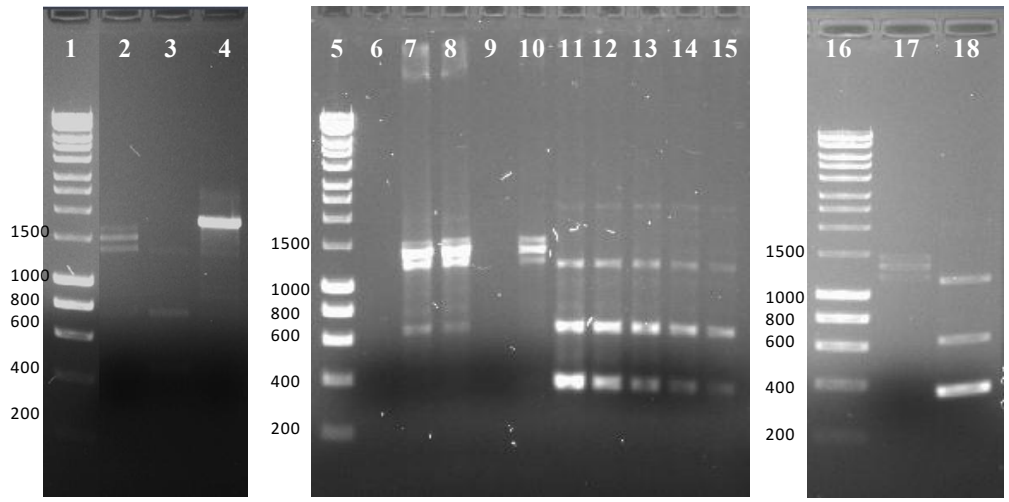


Figure 3.9: *CMO* PCR products for cloning and sequencing

Lanes 1, 5 and 16 denote Hyperladder 1 marker; Lane 2: *Acacia pendula CMO*, lane 3:*Acacia salicina CMO*, lane 4: *Acacia victoriae CMO*; Lanes 6-15 Gradient PCR to confirm amplification of multiple products from primer pair *CMO* F3-R3 amplified at 48, 49.7, 50.9, 51.9 and 53°C (lanes 6-10 *Acacia pendula CMO*; lanes 11 to 15 *Acacia salicina CMO*); Lane 17 and 18: Gel-purified *CMO* PCR products from *Acacia pendula* and *Acacia salicina*.

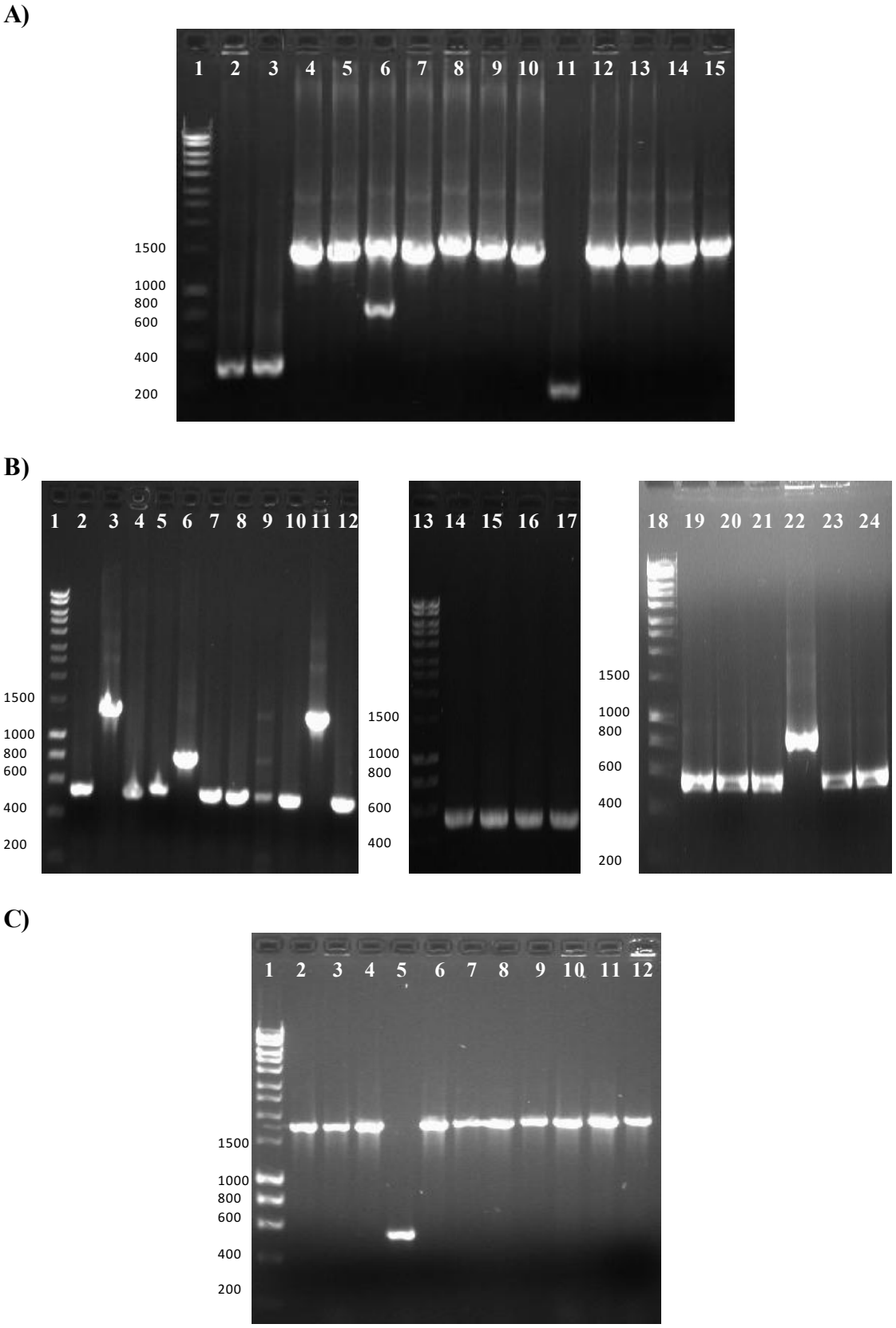


Figure 3.10: Screening of clones by colony PCR
Colony PCR to screen recombinant plasmids with partial (A) *Acacia pendula CMO* gene (lane 1: DNA Hyperladder 1 marker, lanes 2 to 15: clones 1 to 14); (B) *Acacia salicina CMO* gene (lane 1, 13 and 18: DNA Hyperladder 1 marker, lanes 2 to 15: clones 1 to 21); (C) *Acacia victoriae CMO* gene (lane 1: DNA Hyperladder 1 marker, lanes 2 to 12: clones 1 to 11).

3.5.3 Identification and characterisation of partial putative *CMO* gene from *Acacia* species

The sequencing results indicated the length of the amplified sections of the *CMO* genes of *Acacia pendula* and *Acacia victoriae* were 1353 bp and 1556 bp respectively (Appendix IX). Sequencing of *Acacia salicina* partial *CMO* clones 2 and 11, yielded incorrect products due to non-specific primer binding (sequence of clone 2 and its translated product is given in Appendix VIII). PCR product BLAST results confirmed the identity of both sequences as being genomic copies of *CMO* gene. *Acacia pendula CMO* had the highest % identity (based on maximum query sequence coverage) to *Atriplex nummularia* (97%; GenBank No.: AB112481.1) *CMO* cDNA, *Atriplex hortensis CMO* cDNA (97%; GenBank No.: AF270651.1) and *Atriplex prostrata CMO* cDNA (98%; GenBank No.: AY082068.1) (Appendix VII). *Acacia victoriae CMO* showed highest % identity of 93% only to *A. thaliana* choline monooxygenase (GenBank Nos.: NM_119135.4, BT028917.1, AY090377.1 and AB093586.1) (Appendix VII).

Alignment (manually using BioEdit) of the *S. oleracea* and *B. vulgaris CMO* cDNAs with the partial *CMO* genomic gene enabled the prediction of partial gene structures that contained two partial exons, four complete exons and five complete introns (Appendix IX). The predicted exons of the *CMO* genes from *Acacia pendula* and *Acacia victoriae* were then joined to deduce their individual putative cDNA contig. The partial CMO exon contigs were aligned (Figure 3.12) with other plant CMO sequences (trimmed to equal lengths) obtained from GenBank to produce a phylogenetic tree (Figure 3.11). Based on their phylogenetic relationship, *Acacia pendula* CMO was grouped with glycine betaine accumulators such as *Atriplex spp, S. oleracea and O. japonicus*, whereas *Acacia victoriae* was grouped with typical low/non-accumulators such as rice, barley and maize. These observations were further supported by percentage sequence identities of *Acacia pendula* and *Acacia victoriae* CMO with other plant CMOs. *Acacia pendula* CMO showed highest sequence identity to GB accumulators such as *Atriplex nummularia* (96.8%), *Atriplex semibaccata* (96.8%), *Atriplex amnicola* (96.8%) and *Atriplex hortensis* (96.3%). *Acacia victoriae* CMO showed highest percentage sequence identity to *Ricinus communis* (71.8%), *Lycium barbarum* (68.5%) and *Oryza sativa* (67.3%).

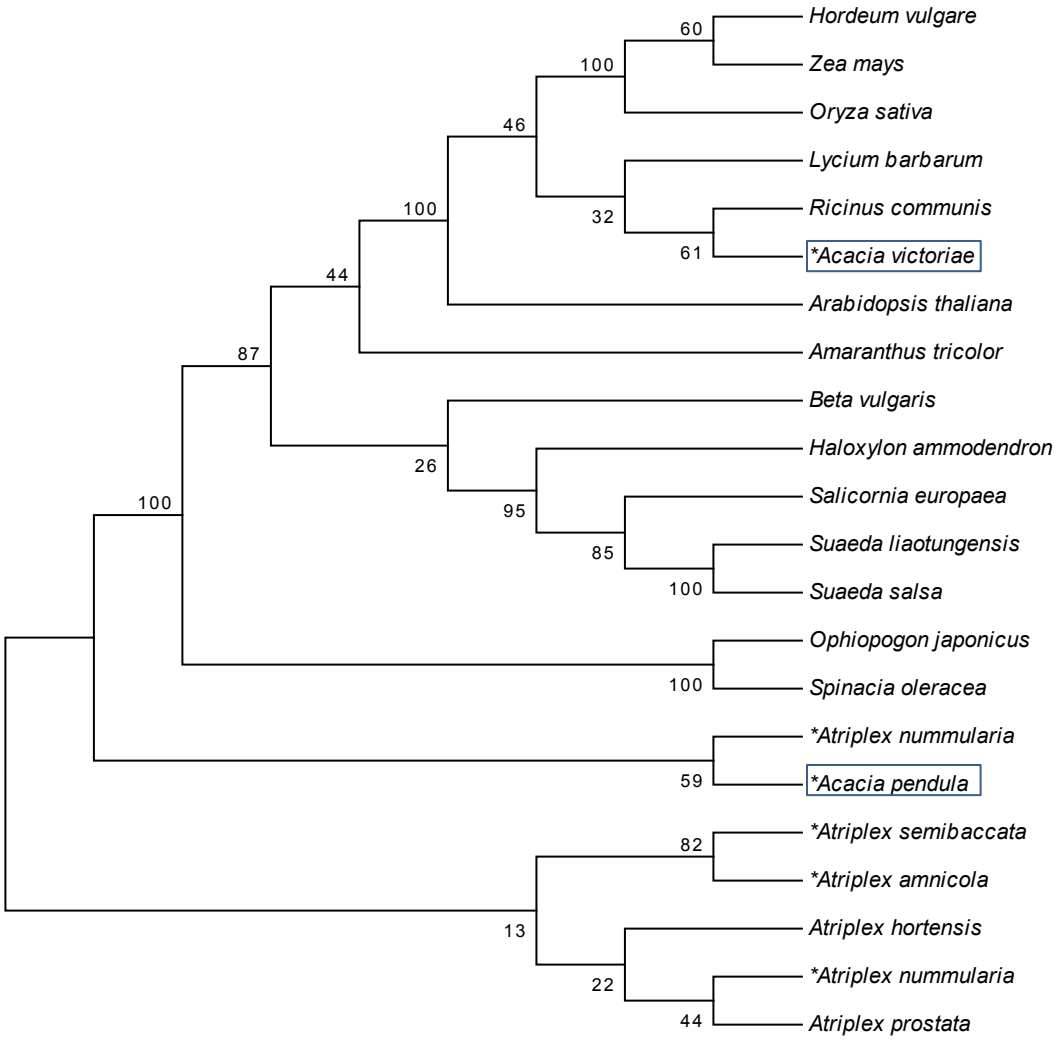


Figure 3.11: Phylogenetic tree showing the relationship between *Acacia pendula* and *Acacia victoriae* putative partial CMO with other partial plant CMOs.

The phylogenetic tree was constructed using the Neighbor-Joining method in MEGA5 with a boot strap replication value of 1000. Numbers at each branching node represents the confidence value of bootstrapping. All positions containing gaps and missing data were eliminated. There were a total of 188 positions in the final dataset. There were a total of 188 positions in the final dataset. An asterisk '*' indicates predicted protein deduced from exon contigs using experimentally obtained *CMO* gene sequence. All other full length protein sequences were obtained from GenBank and the sequence lengths trimmed after alignment. (*Spinacia oleracea* - EF362838.1, *Arabidopsis thaliana* - BAC21260.1, *Amaranthus tricolor* - AB303389.1, *Atriplex hortensis* - AF270651.1, *Atriplex nummularia* - AB112481.1, **Atriplex nummularia* - KC785451, **Atriplex semibaccata* - JX486549, *Atriplex prostrata* - AY082068.1, *Beta vulgaris* - AF023132.1, *Haloxylon ammodendron* - GQ379204.1, *Hordeum vulgare* - AB434467.1, *Lycium barbarum* - FJ514800.1, *Ophiopogon japonicus* - DQ645889.1, *Oryza sativa* Japonica - AJ578494.1, *Ricinus communis* - XM_00251821, *Salicornia europaea* - AY849925.1, *Suaeda liaotungensis* - AF354442.1, *Suaeda salsa* - DQ656523.1, *Zea mays* - DQ864498.1).

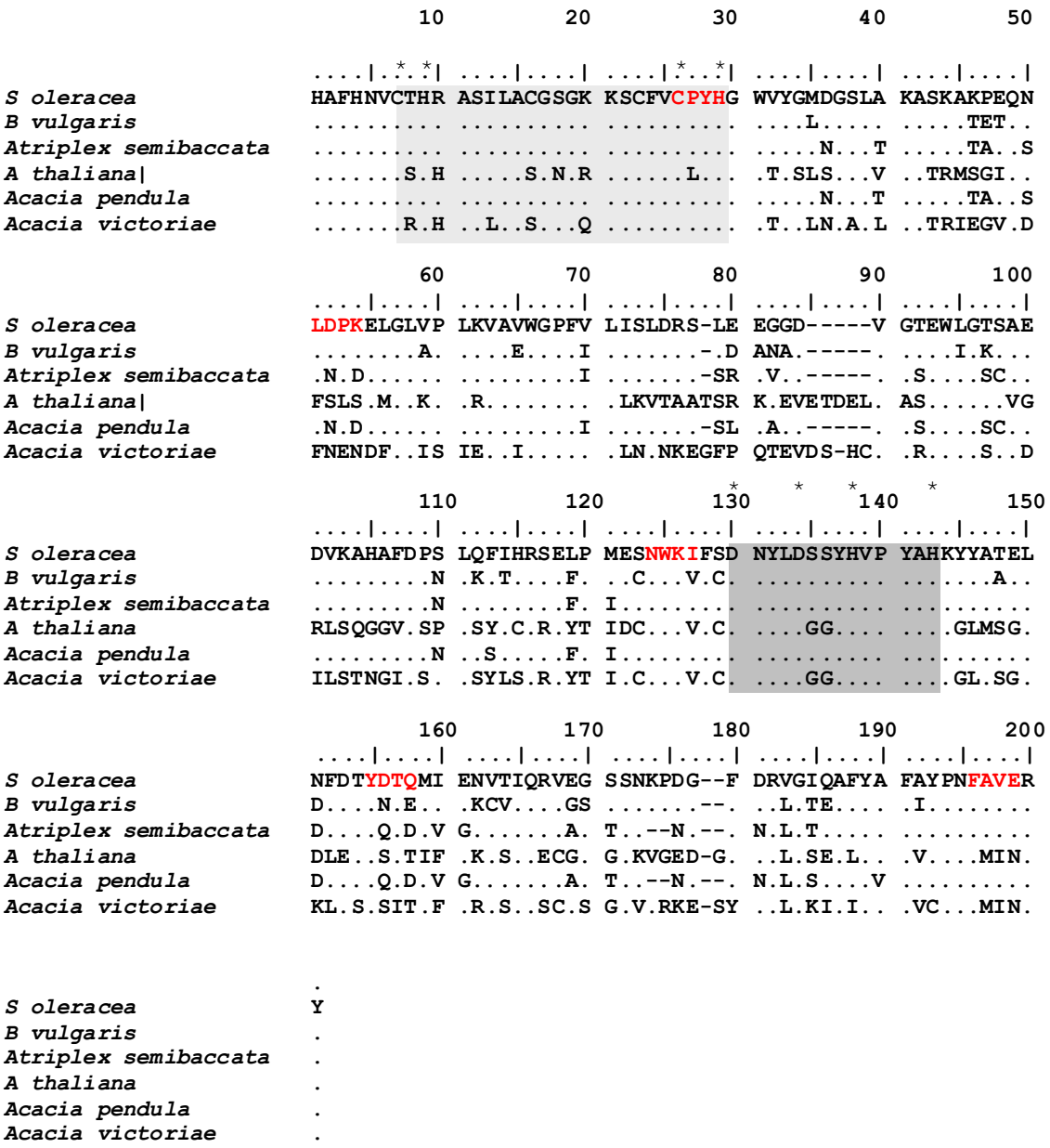


Figure 3.12: Alignment of the deduced amino acid sequences of *Acacia* CMOs.
Amino acid sequence alignment of the putative CMO proteins deduced from exon contigs of *CMO* gene from *Acacia pendula* and *Acacia victoriae* isolated in this study with the reported sequences of *Spinacia oleracea* (spinach) (GenBank ABN43460.1), *Beta vulgaris* AAB80954.1, *Atriplex semibaccata* - JX486549 and *Arabidopsis thaliana* (AEE85689.1). The alignments were created in ClustalW in BioEdit. A dot (.) indicates a conserved residue when aligned with *S. oleracea* CMO. A dash (-) indicates a gap introduced to optimally align the sequences, or a missing residue. The light-grey shaded box indicates the Rieske iron-sulfur center [2Fe-2S] ($CXHX_{16}CX_2H$), with the key cysteine (C7, C26) and histidine (H9, H29) residues, marked with asterisks. The dark-grey shaded box illustrates the conserved mononuclear non-heme Fe binding motif ($DX_3DX_3HX_4H$), with the key aspartic acid (D130, D134) and histidine (H138, H143) residues, marked with asterisks. Residues highlighted in red indicate terminal amino acid residues of an exon.

The translated exon contigs were subjected to CDD-Search to detect conserved sequence motifs. The most characteristic signature motifs present in plant CMOs are the Rieske type cluster 2Fe-2S ($CXHX_{15-17}CX_2H$) and the mononuclear non haeme cluster ($G/DX_{3-4}DX_2HX_{4-5}H$). Both these motifs were identified in the putative CMO cDNA contigs of *Acacia pendula* and *Acacia victoriae*. *Acacia pendula* showed 100% identity to other GB accumulators such as spinach and *Atriplex* spp, but *Acacia victoriae* CMO showed five residues changes in the Rieske type cluster and two changes in Mononuclear non heme cluster (Figure 3.13).

S oleracea	C T H R A S I L A C G S G K K S C F V C P Y H
B vulgaris	C T H R A S I L A C G S G K K S C F V C P Y H
Atriplex semibaccata	C T H R A S I L A C G S G K K S C F V C P Y H
A thaliana	C S H H A S I L A S G N G R K S C F V C L Y H
Acacia pendula	C T H R A S I L A C G S G K K S C F V C P Y H
Acacia victoriae	C R H H A S L L A S G S G Q K S C F V C P Y H
	* *

a) Rieske type cluster

S oleracea	D N Y L D S S Y H V P Y A H
B vulgaris	D N Y L D S S Y H V P Y A H
Atriplex semibaccata	D N Y L D S S Y H V P Y A H
A thaliana	D N Y L D G G Y H V P Y A H
Acacia pendula	D N Y L D S S Y H V P Y A H
Acacia victoriae	D N Y L D G G Y H V P Y A H
	* * * * * * * * * * * * * *

b) Mononuclear non heme cluster

Figure 3.13: Changes in amino acid residues in signature motifs of putative *Acacia pendula* and *Acacia victoriae* CMO.

Amino acid sequence alignment of signature motifs of the putative *Acacia* CMO proteins deduced from exon contigs along with the reported sequences of *Spinacia oleracea* (spinach) (GenBank ABN43460.1), *Beta vulgaris* AAB80954.1, *Atriplex semibaccata* - JX486549*Arabidopsis thaliana* (AEE85689.1).

3.5 Discussion

3.5.1 Glycine betaine biosynthetic genes are highly conserved in Australian saltbushes

Glycinebetaine has been implicated in playing a protective role in plants against various abiotic stresses including salinity, drought, heat, cold and water logging (reviewed in Khan *et al.,* 2009, Giri, 2011). As mentioned earlier, in plants the two major enzymes involved in GB biosynthesis are choline monooxygenase (CMO) and betaine aldehyde dehydrogenamse (BADH). In this study, putative CMO and BADH sequences of three

native Australian saltbush species, *Atriplex nummularia*, *Atriplex semibaccata* and *Atriplex amnicola*, were isolated for the first time (except *Atriplex nummularia* CMO) and analysed. Although *Atriplex nummularia* CMO was published earlier, no in depth analyses of its sequence is available.

CMO belongs to a group of novel Rieske non-heme iron oxygenase (RO) family, catalyzes the first step in GB synthesis in plants, and is not found in animals or bacteria. The presence of all essential conserved regions such as the Reiske-type cluster and mononuclear non-heme cluster in the putative CMOs of the saltbushes (Table 3.1), including a 58-residue chloroplast transit peptide proposed to be required for this subcellular localisation (Rathinasabapathi *et al.,* 1997), suggests these encode functional enzymes. Non-accumulators such as *Arabidopsis* and rice also have the Reiske-type and mononuclear non-heme clusters. However, they exhibit mutations at other residues considered functionally important (e.g., in *Arabidopsis* CMO; Hibino *et al.,* 2002), or process incorrect and shorter transcripts (e.g., rice CMO; Luo *et al.,* 2012), and vary significantly at their N-terminal (Appendix II). The Rieske motif consensus for AnCMO, AsCMO and AaCMO was $CXHX_{16}CX_2H$, identical to the spinach and sugar beet motifs and very similar to the plant consensus ($CXHX_{15-17}CX_2H$). The common signature motif for the mononuclear non-heme Fe cluster in oxygenase enzymes shows two residues after the second Asp (DXX) (Jiang *et al.,* 1996). The Asp and His may act as mononuclear Fe ligands at the site of oxygen activation (Jiang *et al.,* 1996), contributing to catalytic function. This cluster in AnCMO, AsCMO and AaCMO was $DX_3DX_3HX_4H$; however, this extra residue is consistent with all plant CMOs ($G/DX_{3-4}DX_2HX_{4-5}H$) (Appendix II). But several changes in these regions apart from the conserved cysteine (C185, C204; Appendix II) and histidine (H187, H207; Appendix II) residues were observed, persistent among the cereal/crop plants and *Arabidopsis*. Comparison of the *Arabidopsis* Rieske motif with these motifs (Appendix II) showed the changes S186T, R188H, C194S, S196N, K199R and P205L; except for *Hordeum vulgare, Lycium chinensis, Oryza sativa, Lycium barbarum, Ricinus communis* and *Zea mays*. These 6 species also exhibited changes around this region; although these do have a *CMO* gene, they are not known to accumulate GB abundantly. Changes in amino acid residues may affect the physicochemical properties of the protein as well as

protein folding; however the effects need to be investigated through site-directed mutagenesis.

A number of other functionally important sites identified by CDD-search, based on multiple alignments of well-annotated full-length proteins and conserved protein domains, were also noted in the AnCMO, AsCMO and AaCMO sequences, i.e., the active site, substrate (choline + O_2 + reduced ferredoxin + H^+) binding site, Fe-binding sits and a putative α-subunit interface (Table 3.1). The α-subunits are catalytic, their N-terminal binding to the Reiske cluster and the C-terminal binding to the non-heme Fe (CDD Search ID cd08883). Taken together, the observations suggest strongly that the saltbushes encode functional CMO enzymes. Reiske proteins are commonly found in chloroplasts and mitochondria (Balk and Lobreaux, 2005). For AnCMO, AsCMO and AaCMO, ChloroP indicated a cTP, and TargetP also indicated a cTP, with the strongest prediction (reliability class 1- AnCMO, AsCMO and reliability class 2- AaCMO). However, its predicted cTP length is inconclusive due to certain discrepancies. Firstly, the multiple sequence alignment suggested 58 residues (Figure 3.3), as all three putative saltbush CMOs showed the AVA residues found by peptide sequencing at the start of mature spinach CMO (Rathinasabapathi *et al.,* 1997). Secondly, ChloroP suggested the cTP length of 72 for AnCMO, 91 for AsCMO and 67 for AaCMO despite Ala61, Ala59 and Ala61 having the highest prediction scores, respectively. Finally, TargetP predicted the same cTP lengths (72 for AnCMO, 91 for AsCMO, 67 for AaCMO), as both software packages run the same scoring matrix derived from the MEME (Multiple Em for Motif Elicitation) algorithm. Hence, while there is a strong indication of chloroplast localization from the putative sequences, experimental determination of the actual processing sites is necessary.

The biochemical properties of the putative mature CMOs were construed using ProtParam (Table 3.2). The estimated molecular weights of 42.67 kDa, 42.54 kDa and 42.53 for monomers of AnCMO, AsCMO and AaCMO, respectively, are close to the experimentally determined values for other plants, e.g., spinach (42.8 kDa; Rathinasabapathi *et al.,* 1997), *Amaranthus tricolor* (>43 kDa; Meng *et al.,* 2001). However, the instability index of 40 or above predicts a protein to be unstable (Gasteiger *et al.,* 2005), hence these CMOs may not be monomeric. A homodimer or

possible homotrimer of 135 kDa is reported in spinach (Rathinasabapathi *et al.,* 1997), a dicot GB accumulator. The CMO of *A. thaliana,* a dicot non-accumulator, shows an instability index of 31, classifying it as stable. This may be partly due to the prediction of its transit peptide being by UniProt rather than experimentally. Interestingly, *O. japonicus,* a monocot, shows a pattern similar to the dicot accumulators. Although native GB accumulation data for *O. japonicus* is not reported, expression of *O. japonicus* CMO in tobacco led to 2-2.5 fold increase in GB accumulation (Liu *et al.,* 2010), suggestive of this monocot being an accumulator with a functional CMO.

BADHs belong to the class of pyridine nucleotide-dependent dehydrogenases. The putative BADHs identified in this study exhibited motifs of paramount structural and functional importance, including the two most conserved ones, the Glu- and Cys-active site motifs which play a role in the catalytic activity of BADHs. The recent tertiary structure predictions and X-ray crystallography of spinach SoBADH (PDB 4A0M) (Diaz-Sanchez *et al.,* 2012) support some of the residues being essential for substrate binding; mutations at these residues could thus alter the enzyme activity and subsequently GB accumulation. The spinach BADH structure also emphasized the significance of certain other residues that play a direct role in BADH activity, and shed some light on why certain BADHs have higher activity while others have little or none. While Ala441/Cys441 is found in GB accumulators, it is replaced by Ile441 in low/non GB accumulators (Diaz-Sanchez *et al.,* 2012 supplementary data). Tyr160, Trp167, Trp285 and Trp456 form a pocket that allows binding of the substrate betaine aldehyde (BA). This interaction is not affected by Ala441/Cys441; however, Ile441 decreases the size of the pocket, thereby inhibiting substrate binding (Diaz-Sanchez *et al.,* 2012). The Ala443 (equivalent to Ala441) is conserved in AnBADH, AsBADH and AaBADH (note the difference in number is due to deletion of residues 62-64 in spinach BADH; Figure 3.5). This suggests that all three saltbushes are potential GB accumulators, expressing functional BADH. Changes to the substrate-binding site may also facilitate, or prevent, the binding of substrates other than BA, e.g., in BADH-mediated oxidation of other aminoaldehydes such as 3-amino-propionaldehyde, 4-aminobutyraldehyde or 4-guanidinobutyraldehyde in *Avena sativa* (Livingstone *et al.,* 2003). Comparison of the physicochemical properties of saltbush BADHs (Table 3.2) did not reveal any notable differences. The classification of BADHs as stable may be due to the fact that post-

translational processing was not considered, as BADHs lack a typical N-terminal signal peptide, and the peroxisome-targeting SKL was also lacking. The prediction needs experimental investigation, as an atypical, unusually short N-terminal transit peptide has been hypothesised for spinach BADH (Weretilnyk and Hanson 1990).

Studies of enzyme properties and site-directed mutagenesis at selected residues may help engineer salt tolerance further. In addition to the detailed sequence analyses of CMO and BADH from *Atriplex nummularia, Atriplex semibaccata* and *Atriplex amnicola*, their close phylogenetic relationship with other members of the Amaranthaceae family and separation from the Poaceae plants, such as rice, barley and corn, suggested a strong possibility that these three saltbushes are GB accumulators. For CMO, much of the distinction into different groups can be attributed to differences in the protein sequence alignment of the N-terminal signal peptide (Appendix II). It is notable that *Arabidopsis* and cereals, such as rice, are known low or non GB accumulators (Hibino *et al.,* 2002, Luo *et al.,* 2012) and do not exhibit the AVA residues at the start of the mature polypeptide determined experimentally in spinach (Rathinasabapathi *et al.,* 1997), raising the question of whether their CMO pre-proteins are processed correctly. The results presented in this chapter are fundamental to further investigate the functionality of these genes in terms of gene expression and expression of GB.

The presence of genes may not necessarily indicate actual gene expression or high GB accumulation, as seen in *A. thaliana* (Hibino *et al.,* 2002). Several other factors need to be considered, e.g., regulation of substrate synthesis, intracellular synthesis and transport, accumulation and degradation, and physicochemical properties of the enzyme including key residues (Rao *et al.,* 2006). Hence, this study also investigated the gene expression and GB accumulation in the selected saltbushes under salinity stress. Semi-quantitative mRNA expression analysis showed up-regulation of *CMO* in both types of tissue (leaf and root). *BADH* showed substantial upregulation in leaves only, in agreement with previous reports focussing on *BADH* expression in leaves (Nuccio *et al.,* 1998, Ahmad *et al.,* 2008), with only a few on *BADH* expression in roots (Nakamura *et al.,* 2001). In barley, one isozyme of *BADH, BBD1*, showed two-fold increase in expression under salinity, drought and abscisic acid, whereas *BBD2* showed

only a slight increase under salinity, drought and cold (Nakamura *et al.,* 2001). In the mangrove *Avicennia marina*, one type of the root *BADH* mRNA showed a 2-fold increase but no change in another type (Hibino *et al.,* 2001). These observations suggest a possibility of more than one *BADH* isozymes may be present. However, the cDNA sequences of both species did not provide any evidence of this. Expression of *CMO* and *BADH* transgenically into non GB accumulating plants such as *A. thaliana* (Hibino *et al.,* 2002), rice (Shirasawa *et al.,* 2006) and tobacco (*Nicotiana tabacum*) (Yang *et al.,* 2008) led to significant levels of tolerance to salinity. Thus, strong induction of these genes in saltbushes indicates their involvement in the extreme innate salt and drought tolerance of these plants.

3.5.2 Glycine betaine biosynthesis may not occur in *Acacia* species

This study is the first to provide evidence that *Acacia* species do have the underlying genetic mechanism for producing GB. The aim of this study was to investigate the likelihood of a GB biosynthetic pathway in three reportedly salt tolerant *Acacia* species (*Acacia pendula, Acacia salicina, Acacia victoriae*) at both gene isolation and expression levels, and explore the possible involvement of GB in their salinity stress tolerance response. The absence of any amplification of BADH in the tested *Acacia* species may be due to primer degeneracy/specificity or may be its suggestively low gene copy number in the *Acacia* genome. On the other hand, the amplification of *CMO* from the gDNA of *Acacia pendula* and *Acacia victoriae* was successful.

Although not transcriptionally active, the mere presence of the *CMO* gene opens a plethora of future alternatives to manipulate the biochemical pathway in order to synthesise GB. Based on sequence similarity and phylogenetic relationships (Figure 3.11), *Acacia pendula* is a better candidate for further investigating the means to activate the innate GB biosynthetic pathway. *Acacia pendula* CMO showed a higher degree of signature motifs conservation which is an essential factor for a functional CMO. The relationship of *Acacia pendula* CMO to CMOs from other GB accumulating plants is a good indication of its potential GB accumulating ability. On the other hand, *Acacia victoriae* CMO exhibited significant differences in the conserved domains and was more related to the CMO from the non-accumulator *A. thaliana* (Hibino *et al.,* 2002). Considering the fact that acacias are native Australian vegetation that

predominantly grow in water/nutrient deficient and salt degraded lands, enhancing additional qualities such as GB production would contribute to sustainable farm forestry and animal feed. In order to provide substantial evidence that GB may not be synthesised by these *Acacia* species further enzyme and biochemical tests are required. The results of these confirmatory experiments are present in Chapter 4.

Chapter 4

Biochemical analysis of osmoprotectant compounds and/or relevant biosynthetic enzymes in selected salt bush and *Acacia* species

4.1 Abstract

Plant adaptation to environmental stresses is a complex mechanism that is activated by a cascade of genomics, proteomics and metabolomics networks. This study focussed on examining the role of certain metabolites (osmoprotectants) in response to salinity stress in the selected Australian native plants. Reliable methods for HPLC detection of GB and trehalose were developed and optimised. The results showed that under salt stress, the saltbushes accumulated high levels of GB (*Atriplex nummularia* 29.69 mmol kg^{-1} FW, *A. semibaccata* 42.57 mmol kg^{-1} FW, *A. amnicola* 5.20 mmol kg^{-1} FW) compared to reported levels in cereals such as barley (4-9 mmol GB kg^{-1} FW) and wheat (12 mmol GB kg^{-1} FW), owing to their extremely conserved gene sequences as shown in chapter 3. This data was further supported by the high BADH enzyme activity in saltbushes. However, the *Acacia* species did not produce any detectable levels of GB and may be attributed to their transcriptionally inactive/absent genes. Proline content was determined using ninhydrin reagent and was found to be enhanced in *Atriplex* and *Acacia* genera under salt stress. *Acacia victoriae* displayed increased P5CS enzyme activity and no change in PDH enzyme activity (relative to control plants) and produced the highest amount of proline 6.97 µmoles mg^{-1} FW of leaf tissue under salt conditions. Trehalose accumulation was not detected in both groups (*Acacia* and *Atriplex* species) under control and stressed conditions. The results strongly suggest the role of GB and proline in plants under salt stress and may contribute to their inherent salt tolerant nature. The high GB level produced by *Atriplex semibaccata* accentuates its forage qualities on salinity-prone land.

4.2 Introduction

There are several classes of osmoprotectants as mentioned in Chapter 1. This chapter focusses on a quaternary ammonium compound, glycine betaine (GB), and the amino acid proline, because of their reported prominence in abiotic stress tolerance mechanism of plants, and also investigates trehalose accumulation, known to function as an osmoprotectant in other organisms but with little information available on its function in higher plants. As detailed in section 1.8.5, saltbushes make a highly desirable mixed-fodder species and have been successfully used in many large animal grazing trials in Africa and Australia (reviewed in Ben-Salem *et al.,* 2010). Glycine betaine has been shown in independent trials to improve the health of pigs and poultry (reviewed in Ratriyanto *et al.,* 2009). It is thus a logical question whether the high degree of salt tolerance in saltbushes and their food functionality may be related to expression of GB. The results presented in Chapter 3 confirmed the presence of transcriptionally active GB biosynthetic genes in saltbushes paving the way for further biochemical testing.

Proline accumulation is a common response by plants under abiotic stress (section 1.9.3). Previous studies on five *Acacia spp*. have shown significant increase in proline levels in leaves and roots (Yokota, 2003). Proline content increases considerably to maintain cytoplasmic solute potential and then decreases on alleviation of stress (Sharma and Verslues, 2010); hence proline accumulation patterns may indicate the stress condition of plants.

This chapter thus aimed to address the following questions:

- Do the saltbushes (*Atriplex* species) and wattles (*Acacia* species) under study synthesise GB?
- Do they have a proline biosynthetic pathway, and is it responsive to salt stress?
- Do these plants produce trehalose at levels that may play a role in salinity stress?

In the case of *Acacia* species, many are able to withstand harsh environmental conditions, but there are limited reports on the biochemical basis of their tolerance. Addressing these gaps is essential if these species are to be fully exploited for environmental and economic sustainability on saline and/or drought-prone land.

4.3 Experimental design

To ensure a thorough exploration of salinity-induced accumulation patterns of glycine betaine, proline and trehalose in the select group of *Atriplex* and *Acacia* species, the accumulation levels of these compounds was investigated using different experimental approaches. The experimental design differs due to low detection level of metabolites. The first step was the quantification of each of the metabolites. This was accompanied or followed by examining the expression of key enzymes involved in the respective biochemical pathways. In some cases, this led to investigations at both levels i.e. at the biochemical level and enzyme level (as in glycine betaine and proline biosynthesis). When there was no result obtained for biochemical quantitation, studies on enzyme activity were not conducted (e.g., for trehalose analysis).

4.4 Results

4.4.1 Glycine betaine quantitation in native plants

4.4.1.1 HPLC method optimisation for GB detection

The method was developed using 75:25 acetonitrile: water solvent system. The flow rate was optimised at 1 mL/minute and the wavelength for detection was optimal at 190 nm. Glycine betaine standards in 50% ethanol at these conditions showed clear peak resolution and had an estimated retention time of 4.6 to 5.0 minutes on average (Figure 4.1; Table 4.1). These optimised conditions were used for analysis of plant samples. The results obtained for method validation were as per the International Conference on Harmonisation (ICH, 1996) and are given in Table 4.2. The linearity of the method was established from standard curve calibration (Figure 4.2). The typical linear regression value (R^2) was 0.9992 for the concentration range of 50 - 1000 µg/mL. The linear regression equation, used for calculating the amount of GB in the test leaf samples, was x=y-c/m where x is the amount of GB, y is the average peak area (response) of the test sample, C is the intercept (C= 21295) and m is the slope of the standard curve (m or S= 2447.3). The precision of the method was established based on % Relative Standard Deviation (%RSD) for intraday and interday repeatability (ICH, 1996). The intraday precision %RSD was 0.71%, 0.44% and 0.29% for 50, 300 and 500 µg/mL of the standard betaine solutions. The interday precision %RSD was 0.61%, 1.53% and 0.41% for the standards. These values are well below the stipulated ±2% according to ICH

guidelines (ICH, 1996). The limit of detection was 0.58 µg/mL and the limit of quantitation was 1.75 µg/mL.

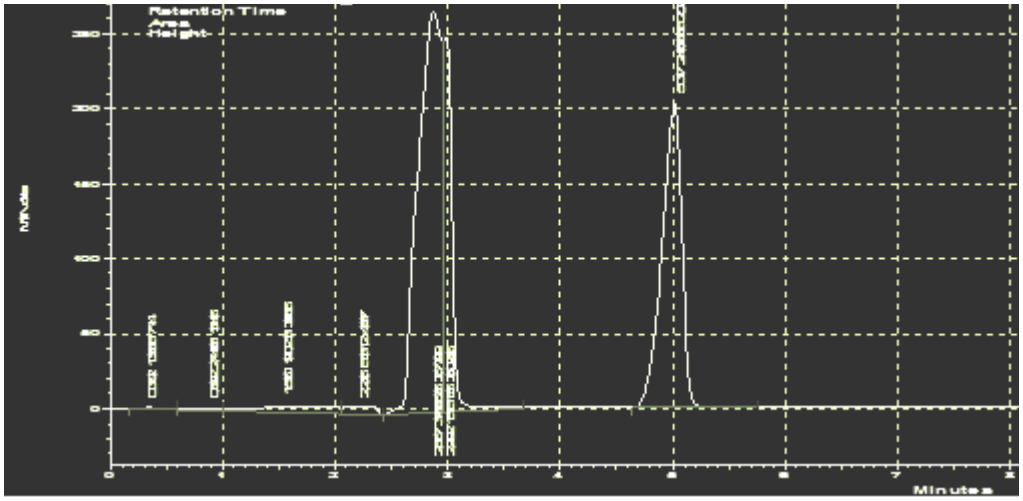


Figure 4.1: HPLC chromatogram of betaine 1 mg/mL in 50% ethanol

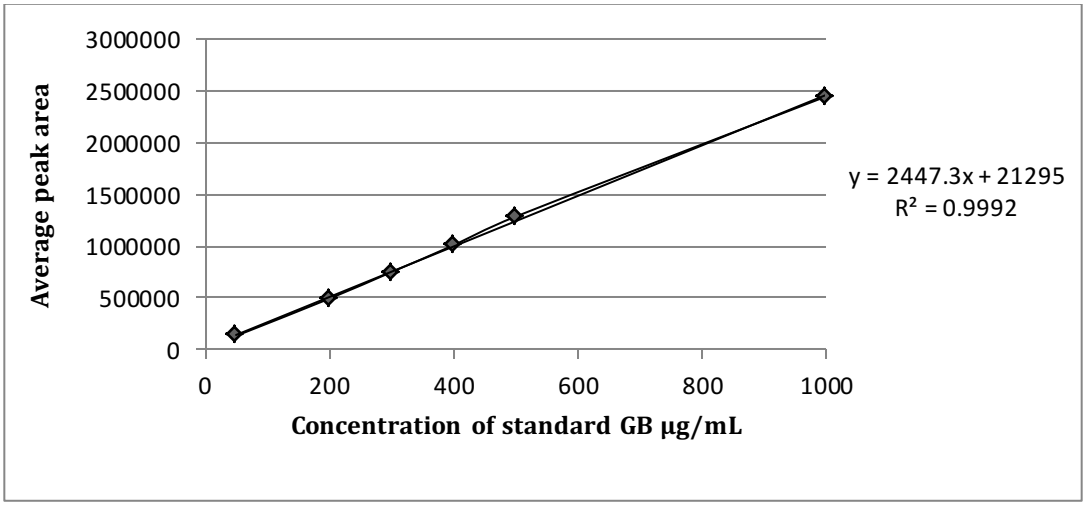


Figure 4.2: Standard curve calibration of glycine betaine (50 to 1000 µg/mL)

Table 4.1: Average peak area* and retention times of standard GB solutions

Average Retention time (minute)	Concentration of GB (µg/mL)	Average peak area ± Standard deviation
5.0	1000	2448904.667 ± 30318.09
4.7	500	1287204.667 ± 3745.969
4.7	400	1010402.667 ± 3863.633
4.6	300	750453 ± 3312.522
4.7	200	493204.667 ± 752.823
4.8	100	328482.667 ± 2135.429
4.8	50	133460.667 ± 316.658

*Average peak area was determined as a mean of three HPLC runs per 10 µL standard.

Table 4.2: Results obtained for method validation based on five parameters defined by International Conference on Harmonisation (ICH)

Parameter	Result
Range	50 - 1000 µg/mL
Linearity	Correlation coefficient R^2 = 0.9992 Slope (S) = 2447.3 Intercept = 21295
Intraday precision %RSD	50 µg/mL = 0.71% 300 µg/mL = 0.44% 500 µg/mL = 0.29%
Interday precision %RSD	50 µg/mL = 0.61% 300 µg/mL = 1.53% 500 µg/mL = 0.41%
Limit of detection	0.58 µg/mL
Limit of quantitation	1.75 µg/mL

4.4.1.2 Quantitation of GB in leaf extracts

Leaf samples were primarily chosen for GB analysis because this tissue is known to accumulate GB and is typically analysed (Rhodes and Hanson, 1993). Other tissues were not analysed due to the inadequate amounts or unsuitable quality for the extensive biochemical analyses. Sample chromatograms of *Atriplex nummularia* control and salt-stressed tissue extracts are shown in Figure 4.4 and its corresponding data on the average peak area used to determine the concentration of GB in ethanolic leaf extracts is shown in Table 4.3. The average retention time for GB in leaf extracts was 4.7–4.9 minutes. The amount of GB expressed per milligram of fresh leaf tissue (µg of GB/mg FW) of all six species tested is shown in Table 4.4. Among them, *A. semibaccata* produced the highest quantities of GB, higher than *A. nummularia* and *A. amnicola* under control as well as salt-stressed conditions (Figure 4.3). There was a small, statistically insignificant difference in the GB levels in control plants of *A. nummularia* (1.33 ± 0.34 µg of GB/mg FW) and *A. semibaccata* (1.96 ± 0.2 µg of GB/mg FW), whereas *A. amnicola* showed a considerably lower amount (0.32 ± 0.05 µg of GB/mg FW). Interestingly, the salt-stressed plants showed 2.6, 2.6 and 1.9 fold higher levels of GB (*A. nummularia* 3.48 ± 0.24, *A. semibaccata* 5.00 ± 0.29, *A. amnicola* 0.61 ± 0.11) than control plants. However, among the three *Acacia* species tested, no peaks were evident around the estimated retention time of standard GB, indicating the absence of

GB. Samples of *Acacia salicina* chromatograms for leaf tissues are shown in Figure 4.4.

Table 4.3: Average peak area and retention times of GB present in 50% ethanolic leaf extracts of *Atriplex nummularia (An)*

Average Retention time (minutes)	Sample	Average peak area ± Standard deviation	Total amount of leaf sample (mg)	Amount of GB in total sample (mg/ml)
4.9	*An* Control 1	259536.333 ± 347.7358512	54.8	97.3486427
4.8	*An* Control 2	306016 ± 2005.479	74.6	116.340865
4.7	*An* Control 3	93745.333 ± 2897.703	45.3	29.6041897
4.9	*An* Stressed 1	438680.667 ± 1190.999	51.7	170.549449
4.9	*An* Stressed 2	603756.667 ± 893.6015	60.3	238.001743
4.8	*An* Stressed 3	541982.333 ± 1031.653	66.7	212.759912

Average peak area was determined as a mean of three HPLC runs per 10 µL leaf extract.

Table 4.4: GB accumulation in native plants

Plant	Amount of GB (µg of GB/mg FW)		Amount of GB (mM of GB/kg of FW)	
	Control	Salt-stressed	Control	Salt-stressed
Atriplex nummularia (An)	1.33 ± 0.344	3.48 ± 0.236*	11.35 ± 2.936	29.69 ± 2.017*
Atriplex semibaccata (As)	1.96 ± 0.196	5.00 ± 0.289*	16.73 ± 1.667	42.57 ± 2.461*
Atriplex amnicola (Aa)	0.32 ± 0.051	0.61 ± 0.106*	2.74 ± 0.432	5.20 ± 0.897*
Acacia pendula (Apen)	ND	ND	ND	ND
Acacia salicina (Asal)	ND	ND	ND	ND
Acacia victoriae (Avic)	ND	ND	ND	ND

FW= Fresh weight of leaf tissue. Glycine betaine (mM) was calculated from the molecular weight of betaine 117.146 (mass/molecular weight). An asterisk (*) indicates significant difference at $p<0.05$ between control and salt-stressed samples. ND: "Not Detected".

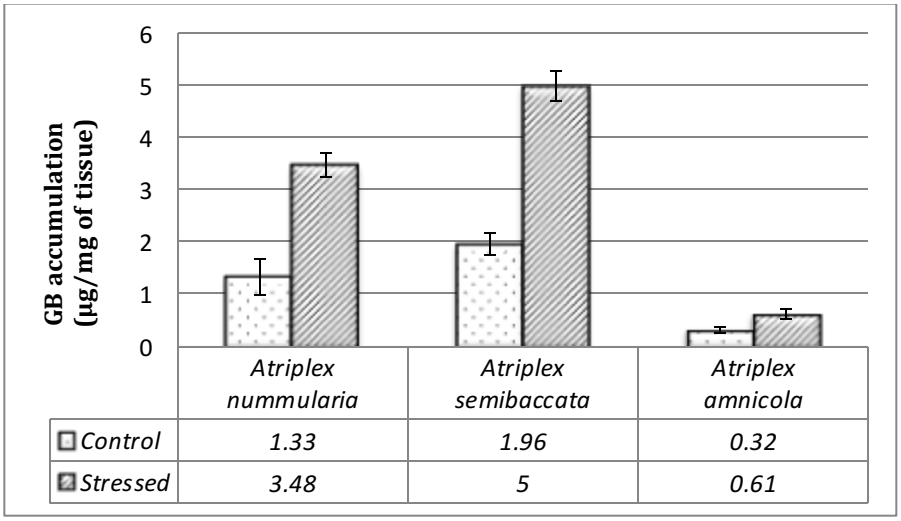


Figure 4.3: Glycine betaine accumulation in saltbush leaf tissues

* indicates statistically significant ($p<0.05$) difference in values between control and salt treatments

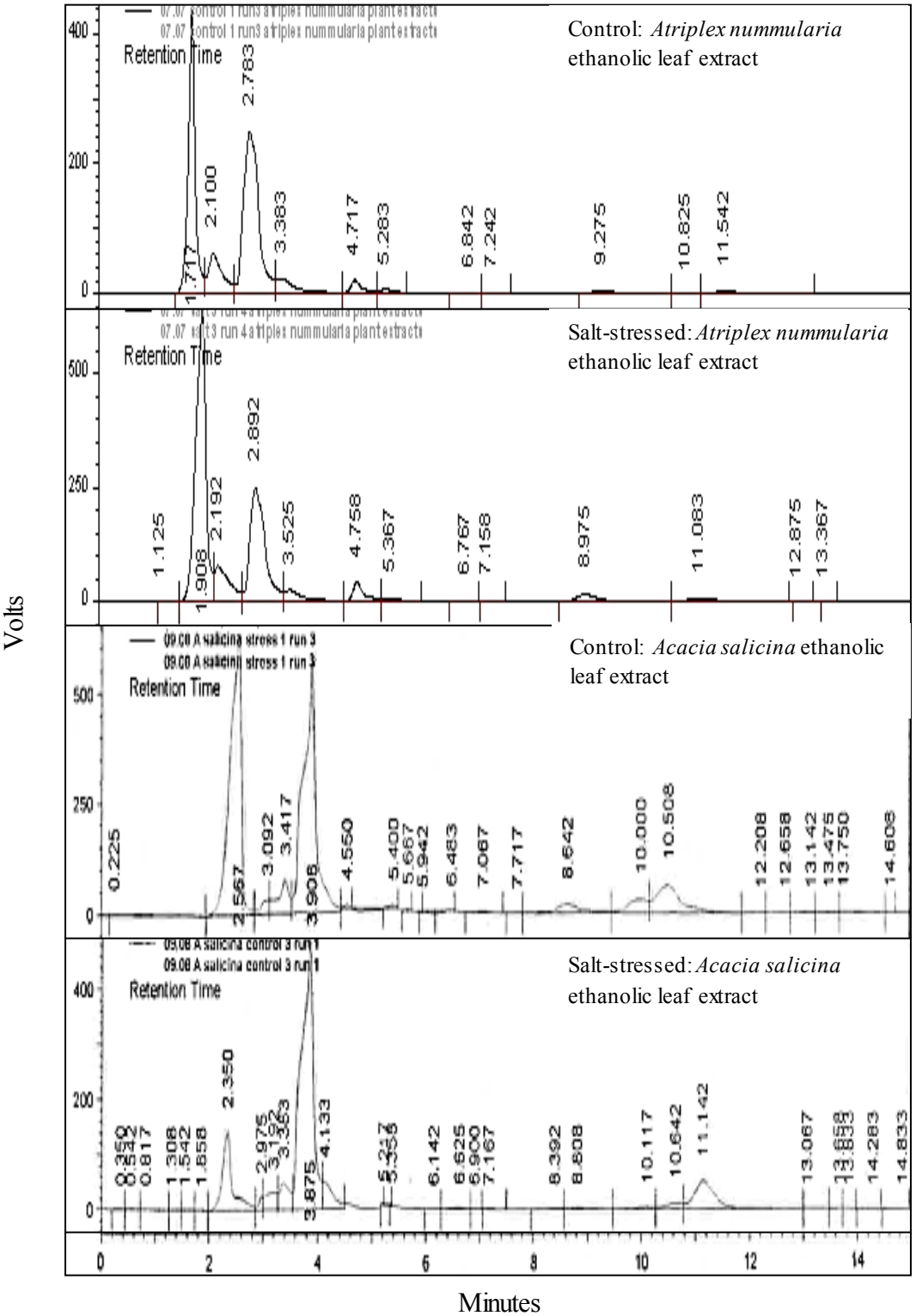


Figure 4.4: Sample HPLC chromatograms of control and salt-stressed *Atriplex nummularia* and *Acacia salicina* ethanolic leaf extracts

Glycine betaine seen with a retention time of 4.717 minutes for control and 4.758 for salt-stressed leaf samples of *Atriplex nummularia*.

4.4.1.3 Total protein content

The protein content in each plant extract was estimated using a standard curve plotted with varying concentrations of BSA and their corresponding absorbance at 595 nm (Figure 4.5). Protein concentrations were calculated using the formula x= (y-C)/m, where m is the slope (m) and C is the intercept of the standard curve. For the *Atriplex* species, m= 3.922 and C= 0.0111; and for the *Acacia* species, m= 4.6783 and C= 0.0728. Table 4.5 shows the estimated concentration of protein in *Atriplex nummularia (An), Atriplex semibaccata (As), Atriplex amnicola (Aa)*; and *Acacia salicina (Asal), Acacia victoriae (Avic), Acacia pendula (Apen).*

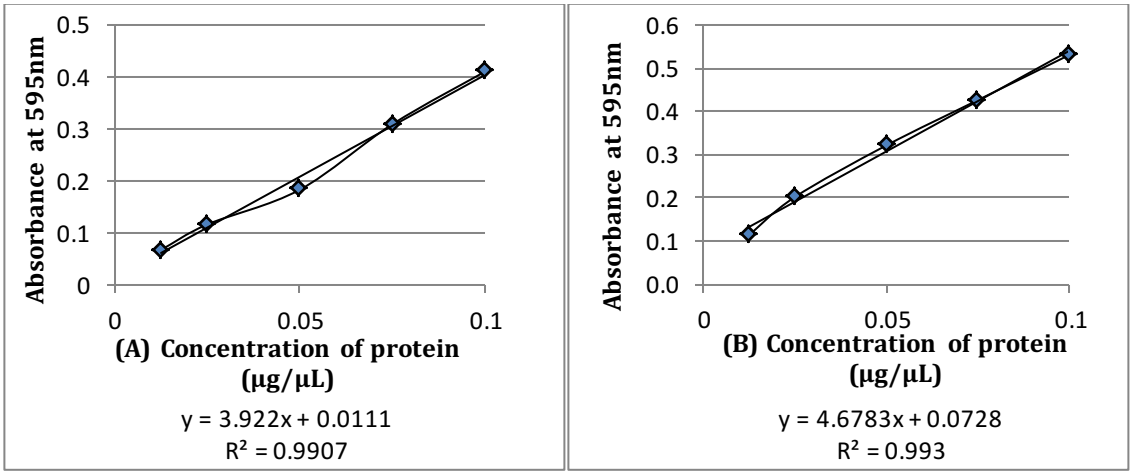


Figure 4.5: Standard curves for estimation of protein concentration in leaf extracts to determine BADH activity

A. Standard curve for *Atriplex* spp. B. Standard curve for *Acacia* spp.

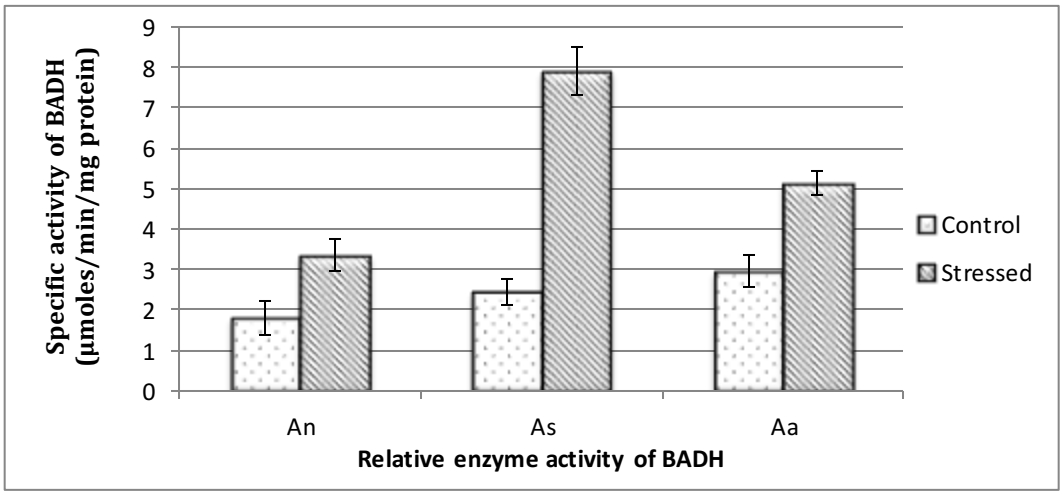
Table 4.5: Total protein concentration from control and salt-stressed leaf tissues

Plant	Average protein concentration (mg/mL)	
	Control	Salt-stressed
Atriplex nummularia	0.162 ± 0.017	0.193 ± 0.006
Atriplex semibaccata	0.140 ± 0.033	0.128 ± 0.007
Atriplex amnicola	0.063 ± 0.029	0.085 ± 0.050
Acacia pendula	0.358 ± 0.110	0.299 ± 0.090*
Acacia salicina	0.313 ± 0.163	0.371 ± 0.077*
Acacia victoriae	0.185 ± 0.050	0.310 ± 0.033*

* indicates statistically significant ($p<0.05$) difference in values between control and salt treatments

4.4.1.4 Activity of BADH enzyme

Following the estimation of total protein concentration, BADH enzymatic activity of the various plant extracts were assayed. Crude leaf extracts from salt-stressed plants showed elevated levels of activity compared to control samples (Figure 4.6). *Atriplex semibaccata* showed three times more specific activity of BADH in salt-stressed leaf tissues (7.905 ± 0.605 µmoles/minute/mg protein), compared to control tissues (2.433 ± 0.313 µmoles/minute/mg protein). *Atriplex amnicola* showed a significant increase in specific activity from 2.952 ± 0.391 µmoles/min/mg protein in control tissues to 5.137 ± 0.309 µmoles/minute/mg protein in salt-stressed tissues. *Atriplex nummularia* also showed a significant increase from 1.803 ± 0.417 µmoles/min/mg protein (control) to 3.346 ± 0.393 µmoles/minute/mg protein.



Plant	Specific activity Units/mg protein	
	Control	Salt-stressed
Atriplex nummularia	1.803 ± 0.417	3.346 ± 0.393*
Atriplex semibaccata	2.433 ± 0.313	7.905 ± 0.605*
Atriplex amnicola	2.952 ± 0.391	5.137 ± 0.309*

Figure 4.6: Specific activity of BADH enzyme in leaf extracts

* indicates statistically significant ($p<0.05$) difference in values between control and salt treatments

4.4.2 Proline quantitation in native plants

4.4.2.1 Proline quantitation

The amount of proline was estimated using the ninhydrin based protocol of Bates *et al.* (1973). A standard curve was developed (sample shown in Figure 4.7) and the amounts expressed as nmoles per mg fresh weight (FW) of leaf tissue. Among the six native species tested, *Acacia victoriae* expressed the highest amount of 6.97 nmoles proline per mg FW of leaf tissue under salt treatment and 1.62 nmoles under control conditions.

On average, the acacias (4.55±2.36 nmoles proline per mg FW of leaf tissue) produced 2-fold more proline compared to the saltbushes under salt treatment (2.24 ± 1.36 nmoles per mg FW) (Figure 4.8). Under salinity, *Atriplex nummularia* (3.01 ± 0.68 nmoles proline per mg FW) produced more proline compared to *Atriplex semibaccata* (2.22 ± 1.16 nmoles per mg FW) and *Atriplex amnicola* (1.48 ± 0.22 nmoles per mg FW). *Acacia victoriae* produced higher levels of proline compared to *Acacia salicina* (3.98 ± 0.80 nmoles per mg FW) and *Acacia pendula* (1.48 ± 0.11 nmoles per mg FW).

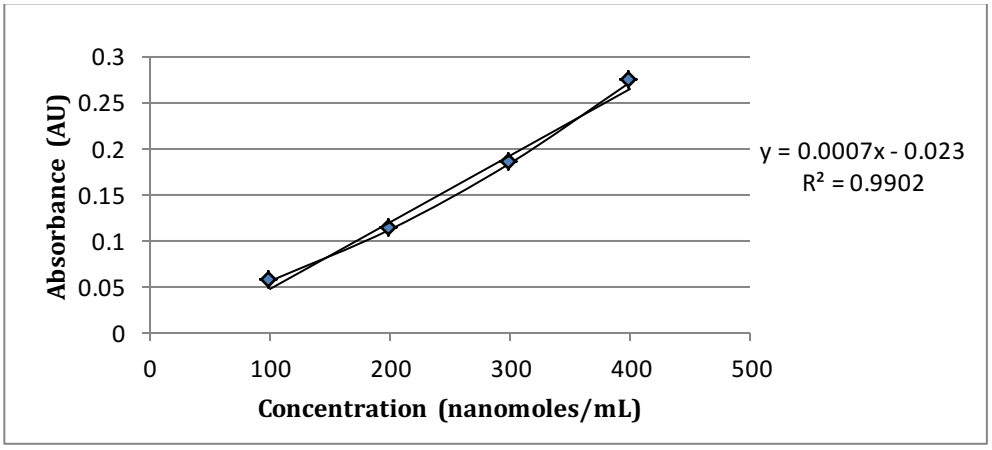


Figure 4.7: Standard curve for estimation of proline concentration in leaf extracts

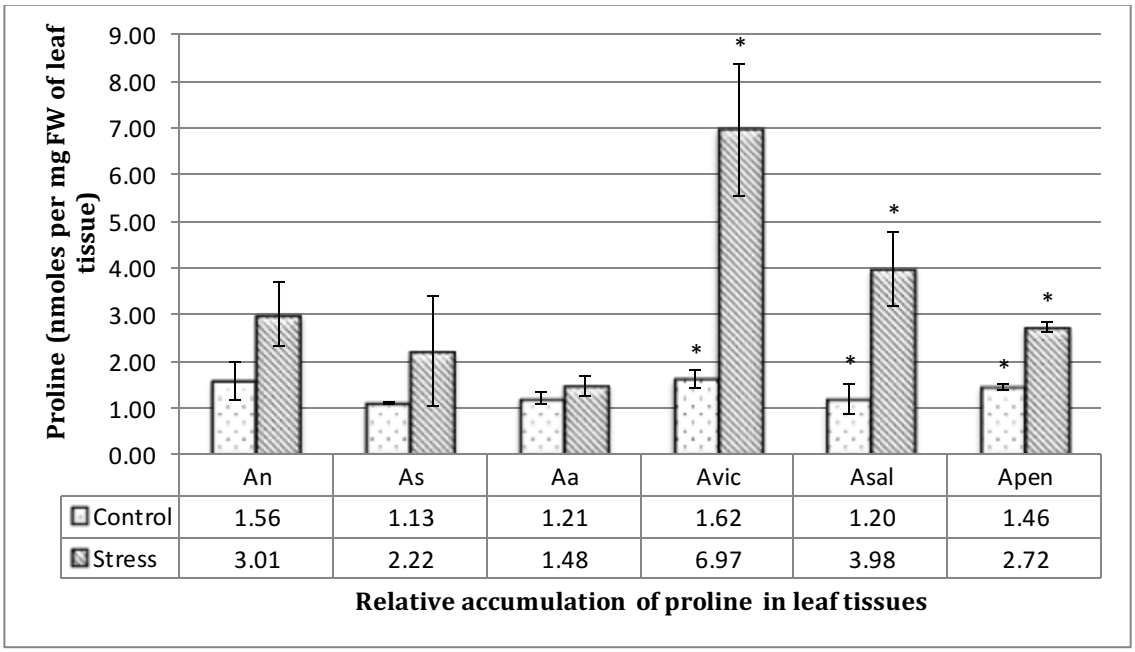


Figure 4.8: Estimation of proline concentration in *Atriplex* spp. and *Acacia* spp. leaf extracts

* indicates statistically significant ($p<0.05$) difference in values between control and salt treatments (An: *Atriplex nummularia*; As: *Atriplex semibaccata*; Aa: *Atriplex amnicola*; Avic: *Acacia victoriae*; Asal: *Acacia salicina*; Apen: *Acacia pendula*)

4.4.2.2 Total protein content

The concentration of protein in each plant extract was estimated using a standard curve plotted with varying concentrations of BSA and their corresponding absorbance unit at 595 nm (Figure 4.9). Protein concentrations were calculated using the formula x = (y-C)/m, where m is the slope (m) and C is the intercept of the standard curve. For the *Atriplex* species, m= 3.922 and C= 0.0111; and for the *Acacia* species, m= 4.6783 and C= 0.0728. Table 4.6 shows the estimated concentration of protein in *Atriplex nummularia (An), Atriplex semibaccata (As), Atriplex amnicola (Aa)*; and *Acacia salicina (Asal), Acacia victoriae (Avic), Acacia pendula (Apen).*

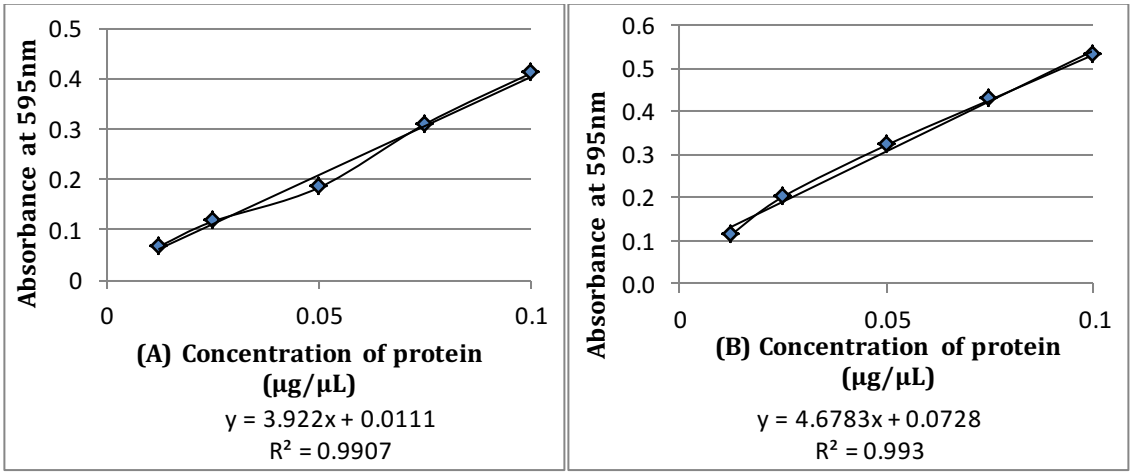


Figure 4.9: Standard curves for estimation of protein concentration in leaf extracts for assaying the activity of proline biosynthetic enzymes

A. Standard curves for *Atriplex* spp. B. Standard curves for *Acacia* spp.

Table 4.6: Estimated total protein concentration from control and salt-stressed leaf tissues

Plant sample	Average protein concentration (mg/mL)	
	Control	Salt-stressed
Atriplex nummularia	0.403 ± 0.135	0.293 ± 0.114
Atriplex semibaccata	0.172 ± 0.010	0.193 ± 0.061
Atriplex amnicola	0.035 ± 0.007	0.078 ± 0.052*
Acacia pendula	0.084 ± 0.020	0.108 ± 0.025
Acacia salicina	0.013 ± 0.003	0.119 ± 0.057*
Acacia victoriae	0.056 ± 0.040	0.056 ± 0.007

* indicates statistically significant ($p<0.05$) difference in values between control and salt treatments

4.4.2.3 Assay of P5CS activity

As seen in Figure 4.10, there was a clear distinction in the P5CS enzyme expression patterns between the genus *Atriplex* and *Acacia*. The *Atriplex* species exhibited limited P5CS enzyme activity, almost 10 times lower than the *Acacia* species. However, comparison of P5CS activity (as fold change) between control and salt treated plants within each species indicated a 1.6 fold change for *Atriplex amnicola*, 2X for *Atriplex nummularia* and 2.5X for *Atriplex semibaccat*a, and 1.2X for *Acacia pendula* and 2.2X each for *Acacia salicina* and *Acacia victoriae*. A positive correlation, i.e. increase in P5CS activity with a concurrent increase in proline content, was observed (Table 4.7), with stressed plants expressing higher levels of proline than control. Under salt conditions, among the saltbushes, *Atriplex amnicola* had the highest enzyme activity, and among the acacias, *Acacia victoriae* had the highest enzyme activity.

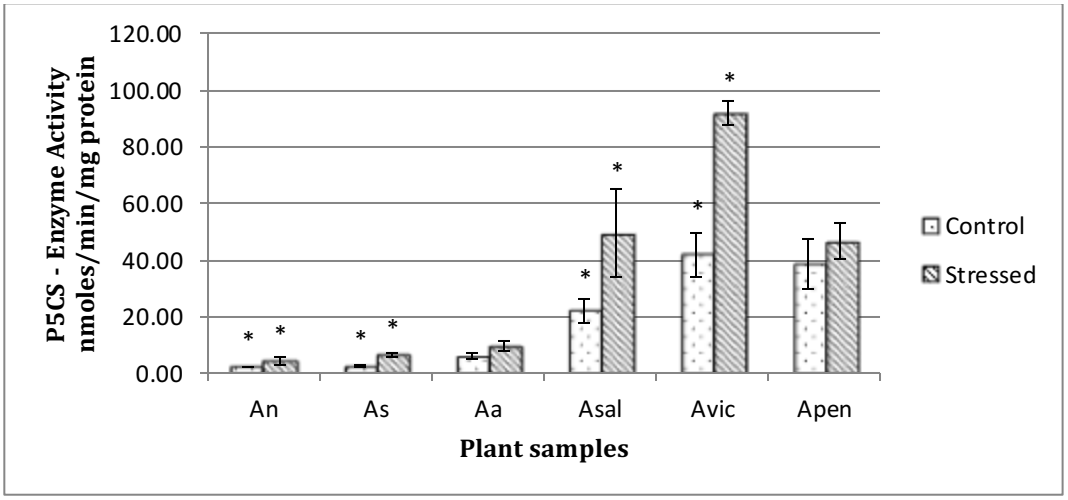


Figure 4.10: Activity of P5CS enzyme in leaf tissues of control and salt-stressed plants

* indicates statistically significant ($p<0.05$) difference in values between control and salt treatments
(An: *Atriplex nummularia*; As: *Atriplex semibaccata*; Aa: *Atriplex amnicola*; Avic: *Acacia victoriae*; Asal: *Acacia salicina*; Apen: *Acacia pendula*)

4.4.2.4 Assay of PDH activity

In terms of PDH activity (Figure 4.11), *Acacia salicina* exhibited the highest activity of 224.07± 26.02 nmoles/minute/mg protein among the tested species under salt treatment. The fold change in PDH enzyme activity in *Atriplex* species was 1.6 (*Atriplex amnicola*), 1.8 (*Atriplex nummularia*) and 5.6 (*Atriplex semibaccata*), and in *Acacia* species -0.6 (*Acacia pendula*), 1 (*Acacia victoriae*) and 2.9 (*Acacia salicina*) (Table 4.7). This translates to *Acacia pendula,* showing a decrease in PDH activity, *Acacia*

victoriae showing no difference, and *Acacia salicina* and all three *Atriplex spp.* showing an increase in PDH activity between control and stress plants.

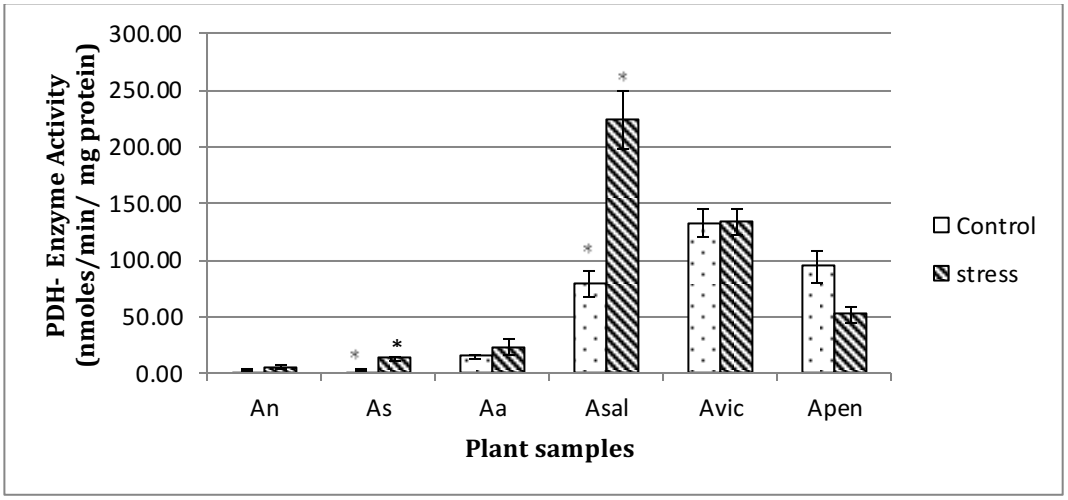


Figure 4.11: Activity of PDH enzyme in leaf tissues of control and salt-stressed plants

* indicates statistically significant ($p<0.05$) difference in values between control and salt treatments (An: *Atriplex nummularia*; As: *Atriplex semibaccata*; Aa: *Atriplex amnicola*; Avic: *Acacia victoriae*; Asal: *Acacia salicina*; Apen: *Acacia pendula*)

Table 4.7: Relative activity of P5CS enzyme in leaf tissues of control and salt-stressed plants

Plant	P5CS activity in leaf tissues (nanomoles/minute/mg protein)		PDH activity in leaf tissues (nanomoles/minute/mg protein)		Accumulation of Proline in leaf tissues (nanomoles Proline/mg of FW)	
	Control	**Salt-stressed**	**Control**	**Salt-stressed**	**Control**	**Salt-stressed**
Atriplex nummularia	2.23 ± 0.27	4.50 ± 1.27*	2.90 ± 0.20	5.27 ± 1.48	1.56 ± 0.41	3.01 ± 0.68
Atriplex semibaccata	2.61 ± 0.31	6.63 ± 0.61*	2.26 ± 0.64	12.67 ± 1.45*	1.13 ± 0.02	2.22 ± 1.16
Atriplex amnicola	5.90 ± 1.20	9.47 ± 1.59	14.63 ± 1.44	22.70 ± 7.37	1.21 ± 0.12	1.48 ± 0.22
Average for saltbushes	**3.58 ±1.55**	**6.87 ± 1.44**	**6.59 ± 4.02**	**13.55 ± 5.05**	**1.90 ± 0.42**	**2.27 ± 1.36**
Acacia victoriae	41.96 ± 7.95	91.71 ± 4.18*	132.26 ± 12.50	133.63 ± 12.04	1.62 ± 0.21	6.97 ± 1.42
Acacia salicina	22.13 ± 4.26	49.49 ± 15.72*	78.72 ± 11.64	224.07 ± 26.02*	1.20 ± 0.33	3.98 ± 0.80
Acacia pendula	38.81 ± 8.79	46.52 ± 6.31	93.95 ± 13.82	52.16 ± 7.25	1.46 ± 0.06	2.72 ± 0.11
Average for wattles	**34.13 ±6.09[a]**	**62.57 ± 14.59[a]**	**101.64 ± 15.93[a]**	**136.62 ± 49.65[a]**	**1.43 ± 0.39**	**4.56 ± 2.36**

* indicates statistically significant ($p<0.05$) difference in values between control and salt treatments

'[a]' indicates statistically significant ($p<0.05$) difference in values between control/stressed *Acacia* and *Atriplex* xpecies.

4.4.3 Analysis of trehalose accumulation in native plants

4.4.3.1 Trehalose quantitation by HPLC

The method was developed using 75:25 acetonitrile: water solvent system on a sugar column, with a flow rate of 1 mL/minute detected using a Refractive Index Detector (RID). The results for method validation are given in Table 4.8. The linearity of the method was established from standard curve calibration (Figure 4.12). The typical linear regression value R^2 was 0.9925 for concentration the range of 0.50 to 3 mg/mL. The linear regression equation used for calculating the amount of trehalose in the test leaf samples was x=y-c/m, where x is the amount of trehalose in the test sample, y is the average peak area (response) of the test sample, C is the intercept (C= 43896) and m is the slope of the standard curve (m = 60679).

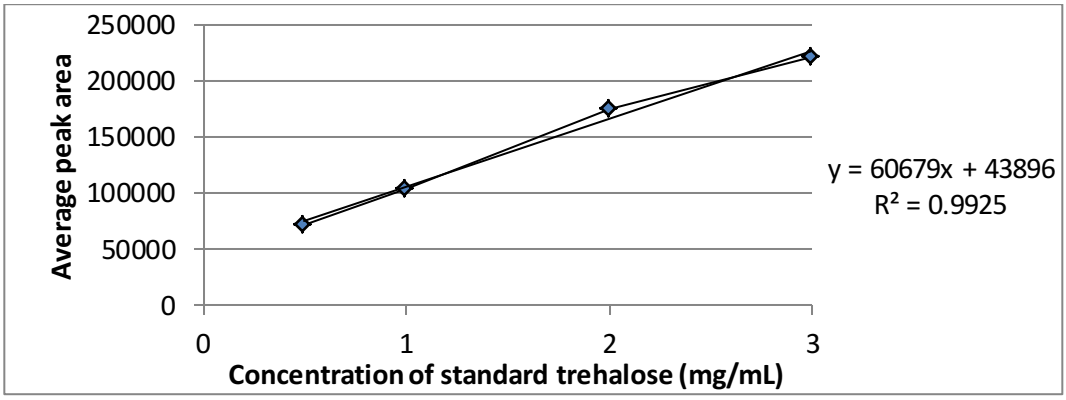


Figure 4.12: Standard curve calibration of trehalose (0.50 to 3 mg/mL)

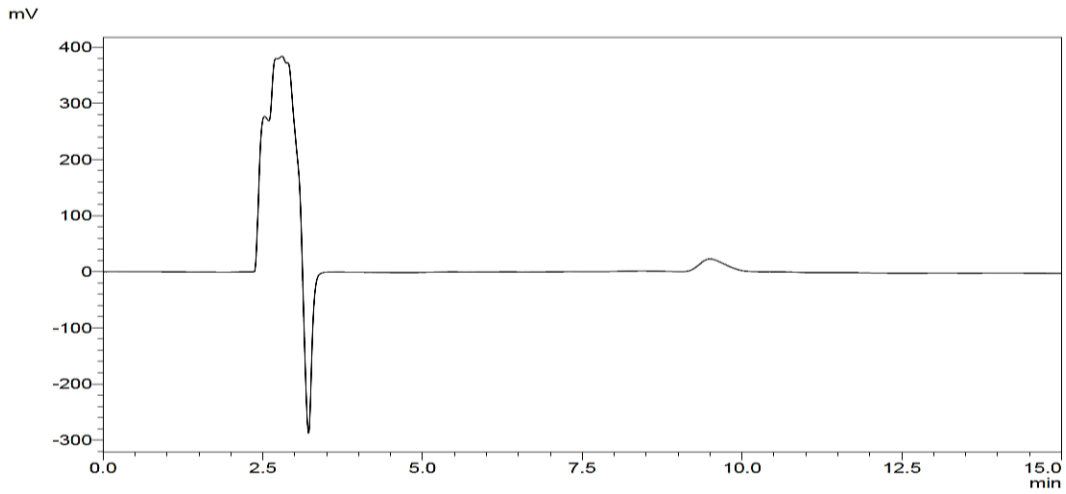


Figure 4.13: HPLC chromatogram of trehalose (1 mg/mL in 50% ethanol) with a retention time of 9.5 minutes

Table 4.8: Results obtained for method validation based on five parameters defined by International Conference on Harmonisation (ICH)

Parameter	Result
Range	0.5 - 3 mg/mL
Linearity	Correlation coefficient R^2 = 0.9925 Slope (S) = 60679 Intercept = 43896
Intraday precision %RSD	0.5 mg/mL = 1.05% 2 mg/mL = 0.10%
Interday precision %RSD	0.5 mg/mL = 1.48% 2 mg/mL = 0.27%
Limit of detection	0.04 mg/mL
Limit of quantitation	0.12 mg/mL

Precision of the method was established based on %RSD for intraday and interday repeatability (as shown for GB) for the 0.5 and 2 mg/mL of trehalose standard solutions. The intraday precision %RSD was 1.05% (0.5 mg/mL) and 0.10% (2 mg/mL). The interday precision %RSD was 1.48% and 0.27% for the above standards. These values are well below the stipulated ±2% according to ICH guidelines (ICH, 1996). The limit of detection was 0.04 mg/mL and the limit of quantitation was 0.12 mg/mL (Table 4.8).

4.4.3.2 Quantitation of trehalose in leaf extracts

Standard trehalose produced a peak at a retention time of 9.5 minutes (Figure 4.13), but no peak was observed corresponding to it in leaf extracts of *Acacia* and *Atriplex* species. The experiment was repeated with the samples two more times on different days; however, no peak was detected between 9 and 10 minutes (tissue chromatogram samples presented in Figure 4.14). The results indicate that no detectable level of trehalose is produced, or trehalose induction and/or accumulation may not occur under the stress conditions applied, in all six species tested.

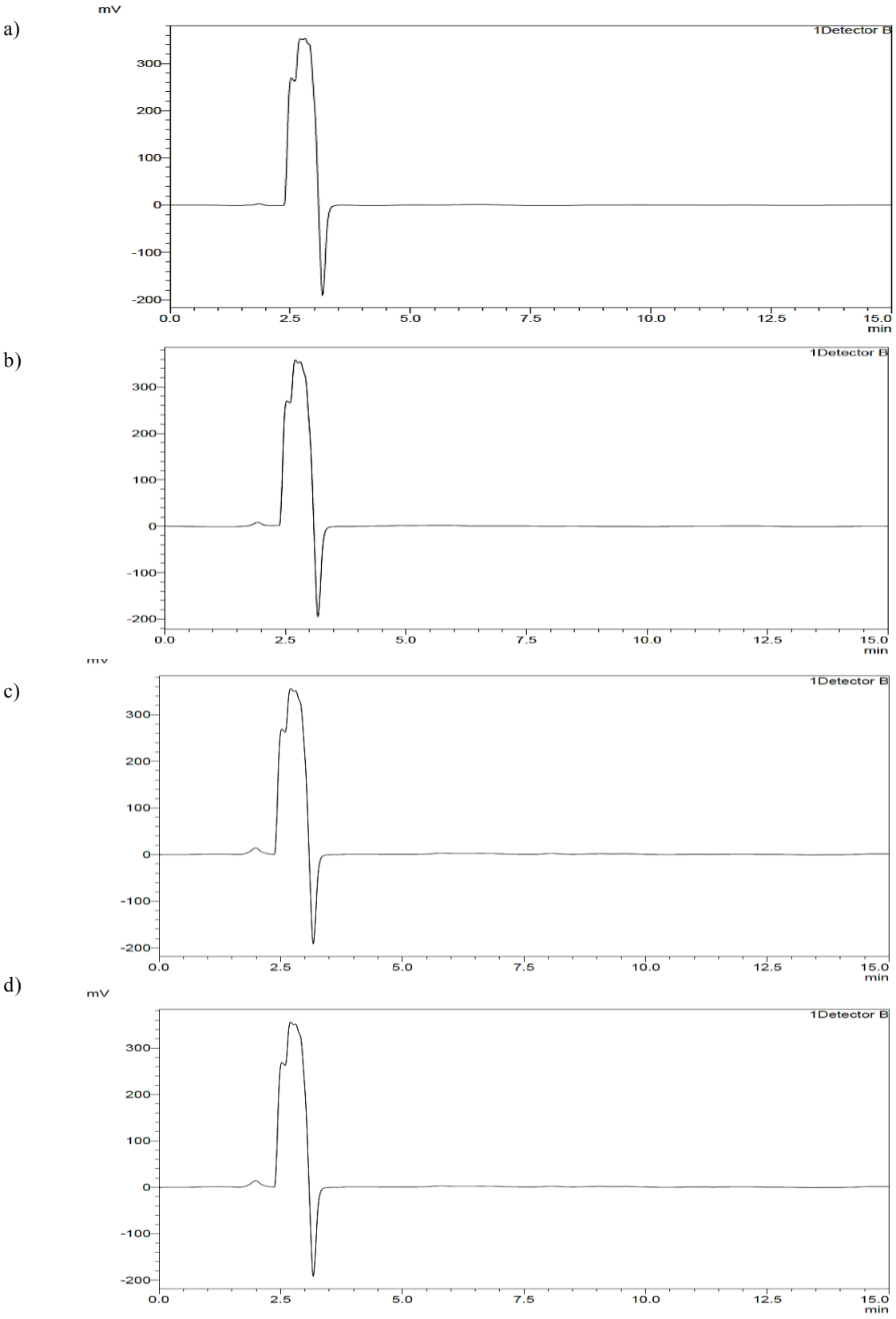


Figure 4.14: Chromatograms of *Atriplex amnicola* and *Acacia salicina* leaf extracts
a) *Atriplex amnicola* control leaf tissue extract; b) *Atriplex amnicola* salt-stressed leaf tissue extract; c) *Acacia salicina* control leaf tissue extract; d) *Acacia salicina* salt-stressed leaf tissue extract.

4.5 Discussion

Osmoprotectants play a significant role in ameliorating tolerance to salt and drought stresses in plants (sections 1.4 – 1.7). Some plants adapt to high salt concentrations by lowering tissue osmotic potential via the accumulation of osmoprotectants. Maintaining osmotic (solute) potential is vital to processes that directly influence plant growth, for instance, leaf expansion and stomatal conductance (Rajendran *et al.,* 2009). Thus, the main aim of this chapter was to investigate the possible induction of osmoprotectants during salinity stress of three *Atriplex* species (*Atriplex nummularia, Atriplex semibaccata, Atriplex amnicola*) and three *Acacia* species (*Acacia salicina, Acacia victoriae, Acacia pendula*), and evaluate their potential as a mixed fodder species with GB accumulation characteristics.

4.5.1 Salinity stress induces accumulation of high levels of glycine betaine in the leaves of saltbushes but not wattles

Glycine betaine has two distinct applications. Firstly, a protective role in plants against multiple abiotic stresses including salinity, drought, heat, cold and water logging (reviewed in Khan *et al.,* 2009; Giri, 2011). Natural GB accumulators, such as halophytes, or transgenic accumulators, such as potato (Ahmad *et al.,* 2008), cotton (Zhang *et al.,* 2009) or bread wheat (He *et al.,* 2010), have shown increased salinity tolerance. Secondly, animal studies suggest GB improves the nutrition and health of pigs and poultry (Ratriyanto *et al.,* 2009). Betaine protects internal organs and improves cardiovascular risk factors, while choline is metabolized to membrane components, like acetylecholine and phosphatidylcholine, that are essential to cell function (Zeisel *et al.,* 2006). Betaine and choline reduce the risk of infant neural tube defects and β-alaninebetaine may have a role in cholesterol reduction (Raman and Rathinasabapathi, 2003).

The salt bushes also have two beneficial traits. At the outset, they are salt-tolerant (see Section 1.9); and secondly they are reported to have positive health effects (improved digestion) in sheep and goats (Ben-Salem *et al.,* 2010) and contributed to 15% increase in live weight of cattle (Fancote *et al.,* 2009). The results presented in this chapter thus address the missing link between saltbushes and their advantageous traits via GB production.

According to earlier classification of plants based on typical analysis of their leaf tissues into GB accumulators and GB non-accumulators by Rhodes and Hanson (1993), members of the family Chenopodiaceae (such as spinach and sugar beet) are natural GB accumulators. Saltbushes (*Atriplex* spp.) belong to this family. The results presented in Chapter 3, confirm that the saltbushes have the necessary genetic machinery for GB synthesis. The HPLC quantitation in this study corroborated that *Atriplex semibaccata* and *Atriplex nummularia* exhibited superior GB accumulating ability compared to crop plants such as barley (Nakamura *et al.,* 1996) and wheat (Raza *et al.,* 2007). They also showed 2 to 3 fold higher levels than the respective controls under salt stress, with *Atriplex semibaccata* showing significant amounts. *Atriplex amnicola* was also confirmed to produce GB, but not as much as *A. semibaccata* and *A. nummularia.*

Some *Atriplex* species have been demonstrated to be GB accumulators, e.g., *A. griffithi* (Khan *et al.,* 1998) and *A. portulacoides* (Bessieres *et al.,* 1999). The data on other halophytic chenopods, such as *Suaeda* were more appropriate for comparison, since the data for previously reported *Atriplex* species were expressed in terms of dry weights, making comparisons of the current results to these data difficult. Park *et al.,* (2009) showed production of 30.8 to 33.0 mmol kg^{-1} FW in the leaves of three *Suaeda* species under salt stress. In the present work, *Atriplex nummularia* and *Atriplex semibaccata* were found to accumulate GB at 29.69 mmol kg^{-1} FW and 42.57 mmol kg^{-1} FW, respectively, proving their excellent innate ability. In comparison, barley is reported to accumulate 4 to 9 mmol GB kg^{-1} FW (Nakamura *et al.,* 1996) and wheat, 12 mmol GB kg^{-1} FW (Raza *et al.,* 2007) under salt stress. The enzyme BADH plays a major role in GB biosynthesis from betaine aldehyde (Section 1.8.1). The production of GB correlated well with BADH activity, the highest being in *Atriplex semibaccata*. The results show the strong potential of saltbushes for revegetation and as a perennial fodder (typically used as mixed fodder) in salinity and drought-affected areas; especially *Atriplex semibaccata* due to its high GB accumulation ability. However, it is unclear at this stage whether the amounts of GB accumulated may have an effect on the nutritional quality of this mixed-feed crop.

In the case of *Acacia* species, no measurable level of GB was observed under the given conditions, suggesting that if at all GB is produced by these species; it is below the

detection limits of the methodology used. In spite of the widespread occurrence of GB (Blunden *et al.,* 2005), some plants that do have the genetic and biochemical pathway do not accumulate GB. In such cases, transgenic approaches have successfully aided the plants to accumulate GB and offer protection against some of the abiotic stresses. For example, expression of *CMO* and *BADH* transgenically in non GB accumulating plants such as tobacco (*Nicotiana tabacum*) (Nuccio *et al.,* 1998, Yang *et al.,* 2008), *A. thaliana* (Hibino *et al.,* 2002) and rice (*Oryza sativa*) (Shirasawa *et al.,* 2006, Takabe *et al.,* 1998) have shown significant levels of tolerance to salinity.

4.5.2 Proline accumulation is highly regulated by P5CS and PDH

Accumulation of proline occurs by *de novo* synthesis from either glutamate or ornithine, along with the suppression of its catabolism (Delauney and Verma, 1993). Pyrroline 5-carboxylate synthetase (P5CS) is a bifunctional enzyme that catalyses the first two steps of proline biosynthesis (see section 1.9.1), whereas proline dehydrogenase (PDH) carries out proline catabolism. The results of this study provide evidence of three distinct patterns of regulating proline accumulation. Firstly, an increase in P5CS and PDH activities, accompanied with an increase in proline levels as seen in *Acacia salicina, Atriplex nummularia, Atriplex semibaccata* and *Atriplex amnicola*. Although these species displayed significantly greater P5CS activity, the simultaneous high PDH activity did not facilitate the accumulation of proline levels to as high as that found in *Acacia victoriae*. This could be because of two possibilities; either these plant species are comparatively less affected by salinity, or they are recovering much sooner after withdrawal of salt application (Sharma and Verslues, 2010). Both these traits are indicative of their salt tolerant nature. High PDH levels may contribute to their tolerance by regulating the amount of proline (Parida *et al.,* 2008) as well as by providing energy, transferring redox potential between cellular organelles, and modulation of reactive oxygen species levels (Servet *et al.,* 2012). Secondly, increase in P5CS and decrease in PDH activities, along with an increase in proline (*Acacia pendula*); similar observations have been reported in several studies where PDH activity decreased when proline accumulation increased. For example, a significant decrease in PDH activity (alongside increased P5CS activity and proline content) was seen in *Solanum tuberosum* (Hmida-Sayari *et al.,* 2005) and *Gossypium hirsutum* (Parida *et al.,* 2008). Thirdly, increase in P5CS activity and no change in PDH activity, accompanied

by the highest level of proline accumulation (*Acacia victoriae* showed a negligible increase from 132.26 ± 12.50 to 133.63 ± 12.04 nanomoles/min/mg protein). Increased P5CS levels have been reported to correspond with increased proline levels in several plants under salt and water stress (*Solanum tuberosum,* Hmida-Sayari *et al.,* 2005; *Oryza sativa,* Choudhary *et al.,* 2005; *Gossypium hirsutum,* Parida *et al.,* 2008).

4.5.3 Trehalose accumulation may not occur in saltbushes and wattles under salinity stress

One of the early views on trehalose accumulation was that it occurred only in early lineages of vascular plants with the resurrection plants being an exception (Muller *et al.,* 1995). Recent studies confirm that other plants also accumulate trehalose, to as much as 2.73 mg/g dry weight (wheat; El-Bashiti *et al.,* 2005). The function of trehalose under stresses such as drought, salt and chilling stress has been linked to the stability of proteins and biological membranes, as detailed in section 1.7. It has been reported to perform substantially better than other sugars (such as sucrose) in preserving membranes and enzymes (Colaco *et al.,* 1992). However, its exact role is still unknown. From the HPLC results in this study, trehalose may not accumulate in the plants tested, or may be below detectable limits, and therefore may not have a significant role as an osmoprotectant. Standard trehalose sugar was detected by HPLC, showing the method of detecting the sugar was successful. Some of the possible explanations for the lack of accumulation can be attributed to the induction of trehalase or inactive genetic machinery. Trehalase is ubiquitous in higher plants, and catabolism of trehalose has been reported thus far to be only by the hydrolytic action of trehalase (Muller *et al.,* 1999). Plants that do not accumulate trehalose do seem to have the required genetic machinery. Avonce *et al.* (2006) showed that *Arabidopsis thaliana* contained 11 copies of the gene encoding TPS (trehalose 6-phosphate synthase) and 10 copies of that encoding TPP (trehalose 6-phosphate phosphatase), the key enzymes involved in trehalose biosynthesis. These genes were expressed in specific tissues and controlled at various developmental stages of the plant, but no actual trehalose could be detected (Avonce *et al.,* 2006). This suggests that trehalose may not play a significant role as an osmoprotectant and raises questions on its roles and regulation of its biosynthesis. However, trehalose biosynthetic genes may play other crucial roles in plant growth and development (Avonce *et al.,* 2006).

CHAPTER 5

Application of molecular markers for identification of potential salt tolerant plants

5.1 Abstract

This chapter focussed on developing a novel approach for preliminary screening of salt tolerance in *Acacia* species based on the principle of comparative biology and molecular phylogenetics using DNA markers. The approach was applied to species in published morphological groups hypothesised to have a close relationship to *Acacia pendula, A. salicina, A. stenophylla* and *A. victoriae* (Flora of Australia Volumes 11 A, and 11 B: Maslin, 2001) which were reported as salt tolerant in independent field trials by NUFG farmers at Kamarooka (Australia). The present study generated nucleotide sequences for DNA marker regions (ITS, ETS, psbA-trnH, rpl32-trnL, trnL-F and matK) for the four target species and their closest relatives. An initial phylogenetic tree was constructed using the 4 chloroplast markers (data not shown) and 2 nuclear ribosomal DNA markers. However, due to the availability of an extensive, previously published (Brown et al, 2012) *Acacia* dataset for the nuclear ribosomal DNA markers, the ITS and ETS sequence data obtained in this study were favoured. This enabled the analysis of a broader framework of *Acacia* phylogenetics, resolved unclear relatedness and identified further species with a potential for salt tolerance. Eleven other species (*Acacia rigens, A. enterocarpa, A. sclerophylla, A. eriopoda, A. papyrocarpa, A. oswaldii, A. ligulata, A. cupularis, A. xanthina, A. rostellifera, A. synchronicia)* collectively identified to be phylogenetically related to *Acacia pendula, A. salicina, A. stenophylla* and *A. victoriae* were predicted to be salt tolerant. Generating huge molecular data, such as in this study, can be applicable in screening for other desirable properties e.g. tolerance to frost, drought, alkalinity and water-logging or even pharmaceutical benefits; as well as contributing new fundamental data to genetics and biodiversity applications.

5.2 Introduction

Many *Acacia* species are capable of growing in harsh conditions, such as salt tolerant *Acacia longifolia* growing in coastal dunes (Morais *et al.* 2012), saline/alkaline tolerant *Acacia ampliceps* (Fagg and Stewart, 1994) and drought tolerant *Acacia aneura* (Fagg and Stewart, 1994). The genus *Acacia* was therefore hypothesised in the current study to include ideal candidates for enabling saline land reclamation, a major environmental priority in Australia, together with a potential for financial returns. However, there are over 1000 acacia species known, and there is little information for most of the Australian species regarding their levels of salinity tolerance. The generation of information regarding a species' salt tolerance is time-consuming and costly, making the development of new methodologies for identifying salinity tolerant *Acacia* species a worthwhile undertaking.

This study aims to use molecular phylogenetics to rapidly identify the close relatives of known salt-tolerant species, in order to create a short-list of species which have potential for agroforestry on saline lands. These short-listed species will then be subjected to testing for salinity tolerance. The four *Acacia* species known to be to be salt tolerant (among other vegetation) (section 1.8.2) were: — *A. pendula, A. salicina, A. stenophylla,* and *A. victoriae*. The closest relatives of these four target species, based on morphology and classification in the Flora of Australia, were then sampled. Ascertaining phylogenetic relationships of these taxa, based on genetic markers, is essential.

The species that were investigated may be split into four groups:

- *Acacia pendula:* more likely to be related to *A. omalophylla, A. melvillei*
- *Acacia stenophylla: A. coriacea, A. sibilans* and *A. calcicola*
- *Acacia salicina:* related to the *A. bivenosa* group- *A. ligulata, A. ampilceps, A. cupularis, A. didyma, A. rostellifera, A. sclerosperma, A. startii, A. telmica, A. tysonii* and *A. xanthina*
- *Acacia victoriae group:* related to *A. alexandri, A. aphanoclada, A. charatacea, A. cuspidifolia, A. dempsteri, A. glaucocaesia, A. pickardii, A. ryaniana, A. synchronicia, A. marramamba* and *A. strongylophylla.*
- *Paraserianthes lophantha* was used as the out-group

5.4 Results

Out of the 30 taxa sampled, 19 yielded good quality data for both ITS and ETS markers. Six species (*A. ampliceps, A. bivenosa, A. didyma, A. sclerosperma, A. startii* and *A. telmica*) could not be analysed due to no genomic DNA being obtained, or the sequence chromatograms were poor, and there were no corresponding sequences in GenBank; hence these were excluded from further analysis. For five other species that did not produce high quality sequences (*A. victoriae, A. alexandri, A. aphanoclada, A. cuspidifolia, A. dempsteri*), the ITS and ETS sequences available in GenBank were included in the preliminary analysis.

Table 5.1 shows the length of the individual subunits, including the aligned versus the unaligned lengths for these 19 species. The aligned length indicates the final length based on the entire alignment file and includes gaps, whereas the unaligned length provides the range of sequence lengths for all individual species. Sequencing near the 5' end of the ITS region had unresolved bases close to the primer binding site, hence the length of the ITS1 subunit (196 bases) given in Table 5.2 is shorter compared to previously reported lengths of *Acacia* ITS1 sequences (Murphy *et al.*, 2003; 2010). The 5.8S and ITS2 subunits gave complete sequence data. The 5.8S subunit was found to be the most conserved region in terms of length; all sequences were 159 bases long and showed no variation with the 5.8S region of other *Acacia* species (Murphy *et al.*, 2003; 2010). The ITS2 subunit varied from 149 to 215 bases. *Acacia pickardii* had the shortest length (149 bp) while *A. xanthina* was 215 bases long. The ETS region was sequenced full length and ranged between 393 bp to 407 bp. The LSU and SSU were partial sequences due to the position of the primer binding sites.

Table 5.1: Sequence characteristics of ITS1, 5.8S, ITS2 and ETS regions

Sequenced region	ITS1	5.8S	ITS2	LSU	ETS	SSU
	(1-196)	(197-355)	(356-571)	(572-712)	(713-1129)	(1130-1147)
Aligned length	196	159	216	141	416	18
Unaligned length	108-188	159	149-215	0-137	393-407	17-18

Numbers indicate length in basepairs. The aligned length indicates an overall range of lengths, based on the alignment of all individual raw sequences and including gaps. The unaligned length denotes the lengths of raw sequences from individual species without gaps.

For the chloroplast regions, out of the 30 taxa sampled, 24 yielded good quality sequence data for the psbA-trnH marker, 25 for rpl32-trnL and trnL-F and 28 for matK. The aligned and unaligned length sequence lengths for the four regions are shown in Table 5.2. However, the availability of larger ITS and ETS datasets from Brown *et al.* (2012) favoured the interpretation of the phylogenetic data on a broader scale. Also a few gaps were noticed with the chloroplast data such as unavailability of sequence data for a suitable out group as well as taxa that did not yield any PCR amplification products and poor resolution of the phylogenetic trees because of the limited taxa used. Hence, a preliminary parsimony analysis of the four chloroplast regions was done (data shown in Appendices X-XIII) and would be utilised in future work when the expected larger dataset becomes available.

Table 5.2: Sequence length of chloroplast regions

Sequenced region	psbA-trnH	rpl32-trnL	trnL-F	matK
Aligned length	437	683	1152	833
Unaligned length	305-387	540-663	746-1105	736-786

Numbers indicate length in basepairs. The aligned length indicates an overall range of lengths, based on the alignment of all individual raw sequences and including gaps. The unaligned length denotes the lengths of raw sequences from individual species without gaps.

5.4.1 Parsimony analysis for the combined ITS and ETS regions

A phylogenetic tree with the 24 *Acacia* taxa and one out-group was constructed using MEGA 4 (Tamura *et al.*, 2007), and the evolutionary history inferred using the Maximum Parsimony optimisation criterion. Six most parsimonious trees (length = 432) were found, of which tree number one is shown in Figure 5.1. For these trees, the consistency index was 0.513, retention index was 0.749, and the composite index for all sites = 0.491, and for parsimony-informative sites was 0.384. The Maximum Parsimony tree was generated using the Close-Neighbor-Interchange algorithm, with search level 3, in which the initial trees were obtained with random addition of sequences (10 replicates).

As seen in Figure 5.1, *Acacia pendula* is sister to *A. sibilans,* and *A. stenophylla* is sister to *A. omalophylla*. This observation is slightly different to that based on morphological similarity; *A. pendula* was expected to be most closely related to *A. omalophylla* and in turn to *A. melvillei,* with *A. sibilans* most similar morphologically to *A. stenophylla*

(Flora of Australia, Vol. 11B: Orchard and Wilson, 2001). However, although *A. pendula* and *A. stenophylla* fall in the same clade, *A. stenophllya* appears more closely related to *A. omalophylla* (bootstrap support 87%). *Acacia salicina* was resolved and well supported as being sister to the *A. bivenosa* group (comprising *A. cupularis, A. xanthina, A. tysonii, A. ligulata, A. rostellifera*), which forms a monophyletic clade (100% bootstrap).

The *A. victoriae* group is reportedly a well characterised group, forming a monophyletic clade along with the *A. pyrifolia* group (Ariati *et al.*, 2006). Maslin (1992) had revised this group and included ten species that were found mainly in dry parts of Australia: *A. victoriae* Benth., *A. alexandri* Maslin, *A. aphanoclada* Maslin, *A. chartacea* Maslin, *A. cuspidifolia* Maslin, *A. dempsteri* F. Muell., *A. glaucocaesia* Domin, *A. pickardii* Tind., *A. ryaniana* Maslin, and *A. synchronicia* Maslin (Maslin, 1992). The species are mostly characterised by spinose stipules (Ariati *et al.*, 2006), a feature that rarely occurs in other members in section *Phyllodineae.* Except for *A. glaucocaesia*, the other nine species were included in the present analysis. *Acacia victoriae* was found to be sister to a clade comprising *A. synchronicia,* which together were sister to *A. chartacea* and *A. ryaniana* (Figure 5.1). The relationship of *A. chartacea* and *A. ryaniana* is strongly supported by a bootstrap value of 96% and previously reported by Ariati *et al.* (2006). These species share morphological characteristics in their phyllodes and a minute gland near the apical mucro (http://www.anbg.gov.au/abrs/online-resources/flora/stddisplay.xsql?pnid=41065).

The results obtained from this phylogenetic tree thus form a predictive method of identifying closes relatives that may be potentially salt tolerant. However, it was identified that a broader sampling of species from across the *Acacia* classification, would improve the potential predictive powers of this study for identifying new salinity-tolerant *Acacia* species. This analysis is described below (5.4.2) and used Bayesian analysis methods.

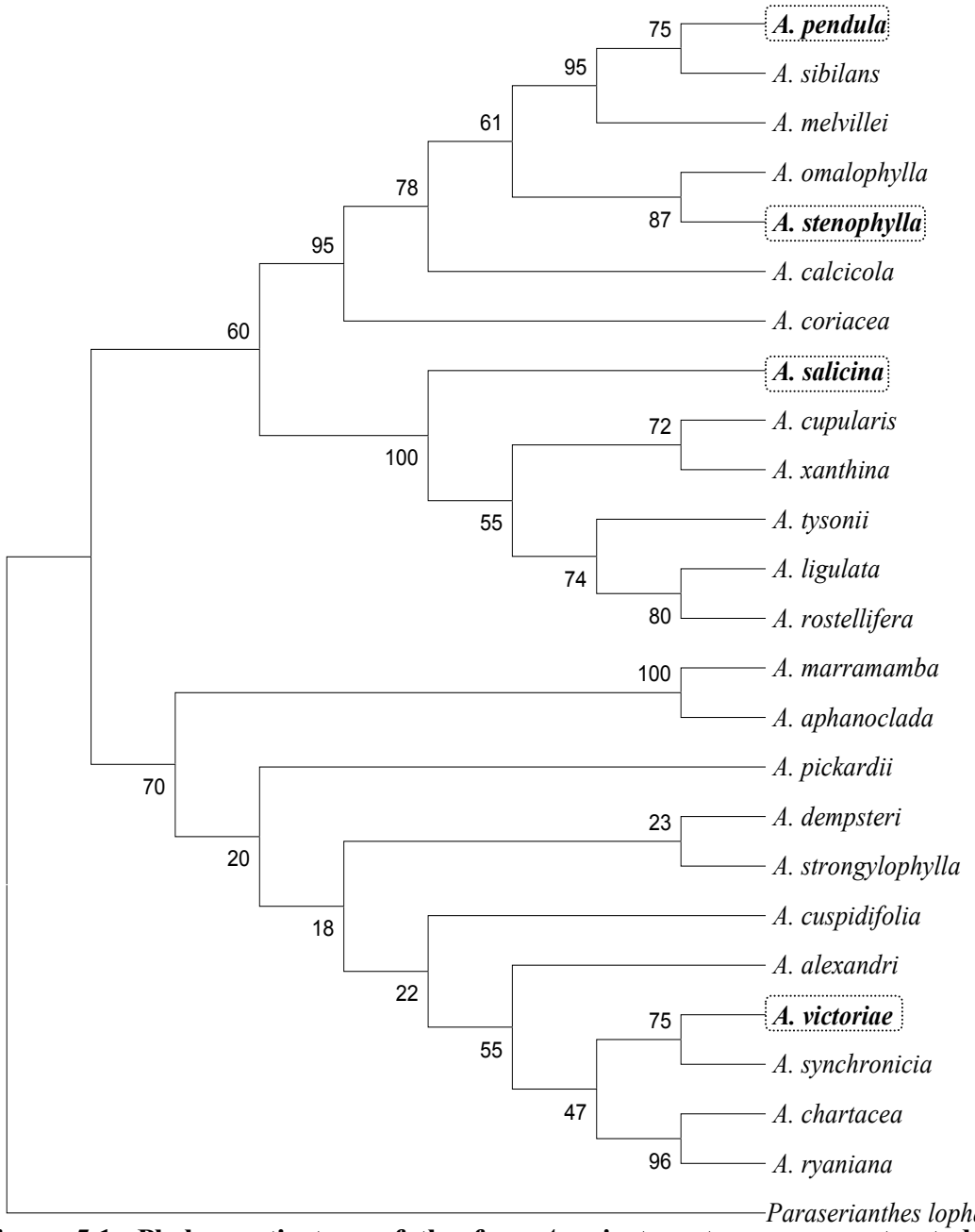


Figure 5.1: Phylogenetic tree of the four *Acacia* target groups constructed using the ITS and ETS markers on MEGA4 using Maximum Parsimony algorithm

Bootstrap values with 1000 replicates (indicated as % of replicate trees) is shown next to each branch. Species in bold were the four species currently in use for agroforestry in saline degraded lands at Bendigo, Victoria, Australia.

5.4.2 Bayesian analysis of the combined ITS and ETS regions incorporated into the larger dataset

The sequence data generated for the 24 taxa targeted in the parsimony analysis, were subsequently incorporated into a larger dataset of Brown *et al.* (2012), for a total of 178 sequences (including the out-group *P. lophantha*). This data was then analysed using Bayesian analysis methods because of the efficiency of the software program (MrBayes) to process large datasets. The length of the concatenated ITS and ETS sequences was 1290 bp. A total of 8 indel characters (six from ITS and two from ETS regions) were scored. The average standard deviation of split frequencies was 0.010 and the average potential scale reduction factor was 1.001. Incorporation of the new dataset showed that some of the species fall into clades that were found to be resolved in previous studies and the placement of groups of interest are outlined below (Murphy *et al.,* 2010; Miller *et al.,* 2011; Brown *et al.,* 2012) (Figure 5.2).

As seen in Figure 5.2, *A. pendula* is sister to *A. sibilans*, whereas *A. stenophylla* falls into quite a separate phylogenetic placement comprising a weakly supported (PP = 0.545) clade of *A. omalophylla*, *A. elongata*, *A. oswaldii*, *A. cognata* and *A. verticillata*. This observation varies slightly from that based on morphological similarity; according to the Flora of Australia *Acacia* treatment (Orchard and Wilson, 2001b), *A. pendula* was expected to be related to *A. omalophylla*, and in turn to *A. melvillei*. In contrast, *A. sibilans* is morphologically expected to be related to *A. stenophylla* and *A. pendula*; however, in this study *A. pendula* is sister to *A, sibilans*, with both of these closely related to a clade of *A. papyrocarpa*, *A. enterocarpa*, *A. eriopoda*, *A. sclerophylla*, and *A. calcicola* sister to *A. rigens*. Species in this group share a common morphologically similar, narrow, sclerophyllous phyllodes and grow mostly in arid and semi-arid regions (Murphy *et al.,* 2010). The results of the present study suggest that better understanding of morphological synapomorphies and further molecular sampling of these groups is necessary, as specimens in herbaria may be cryptic and misidentification cannot be ruled out for non-fruiting material.

The *A. victoriae* group, which is named after the taxon *A. victoriae,* is closely related to the *A. pyrifolia* group, and these two groups were informally named as the '*A. victoriae* and *A. pyrifolia* clade' by Murphy *et al.* (2010). In this study, *A. victoriae* is found to be sister to *A. synchronicia*, and in turn is placed in a larger clade with *A. chartacea*, *A.*

ryaniana and *A. alexandri*. This strongly supported clade (PP = 0.999) has been resolved in previous reports (Ariati *et al.,* 2006; Murphy *et al.,* 2010; Miller *et al.,* 2011; Brown *et al.,* 2012), but the salinity tolerance of these species has not been assessed.

The fourth grouping resolved *A. salicina* in a strongly supported clade (PP =1.0) with *A. cupularis, A. ligulata, A. rostellifera, A. xanthina* and *A. tysonii*, as expected based on their morphological grouping as the '*A. bivenosa'* group, as per Flora of Australia (Orchard and Wilson; 2001a; b). However, *A. cupularis* is sister to *A. ligulata* and *A. rostellifera* rather than to *A. xanthina* as predicted.

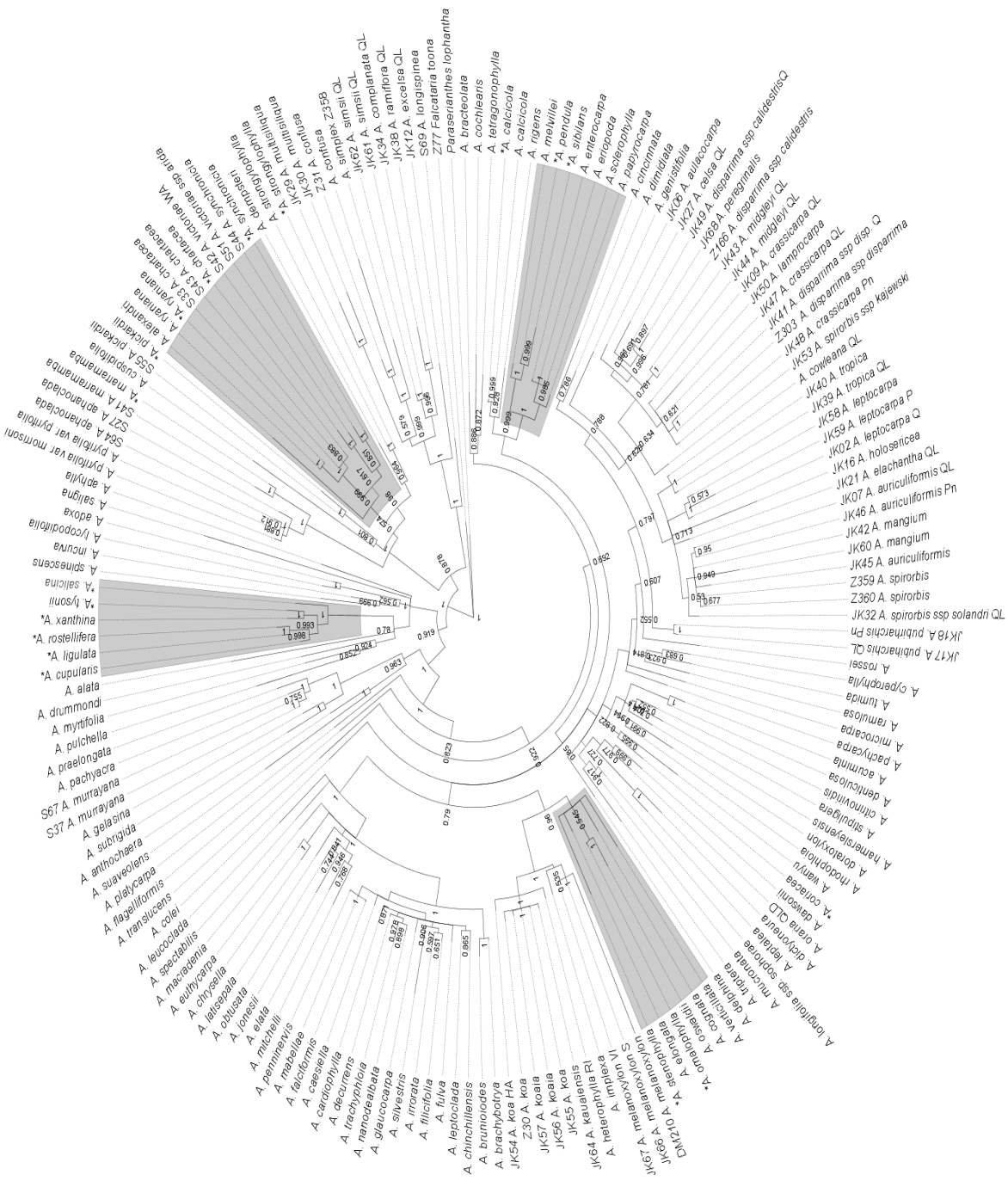


Figure 5.2: Phylogenetic tree of *Acacia* species
The circular tree is displayed using Fig Tree (Rambaut and Drummond, 2008), with data from Bayesian analysis of 178 *Acacia* species. The four known salinity-tolerant species of interest (*A. victoriae*, *A. salicina*, *A. pendula*, *A. stenophylla*) and their closest relatives are shaded in grey.

5.5 Discussion

Comparative biology is based on the expectation that closely related organisms share traits, such as salinity tolerance, that are less common in more distantly related organisms (Cracraft, 2002). Therefore, clarifying molecular phylogenetic relationships can aid in selecting candidate species that may display a particular trait. Miller *et al.,* (2011) used plastid and nuclear rDNA data to test for invasiveness of species across a broad framework of 110 acacias. Although the invasive species did not form a monophyletic group, some evidence for phylogenetic grouping of invasive species was found. That study also identified sister species of the known invasive species, and these were inferred to have increased potential for invasiveness and require extra care if proposed to be introduced to new regions. The present study takes a similar approach, to rapidly identify sister species of known salt tolerant taxa. Unspecific markers such as the ITS and ETS were used to avoid characters potentially subject to homoplasy/ covergent evolution. This strategy is commonly used for certain phylogenetic studies. ITS and ETS sequence data were obtained for species in morphological groups closely related to the four known salt-tolerant species, *A. pendula, A. salicina, A. stenophylla* and *A. victoriae*, mentioned above. These data, combined with an extensive *Acacia* dataset of ITS and ETS markers, was used to generate phylogenetic relationships and identify further species with a potential for salt tolerance. These findings could then be used to test the salinity tolerance of select *Acacia* species at a hydroponic level (under controlled conditions in the laboratory) and eventually at field level.

In this chapter, new ITS and ETS molecular data was generated for 19 taxa of specific interest, due to their putative close relationship to four known salt-tolerant species is reported. The initial study began with results based on a parsimony analysis of a smaller sample of 24 species created based on morphological characteristics. The more recent availability of data from Brown *et al.* (2012) enabled the incorporation of these samples into a larger molecular phylogenetic study and used a more rigorous model-based Bayesian analysis, using MrBayes. The results obtained in this latter study serve as a model for the use of molecular phylogenetics data, not only for testing of phylogenetic relationships, but also for species selection for other application-oriented outcomes. Analysis of literature pertaining to the putative potential species also indicate

that there is very little salt tolerance data for any *Acacia* species, or indeed, many other native Australian plants, substantiating the need for such work.

The four main salt-tolerant species, *A. pendula, A. stenophylla, A. salicina* and *A. victoriae,* were selected based on the Kamarooka Project (NUFG, Bendigo, Australia; NUFG, 2013), and have other potential applications, but overall are currently under-utilized. *Acacia pendula* is known for its strong and dense wood, and used as an ornamental tree, or for firewood, and for making small wooden articles (Department of Primary Industries, Victoria; http://www.dpi.vic.gov.au/forestry/investment-trade/regional-information/farm-forestry-in-the-north-central-region/myall). *Acacia pendula* is also a useful stock fodder in drought (World Wide Wattle; http://www.worldwidewattle.com/). Some species resolved in this study as related to *A. pendula* are salt tolerant, e.g., *A. calcicola,* with a salinity tolerance of 16.5 dS/m under hydroponic conditions (Malik and Ahmed, 2002) and 19.9 dS/m of root zone salinity (Akhter *et al.,* 2003), while others such as *A. papyrocarpa* may be suitable for salty land (Government of South Australia, http://www.pir.sa.gov.au/__data/assets/pdf_file/0005/139163/salinity.pdf), but a definitive range of their tolerance is yet to be determined. *Acacia calcicola,* with a tolerance range of 16.5-19.9 dS/m, can be classified as highly salt tolerant, with soil electrical conductivity (EC) >16 dS/m being considered extremely saline (Food and Agricultural Organisation (FAO) http://www.fao.org/docrep/x5871e/x5871e04.htm). Additionally, *A. calcicola* can be used for fuel, shade, shelter and windbreaks (FAO; http://ecocrop.fao.org/ecocrop/srv/en/cropView?id=2591). It is notable that *A. papyrocarpa* and *A. ligulata* have been recorded as growing on salty land, although no definitive range of salinity tolerance has been determined. *Acacia enterocarpa*, another close relative, is a nationally 'Endangered' species (Moritz and Bickerton, 2011), and both species have been reported as frost tolerant (Bird *et al.,* 1996). Thus, certain favourable characteristics may be shared within clades identified in phylogenetic studies.

Acacia stenophylla falls into a weakly supported clade with *A. verticillata, A. cognata, A. elongata, A. oswaldii* and *A. omalophylla* (Figure 5.2), although the relationships within this clade are not fully resolved. *Acacia stenophylla* is capable of growth in

extremely saline sites (EC >16 dS/m) (FAO). It is highly frost tolerant (Bird *et al.,* 1996) as well as tolerant to highly alkaline soils and waterlogging (Marcar and Crawford, 2004). The salinity tolerance of these closely related species (*A. verticillata, A. elongata, A. oswaldii* and *A. omalophylla*) is yet to be determined.

The *A. victoriae* group is a well-characterized monophyletic clade (Ariati *et al.,* 2006). Maslin (1992) had revised this group and included ten species (nine of which are included in the present analysis): *A. victoriae* Benth., *A. alexandri* Maslin, *A. aphanoclada* Maslin, *A. chartacea* Maslin, *A. cuspidifolia* Maslin, *A. dempsteri* F. Muell., *A. glaucocaesia* Domin, *A. pickardii* Tind., *A. ryaniana* Maslin and *A. synchronicia* Maslin. Most of these are found in arid parts of Australia, and are characterized by spinose stipules, similar phyllode characteristics and a minute gland near the apical mucro (Ariati *et al.,* 2006; Orchard and Wilson, 2001a; b). *Acacia victoriae* was found to be sister to a clade comprising *A. synchronicia,* and in turn to *A. chartacea* and *A. ryaniana*. The relationship of *A. chartacea* and *A. ryaniana* is strongly supported (PP =1.0). No data exists yet on the salinity tolerance of species closely related to *A. victoriae*. *Acacia victoriae* is also of interest due to its seed pods being a source of triterpenoid saponins called Avicins (Haridas *et al.*, 2001a; 2001b) which have strong potential as anti-tumor drugs (Lemeshko *et al.*, 2006).

Acacia salicina, similarly to *A. victoria*, has strong potential in medicinal chemistry, within its leaves are bioactive compounds that have anti-mutagenic, anti-genotoxic and antioxidant potency (Chatti *et al.,* 2011; Boubaker *et al.,* 2012). *Acacia salicina* has been shown to be sister (PP = 1.0) to a clade comprising *A. cupularis, A. ligulata, A. rostellifera, A. xanthina* and *A. tysonii* (Figure 5.2), which flags these taxa as potential candidates for bioactive compounds, in addition to having the potential for salt-tolerance. *Acacia ligulata* is described as 'somewhat salt tolerant' based on the salt tolerance of its seedlings (Yokota, 2003). *Acacia ligulata* is a prospective candidate for revegetation of areas with slight to moderate salinity. *Acacia xanthina* is recorded to grow on arid lands and limestone, while *A. tysonii* is a species with hard wood, and is advantageous for soil stabilisation in saline sites (World Wide Wattle; http://www.worldwidewattle.com/). Thus, a number of species in the *A. salicina* clade may be suitable for revegetation purposes.

Ascertaining the relationships of the four target species to their closest relatives based on phylogenetics assists in narrowing the identification process of putative salt tolerant species, especially in such a large genus like *Acacia*. This is significant since there is such limited data on salinity tolerance for the vast number (> 1000) of Australian *Acacia* species, as large-scale testing of salinity tolerance parameters is time consuming and prohibitively expensive. As such, a rapid and inexpensive methodology to flag candidate species, as described here, is highly significant. The methodology will also be applicable to the identification of threatened Acacia species (Powell *et al.,* 2012). The utilization of DNA markers such as ITS and ETS, is informative due to the large amount of already available sequence data, the informativeness of these DNA regions, and the relative ease of these markers for sequencing via Sanger sequencing methods (analogous to DNA barcodes). This molecular phylogenetic screening can be followed by testing of select species for physiological markers, such as biomass and ion accumulations, in laboratory and eventually field conditions. This is a more efficient method than randomly screening hundreds of taxa, many of which are difficult to obtain seed or other plant material for testing. A report on these findings is presented in the following chapter (Chapter 6). The selected molecular phylogenetic approach could be extended to other taxa, or tolerance to other traits such as frost, drought, alkalinity or water-logging often associated with salinity tolerance, or phytochemical composition, weediness potential, forage potential, and other characteristics important for utilization. Thus, the results presented here may help in rational selection of candidate plants that not only provide a 'green cover' for the landscape but also contribute to its productivity.

CHAPTER 6

Salinity testing of potential salt tolerant *Acacia* species

6.1 Abstract

This chapter provides experimental evidence to validate the hypothesis of salt tolerant species selection based on phylogenetic screening (presented in Chapter 5). In this study, the salinity tolerance of 15 different *Acacia* species was evaluated for saline land reclamation and agroforestry. Four species (*Acacia pendula, A. salicina, A. stenophylla* and *A. victoriae*) capable of growth in saline soils but without any experimentally investigated data, and eleven other closely related species (*Acacia rigens, A. enterocarpa, A. sclerophylla, A. eriopoda, A. papyrocarpa, A. oswaldii, A. ligulata, A. cupularis, A. xanthina, A. rostellifera, A. synchronicia)* were tested. The effects of 300 mM NaCl on plant physiological parameters such as growth, biomass accumulation and concentrations of major cations of these species were investigated. A method was developed for comprehensive analyses of the datasets, leading to the use of three different salt tolerance indices or STI to rank the species (based on increases in growth, $STI_G>1$; fresh weight, $STI_{FW}>1$ and dry weight, $STI_{DW}>1$). The species tested exhibited distinct responses to the various parameters consistently indicative of salt tolerance e.g. eight species showed an increase in fresh weight, 11 species showed significant increase in root length but only two species (*Acacia cupularis* and *Acacia enterocarpa*) showed an increase in dry weight. All the species showed alterations in their ionic ratios essential for osmotic balance. However, none of the species exhibited any symptoms of severe salt toxicity/injury. Two highly tolerant (*Acacia cupularis*, *Acacia enterocarpa;* $STI_{G/FW/DW}>1$) and three moderately salt-tolerant (*Acacia xanthina*, *Acacia eriopoda*, *Acacia stenophylla;* $STI_{G/FW}>1$) species were thus identified; supporting the use of initial phylogenetic screening for large genera, such as *Acacia*, and also provides an experimental methodology for identifying candidate species for environmental applications.

6.2 Introduction

Salinity affects plants in several physiological ways as mentioned in Figure 1.3 (section 1.3). The pronounced inhibitory effects are due to changes in osmotica, ion toxicity, nutritional and hormonal imbalance that ultimately impact plant growth and yield (reviewed in Munns and Tester, 2008; Ahmad *et al.,* 2013). Hence, investigating the eco-physiological traits, such as plant growth, biomass accumulation and relative water content, together with the accumulation patterns of ions, are vital in determining a plant's tolerance to salt. The aim of this study was to test the hypothesis that *Acacia* species identified by molecular phylogenetic analysis (Chapter 5) as being closely related to known salt tolerant species may also be salt tolerant, and could be suitable for sustainable agriculture in saline lands.

Four *Acacia* species (*A. pendula, A. salicina, A. stenophylla* and *A. victoriae*) had been identified as salt tolerant and were the targets of a broad phylogenetic study (Chapter 5) incorporating 178 Acacia species. Following Bayesian analysis, the phylogenetic tree resolved twenty species as closely related to the four initial species, and hence also hypothesised to be salt-tolerant. Upon investigation, these species had other historically recorded known uses, making them potential candidates for agroforestry (Table 6.1). The present study undertook experimental assessment to test whether these species include salt-tolerant candidates. The effects of salinity were evaluated on key criteria of plant growth, relative water content, biomass and cation accumulation (Kauser *et al.*, 2012; Morais *et al.*, 2012), leading to a comprehensive picture of their biochemical response to salinity stress. Species from the molecular selection data that were tested for salt tolerance are *A. rigens, A. enterocarpa, A. sclerophylla, A. eriopoda, A. papyrocarpa, A. oswaldii, A. ligulata, A. cupularis, A. xanthina, A. rostellifera and A. synchronicia,* along with *A. pendula, A. salicina, A. stenophylla* and *A. victoriae* (refer to section 2.7 for methods).

Table 6.1: *Acacia* spp. of economic value for potential in land reclamation projects

Species	Benefits/Applications/Importance	Reference*
Acacia pendula		
A. melvillei	*Acacia melvillei* Shrubland in the Riverina and Murray-Darling Depression bioregions (Australia) was designated as an 'endangered ecological community' in 2007.	http://www.environment.nsw.gov.au/determinations/acacamelvilleiFD.htm
A. calcicola	Wood can be used as a source of fuel Timber used to make artefacts, burnt leaf ash mixed with pituri (*Nicotiana* species) is used as tobacco.	http://ecocrop.fao.org/ecocrop/srv/en/cropView?id=2591 http://www.opbg.com.au/pdfs/selfguided%20walks/WATTLE%20WALK.pdf
A. rigens	Frost tolerant (up to -7° C), Recommended for its foliage in horticulture, used as a feature plant.	http://www.anbg.gov.au/acacia/species/A-rigens.html
A. enterocarpa	Listed as 'nationally endandered species'.	http://www.environment.gov.au/cgi-bin/sprat/public/publicspecies.pl?taxon_id=17615
A. sclerophylla	Decorative species, suitable for hydroseeding/hydro-mulching on roadside batters.	http://www.anbg.gov.au/acacia/species/A-sclerophylla.html
A. eriopoda	Edible gum which is rich in protein [42%] and an unusually high quantity of arabinose content for potential use in the food industry. Wood utilised to create spears and burnt leaf ash used for chewing along with tobacco.	http://www.stmarysbroome.wa.edu.au/home/nature/eri.html
A. papyrocarpa	Musical instruments, Craftwood/Turnery	Maslin, 1997
Acacia stenophylla		
A. omalophylla	Myall wood is fragrant and durable, used as craft wood and to make furniture and fine joinery.	http://www.wisegeek.com/what-is-acacia-wood.htm
A. oswaldii	The seeds are recorded as edible. Foliage browsed by pasture-animals.	http://brg.cma.nsw.gov.au/uploads/MurriFoodPlants.pdf http://bie.ala.org.au/species/Acacia+amaliae#tab_literature
A. elongata	Feature plant, capable of growth in saline soils along water courses.	http://plantsandlandscapes.com.au/prov_site/Acacia_elongata
A. cognata	Wet land indicator species	http://www.epa.qld.gov.au/wetlandinfo/site/factsfigures/FloraAndFauna/Species/14894.html

(table 6.1 continued)

Species	Benefits/Applications/Importance	Reference*
Acacia salicina		
A. ligulata	Leaves and branches used as a diaphoretic by aborigines Sand stabilisation and low windbreaks in arid areas.	http://www.worldwidewattle.com/speciesgallery/ligulata.php http://ecocrop.fao.org/ecocrop/srv/en/cropView?id=2647
A. cupularis	Covers large area and an effective natural weed suppressant under controlled environments.	http://www.aaev.net/management/nec/assets/Indigenous%20plants%20at%20AAEV.pdf
A. xanthina	Capable of growing in phosphate impoverished, low nutrient landscapes.	de Campos *et al.,* 2013
A. rostellifera	Suited for sand dune stabilisation	http://www.worldwidewattle.com/infogallery/utilisation/acaciasearch/pdf/rostellifera.pdf
A. tysonii	Hard wood species, moderately suited for soil stabilisation in saline sites	http://www.worldwidewattle.com/speciesgallery/descriptions/kalannie/tysonii.pdf
Acacia victoriae		
A. ryaniana	Priority 2 species	http://florabase.dec.wa.gov.au/conservationtaxa
A. synchronicia	Edible seeds, suggested for trial in tropical dry Africa	Thomson *et al.*, 1998
A. strongylophylla	Ornamental plant (Whibley, 1980)	http://aciar.gov.au/files/node/13315/australian_acacias_in_developing_countries_part_1_57558.pdf
A. pickardii	Threatened/vulnerable species	http://www.worldwidewattle.com/speciesgallery/pickardii.php

*All references were reviewed on 01 October 2013.

6.3 Results

Twenty species were found to exhibit close molecular phylogenetic relationships with the initial four salt-tolerant species of interest, i.e., *A. pendula, A. salicina, A. stenophylla* and *A. victoriae* (Chapter 5). These were resolved into four clades and were not monophyletic, i.e., *A. pendula* was closely related to taxa in two smaller clades (*A. papyrocarpa, A. enterocarpa, A. eriopoda* and *A. sclerophylla,* and *A. calcicola* and *A. rigens*); *A. stenophylla* was grouped into a weakly supported clade with *A. verticillata, A. cognata, A. elongata, A. oswaldii* and *A. omalophylla; A. salicina* was shown to be sister to a clade comprising *A. cupularis, A. ligulata, A. rostellifera, A. xanthina* and *A. tysonii*; and *A. victoriae* which was found to be sister to *A. synchronicia* and placed in a larger clade with *A. chartacea, A. ryaniana* and *A. alexandri.* After considerations of seed availability and % germination, fifteen species were tested for their salinity tolerance: *A. pendula* and five species identified as being phylogenetically closely related *(A. enterocarpa, A. eriopoda, A. papyrocarpa, A. rigens, A. sclerophylla), A. stenophylla* and its close relative *A. oswaldii, A. salicina* and its four close relatives *(A. cupularis, A. ligulata, A. rostellifera, A. xanthina)* and *A. victoriae* and its close relative *A. synchronicia.* Literature indicated that many of these species are historically commonly used for food purposes, fuel wood, craft wood, biodiversity values, and/or land management programs (Table 6.1).

6.3.1 Effect of salinity on shoot and root length

Some plants exhibited reductions in growth, while others showed an increase. No severe effects such as leaf burn or chlorosis were observed. A majority of stressed plants (11 species) showed a reduction in shoot length compared to their controls, while four species (*A. salicina*, *A. cupularis*, *A. papyrocarpa, A. stenophylla*) showed an increase in shoot length compared to controls (Figure 6.1A, Table 6.2). The changes were found to be statistically significant ($p < 0.05$) for three species showing reductions, i.e., *A. rostellifera, A. ligulata* and *A. eriopoda*. The trend of increased root length was observed in all species, (Figure 6.1B, Table 6.3), except in *A. ligulata, A. rigens, A. sclerophylla* and *A. oswaldii*. Interestingly, two species (*A. cupularis* and *A. stenophylla*) exhibited a statistically significant increase in root length in stressed compared to control plants.

Table 6.2: Effect of salinity on shoot physiology parameters

Identification		Shoot physiology parameters									
S. no.	Plant	Length (cm)		FW (gram)		DW (gram)		LRWC (%)		TWC (%)	
		C	S	C	S	C	S	C	S	C	S
1	*A. salicina*	23.00±2.646	24.70±1.818	3.280±0.386	3.010±0.260	0.935±0.107	0.552±0.032*	63.2	62.5	71.5	81.6
2	*A. cupularis*	11.47±1.410	11.90±1.537	1.548±0.156	1.569±0.119	0.248±0.030	0.318±0.012	81.0	60.9	84.0	79.6
3	*A. rostellifera*	22.07±0.481	13.07±1.618*	3.754±0.404	1.294±0.747*	0.855±0.069	0.304±0.167*	80.0	63.3	77.0	71.7
4	*A. ligulata*	18.60±0.666	8.15±0.950*	1.579±0.215	1.575±0.003	0.308±0.028	0.246±0.025	75.0	78.0	80.2	84.4
5	*A. xanthina*	4.87±0.536	3.83±0.406	0.596±0.034	0.650±0.033	0.167±0.005	0.150±0.018	60.0	50.0	71.8	77.0
6	*A. pendula*	17.20±1.000	15.70±1.200	2.482±0.170	1.834±0.122	0.730±0.022	0.530±0.032*	56.3	60.0	70.5	71.1
7	*A. papyrocarpa*	2.77±0.869	2.93±0.348	0.260±0.045	0.141±0.010	0.051±0.005	0.039±0.004	57.1	60.0	80.1	72.6
8	*A. enterocarpa*	2.70±0.208	2.60±0.173	0.106±0.004	0.179±0.031	0.020±0.002	0.045±0.008	88.9	73.3	81.5	74.8
9	*A. eriopoda*	8.30±0.321	4.33±0.233*	0.295±0.010	0.411±0.012	0.090±0.003	0.094±0.005	53.4	69.6	69.4	77.3
10	*A. rigens*	2.50±0.379	2.30±0.379	0.283±0.033	0.226±0.028	0.068±0.008	0.059±0.007	81.5	69.6	76.1	73.6
11	*A. sclerophylla*	3.33±0.674	0.77±0.233	0.214±0.024	0.097±0.011	0.050±0.003	0.020±0.002	80.0	87.5	76.3	79.2
12	*A. stenophylla*	27.90±0.781	29.93±1.312	4.194±0.186	3.822±0.193	1.335±0.072	1.123±0.036*	66.7	80.0	68.2	70.5
13	*A. oswaldii*	11.23±0.433	10.53±0.371	1.388±0.090	1.067±0.059	0.374±0.025	0.257±0.054	61.4	77.8	73.0	76.3
14	*A. victoriae*	12.57±0.529	10.60±0.513	1.395±0.084	0.992±0.083	0.350±0.066	0.156±0.046*	35.3	35.7	75.2	84.8
15	*A. synchronicia*	7.33±0.762	7.10±0.462	0.724±0.062	0.554±0.003	0.196±0.010	0.135±0.013	58.3	60.0	72.8	75.8

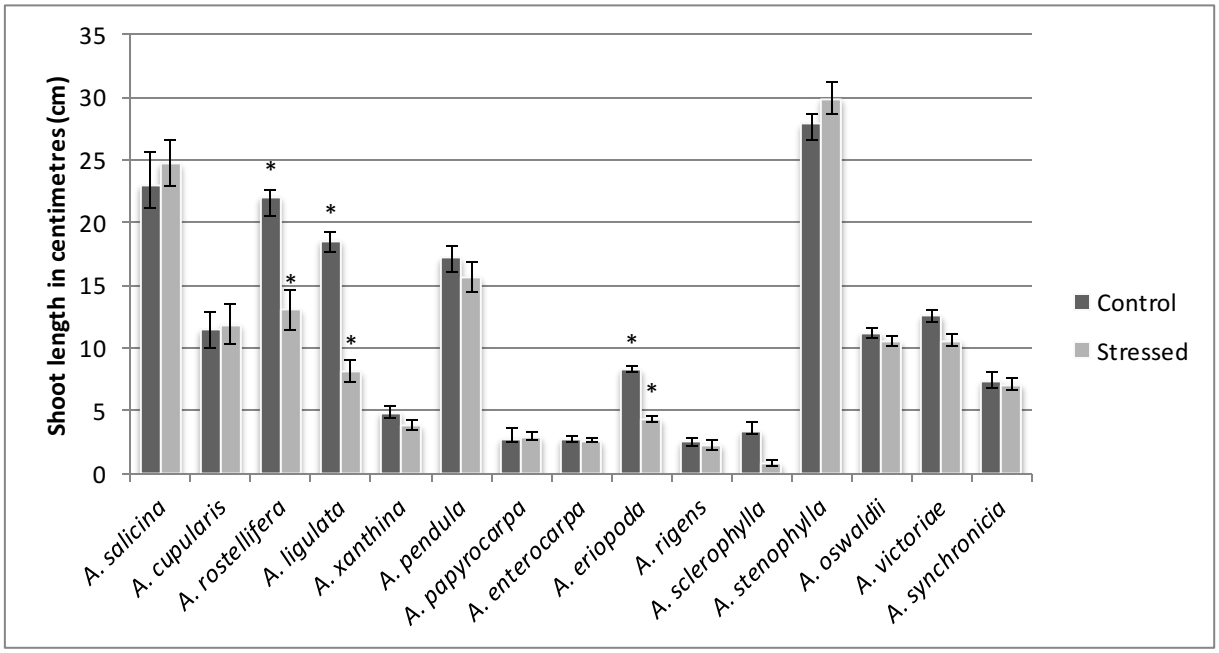
C: control plants, S: plants stressed with 300 mM salt.
FW: Fresh weight in gram, DW: Dry weight in gram, LRWC: Leaf relative water content (%), TWC: Tissue water content (%)
Values are mean ± SEM of three biological replicates
An asterisk '*' indicates mean difference between control and stressed sample is significant at $p < 0.05$

Table 6.3: Effect of salinity on root physiology parameters

Identification		Root physiology parameters							
S. no.	Plant	Length (cm)		FW (gram)		DW (gram)		RWC (%)	
		C	S	C	S	C	S	C	S
1	*A. salicina*	34.10±5.456	38.93±2.058	1.520±0.123	1.322±0.098	0.469±0.051	0.368±0.028*	69.3	68.8
2	*A. cupularis*	23.10±1.986	40.57±1.059*	0.250±0.032	0.263±0.029	0.043±0.005	0.085±0.012	82.6	66.6
3	*A. rostellifera*	24.33±3.571	29.97±15.219	1.551±0.095	0.408±0.281*	0.404±0.067	0.107±0.036*	74.2	68.7
4	*A. ligulata*	17.67±0.338	14.05±1.050	5.380±0.1437	0.143±0.007*	0.113±0.001	0.026±0.003*	97.9	82.0
5	*A. xanthina*	13.07±1.241	22.73±1.386	0.030±0.003	0.026±0.001	0.007±0.000	0.005±0.000	77.3	79.2
6	*A. pendula*	22.40±0.800	23.30±1.200	1.099±0.112	0.967±0.057	0.291±0.020	0.273±0.022	75.1	69.9
7	*A. papyrocarpa*	15.83±2.697	22.97±3.405	0.028±0.010	0.020±0.006	0.008±0.001	0.004±0.001	73.4	78.0
8	*A. enterocarpa*	18.77±0.825	19.40±0.045	0.019±0.002	0.103±0.022	0.005±0.000	0.017±0.003	71.2	83.1
9	*A. eriopoda*	26.17±0.555	30.10±0.635	0.125±0.008	0.058±0.004	0.054±0.004	0.020±0.001	56.4	66.5
10	*A. rigens*	18.03±1.506	17.77±0.623	0.085±0.008	0.032±0.005	0.020±0.003	0.014±0.003	75.6	58.3
11	*A. sclerophylla*	18.13±4.476	16.43±2.919	0.029±0.009	0.019±0.006	0.010±0.002	0.005±0.001	59.9	71.0
12	*A. stenophylla*	27.60±1.021	38.30±1.504*	1.446±0.037	1.734±0.085*	0.575±0.024	0.515±0.060	60.3	69.8
13	*A. oswaldii*	16.67±0.328	13.87±0.902	0.873±0.045	0.761±0.051	0.225±0.055	0.177±0.019	74.8	76.8
14	*A. victoriae*	13.10±0.666	19.00±0.721	0.521±0.058	0.489±0.018	0.197±0.042	0.095±0.004*	63.2	80.5
15	*A. synchronicia*	15.30±1.136	18.07±1.010	0.041±0.007	0.037±0.006	0.011±0.002	0.007±0.001	73.5	80.8

C: control plants, S: plants stressed with 300 mM salt.
FW: Fresh weight in gram, DW: Dry weight in gram RWC: Root water content (%)
Values are mean ± SEM of three biological replicates
An asterisk '*' indicates mean difference between control and stressed sample is significant at $p < 0.05$

A.



B.

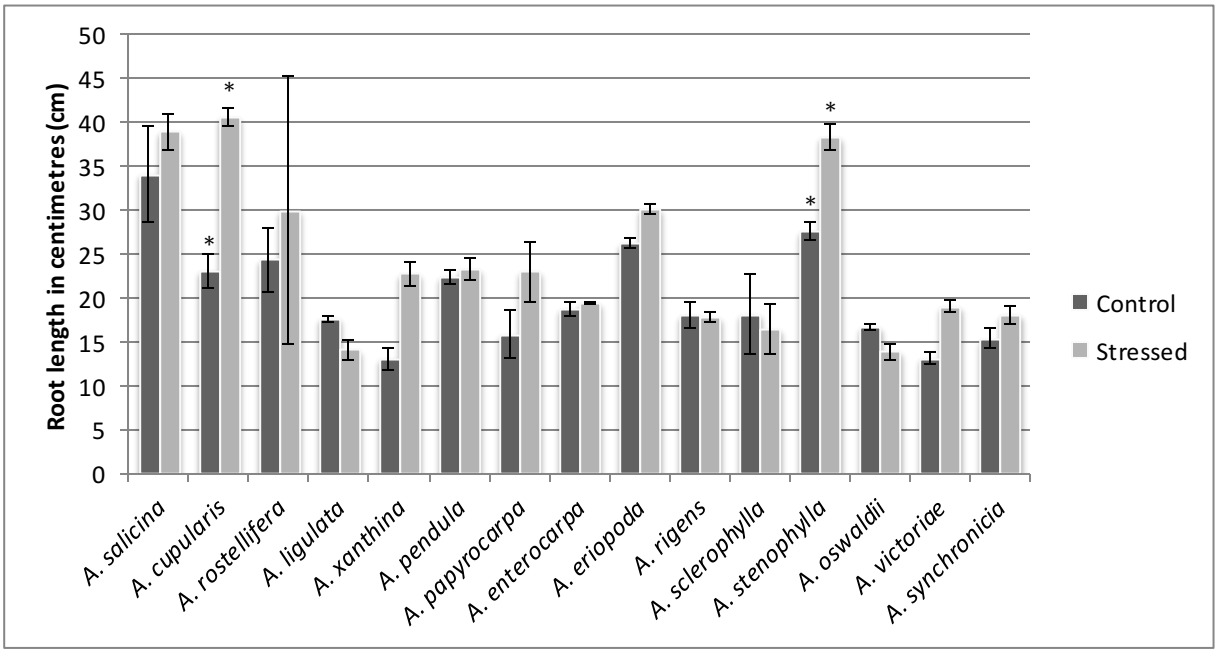


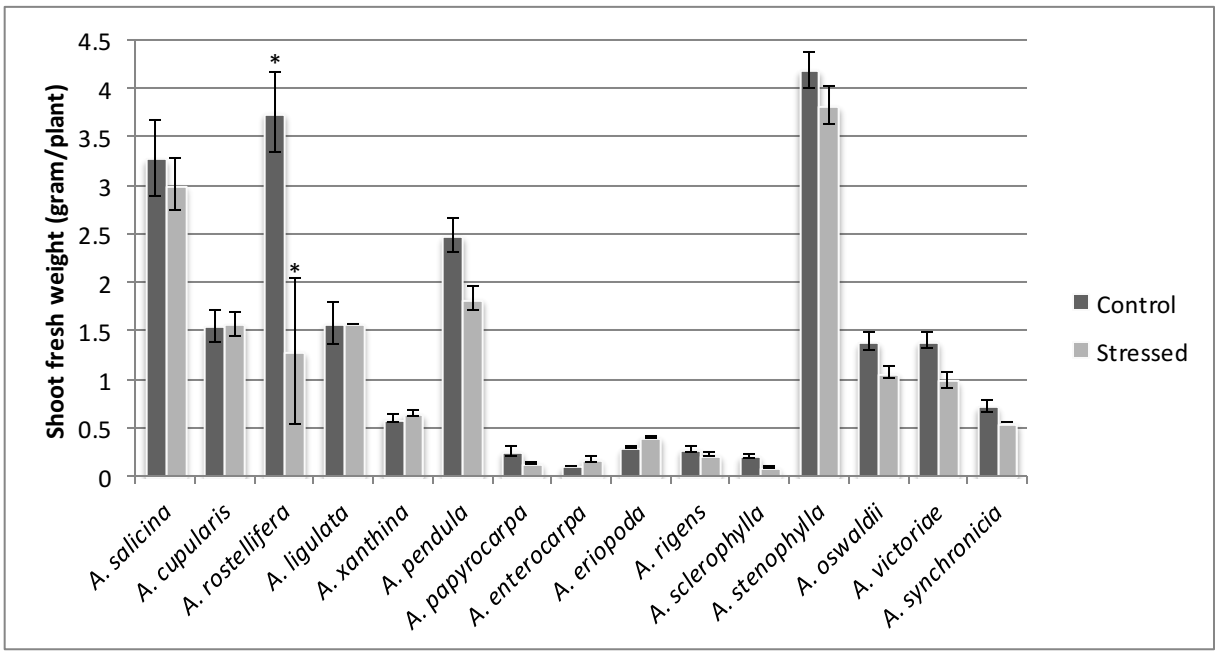
Figure 6.1: Effect of salinity on shoot and root lengths

A. Effect of salinity on shoot length.
B. Effect of salinity on root length.
Vertical bars represent standard error of mean (SEM) of three biological replicates. An asterisk indicates significant change ($p < 0.05$) between treatments.

6.3.2 Effects of salinity on plant biomass

Almost all species showed a decrease in shoot fresh weight under salt stress, with a significant change observed in *A. rostellifera* (p=0.000) (Figure 6.2A, Table 6.2). *A. cupularis*, *A. xanthina, A. enterocarpa* and *A. eriopoda* showed a small but negligible ($p > 0.05$) increase. All species showed a decrease in root fresh weight compared to the controls, especially *A. rostellifera* and *A. ligulata* (both p=0.000), while *A. stenophylla* (p=0.009) and *A. enterocarpa* (p=0.428) showed slightly higher root fresh weight (Figure 6.2B, Table 6.3). *A. salicina, A. rostellifera, A. pendula, A. stenophylla* and *A. victoriae* showed a significant ($p < 0.05$) reduction in shoot dry weight (Figure 6.3A, Table 6.2). Many other species also exhibited a small ($p > 0.05$) decrease, except *A. cupularis*, *A. enterocarpa* and *A. eriopoda* which showed increases which were non-significant ($p > 0.05$). In terms of root dry weight, a statistically significant ($p < 0.05$) decline was observed in four species, *A. salicina, A. rotesllifera, A. ligulata* and *A. victoriae* ($p < 0.05$), and minor decreases in four species (*A. eriopoda, A. pendula, A. stenophylla, A. oswaldii*) (Figure 6.3B, Table 6.3). On the contrary, *A. cupularis* and *A. enterocarpa* showed a non-significant ($p > 0.05$) increase.

A.



B.

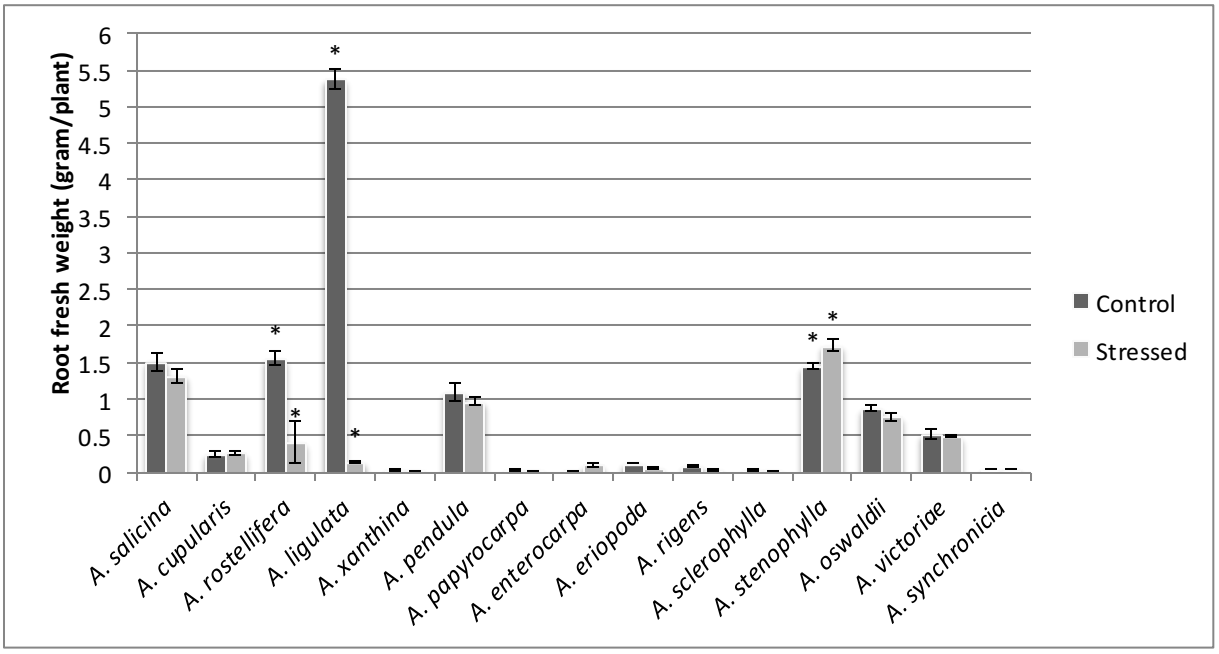


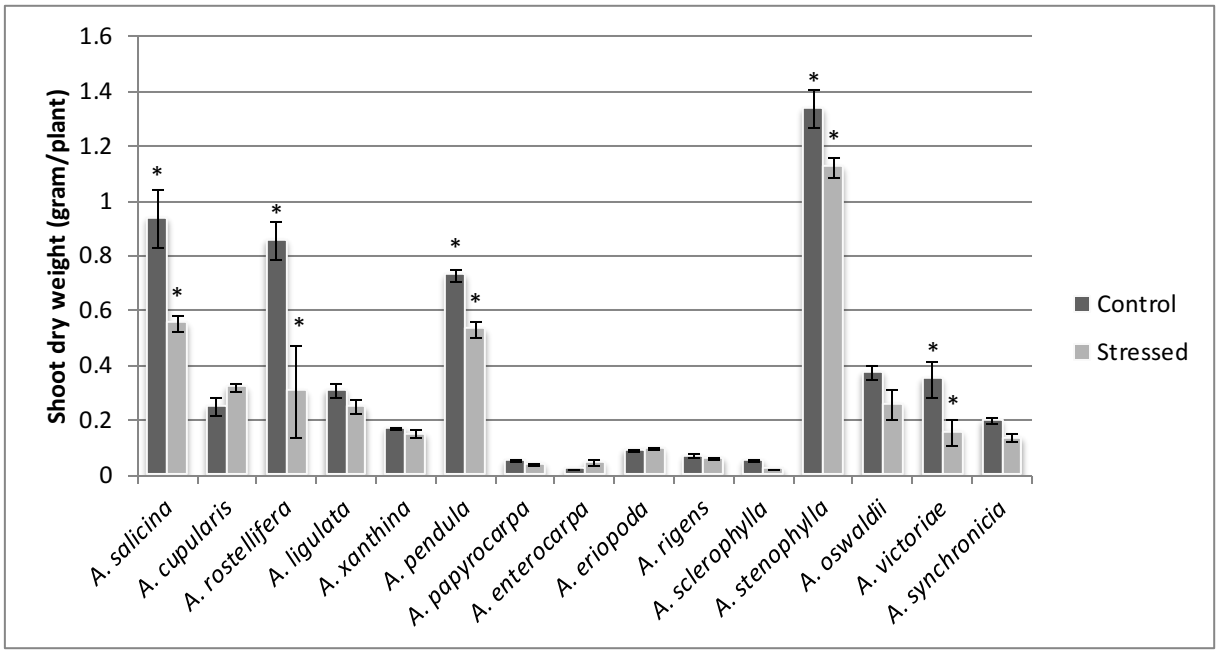
Figure 6.2: Effect of salinity on shoot and root fresh weights

A. Effect of salinity on shoot fresh weight.

B. Effect of salinity on root fresh weight.

Vertical bars represent standard error of mean (SEM) of three biological replicates. An asterisk indicates significant change ($p < 0.05$) between treatments.

A.



B.

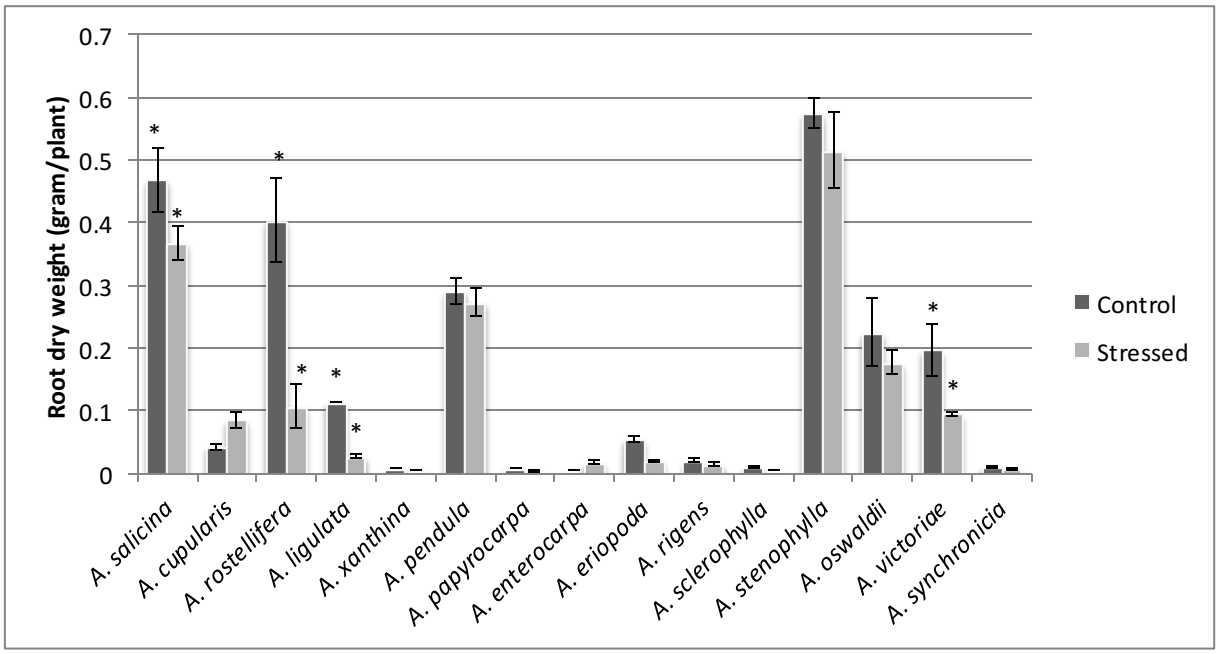


Figure 6.3: Effect of salinity on dry weight of shoot and root

A. Effect of salinity on dry weight of shoot.

B. Effect of salinity on dry weight of root

Vertical bars represent standard error of mean (SEM) of three biological replicates. An asterisk indicates significant change ($p < 0.05$) between treatments.

6.3.3 Effect of salinity on water content

The leaf relative water content % (LRWC) calculated using the fresh, dry and turgid weight of the leaf of individual plant relate to the increase or decrease in water content of the leaf. Some species showed an increase in LRWC% (*A. ligulata, A. pendula, A. papyrocarpa, A. eriopoda, A. sclerophylla, A. stenophylla, A. oswaldii, A. victoriae* and *A. synchronicia*) while some showed a decrease (*A. salicina, A. cupularis, A. rostellifera, A. xanthina, A. enterocarpa* and *A. rigens*). The root water content (computed as tolerance index [TI_{RWC}]) of the plants was derived based on fresh and dry weights of roots (Table 6.3). A TI_{RWC} value of 1 indicates no change in root water content between control and stressed plants, >1 indicates increased root water content, and <1 denotes a reduction.

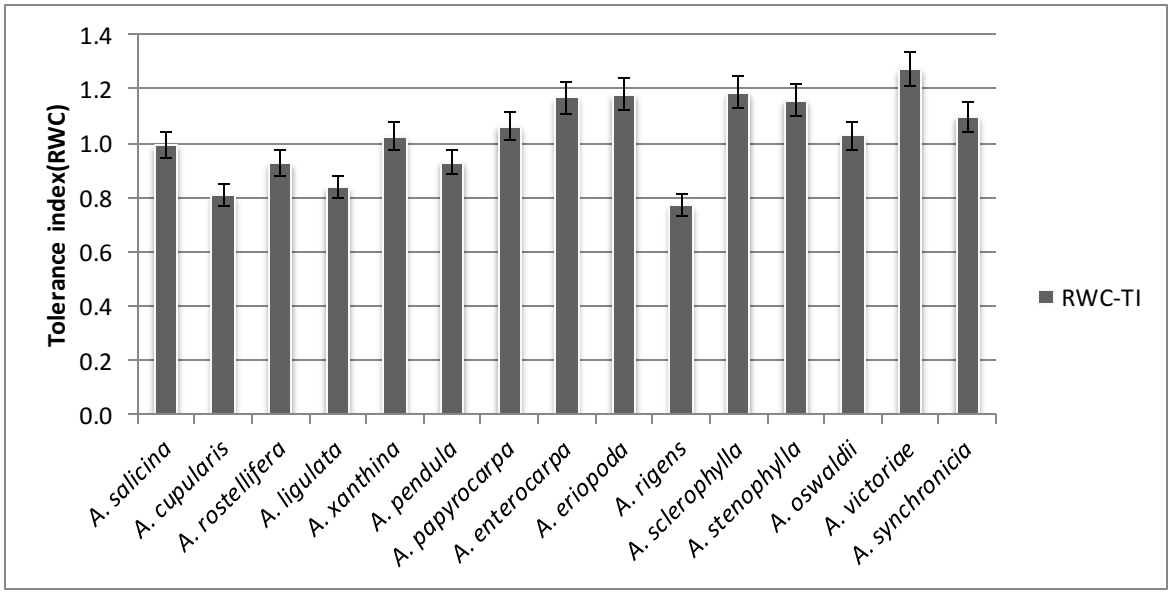


Figure 6.4: Effect of salinity on RWC in terms of tolerance index (TI_{RWC})
Vertical bars represent standard error of mean (SEM) of three biological replicates.

Accordingly, species that indicated increased root water content (TI_{RWC} >1) were: *A. xanthina, A. papyrocarpa, A. enterocarpa, A. eriopoda, A. sclerophylla, A. stenophylla, A. oswaldii, A. victoriae,* and *A. synchronicia* (Figure 6.4, Table 6.3). Species with a reduction in root water content (TI_{RWC} < 1) were *A. cupularis, A. rostellifera, A. ligulata, A. pendula,* and *A. rigens*; while *A. salicina* showed no change (TI_{RWC} = 1). The tissue water content % (TWC%) indicates the overall total water content of the plant including the shoot and the roots. Salinity treatment had caused an increase in the water content of a majority of species, namely *A. salicina, A. ligulata, A. xanthina, A.*

pendula, A. eriopoda, A. sclerophylla, A. stenophylla, A. oswaldii, A. victoriae and *A. synchronicia.* However, some species showed a decrease in TWC (*A. cupularis, A. rostellifera, A. papyrocarpa, A. enterocarpa,* and *A. rigens*).

6.3.4 Overall salt tolerance index

The data obtained above for the key parameters of plant growth (shoot + root lengths) and plant biomass, i.e., total (shoot + root) FW and total (shoot + root) DW (Tables 6.2 and 6.3), were used to compute the overall stress tolerance indices (STI) (Table 6.4). STI >1 indicates a positive correlation of tolerance to stress; and STI <1 indicates a negative correlation and an STI of 1 indicates no notable change in a particular parameter between the two treatments. Under salinity, some species had higher growth compared to control plants, with the highest growth (1.5 times) observed in *A. cupularis* and *A. xanthina*. A 40% decline (STI=0.6) was seen in *A. ligulata*, but no change in *A. pendula, A. enterocarpa, A. eriopoda* or *A. rigens*.

In terms of fresh weight, only five species (*A. cupularis, A. xanthina, A. enterocarpa, A. eriopoda* and *A. stenophylla*) showed an increase under salt stress (STI >1). Ten species showed a decrease, including *A. ligulata,* which showed a steep decline of 80%, whereas *A. cupularis* and *A. stenophylla* had no notable difference. Salt treatment also reduced dry weights; except for *A. cupularis* and *A. enterocarpa,* all other species had a reduction in DW, the highest being in *A. rostellifera*. Based on the STIs, the species exhibiting superior performance (STI>1) in all three parameters (growth, FW, DW) compared to other species were given an arbitrary rank of 1 (Table 6.4), species with two STI >1 given a rank of 2, species with one STI>1 given a rank of 3, and no rank given to species with a poor performance in all parameters. This comparison was ultimately used to predict prospective candidates for field trials. Candidates that performed on all three levels, i.e., *A. cupularis* and *A. enterocarpa,* seems to be the most tolerant or adaptable and hence the most suitable species for trials on saline lands. These were followed by species that performed well in two parameters (*A. xanthina*, *A. eriopoda* and *A. stenophylla*), then those that performed well in at least one of the parameters (*A. pendula*, *A. papyrocarpa*, *A. rigens*, *A. victoriae* and *A. synchronicia*). Only four species seemed to have less desirable qualities of salt tolerance (*A. rostellifera*, *A. ligulata*, *A. sclerophylla* and *A. oswaldii*).

Table 6.4: Combined data (Shoot+Root) used for predicting comparative ranking of salinity tolerant species

Identification		Combined data on physiology parameters[1]						Salt tolerance index (STI)[2]			Ranking[3]	Comparative ranking[4]
S. no.	Plant	G (cm)		FW (gram)		DW (gram)		STI_G	STI_{FW}	STI_{DW}		
		C	S	C	S	C	S					
1	*A. salicina*	57.1	63.63	4.8	4.332	1.40	0.92	1.1	0.9	0.7	A^G	**3**
2	*A. cupularis*	34.57	52.47	1.798	1.832	0.29	0.40	1.5	1.0	1.4	$A^GA^{FW}A^{DW}$	**1**
3	*A. rostellifera*	46.4	43.04	5.305	1.702	1.26	0.41	0.9	0.3	0.3	-	**-**
4	*A. ligulata*	36.27	22.2	6.959	1.718	0.42	0.27	0.6	0.2	0.6	-	**-**
5	*A. xanthina*	17.94	26.56	0.626	0.676	0.17	0.16	1.5	1.1	0.9	A^GA^{FW}	**2**
6	*A. pendula*	39.6	39	3.581	2.801	1.02	0.80	1.0	0.8	0.8	A^G	**3**
7	*A. papyrocarpa*	18.6	25.9	0.288	0.161	0.06	0.04	1.4	0.6	0.7	A^G	**3**
8	*A. enterocarpa*	21.47	22	0.125	0.282	0.03	0.06	1.0	2.3	2.5	$A^GA^{FW}A^{DW}$	**1**
9	*A. eriopoda*	34.47	34.43	0.42	0.469	0.14	0.11	1.0	1.1	0.8	A^GA^{FW}	**2**
10	*A. rigens*	20.53	20.07	0.368	0.258	0.09	0.07	1.0	0.7	0.8	A^G	**3**
11	*A. sclerophylla*	21.46	17.2	0.243	0.116	0.06	0.03	0.8	0.5	0.4	-	**-**
12	*A. stenophylla*	55.5	68.23	5.64	5.556	1.91	1.64	1.2	1.0	0.9	A^GA^{FW}	**2**
13	*A. oswaldii*	27.9	24.4	2.261	1.828	0.60	0.43	0.9	0.8	0.7	-	**-**
14	*A. victoriae*	25.67	29.6	1.916	1.481	0.55	0.25	1.2	0.8	0.5	A^G	**3**
15	*A. synchronicia*	22.63	25.17	0.765	0.591	0.21	0.14	1.1	0.8	0.7	A^G	**3**

C: control plants, S: plants stressed with 300 mM salt; G: Growth, FW: Fresh Weight, DW: Dry Weight.

[1] Values are given as the combined average of mean values of shoot and root data for each parameter.

[2] STI >1 indicates a measured increase in a specific parameter while STI<1 indicates a measured decrease; STI=1 indicates no measured change in a specific parameter.

[3] Ranking: the letter 'A' denotes superior performance of the plant under 300 mM NaCl stress compared to the other species tested. The letters 'G', 'FW' and 'DW' indicate the parameters used for evaluation of salt tolerance.

[4] Comparative ranking: species that had an STI >1 for all three parameters were ranked number 1, species that had an STI >1 for two parameters were ranked as number 2, and species that had an STI >1 for only one parameter were ranked as number 3.

6.3.5 Effect of salinity on ion concentrations

Sodium: Salt treatment resulted in elevated shoot and root Na^+ concentration compared to control plants (Tables 6.5 and 6.6). The level of accumulation was significant across all species (shoot: $p = 0.002$; root: $p = 0.0001$). Sodium accumulation in the shoot tissues of control plants ranged from 0.50-10.07 mg/g DW (0.02-0.44 mM/g DW), whereas in stressed shoots it ranged from 1.54-38.37 mg/g DW (0.07-1.67 mM/g DW). The Na^+ content in stressed shoot was highest in *A. ligulata* (38.37 ± 3.452 mg/g DW) and lowest in *A. cupularis* (1.54 ± 0.314 mg/g DW). In the root tissues of control plants the Na^+ range was 0.52 – 7.01 mg/g DW (0.02-0.31 mM/g DW) and in stressed plants it was 1.73-29.39 mg/g DW (0.08-1.28 millimoles/g DW). In stressed root tissues, the Na^+ content was highest in *A. rostellifera* (29.39 ± 10.00 mg/g DW) and lowest in *A. xanthina* (1.73 ± 0.216 mg/g DW). In terms of fold change (Table 6.7), *A. eriopoda* had a substantial rise of 22.8 times in stressed shoot tissues and *A. rostellifera* accumulated 56.0 times more Na^+ in its roots under stress.

Potassium: The concentration of potassium ions was altered in most species between the two treatments (Tables 6.5 and 6.6); although the differences were not statistically significant for all 15 species (shoot: $p = 0.961$; root: $p = 0.457$). The general range of accumulation in these species was 2.52 – 41.97 mg/g DW or 0.06-1.07 mM/g DW (control shoot), 1.23-44.53 mg/g DW or 0.03-1.13 mM/g DW (stressed shoot), 3.19-25.38 mg/g DW or 0.08-0.65 mM/g DW (stressed shoot) and 1.93-37.53 mg/g DW or 0.05-0.96 mM/g DW (Tables 6.5 and 6.6). The only exceptions to this range were *A. ligulata* (control shoot) and *A. cupularis* (stressed root) which had a very high K content of 93.86 mg/g DW (2.4 mM/g DW) and 87.87 mg/g DW (2.25 mM/g DW) respectively. In terms of fold change (Table 6.7), *A. stenophylla* had the highest fold change of 6.8 in shoot and 4.3 in root. A decrease in fold change was seen in *A. ligulata* shoot (0.4) and in the root tissues of *A. rostellifera*, *A. eriopoda* and *A. rigens* (0.6) respectively.

Table 6.5: Effect of salinity on ion accumulation in shoots of *Acacia* species

Identification		Ion accumulation levels in shoot (mg/g DW)							
S. no.	Plant	Na		K		Ca		Mg	
		C	S	C	S	C	S	C	S
1	*A. salicina*	0.583±0.096	12.639±0.219*	5.284±0.174	17.951±0.951*	52.032±2.832	94.342±2.146*	1.427±0.069	1.432±0.283
2	*A. cupularis*	1.035±0.532	1.535±0.314	41.970±0.571	44.53±0.47	69.649±13.773	50.041±1.923	11.512±2.75	8.526±0.293
3	*A. rostellifera*	2.797±0.716	17.699±4.627*	11.727±2.862	16.976±1.1	31.481±0.72	30.024±5.715	9.222±0.951	12.355±2.858
4	*A. ligulata*	3.077±2.249	38.366±3.452*	93.857±3.875	39.58±0.557*	57.806±16.679	73.909±0.294	18.024±5.213	24.421±3.231
5	*A. xanthina*	0.663±0.12	4.508±0.52*	4.116±0.264	2.123±0.579*	17.306±1.646	14.512±0.891	7.439±0.613	7.019±0.842
6	*A. pendula*	2.785±0.431	7.05±0.696*	3.898±0.644	13.352±1.8*	32.49±2.832	27.64±0.515	13.909±0.717	12.91±0.544
7	*A. papyrocarpa*	4.188±2.295	21.557±2.211*	38.453±2.277	30.966±3.097	22.279±4.449	25.852±3.067	20.864±2.558	18.906±0.102
8	*A. enterocarpa*	1.756±0.686	8.294±0.431*	5.975±0.802	3.325±0.579	40.95±1.913	5.147±1.11*	7.317±4.707	2.591±2.135*
9	*A. eriopoda*	0.721±0.145	16.462±0.108*	3.403±0.568	1.959±0.232	38.213±1.959	11.531±1.275*	2.629±0.049	1.419±0.037
10	*A. rigens*	10.071±1.944	14.705±2.851	4.436±0.439	2.218±0.180*	40.31±1.146	40.357±3.115	3.454±0.402	1.389±0.64*
11	*A. sclerophylla*	3.921±1.856	2.287±0.239	24.71±1.37	34.711±0.742	18.438±0.824	18.425±1.853	9.44±1.111	12.613±1.417
12	*A. stenophylla*	4.197±0.551	22.639±1.791*	2.642±0.086	17.951±0.951*	70.968±2.744	64.015±2.913	17.685±1.193	21.554±0.828
13	*A. oswaldii*	1.706±0.318	3.749±0.691*	5.904±0.29	11.168±0.502*	47.211±1.884	46.407±1.373	12.462±1.504	18.047±2.553
14	*A. victoriae*	1.846±0.164	5.34±0.4*	3.086±0.097	8.458±0.575*	32.436±3.469	24.243±3.454	6.052±0.763	4.558±0.386
15	*A. synchronicia*	0.497±0.128	3.731±0.283*	2.515±0.368	1.229±0.415	7.065±0.522	5.093±0.151	3.394±0.221	1.405±0.254*

C: control plants, S: plants stressed with 300 mM salt.
Na: Sodium, K: Potassium, Ca: Calcium, Mg: Magnesium
Values are mean ± SEM of three biological replicates
An asterisk '*' indicates mean difference between control and stressed sample is significant at $p < 0.05$

Table 6.6: Effect of salinity on ion accumulation in roots of *Acacia* species

Identification		Ion accumulation levels in root (mg/g DW)							
S. no.	Plant	Na		K		Ca		Mg	
		C	S	C	S	C	S	C	S
1	*A. salicina*	1.458±0.153	7.408±0.545*	10.079±1.91	14.008±1.206	25.163±4.744	50.237±4.876*	5.878±1.022	5.131±0.285
2	*A. cupularis*	6.116±0.807	18.391±3.464*	21.026±1.586	87.867±7.125*	65.784±2.536	56.127±2.954	21.95±2.38	34.137±3.613
3	*A. rostellifera*	0.525±0.027	29.385±10.003*	23.223±4.432	14.052±2.54	70.893±5.605	68.284±25.832	50.46±2.76	24.278±6.978*
4	*A. ligulata*	7.013±0.049	10.002±0.432	25.379±2.237	37.527±23.226	59.51±13.885	71.078±5.968	59.51±7.028	22.925±6.699*
5	*A. xanthina*	0.563±0.125	1.729±0.216*	13.655±0.43	15.474±0.888	21.065±1.192	15.109±1.628	3.755±0.778	3.166±0.814
6	*A. pendula*	2.745±0.31	10.792±0.352*	16.77±0.445	11.28±1.045	23.16±0.921	19.426±0.749	8.113±0.512	5.244±0.302
7	*A. papyrocarpa*	2.199±0.43	13.909±0.717*	25.358±4.412	21.056±5.607	43.402±15.327	10.686±4.902*	32.216±8.242	13.196±1.46*
8	*A. enterocarpa*	0.523±0.102	3.295±2.196*	3.193±0.551	2.326±0.375	64.326±8.814	19.931±0.88*	2.26±0.427	1.418±0.162
9	*A. eriopoda*	3.625±0.704	19.381±1.612*	4.977±0.676	2.835±0.277	76.097±1.881	45.295±4.607	4.583±0.766	3.101±0.209
10	*A. rigens*	0.804±0.033	15.054±6.766*	3.371±0.544	1.934±0.29	48.088±9.369	17.908±2.385*	2.494±0.315	6.477±0.226*
11	*A. sclerophylla*	0.517±0.021	11.302±2.049*	24.96±2.473	30.857±2.124	73.04±17.451	43.493±2.985	23.369±3.326	34.965±19.665
12	*A. stenophylla*	5.335±0.566	23.617±1.38*	3.271±0.616	14.008±1.206*	46.302±1.526	46.484±0.678	13.75±0.525	11.525±1.385
13	*A. oswaldii*	3.159±0.462	6.333±0.567*	25.155±0.853	18.154±1.245	35.592±2.862	26.511±0.66	10.009±0.878	6.61±0.188
14	*A. victoriae*	1.741±0.292	7.086±0.377*	7.532±0.845	6.386±0.628	16.505±1.513	14.502±0.454	4.245±0.734	2.01±0.356*
15	*A. synchronicia*	0.935±0.109	2.041±0.257*	8.617±0.892	7.079±0.327	19.453±1.468	19.304±0.66	2.95±0.332	1.147±0.193

C: control plants, S: plants stressed with 300 mM salt.
Na: Sodium, K: Potassium, Ca: Calcium, Mg: Magnesium
Values are mean ± SEM of three biological replicates
An asterisk '*' indicates mean difference between control and stressed sample is significant at $p < 0.05$

K^+/Na^+ selectivity ratio: The selective uptake of K^+ in relation to Na^+ was expressed as a ratio (Ashraf and McNeilly, 2004). Several studies have used this ratio as an important measure for determining the tolerance/susceptibility nature of plants to salt stress (Ashraf and McNeilly, 2004). This ratio was altered in most species under salinity stress (Table 6.7, Figure 6.5). A ratio of 1 indicates no change in the accumulation of K^+ relative to Na^+ under control or stress conditions. A ratio of < 1 indicates higher accumulation of Na^+ (or low K^+) and > 1 indicates lower accumulation of Na^+ (or high K^+). In terms of shoot K^+/Na^+ selectivity ratio, *A. sclerophylla*, *A. stenophylla* and *A. pendula* maintained a safe proportion of K^+: Na^+ (> 1) under stress. In terms of root K^+/Na^+ selectivity ratio, *A. cupularis* and *A. ligulata* had $K^+/Na^+ > 1$. A major disturbance in ion selectivity ratio in shoot and root (K^+: Na^+ < 1) was observed in *A. synchronicia*, *A. rigens*, *A. eriopoda*, *A. enterocarpa*, *A. papyrocarpa*, *A. xanthina*, *A. rostellifera* and *A. salicina.*

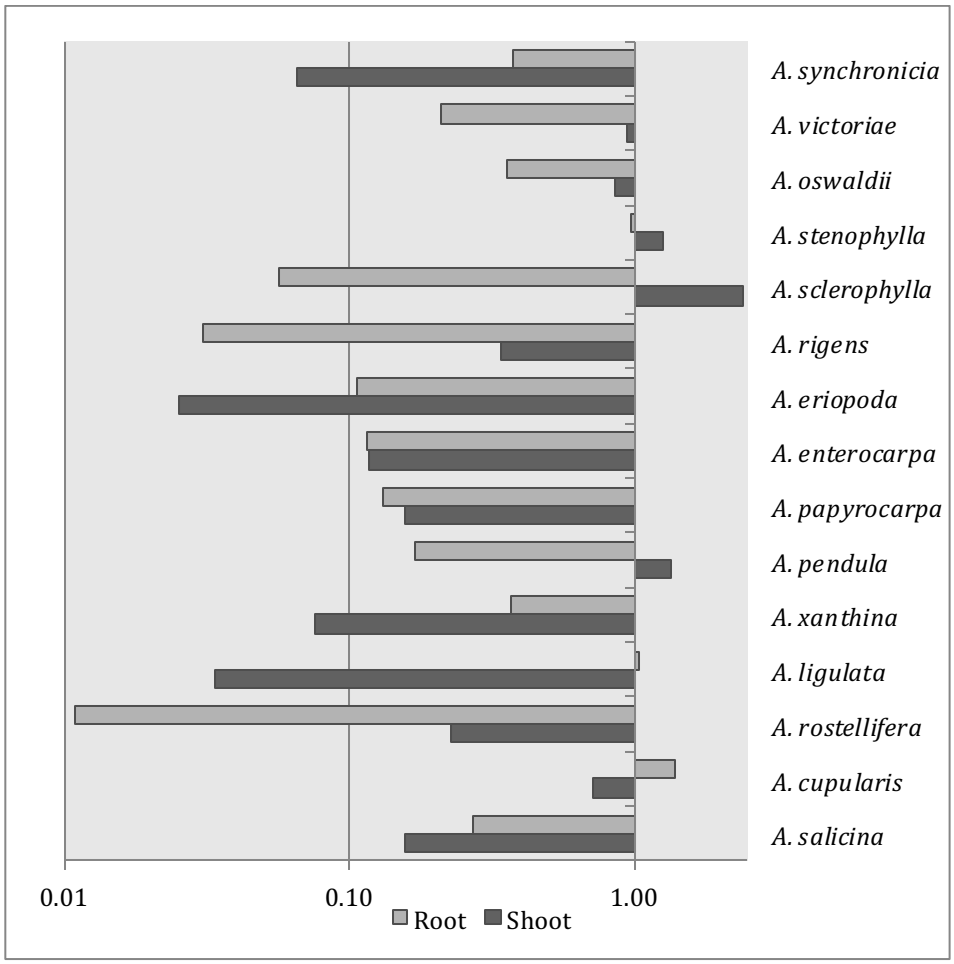


Figure 6.5: Selective uptake of K^+ in relation to Na^+ (K^+/Na^+ ratio) of *Acacia* species under salinity stress

Calcium (Ca^{2+}): The range of calcium accumulation under control was 7.07-70.97 mg/g DW (or 0.03-2.92 mM/g DW) in shoot and 16.51-76.10 mg/g DW (or 0.68-3.13 mM/g DW) in root; whereas under salt stress, Ca^{2+} accumulation was 5.10-94.34 mg/g DW (or 0.21-3.88 mM/g DW in shoot and 10.69-71.08 mg/g DW (or 0.44-2.92 mM/g DW) in root tissues (Tables 6.5 and 6.6). In terms of fold change, Ca^{2+} accumulation was highest in *A. salicina* in the shoot (1.8) and root (2.0) (Table 6.7). Ca^{2+} levels were lowered under stress by almost 90% in *A. enterocarpa* shoots and by 80% in *A. papyrocarpa* roots. Calcium levels were generally higher than those of Na^{+}, K^{+}, Mg^{2+} in all species, and not significantly different between the control and stressed groups (shoot: $p = 0.708$; root: $p = 0.165$).

Table 6.7: Accumulation of cations in *Acacia* spp

S. no.	Plant	Fold change of cation accumulation*								K^+/Na^+ ratio**	
		Na^+		K^+		Ca^{2+}		Mg^{2+}			
		S	R	S	R	S	R	S	R	S	R
1	***A. salicina***	21.7	5.1	3.4	1.4	1.8	2.0	1.0	0.9	0.16	0.27
2	***A. cupularis***	1.5	3.0	1.1	4.2	0.7	0.9	0.7	1.6	0.72	1.39
3	***A. rostellifera***	6.3*	56.0	1.4	0.6	1.0	1.0	1.3	0.5	0.23	0.01
4	***A. ligulata***	12.5*	1.4	0.4	1.5	1.3	1.2	1.4	0.4	0.03	1.04
5	***A. xanthina***	6.8	3.1	0.5	1.1	0.8	0.7	0.9	0.8	0.08	0.37
6	***A. pendula***	2.5	3.9	3.4	0.7	0.9	0.8	0.9	0.6	1.35	0.17
7	***A. papyrocarpa***	5.1	6.3	0.8	0.8	1.2	0.2	0.9	0.4	0.16	0.13
8	***A. enterocarpa***	4.7	6.3	0.6	0.7	0.1	0.3	0.4	0.6	0.12	0.12
9	***A. eriopoda***	22.8	5.3	0.6	0.6	0.3	0.6	0.5	0.7	0.03	0.11
10	***A. rigens***	1.5	18.7	0.5	0.6	1.0	0.4	0.4	2.6	0.34	0.03
11	***A. sclerophylla***	0.6	21.9	1.4	1.2	1.0	0.6	1.3	1.5	2.41	0.06
12	***A. stenophylla***	5.4	4.4	6.8	4.3	0.9	1.0	1.2	0.8	1.26	0.97
13	***A. oswaldii***	2.2	2.0	1.9	0.7	1.0	0.7	1.4	0.7	0.86	0.36
14	***A. victoriae***	2.9	4.1	2.7	0.8	0.7	0.9	0.8	0.5	0.95	0.21
15	***A. synchronicia***	7.5	2.2	0.5	0.8	0.7	1.0	0.4	0.4	0.07	0.38

*The differential accumulation of major cations under control and salt stress conditions are expressed in terms of fold change. Na^+: Sodium, K^+: Potassium, Ca^{2+}: Calcium, Mg^{2+}: Magnesium. S: Shoot tissues, R: Root tissues.

**The K^+/Na^+ ratio indicates selectivity of ions by plants under salt stress conditions (Ashraf and McNeilly, 2004)

Magnesium (Mg^{2+}): Magnesium levels were less altered in the shoots as compared to the roots (Tables 6.5 and 6.6). Like the K^+ and Ca^{2+} accumulation patterns, levels of Mg^{2+} did not differ significantly between the control and stressed plants (shoot: $p = 0.912$; root: $p = 0.408$). The range of Mg^{2+} accumulation under control was 1.43-20.86 mg/g DW (0.04-0.52 mM/g DW) in shoot and 2.26-59.51 mg/g DW (0.06-1.48 mM/g DW) in root; whereas under salt stress, Mg^{2+} accumulation was 1.39-24.42 mg/g DW (0.03-0.61 mM/g DW) in shoot and 1.15-34.97 mg/g DW (0.03-0.87 mM/g DW) in root tissues. Mg^{2+} accumulation was highest, in terms of fold change (Table 6.7), in shoots of *A. ligulata* and *A. oswaldii* (1.4) and roots of *A. rigens* (2.6). The highest decline (60%) in Mg^{2+} levels occurred in the shoots of *A. enterocarpa*, *A. rigens* and *A. synchronicia* and roots of *A. ligulata*, *A. papyrocarpa* and *A. synchronicia*.

6.4 Discussion

Salinity tolerance is a complex, multi-genic and multi-dimensional trait (Parida and Das, 2005; Mansour and Salama, 2004) and a single parameter by itself cannot define the tolerance status of a plant. The two most common physiological parameters used to assess salt tolerance are growth (e.g. shoot and root length) and biomass (e.g. shoot and root weight) (Ahmed *et al.*, 2013; Morais *et al.*, 2012; Jha *et al.*, 2010). Salinity stress has several implications on plant physiology, including, but not limited to, reduced plant growth, ionic imbalance, photosynthesis and mineral distribution (reviewed in Shabala and Munns, 2012; Nawaz *et al.*, 2010; Munns and Tester, 2008). Many plants genetically vary in their sensitivity/tolerance to salinity and mechanisms of adaptation, including species belonging to the same genus. Kao *et al.*, (2006) showed that three species of soybean (*Glycine* spp.) responded differently to salinity stress. Australian native acacias occupy around 20% of Australian native vegetation, and have many contemporary and historically recorded uses. As a large genus, *Acacia* is comprised of ca. 1000 species in Australia but very little published data exists on salinity tolerance for most Australian native acacia species. However, many acacias are known to be adapted to salinity (Morais *et al.* 2012), alkalinity and drought tolerance (Fagg and Stewart, 1994).

In order to identify species to investigate in details for tolerances, and considering the large number of species in the genus, we first undertook molecular phylogenetic studies

of a subset taxonomically known to be closely related to *Acacia pendula*, *A. salicina*, *A. stenophylla* and *A. victoriae*. This led to the identification of morphological and phylogenetically closely related species (*A. rigens, A. enterocarpa, A. sclerophylla, A. eriopoda, A. papyrocarpa, A. oswaldii, A. ligulata, A. cupularis, A. xanthina, A. rostellifera and A. synchronicia,* along with *A. pendula, A. salicina, A. stenophylla* and *A. victoriae)*. The present work investigated the effects of salinity on plant growth characteristics and ion accumulation in these species under salinity stress, so as to obtain experimental data to identify tolerant species.

6.4.1 Salinity stimulates plant growth in some *Acacia* species

On the basis of Salt Tolerance Index (STI) dependent on plant physiology, *A. cupularis* and *A. enterocarpa* did exceptionally well in terms of increased growth and biomass accumulation (FW and DW). Several agronomically important plants exhibit a decrease in growth under salinity, e.g. potato (Jaarsma *et al.,* 2013), tomato (Ghanem *et al.,* 2011), faba bean (Tavakkoli *et al.,* 2010) and eggplant (Unlukara, 2010). There are a limited number of studies on *Acacia* species, but a negative correlation has been shown between salinity and plant growth e.g. *A. auriculiformis* showed a significant decrease in dry weight (Nguyen *et al.*, 2004) and seed germination (Patel *et al.* 2010). However, it is also not uncommon for salt to induce growth. Glenn *et al.* (1999) suggested that salt-tolerant dicot halophytes such as *Spartina* and *Salicornia* require at least 180 mM NaCl to stimulate growth. The halophytic salt bush *Atriplex nummularia* showed increased growth up to 300 mM NaCl (Araujo *et al.* 2006) and *Pennisetum alopecuroides* (fountain grass or swamp foxtail) also showed an increase in shoot length under 300 mM NaCl (Mane *et al.*, 2011).

A tacit explanation for the positive correlations could be the ability of the plant to utilise, dilute, or partition, the Na^+ to maintain its level below toxicity and/or increase water uptake and FW. Such adaptations enable a plant to adjust its osmoticum, maintain turgidity and avoid Na^+ toxicity (Munns and Tester, 2008). In the current study, *A. salicina*, *A. cupularis, A. xanthina, A. papyrocarpa, A. enterocarpa, A. stenophylla, A. victoriae* and *A. synchronicia* had a STI >1 for growth parameters, suggesting that salt had not affected their growth. *Acacia cupularis, A. xanthina, A. enterocarpa, A. eriopoda* and *A. stenophylla* showed an increase in FW, and *A.*

cupularis and *A. enterocarpa* did exceptionally well both in growth and biomass accumulation (FW and DW) under salt stress. These observations suggest the presence of adaptive mechanisms in these species.

In addition to shoot length increases, eleven species exhibited a remarkable increase in root length. The observation is in accordance with the findings of Hsiao and Xu (2000) that mild osmotic stress may inhibit the growth of leaves and stems but root elongation may continue (e.g. Maize). Continued root growth is a common adaptive mechanism of plants in dry habitats (Ramoliya *et al.*, 2004) and may enable the root to penetrate deeper into the soil to optimise water uptake. Dry weight is another critical parameter commonly used to evaluate salt tolerance (Nguyen *et al.,* 2004; Jaleel *et al.,* 2008; Mane *et al.,* 2011). Many reports show that dry weight decreased as a consequence of salinity stress e.g. in *Pennisetum alopecuroides* (Mane *et al.,* 2011); *Catharanthus roseus* (Jaleel *et al.,* 2008); *Acacia* species (Table 1.1). Reduction in dry weight may be due to a decrease in cell division and cell expansion affecting leaf/root development and elongation (Elhadi *et al.,* 2009). On the other hand, an increase in dry weight under salinity stress may be due to accumulation of solutes for osmotic adaptation and/or increased growth (Mane *et al.*, 2011), indicating better tolerance. On this basis, only *Acacia cupularis* and *A. enterocarpa* ($STI_{DW} > 1$) showed an increase in dry weight, suggesting their relatively higher degree of salt tolerance.

6.4.2 Salinity affects cation balances in *Acacia* species

Osmotic effects lead to decreased cell expansion in root tips and juvenile leaves, affecting stomatal closure (Munns and Tester, 2008). Ion toxicity can be caused due to increased accumulation of ions such as, but not limited to, Na^+, Cl^-, SO^{2-}_4, HCO^{3-}, Ca^{2+}, Mg^{2+} and rarely NO^{3-} or K^+ (reviewed in Munns and Tester, 2008; Chen and Jiang, 2010; Nawaz *et al.*, 2010, Shabala and Munns, 2012). However, accumulation of Na^+ and Cl^- can disrupt water transport and interfere with stomatal conductance (*Atriplex portulacoides,* Redondo-Gómez *et al.*, 2007), and can be especially toxic to susceptible species, causing the characteristic leaf burn and leaf curling, as noted in *Cercis canadensis* var. *mexicana* (Niu *et al.*, 2010). On the contrary, Na^+ was found to stimulate growth in major crop plants such as cotton (Ali *et al.*, 2009) and sugarbeet (Wakeel *et al.*, 2009), and was suggested to be essential for growth at relatively low

levels compared to other macro- and micronutrients (Wakeel *et al.*, 2011). The underlying mechanism for increased growth was attributed to maintenance of osmotic potential by low levels of Na^+ under limited K^+ supply. The results presented in Table 6.7 clearly portray increased Na^+ and K^+ accumulation as well as changes in ionic balance in *Acacia* species under salt stress. This suggests that the extent to which such alterations are not damaging may vary from species to species.

6.4.3 Cation accumulation patterns may be species-specific

Salt tolerance can be denoted by decreased absorption of these ions (Na^+, Cl^-) by roots and their subsequent transfer to the stem and leaves. This is the principle behind 'salt exclusion' seen in plants such as *A. ampliceps* (Marcar *et al.*, 1991), *Senegalia senegal* (Hardikar and Pandey, 2008) and *A. auriculiformis* (Patel *et al.*, 2010). Conversely, 'salt inclusion' may also be a characteristic of salt tolerance. This mode of action involves tissue tolerance and the ability to accumulate and compartmentalise the Na^+ ions into vacuoles, and has been credited for the salt tolerance of several halophytes such as *Salicornia herbacea* (Amiri and Rasouli, 2011); *Atriplex vesicaria, Atriplex nummularia, Atriplex papula, Atriplex rosea, Inula crithmoides, Salicornia rubra, Salicornia utahensis* and *Suaeda occidentalis* (cited in Koyro *et al.*, 2011); and *Atriplex amnicola*, *Atriplex calotheca*, *Atriplex hortensis*, *Chenopodium album*, *Salsola kali* and *Suaeda nudiflor* (Shekhawat *et al.*, 2006).

Given the above reports, it was interesting to note the variations in trends in the *Acacia* species studied. *Acacia cupularis* had a small fold change in Na^+ accumulation (1.5X more in shoot and 3X more in root) and K^+ accumulation (1.1X more shoot and 4.2X more in root) under salt. However, the actual K^+ content was several times higher (44.53 mg/g of stressed shoot, 87.87 mg/g of stressed root) than Na^+ (1.54 mg/g of stressed shoot and 18.39 mg/g of stressed root), suggesting K^+ was the preferred ion under saline conditions. It thus appears that *A. cupularis,* like *A. ampliceps* (Marcar *et al.* 1991), *Senegalia senegal* (Hardikar and Pandey, 2008) and *A. auriculiformis* (Patel *et al.*, 2010), may be a 'salt excluder'. In contrast, *A. enterocarpa* accumulates Na^+ in preference to K^+ in salt conditions, suggesting it may be a 'salt includer'. These differences suggest the use of diverse mechanisms in terms of ion accumulation and

regulation by individual species. The differences in Na^+ movement may be attributed to sodium transporters such as the HKT or SOS1 family (Munns and Tester, 2008).

In addition to accumulation of individual ions, ionic ratios are also imperative in maintaining osmotic and nutrient balance. Ashraf and McNeilly (2004) proposed that the maintenance of high K^+:Na^+ ratio is an important criterion for salt tolerance in brassicas. Maintaining a high K^+:Na^+ ratio is critical for enzyme activity, upholding membrane potential and cell volume regulation (Munns and Tester, 2008). Of the fifteen species tested, only *A. pendula*, *A. sclerophylla*, *A. stenophylla* and *A. victoriae* maintained a positive K^+: Na^+ ratio in shoots, while *A. cupularis*, *A. ligulata* and *A. stenophylla* maintained a positive K^+: Na^+ ratio in roots indicating their preference to K^+ over Na^+ (Table 6.7). *Acacia stenophylla* was the only species among the four to have a positive K^+: Na^+ ratio in shoot and root tissues, suggesting its high salt tolerance capacity.

Increases in cellular levels of K^+, Ca^{2+} and Mg^{2+} can occur as an immediate response, for osmotic adjustment and ionic balance (Bernstein, 1975). Under salt stress, several species with altered K^+:Na^+ selectivity ratios (higher Na+ than K+ under salt) showed decreased uptake of the essential minerals K^+, Ca^{2+} and Mg^{2+} (e.g., *Kochia prostrata*; Karimi *et al.*, 2005). It is also suggested that the distribution of ions between root and shoot can differ (Nawaz *et al.*, 2010). In the species tested, the levels of Mg^{2+} and Ca^{2+} did not vary notably between control and salt-treated plants, with some exceptions; *A. papyrocarpa* exhibited a 6X decrease in Ca^{2+} while *A. rigens* exhibited a 6.5X increase in Mg^{2+} levels. Thus although these species are salt tolerant, they differ in their ability to modulate such contributory factors.

6.4.4 Salinity tolerance may be shared among phylogenetically closely related species

Acacia species have evolved to cope with diverse abiotic stresses and may enable maximum utilisation of lands not fit for conventional agriculture. The outcomes of the present work demonstrate that most of the species that were found to be genetically related to four key species with environmentally-tested salinity tolerance in the previous chapter, did show tolerance through at least some parameters considered to be reflective

of this trait. The data have also provided an insight into the unique responses of different species and the underlying likely biochemical mechanisms such as plant growth patterns, cation accumulations and ratios. The study thus strongly supports the use of molecular phylogenetics for preliminary identification of candidates from little-studied native species for environmental applications.

CHAPTER 7

General discussion and future directions

7.1 General discussion

The deleterious effects of salinity on agriculture, biodiversity and economy have prompted serious action in terms of land, water and resource management. It is of paramount importance not only to recognise the root cause of the problem but to devise strategies that would reduce its impact and prevent it from recurring. In an Australian initiative, thousands of native plants were used to reclaim salt-affected lands in Kamarooka. Amongst them were three saltbush species (*Atriplex nummularia, Atriplex semibaccata and Atriplex amnicola*) and four wattles (*Acacia pendula*, *Acacia salicina*, *Acacia stenophylla* and *Acacia victoriae*). Native plants such as these are ideal candidates to reclaim salt affected lands and re-establish biodiversity, since they are expected to be well-adapted to the local soil conditions.

In order to fully utilise the potential of native plants, it is essential to gain an insight into their stress tolerance mechanisms (e.g. accumulation of osmoprotectants); and to further encompass the role of native plants in agroforestry-related applications, it is essential to widen the search for available genetic resources. Thus, the specific aims of this project were to:

(i) Identify and analyse by molecular and bioinformatics methods, the genes in saltbushes and salt-tolerant acacias that regulate the synthesis of glycine betaine, an osmoprotectant molecule implicated in various stress-responsive mechanisms. Compare these genes and their encoded proteins to those of salinity sensitive/tolerant plants, to identify any sequence changes that may contribute to any unique genetic features.

(ii) Identify candidate salinity-tolerance related genes of these native plants that may be suitable for transfer into legumes and cereal crop plants.

(iii) Analyse whether the reported health benefits of saltbushes to sheep and other grazing animals are due to certain biochemical compounds (e.g. glycine betaine), which are produced in large quantities under salinity stress. If so, this will make the saltbushes a low cost, long-life nutritious feed crop.

(iv) Analyse proline and trehalose under salt stress. Proline has been reported to be an essential biomolecule in plants to alleviate the effects of stress in plants as well as indicate their stress condition (under stress or recovery after stress).

Trehalose has been detected in a limited number of plants but its direct role in salt stress is still questionable.

(v) Identify the phylogenetic barcode (relatedness) of the known salt-tolerant species to other acacias of economic value (e.g. for timber, food, certain chemicals of medical interest), so as to rapidly identify further species suitable for agroforestry in salinity-affected areas and to provide a comprehensive outlook of *Acacia* phylogenetics.

(vi) Test the putative salt tolerant plants under controlled laboratory conditions and eventually at a field level.

The above aims were addressed using a combination of bioinformatics, molecular, biochemical and phylogenetics approaches as detailed in Chapter 2. The bioinformatics approaches included DNA and protein multiple sequence alignments and analysis, primer design, BLAST and conserved domain database searches, prediction of physico-chemical properties of putative proteins using TargetP, WolfPSORT, Protparam and CD-search. The molecular methods included genomic DNA extraction, total RNA extraction, cDNA synthesis, PCR, sequencing and reverse transcriptase semi-quantitative PCR. The biochemical techniques include high performance liquid chromatography, atomic absorption spectroscopy and standard biochemical and enzyme assays. The phylogenetics analysis involved DNA sequencing, contig assembly using Sequencher software, multiple sequence alignment using ClustalW and phylogenetic tree reconstruction using MEGA4 and MrBayes programs.

Chapter 3 addressed the first 2 aims mentioned above. One of the critical gaps identified in literature was the absence of an association of native plants, like saltbushes, that can be potentially used for the dual prospect of saline land reclamation as well as a fodder source, considering their observed role in improving animal health, likely, through production of glycine betaine as postulated in this study. Exploring this link between saltbushes and improvement of animal health via glycine betaine is essential for saline land utilisation with a fodder species. In brief, choline monooxygenase and betaine aldehyde dehydrogenase cDNAs were amplified from control (not subjected to any stress) plant leaf tissues from *Atriplex nummularia, Atriplex semibaccata and Atriplex amnicola* and analysed using various bioinformatics tools. The results,

provided in Chapter 3, showed several lines of evidence that GB biosynthesis can occur and may be involved in the high levels of innate salinity tolerance of these species (*Atriplex* spp.), i.e., conservation of signature residues/motifs in key enzymes and up-regulation of genes encoding these enzymes under salinity stress. The predicted properties (molecular weight, isoelectric point, subcellular localisation) and the phylogenetic relatedness of the saltbushes CMO and BADH to other GB accumulators confirmed their potential to accumulate high GB levels, as compared to cereals and monocots. Nevertheless, at the genetic level, there was not enough data to explain these unique characteristics of GB in saltbushes compared to other salt tolerant plants. Hence, this study further inspected the subsequent processes of GB accumulation under salinity stress. Identification of GB biosynthetic genes and their expression in *Atriplex nummularia* and *Atriplex semibaccata* has been published (Joseph *et al.,* 2013a).

In the case of *Acacia* species, several attempts were made to amplify *CMO* and *BADH* cDNAs using different primer pairs and various PCR cycle conditions. However, the attempts yielded no results. This prompted the question of whether the acacias have any genetic basis for GB biosynthesis. Hence, subsequent attempts to amplify *CMO* and *BADH* genes from genomic DNA were carried out. Again, only *CMO* could be amplified, from *Acacia pendula* and *Acacia victoriae*. The *BADH* gene could not be amplified from any of the three *Acacia* species. These results point to the finding that GB may not be synthesised by the *Acacia* species. Since salt tolerance is a synergistic response of various molecular and biochemical mechanisms, the acacias may employ a different mechanism of salt tolerance rather than via GB accumulation. These mechanisms need to be explored in order to facilitate the greatest outcome of planting these species in areas affected by environmental issues (e.g. drought, heat) that may have similar physiological effects on plants affected by salinity-prone lands.

Chapter 4 reported on the accumulation/non accumulation of GB, proline, and trehalose in the selected native plants. Saltbushes produced high amounts of GB, whereas the acacias did not produce any. The high GB accumulation in saltbushes may explain their survival and longevity in drought-prone areas (in addition to salinity), as GB imparts osmoprotection under various dehydration stresses. Further, the positive effects on animal health, reported in various saltbush feed trials, may also be related to the

production of GB. Thus, the saltbushes have strong potential for the dual prospect of saline land reclamation as well as a functional fodder, and also serve as a genetic resource for manipulation of other important crops (transgenic applications). However, it will be desirable to establish the definitive amounts of GB that can produce stress-mitigating effects in other plant species and health benefits in different grazing animals.

The results presented in Chapter 4 also showed evidence of proline accumulation under salinity stress. Proline accumulation has been reported in other studies to be a useful indicator in assessing the physiological status of the plant and is vital to combat salinity induced damages. The accumulation of high levels of proline has been attributed as a salt tolerance mechanism by some researchers, while others argue that it is more of an adaptive response. Yokota (2003) suggests that proline accumulation in *Acacia* species (*Acacia ampliceps*, *A. salicina*, *A. ligulata*, *A. holosericea*, and *A. mangium*) may be an adaptive response, since the species that accumulated the highest level of proline was a salt-sensitive species (based on decline in dry weight when subjected to salt stress) and was previously reported as such in another research by Marcar *et al.* (1991). The results presented in Chapter 4 of this study also point to a similar observation. The salt bushes, as the name suggests, are salt tolerant halophytes and they accumulated lower levels of proline compared to the acacias, suggesting they are comparatively less affected by salinity or they recover soon after withdrawal of salt application (Sharma and Verslues, 2010). Among the acacias, *Acacia victoriae* accumulated the highest level of proline indicating its relatively lower level of salt tolerance in contrast to halophytes, the proline likely having a role in osmotic adjustment and reduced cellular damages.

These results provide evidence that proline accumulation patterns can be used as a preliminary screening tool along with conventional physiological tests to assess salt tolerance characteristics in plants. Investigations into trehalose accumulation showed that neither the *Atriplex* species nor the *Acacia* species accumulated any detectable levels of trehalose, compelling further examination of trehalose and trehalose biosynthetic genes (i.e. *TPS* and *TPP*) and their role in salinity stress. Analysing the mechanism of stress tolerance via osmoprotection, through various osmoprotectant molecules in these native plants, will translate to significant contributions to Australian agriculture.

The first 2 results chapters (Chapter 3 and Chapter 4) established the salt tolerant nature of these native plants and addressed aims one to four, widening the scope of the study to investigate other possible candidate species for similar applications. In order to accomplish this, a novel application of molecular phylogenetics and comparative biology was undertaken (presented in Chapter 5). The most common application of molecular phylogenetics is in taxonomy and systematics. But in this study, molecular phylogenetics was used as a catalyst in identifying new putative salt tolerant candidates. The hypothesis behind this study is that, relatives of known salt tolerant plants may also be salt tolerant. In order to test this hypothesis, *Acacia* species were chosen as the test group given the evidence regarding salt-tolerance presented via field trials of the Kamarooka project by NUFG farmers, and given the large species number (ca. 1000 species), their widespread occurrence in Australia, and the access to extensive nucleotide sequence data on public databases such as GenBank. As a starting point it was essential to resolve any unclear genetic relationships that may exist around the species of interest (target species used in the Kamarooka project), i.e. *Acacia pendula*, *Acacia salicina*, *Acacia stenophylla* and *Acacia victoriae*. Closely related species were identified based on their morphological relationships with the target species as detailed in the Flora of Australia (Table 2.3). However, the availability of molecular sequence data showed certain discrepancies in their allocation to taxonomic subgroups (detailed in Chapter 5). Molecular phylogenetics based on ITS and ETS data were used to investigate these uncertainties and establish their phylogenetic relatedness addressing aim five. This section has been published (Joseph *et al.,* 2013b)

In order to test for salinity tolerance in phylogenetically related species closest to the target species, the molecular phylogenetics data obtained in Chapter 5 was substantiated with data mining of extant literature (Table 6.1) and their utility in biodiversity and agroforestry-related applications (economical, medicinal and agricultural). The selected species were evaluated based on their performance in terms of growth, relative water content, biomass accumulation, cation concentrations and ionic balance under salt treatment were examined. The results of this chapter identified *Acacia cupularis* and *Acacia enterocarpa*, as salt tolerant under laboratory conditions. It also provides evidence that comparative biology is a valuable tool in predicting the properties of

genetically closely related groups. A manuscript based on these data is currently under peer review. Seed samples of these two species (and a few others ranked next in Table 6.4) have been sent to the NUFG farmers (Bendigo, Australia) for testing in the salinity affected lands of Kamarooka.

7.2 Future directions

There are several reports that illustrate the favourable roles of osmoprotectants such as glycine betaine in plant response to abiotic stress, as well as in improving animal health. Additionally, there are other compatible solutes that offer protective properties to plants under abiotic stress. However, to further exploit their potential the following future directions are suggested:

(i) A recent study has demonstrated that exogenous application of crude sugarbeet extracts including glycinebetaine on tomato under high temperature stress enhanced root growth, leaf photosynthesis and fruit yield (Kanechi *et al.*, 2013). The saltbush *Atriplex semibaccata* as shown in Chapter 4 also produces high levels of glycine betaine. Hence, it would be interesting to investigate if the application of crude saltbush extracts would have similar benefits.

(ii) Study the expression of saltbush *CMO* and *BADH* genes by real time PCR under other abiotic stresses such as drought, heat, alkalinity and water-logging to evaluate their potential as candidates for transgenic applications.

(iii) Isolate the promoter regions (by inverse PCR) and transcription factors (using chromatin immunoprecipitation-sequencing (ChIP-seq) or DNase-hypersensitivity assays) of saltbush *CMO* and *BADH* genes and identify cis-acting elements potentially regulating gene expression.

(iv) Investigate the spatial and temporal expression of the *CMO* and *BADH* genes from saltbushes using reporter genes like GFP (green fluorescent protein) and GUS (β-glucuronidase) in order verify the predicted subcellular localisation of these genes.

(v) Undertake transcriptome sequencing using next-generation mRNA-Seq of *Atriplex nummularia* under salinity stress to find other targets that may contribute to its halophytic nature.

(vi) To investigate if the *Atriplex* and *Acacia* species, like arabidopsis and rice, have trehalose biosynthesis genes, *TPS* and *TPP*, which may be expressed

and have other molecular functions in respect to vegetative development and flowering.

(vii) To construct a broader framework of *Acacia* phylogenetics using data from the two nuclear ribosomal markers and four chloroplast markers of the 178 *Acacia* species already included as part of this study.

(viii) Extend the utility of the molecular phylogenetics data by identifying and evaluating other species that can be used in reclaiming lands affected by other abiotic stresses such as alkalinity and drought.

(ix) To compare the performance of the two newly identified salt tolerant *Acacia* species under field conditions and examine the correlation between laboratory testing and field testing.

To summarise, this thesis has established the untapped potential of native plants in agroforestry and biodiversity, particularly in salinity affected landscapes. Data from the *Acacia* phylogenetics will assist in selecting appropriate genotypes for reclamation of salt affected lands and facilitate potential environmental and economic outcomes. The study has also suggested one of the many scientific foundations to utilise saltbushes as part of a mixed fodder diet.

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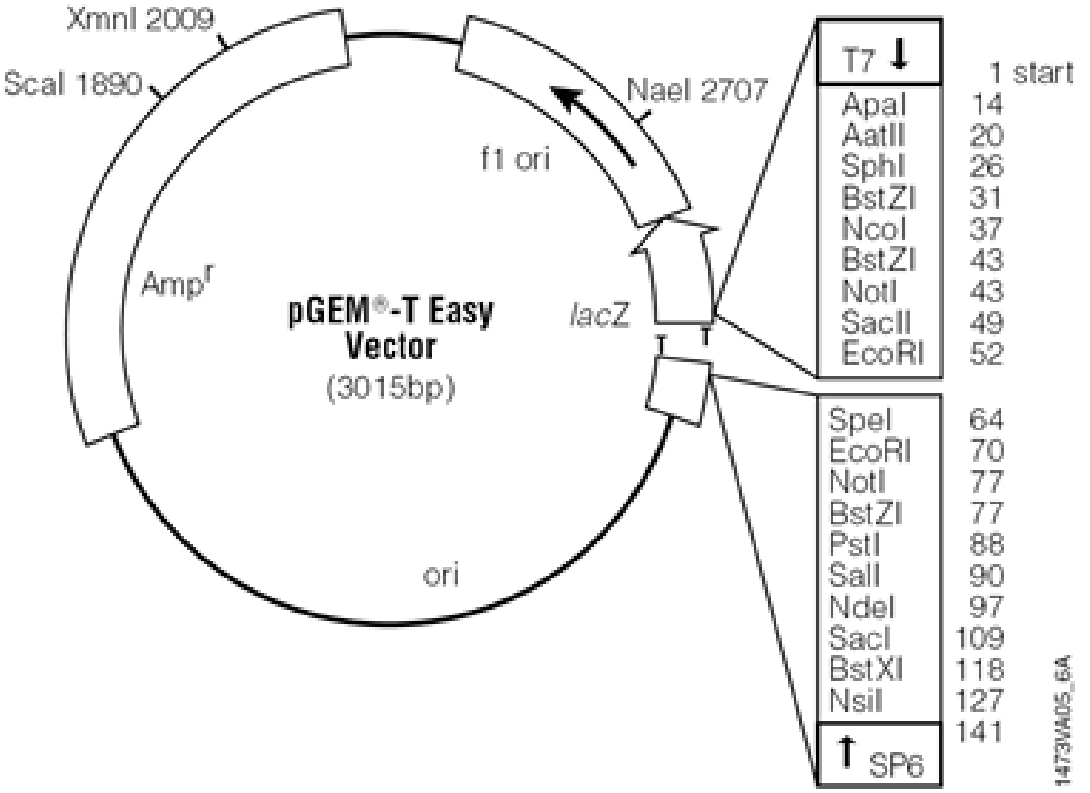
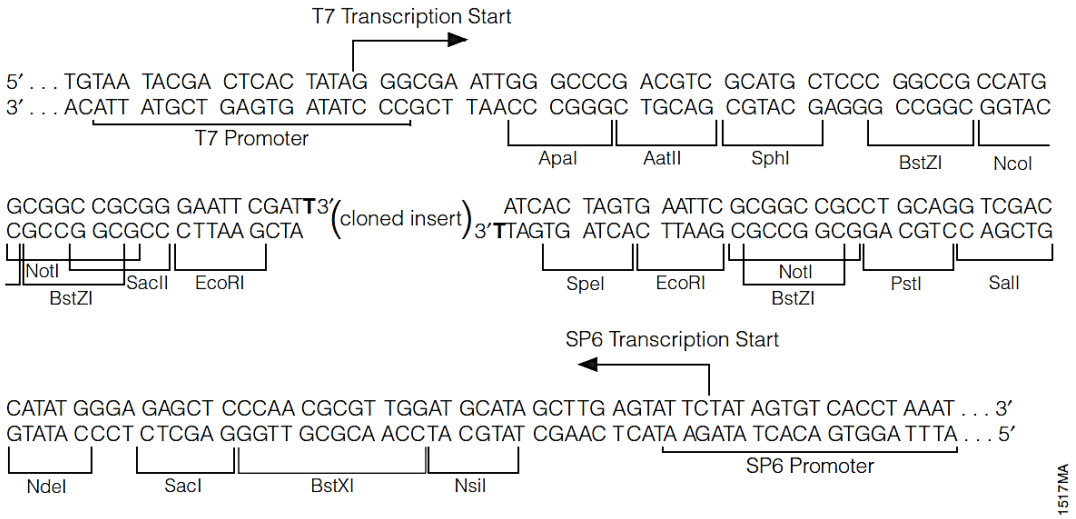
Appendices

Appendix I: pGEM®-T Easy vector map





The T7 and SP6 promoter sequences were used as primer annealing sites for amplification, insert sequencing and colony PCR.

Source:
http://au.promega.com/~/media/Files/Resources/Protocols/Technical%20Manuals/0/pGEM-T%20and%20pGEM-T%20Easy%20Vector%20Systems%20Protocol.pdf

Appendix II: Alignments of the putative protein sequences of saltbush CMOs

```
                                10        20        30        40        50
                        ....|....| ....|....| ....|....| ....|....| ....|....|
Spinacia oleracea       -MAASASATT MLLKYPTTVC GIP------N PSSNNNND-- ----PSNNIA
Arabidopsis thaliana    M.TTLTATVP EF.PPSLKST R--------- ---------- ----------
Amaranthus tricolor     ----M..SAS ..IN...F. .VR------- N...P...-- ----QFSDQI
Atriplex hortensis      ---MA..... .......... ...------. S.A..ST.-- ----.....V
Atriplex nummularia     ---MA..... .......... ...------. S....ST.-- ----.....V
Atriplex prostrata      ---MA..... .......... ...------. S....ST.-- ----.....V
*Atriplex nummularia    ---MA..... .......... ...------. S....ST.-- ----.....V
*Atriplex semibaccata   ---MA..... .......... ...------. S....ST.-- ----.....V
*Atriplex amnicola      ---MA..... .....R.... ...------. S....ST.-- ----.....V
Beta vulgaris           ---MA..... ......-.L. AM.------. S..SS..NDL PTSI.L..NN
Haloxylon ammodendron   -..GA..... ......A.L. SNSGVSNNN. E..LSRDN-- ----NNLS.P
Hordeum vulgare         -..TAQFRPL SSSSSASAAA R--------- ---------- ----------
Lycium barbarum         -..LLQQLSS FHQFKKPKL. C--------- ---------- ----------
Ophiopogon japonicus    -......... .......... ...------. .........-- ----.....V
Oryza sativa Japonica   -..IAQ.--A AAVSSAARAS R--------- ---------- ----------
Ricinus communis        -.TIITV.AM IT.T--PI.S R--------- ---------- ----------
Salicornia europaea     -...A..... ......-SL. SL.----NSS S..SPS.N-- ----NECSR-
Suaeda liaotungensis    -......... ......-.I. .V.----NNE S..CSPK.-- ----NHL.--
Suaeda salsa            -......... ......-.I. .V.----NNE S..CSPK.-- ----NHL.--
Zea mays                M..TGR.--- LAGV.SARAT R--------- ---------- ----------

                                60        70        80        90       100
                        ....|....| ....|....| ....|....| ....|....| ....|....|
Spinacia oleracea       SIPQNTTN-- PTLKSRT-PN KITTNAVAA- --PSFPS-LT TT----TPSS
Arabidopsis thaliana    --GYFNSHSE FGVSISKFSR RRFH.PTRV- ----.AV--- --------.D
Amaranthus tricolor     N..SSLN.NI NIS.ITSKT. ..IPK...S- --.VI..SIN SNNITT.TPN
Atriplex hortensis      Q...T..TNS .L..F..-.. .P-V.....- --.A...-V. ..TTT-....
Atriplex nummularia     QT..T..TNS .L..F..-T. .P-I.....- --.AP..-V. ..TTS-..P.
Atriplex prostrata      Q...TN.TKS .L..C..-.. .P-V.....- --.A...-A. .ITTT-..P.
*Atriplex nummularia    Q...T..TNS .L..F..-.. .P-I.....- --.AS..-V. ..KAT-..P.
*Atriplex semibaccata   Q...T..TNS .L..F..-T. .P-I.....- --.AP..-V. ..TTT-..P.
*Atriplex amnicola      Q...T..TNS .L..F..-.. .P-I.....- --.AS..-V. ..KTT-..P.
Beta vulgaris           NLLS.KNKIL Q.PNIN.ST. ..I.K...S- --.V..T-.K ..SNT--...
Haloxylon ammodendron   QTNN.NN.NN .M..F.AQ.. ..V.....SS --.V..A-IK ..TTTP--..
Hordeum vulgare         --.RA----- ----F.AA.S RVAAA.S.S- ---GE.---- ----------
Lycium barbarum         --.L.PLKNQ IFTYFIKPRK HLSSFQ.SSS LDY.NNH--- --------NY
Ophiopogon japonicus    ........-- .......-.. .........- --.....-.. ..----....
Oryza sativa Japonica   --.RP----- ----T.AA.R R.AAS.SSV- ---AP.E--- --------PA
Ricinus communis        --SLQ-IKNQ --SSVTAQHR SFHSLPKNS- ---HSLQ--- --------TH
Salicornia europaea     DLNIPQ..TP .L..F.AQ.. .LVA....S- --.V...-.. ..TTP--S..
Suaeda liaotungensis    -VS.QQN.NN .L..F..Q.T .LVA....S- --.V..A-SS ..TSSPSS..
Suaeda salsa            -VS.QQN.NN .L..F..Q.T .LVA....S- --.V..A-SS ..TSSPSS..
Zea mays                --.MP----- ----L.AGAR APCAG.A..- ---EPAA--- --------EH

                               110       120       130       140       150
                        ....|....| ....|....| ....|....| ....|....| ....|....|
Spinacia oleracea       IQSLVHEFDP QIPPEDAHTP PSSWYTEPAF YSHELERIFY KGWQVAGISD
Arabidopsis thaliana    .SK..T.... K..L.R.S.. ......D.Q. ..F..D.V.. G...AV.Y..
Amaranthus tricolor     .KRII..... KV.A..GF.. ..T...D.SL .....D.... .......Y..
Atriplex hortensis      .....KD... LV.A...L.. .......... .A...D.... .......Y..
Atriplex nummularia     .....KD... LV.A...L.. .......... .A...D.... .......Y..
Atriplex prostrata      .....KD... S..A...F.. .......... .A...D.... .......Y..
*Atriplex nummularia    .....KD... LV.A...L.. .......... .A...D.... .......Y..
*Atriplex semibaccata   .....KD... LV.A...L.. .......... .A...D.... .......Y..
*Atriplex amnicola      .....KD... LV.A...L.. .......... .A...D.... .......Y..
Beta vulgaris           .R........ E......L.. ..T....... .......... .......Y.E
Haloxylon ammodendron   V.E..YK... T..A...L.. ..T....... .......... ....I..Y..
Hordeum vulgare         ARR.AA.... AV.LAS.V.. ..G...D.G. LRL..D.V.L R...AV.HIG
Lycium barbarum         TKK..Q.... N..I.E.V.. ......DTS. .T...NQV.F .....V.Y.E
Ophiopogon japonicus    .......... .......... .......... .......... ..........
Oryza sativa Japonica   ARR..AA... AV.LAS.V.. ..G...D.D. LRL..D.V.L R...AV.HIW
Ricinus communis        F.N..NK... H..V.E.F.. ......D.S. .DY..HCV.. ....AV.FTK
Salicornia europaea     VNQ....... K..A...L.. .......... .....D.... ..........
```

```
Suaeda liaotungensis   .NQ..... K.....F.. ..... .....D.... .....
Suaeda salsa           .NQ..... K.....F.. ..... .....D.... .....
Zea mays               VRR..A.... AV.LDS.V.. ..G...D.D. LQL.IDSV.F R...AV.HIW

                               160        170        180        190        200
                       ....|....| ....|....| ....|....| ....|....| ....|....|
Spinacia oleracea      QIKEPNQYFT GSLGNVEYLV SRDGEGKVHA FHNVCTHRAS ILACGSGKKS
Arabidopsis thaliana   ....SRDF.. .R..D.DFV. C..EN..I.. .....S.H.. ...S.N.R..
Amaranthus tricolor    ..........  .........  C...Q.....  .........  .....T....
Atriplex hortensis     .V..A.....  .T........  C.........  .........  .........
Atriplex nummularia    .V..A.....  .T........  C.........  .........  .........
Atriplex prostrata     .V..A.....  .T........  .........  .........  .........
*Atriplex nummularia   .V..A.....  .T........  C.....N...  .........  .........
*Atriplex semibaccata  .V..A.....  .T........  C.....N...  .........  .........
*Atriplex amnicola     .V..A.....  .T........  C.........  .........  .........
Beta vulgaris          .V..K.....  .........  ....Q.EL..  .........  .........
Haloxylon ammodendron  .V..K.....  .R.....F..  C.....Q...  .........  .........
Hordeum vulgare        .V.N..DF..  .......FVI  C..AN..LQ.  .....R.H..  L......Q.T
Lycium barbarum        .....S....  .R......V.  C..DG..IY.  .....R.H.T  L..S....S.
Ophiopogon japonicus   ..........  .........  .........  .........  .........
Oryza sativa Japonica  .V.N..D...  .R.....FVI  C..AN.EL..  .....R.H..  L......Q.T
Ricinus communis       ...S.RDF..  .R.....FV.  C..DN.....  .....R.HG.  V..S.C.Q..
Salicornia europaea    ....K.....  .R......V.  C.........  .........  .........
Suaeda liaotungensis   ....K.....  .T......V.  .........  .........  .........
Suaeda salsa           ....K.....  .T......V.  .........  .........  .........
Zea mays               .V.N..DF..  .R.....FV.  C..AN..L..  .....R.H..  L......H.T

                               210        220        230        240        250
                       ....|....| ....|....| ....|....| ....|....| ....|....|
Spinacia oleracea      CFVCPYHGWV YGMDGSLAKA SKAKPEQNLD PKELGLVPLK VAVWGPFVLI
Arabidopsis thaliana   ....L....T .SLS...V.. TRMSGI..FS LS.M..K..R .........L
Amaranthus tricolor    .......... F.L....M.. T.-TEN.VF. .Q.....T.. ..I.......
Atriplex hortensis     .......... ...N...T.. ...T...S.N .D........ .......I..
Atriplex nummularia    .......... ...N...T.. ...TA..S.N .D........ ..E....I..
Atriplex prostrata     .......... ...N.T.T.. ...TA..S.N .D........ ..E....I..
*Atriplex nummularia   .......... .D.N...R.. ...TA..S.N .D....L... .......I..
*Atriplex semibaccata  .......... ...N...T.. ...TA..S.N .D........ .......I..
*Atriplex amnicola     .......... ...N...T.. ...TA..S.N .D........ .......I..
Beta vulgaris          .......... ..L....... ...TET.... ......A... ..E....I..
Haloxylon ammodendron  .......... F..N.D.T.. TQ-AET.TF. .......A.. ..........
Hordeum vulgare        ..Q......T ..L..T.L.. TRISGIK.FN KNDF..L.I. ..T......A
Lycium barbarum        .........T ..L..A.L.. TRITGIK.FK VN.M....MR .......I.L
Ophiopogon japonicus   .......... .......... .......... .......... ..........
Oryza sativa Japonica  ..Q......T ..L..V.L.. AQISGIK.FN KNDF..I.I. ..T......A
Ricinus communis       .......A.T ..L..A.L.. TRITGM..FS LD.Y..L.IN ..A......L
Salicornia europaea    .......... F.L..D.T.. TQTTDA.TF. ...Y...... ..E.......
Suaeda liaotungensis   .......... F....D.T.. TQTTDA.TF. ...Y..K... ..........
Suaeda salsa           .......... F....D.T.. TQTTDA.TF. ...Y..K... ..........
Zea mays               ..Q......T ..L..T.L.. TRISGIK.FN KNDF..I.I. ..T.......

                               260        270        280        290        300
                       ....|....| ....|....| ....|....| ....|....| ....|....|
Spinacia oleracea      SLDRS---LE EGG---DVGT EWLGTSAEDV KAHAFDPSLQ FIHRSELPME
Arabidopsis thaliana   KVTAATSRKG .VETDEL.AS ......VGRL SQGGV.SP.S Y.C.R.YTID
Amaranthus tricolor    .....G--S. GTE---...K ..I.SC..E. .K........ ..N...F...
Atriplex hortensis     .....---SR .V.---...S ....SC.... .......N.. ..N...F.I.
Atriplex nummularia    .....---SR .V.---...S ....SC.... .......N.. ......F.I.
Atriplex prostrata     .....---SR .V.---...S ....SC.... .......N.. ..N...F.I.
*Atriplex nummularia   .....---SR .V.---...S ....CC.... .......N.. V.....F.I.
*Atriplex semibaccata  .....---SR .V.---...S ....SC.... .......N.. ......F.I.
*Atriplex amnicola     .....---SR .V.---...S ....SC.... .......N.. ......F.IG
Beta vulgaris          .....---.D ANA---.... ..I.K..... .......N.K .T....F...
Haloxylon ammodendron  .V.KD---.P .T----.... ....KT.... .......A.. ......F...
Hordeum vulgare        RF.D---SSQ DTV-HDV..D ....SASDLL SRSGINT..P H.C.R.YII.
Lycium barbarum        NFENGALSEQ KSD-FDL..N ....S.SQIL ADGGV.S..S .LCGR.YTV.
Ophiopogon japonicus   .....---.. ...---.... .......... .......... ......F...
Oryza sativa Japonica  KF.SG--FSQ .TA-DNT..D ....SASDLL SRNGI.T..P H.C.R.YII.
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Ricinus communis          NV..ESSPQQ .VD-GNM.EN ....SCSGLL .TNGV.S..S YVC.RTYNI.
Salicornia europaea       .V.KN---.P .T----.P.. ....S..... .......N.. ......F...
Suaeda liaotungensis      ...KT---.P .S----.... ....S..... .........K ......F...
Suaeda salsa              ...KT---.P .S----.... ....S..... .........K ......F...
Zea mays                  RF.DE--ST. DNV-YDT..N ....SASDLL GTNGI.T..P H.C.R.YIIN

                                  310        320        330        340        350
                          ....|....| ....|....| ....|....| ....|....| ....|....|
Spinacia oleracea         SNWKIFSDNY LDSSYHVPYA HKYYATELNF DTYDTQMIEN VTIQRVEGSS
Arabidopsis thaliana      C...V.C... ..GG...... ..GLMSG.DL E..S.TIF.K .S..ECG.G.
Amaranthus tricolor       ....V.C... ...A...... .....A..D. ...K.DLL.K .V....AS..
Atriplex hortensis        .......... .......... ........D. ...Q.D.VG. ......A.T.
Atriplex nummularia       .......... .......... ........D. ...Q.D.VG. ......A.T.
Atriplex prostrata        .......... .......... ........D. ...Q.D.VG. ......A.T.
*Atriplex nummularia      .......... .......... ........D. ...Q.D.VG. ......A.T.
*Atriplex semibaccata     .......... .......... ........D. ...QAD.VG. ......A.T.
*Atriplex amnicola        .......... .......... ........D. ...Q.D.VG. ......A.T.
Beta vulgaris             C...V.C... .......... .....A..D. ...N.E...K CV....GS..
Haloxylon ammodendron     C...V.C... .......... ........D. .........K .V....GS..
Hordeum vulgare           C...V.C... ..GG...... .GAL.SG.QL QS.E.LTY.K .SV..C.SAP
Lycium barbarum           C...V.C... ..GG...... ..DL.SG.TL .S.S.TIL.K .S...C.TG.
Ophiopogon japonicus      .......... .......... .......... .......... ..........
Oryza sativa Japonica     C...V.C... ..GG...... .GTL.SG.QL QS.E.HTY.R .SV..C.SVQ
Ricinus communis          C...V.C... ..GG....F. ..SL.SG.KL .S.S.TIF.R AS..KC..G.
Salicornia europaea       C...V.C... V......... ........D. ......T.GK .V....A.N.
Suaeda liaotungensis      C...V..... .......... ........D. ......T.GK .V....GSNT
Suaeda salsa              C...V..... .......... ........D. ......T.GK .V....GSNT
Zea mays                  C...V.C... ..GG...... .GDL.SG.QL QS.E.LTY.R .SV..C.SAP

                                  360        370        380        390        400
                          ....|....| ....|....| ....|....| ....|....| ....|....|
Spinacia oleracea         NKPDG--FDR VGIQAFYAFA YPNFAVERYG PWMTTMHIHP LGPRKCKLVV
Arabidopsis thaliana      KVGED-G... L.SE.L...V ....MIN... ...D.NLVL. .......V.F
Amaranthus tricolor       ...N.--... L.SE.....I .......... ........V. ..........
Atriplex hortensis        .--N.--.N. L.T..... .......... ........V. ..........
Atriplex nummularia       .--N.--.N. L.T..... .......... ........V. ..........
Atriplex prostrata        .--N.--.S. I.T..... .......... ........V. ..........
*Atriplex nummularia      .--N.--.N. L.S..... .......... ........V. ..........
*Atriplex semibaccata     .--N.--.N. L.T..... .......... ........V. ..........
*Atriplex amnicola        .--N.--.N. L.T..... .......... ........V. ..........
Beta vulgaris             .....--... L.TE.....I .......... T......VV. M.Q.......
Haloxylon ammodendron     .....--... L.S..... .......... .......VQ. ..L.......
Hordeum vulgare           AEQED--I.. L.TK.T...V ....MIN... ...D.NLAV. .DATR..V.F
Lycium barbarum           AER.DQE... L.SK.L...V ....MIN... ...D.NLVL. Q.....LVIF
Ophiopogon japonicus      .....--... .......... .......... .......... ..........
Oryza sativa Japonica     AEQND--... L.TK.I...V ....MIN... ...D.NLVV. .DATR..VIF
Ricinus communis          MGSVD-E... L.SK.I...I F...MIN... ...D.NLVL. ...S..QVIF
Salicornia europaea       .....--... L.N..... .......... .......V.. IAQ.......
Suaeda liaotungensis      .R...--... L.EK.....T .......... .......VQ. IAQ.......
Suaeda salsa              .R...--... L.EK.....T .......... .......VQ. IAQ.......
Zea mays                  AE..D--... L.TK.L...V ....MIN... ...D.NLAV. .DSTR..V.F

                                  410        420        430        440        450
                          ....|....| ....|....| ....|....| ....|....| ....|....|
Spinacia oleracea         DYYIENSMLD DKDYIEKGIA INDNVQREDV VLCESVQRGL ETPAYRSGRY
Arabidopsis thaliana      ..FLDP.LK. .EAFVKRSLE ES.R..M... M......... .SQ..DK...
Amaranthus tricolor       ...LDK..MN ..P....S.M ......K... .......... ..........
Atriplex hortensis        .....K.K.. .......... ......K... .......K.. ..........
Atriplex nummularia       .....K.K.. .......... ......K... .......K.. ..........
Atriplex prostrata        .....K.K.. .......... ......K... .......K.. ..........
*Atriplex nummularia      .....K.K.. .......... ......K... .......K.. ...T......
*Atriplex semibaccata     .....K.K.. .......... ......K... .......K.. ..........
*Atriplex amnicola        .....K.K.. .......... ......K... .......K.. ..........
Beta vulgaris             ...L.KA... ..A..D.... ......K..K .......... ..........
Haloxylon ammodendron     ...L.E.K.G ..E....... ......A..K .......... .....S....
Hordeum vulgare           ..FLDK.L.. .Q.F.NRSLK DSEQ..I..I A...G..... AS...GV...
Lycium barbarum           ..FLDA.LKG .ESF.AQSLQ DSET..I..I K...A..... .S....T...
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Ophiopogon japonicus    ........W. .......... .......... .......... ..........
Oryza sativa Japonica   ..FLDK.LM. .QNF..SSLK DSEQ..M..I A...G..... .S...SV...
Ricinus communis        ..FV.ADYKN ..TF.DRSLI DSER..M..I M...G..... .S...CR...
Salicornia europaea     .....K.... ..E..DR... .........K .......N.. ..........
Suaeda liaotungensis    .....D.L.. N......... ......K..K .......K.. ..........
Suaeda salsa            .....D.L.. N......... ......K..K .......K.. ..........
Zea mays                ..FLDK.L.. .QCF.K.SLE DSEQ..I..I A...G..... .S...SV...

                                  460        470
                        ....|....| ....|....| ...
Spinacia oleracea       VMPIEKGIHH FHCWLQQTLK /--
Arabidopsis thaliana    A-LV..PM.. ...L.HHN.. L/-
Amaranthus tricolor     .......... .....H...N /--
Atriplex hortensis      .......... .....H.V.. /--
Atriplex nummularia     .......... .....H.V.. /--
Atriplex prostrata      .......... .....H.V.. /--
*Atriplex nummularia    .......... .....H.V.. /--
*Atriplex semibaccata   .......... .....H.V.. /--
*Atriplex amnicola      .......... .....H.V.. /--
Beta vulgaris           .......... .....HE..Q /--
Haloxylon ammodendron   .......... .....H.I.. /--
Hordeum vulgare         APSV.MAM.. ...L.HAN.S GQ/
Lycium barbarum         APQV..AM.. ..SL.YEN.H N/-
Ophiopogon japonicus    .......... .......... L/-
Oryza sativa Japonica   APSV.MAM.. ...L.HAN.S G--
Ricinus communis        APTV..AM.. ..QL.HGK.. I/-
Salicornia europaea     .......... .....H.I.Q /--
Suaeda liaotungensis    .......... .....H.I.. /--
Suaeda salsa            .......... .....H.I.. /--
Zea mays                APSV.MAM.. ..RLMHAN.S /--
```

The putative CMO protein sequences were deduced from cDNA (indicated by an asterisk '*') and compared to those of other plant CMOs (*Spinacia oleracea*: EF362838.1; *Arabidopsis thaliana*: BAC21260.1; *Amaranthus tricolor*: AB303389.1; *Atriplex hortensis*: AF270651.1; *Atriplex nummularia:* AB112481.1; *Atriplex prostrata*: AY082068.1; *Beta vulgaris:* AF023132.1; *Haloxylon ammodendron:* GQ379204.1; *Hordeum vulgare*: AB434467.1; *Lycium barbarum:* FJ514800.1; *Ophiopogon japonicus*: DQ645889.1; *Oryza sativa* Japonica: AJ578494.1; *Ricinus communis*: XM_00251821; *Salicornia europaea*: AY849925.1; *Suaeda liaotungensis*: AF354442.1; *Suaeda salsa*: DQ656523.1; *Zea mays*: DQ864498.1). The alignments were created in ClustalW under the 'Accessory Application' tab in BioEdit. The **bold** and *italicized* "***M***" at the start of the sequence denotes the start codon. The downward arrow indicates the start of putative the mature spinach CMO (residue A69; see Figure 3.3). A dot (.) indicates a conserved amino acid residue at the position aligned with *Spinacia oleracea* CMO. A dash (-) indicates a gap introduced to align the sequences, or a missing residue. The "/" symbol denotes the stop codon at the end of the sequence.

Appendix III: Alignments of the putative BADH protein sequences of saltbushes

```
                                10         20         30         40         50
                       ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola      ---MAFPMPV RQLFIDGEWR EPLLKNRIPI INPSTEEIIG DIPAATAEDV
Atriplex centralasiat  ---....... .......... .......... .......... ..........
Atriplex hortensis     ---....I.A .......... .......... .......... ..........
Atriplex micrantha     ---....I.A .......... .......... .......... ..........
Atriplex semibaccata   ---....... .......... .......... .......... ..........
Atriplex nummularia    ---.....S. H......... .......... .......... ..........
Atriplex prostrata     ---....... ........R. .......... .......... ..........
Atriplex tatarica      ---....IS. .......... .......... .......... ..........
Amaranthus hypochondr  ---..IRV.S .......... ..IK...... .......... ..........
Arabidopsis thaliana   ---..I...T .......... ..I..K.... V..A...V.. ......T...
Avicennia marina1      -------..T .......... ..VQRK.... ...AN.QT.. ........EM
Avicennia marina2      ---..IRI.S .......K.. ..VNRK.L.. V..A...T.. ..........
Brassica napus         ---..ITV.R .....G.Q.T ...RRQTL.V V..A..D... Y.....S...
Chorispora bungeana    ---..I...T .......... ..I..K.... V..A...V.. ......T...
Chrysanthemum lavandu  -MTTTI.I.S .......... ..VK....SV ......Q.V. ..........
Halostachys caspica    ---....I.S .......... ..IKR..... ...A...TV. .....N....
Haloxylon ammodendron  ---.SI.I.C .......... ..IK...... .......... E.........
Helianthus annuus      ---..ISI.F .......... ..VR.....V ...A....V. .........I
Hordeum brevisubulatu  -MAAPPAI.R .G...G.G.. ..T.GRH..V ...A..AT.. ..........
Hordeum vulgare        -MAAPPAI.R .G...G.G.. ..T.GRH..V ...A..DT.. ..........
Jatropha curcas        ---....I.N .....G.... ..V..K.... .......S.. ..........
Kalidium foliatum      ---....I.S .........T ..IK...... ...A...... ..........
Lycium barbarum        MAMSNVGI.S ...Y...... ..VK...... .......... ......S...
Ophiopogon japonicus   ---....I.A .......... .......... .......... ..........
Oryza sativa           -MAAPSAI.R .G...G.VVG ..S.GR.L.V V..A..AT.. ..........
Oryza sativa_Indica    ---..TAI.Q ....VA.... A.A.GR.L.V V..A..SP.. E...G.....
Panax ginseng          ---..IRI.T .......... ..AK....A. ...A..Q... N.....S...
Populus trichocarpa    ---..IHL.I .......... ..V..K...V ...A..Q.V. ..........
Solanum lycopersicum   MAIPNIRI.C .......... ...K...L.. ...AN..... Y.....E...
Sorghum bicolor        --MA.ADV.R P-S..G.D.. ..----CL.V CQ....AT.. ....G.....
Spinacia oleracea_AAA  ---....I.A .......... ..IK.....V .......... ..........
Suaeda salsa           ---.SI.I.S .......... ..IKR..L.. .......T.. E.........
Suaeda liaotungensis   ---.SI.I.S .......... ..IKR..L.. .......T.. E.........
Triticum aestivum      -MVAPAAI.Q .......D.. A.A.GR.L.V ...T..VT.. E...G.S...
Zea mays               -MASQAMV.L ....V..... P.AQGR.L.V V..T..AH.. E...G.....
Zoysia tenuifolia      MAAAPRDV.R .G...G.G.. ..S.GR.L.V V..A..TT.. .V........
Zoysia tenuifolia_BAD  MAAAPRDV.R .G...G.G.. ..S.GR.L.V V..A..TT.. .V........

                                60         70         80         90        100
                       ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola      EVAVVAARKA FKRNKGRDWA ATSGAHRARY LRAIAAKITE RKDH-FVKLE
Atriplex centralasiat  .......... .......... .......... .......... ....-.....
Atriplex hortensis     .......... .......... .LW-S...K. .......... K...-.....
Atriplex micrantha     .......... .......... ........K. .......... K...-.....
Atriplex semibaccata   .......... .......... .......... .......... ....-.....
Atriplex nummularia    .......... .......... .......... .......... K...-.....
Atriplex prostrata     .......... .......... ........KH .......... ....-.....
Atriplex tatarica      ........R. .......... .......... .......... ....-.....
Amaranthus hypochondr  .L..A...R. L.....E... SA......K. .......... K..Y-.A...
Arabidopsis thaliana   D...N...R. LS....K... KAP..V..K. .......VN. ..TD-LA...
Avicennia marina1      DI..E..... .F..S.K..S S.T.....K. ........KD ..VE-L.E..
Avicennia marina2      NI..E..... .FH.G.K... TA......K. .......V.. K.EE-LA...
Brassica napus         .L..E..... LT....N..S KA...V.... .......V.. ..SE-LAN..
Chorispora bungeana    ....N...R. .S....K... KAP..L..KH .......V.. ..SD-LA...
Chrysanthemum lavandu  D...K..... L...G.K... SAT.....K. .......... K.EL-.S...
Halostachys caspica    .A..S..... L......... .......... .......VS. ....-.....
Haloxylon ammodendron  .L..G...Q. L......... .......... ........S. KR.Y-.....
Helianthus annuus      DI..K...R. L..DG.KE.. SA......K. .......V.. K..M-.A...
Hordeum brevisubulatu  .L..A.GGPV LA-RRREH.. RA...V..K. .N......K. KISY-LAL..
Hordeum vulgare        .L..A.GGPV LA-RRREP.. RA...T..K. .N.......G KIAY-LAL..
Jatropha curcas        .I..E..Q.. .T........ F....V..K. .......... K.AE-LA...
Kalidium foliatum      .L..A...R. L.....E... SA......K. ........M. ..GQ-IS...
Lycium barbarum        DI..E..... LA.D---..G S.T..Q..K. .......VL. ..SE-LAT..
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```
Ophiopogon japonicus  ......... ......... ........K. .......... K...-.....
Oryza sativa          .L..S...D. .G.DG..H.S RAP..V..K. .K......KD K.SY-LAL..
Oryza sativa_Indica   DA..A...E. L...R..... RAP..V..K. ........I. ..SE-LAR..
Panax ginseng         DI..E...R. LA..G.S... SA...Y..K. .......... ..SE-LA...
Populus trichocarpa   .I..E..... .S....Q..S S....Y..K. .......... K.SE-LG...
Solanum lycopersicum  DM..K...S. LR.D---..G S.T..Q..K. .......VL. K.PE-LAT..
Sorghum bicolor       .MP.ARG.VS ---DG.ALV. CLW.RA-SQL SHT.....KD ..SESLAL..
Spinacia oleracea_AAA ........R. .R..---N.S ........T. .......... K...-.....
Suaeda salsa          .A..S...R. L......... .......... .......VS. K...-.....
Suaeda liaotungensis  .A..S...R. L......... .......... .......VS. K...-.....
Triticum aestivum     DA..A...A. L...R....S RAP..V..K. .......MI. ..SD-LAR..
Zea mays              DA..A...A. L...R..... RAP..V..K. .......VI. ..QE-LA...
Zoysia tenuifolia     .L..A...E. .R.DG..H.S CA...V..KF ........K. K.SD-LAL..
Zoysia tenuifolia_BAD .L..A...E. .R.DG..H.S CA...V..KF ........K. K.SD-LAL..

                              110        120        130        140        150
                      ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola     TLDSGKPLDE AVLDIDDVAT CFDY---FAG QAEALDAKQK APVTLPMDRF
Atriplex centralasiat .......... .......... ..E.---... .......... ..........
Atriplex hortensis    .......R.. .......... ..E.FEY... .......... .......E..
Atriplex micrantha    .......R.. .......... ..E.---... .......... .......E..
Atriplex semibaccata  .......... .......... ..E.---... .......... ........G.
Atriplex nummularia   .......... .......... ..E.---... .......... ..........
Atriplex prostrata    .......F.. .........S ..E.---... .......... ..........
Atriplex tatarica     .......... .......... ..E.---... .......... ..........
Amaranthus hypochondr AM.C...... .AR......G ..E.---Y.D .......... ..IA....T.
Arabidopsis thaliana  A..C...... ..W.M....G ..EF---Y.D L..G..... ...S...ES.
Avicennia marina1     AI......E. .S..M.N.IG ..E.---... I..R..SE.R T..S...ET.
Avicennia marina2     V..C....E. .AW.M....G ..E.---..D L..R..SN.- I..S....T.
Brassica napus        AI.C...... .AW.M....G ..E.---Y.D L.QG..S... ..LS..L.T.
Chorispora bungeana   A..C...... ..W.ME...G ..EF---Y.D L..G..... ...S...EN.
Chrysanthemum lavandu AI.C...Y.. .AW......G ..E.---N.D L.....K..N ..IE....T.
Halostachys caspica   .M........ VA......S. .SE.---... ......D... Y..K......
Haloxylon ammodendron .M........ VM......S. ..E.---... ..K...T... Y..K...E..
Helianthus annuus     AI.C...... .AW.M....G ..E.---N.D L........N ...N....T.
Hordeum brevisubulatu .V.....K.. ..A.M....A ..E.---Y.. L.....G..H ..IS...EE.
Hordeum vulgare       .V.....K.. ..A.M....A ..E.---Y.A L.....G..H ..IS...EE.
Jatropha curcas       AI.C...... .AW......G ..E.---Y.. L..G..... ...S...ET.
Kalidium foliatum     AM........ TEW......G ..E.---Y.E .......... ..IS....T.
Lycium barbarum       S.....TSF. SAA.M....A ..E.---Y.D L.....S.R. T..N.HL.S.
Ophiopogon japonicus  .......R.. .......... ..E.---... .......... .......E..
Oryza sativa          .F........ .AG.ME...A ..E.---Y.D L.....G..R ..IS...EK.
Oryza sativa_Indica   ...C...... .AW.M....G ..E.---..D L..S..KR.N ...S...EN.
Panax ginseng         ...C....E. .AW......A ..E.---N.D L.....G... S.IS...ET.
Populus trichocarpa   VI.C...... .LW.M....G ..E.---Y.D L..G..... ...S...ET.
Solanum lycopersicum  .I.N...WF. .AS.....VA ..E.---Y.D L.....S.KQ TE.K.HL.S.
Sorghum bicolor       .......... .SA.M....A ..E.---Y.D L.....G..R S.IS...EN.
Spinacia oleracea_AAA .I.....F.. .........S ..E.---... ......G... .......E..
Suaeda salsa          .M........ ........S. ..E.---..D ......N... Y..K......
Suaeda liaotungensis  .M........ ........S. ..E.---..D ......N... Y..K......
Triticum aestivum     A..C...... .AW.M....G ..EF---... H.....KR.N .A.A..-EN.
Zea mays              A..C...Y.. .AW.M....G ..E.---..D ......KR.N S..S...ET.
Zoysia tenuifolia     .......... .NA.M....A ..E.---Y.D L...F.G..R L.IS...EN.
Zoysia tenuifolia_BAD .......... .NA.M....A ..E.---Y.D L...F.G..R L.IS...EN.

                              160        170        180        190        200
                      ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola     KSHVLRQPIG VVGLISPWNY PLLMATWKIA PALAAGCTAV LKPSELASVT
Atriplex centralasiat .......... .......... .......... .......... ..........
Atriplex hortensis    .......... .......... ....D..... ........T. ..........
Atriplex micrantha    .......... .......... .......... .......... ..........
Atriplex semibaccata  .......... .......... .......... .......... ..........
Atriplex nummularia   .......... .......... .......... .......... ..........
Atriplex prostrata    .......... .......... .......... .......... ..........
Atriplex tatarica     .......... .......... .......... .......... ..........
Amaranthus hypochondr .C...K.... .......... ........V. .......S.. ..........
Arabidopsis thaliana  ..Y..K..L. .....T.... .....V..V. .S.......I ..........
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Avicennia marina1      .C.L.KE... ......... .....I..V. ....S....I .........
Avicennia marina2      .C...KE... ....VX.... ........V. .........I .....I....
Brassica napus         .GY..KE... .....T.... .....V..V. .........I .........
Chorispora bungeana    ..Y..K.... .....T.... .....V..V. .S.......I .........
Chrysanthemum lavandu  .C..IKE... .....T.... ........V. .......A.I ........L.
Halostachys caspica    ......... ......... ......... ......... .........
Haloxylon ammodendron  ......... ......... ......... ......... .........
Helianthus annuus      .C.II.E... .....T.... ........V. S......A.. .........
Hordeum brevisubulatu  .TY..KE... .....T.... ........V. ......... ........L.
Hordeum vulgare        .TY..KE... .....T.... ........V. ......... ........L.
Jatropha curcas        ..Y..KE... ..A..T.... ........V. .........I .........
Kalidium foliatum      .C........ ......... ........V. .....S.ATI .....T....
Lycium barbarum        .TY...E.L. .....T.... ........V. .......A.I ........I.
Ophiopogon japonicus   ......... ......... ......... ......... .........
Oryza sativa           E.Y..KE... .....T.... ........V. .V..V..... .E..G...L.
Oryza sativa_Indica    .CYLRKE... .....T.... ........V. ......... .........
Panax ginseng          ..YI.KE... .....T.... .M......V. .......A.I .........
Populus trichocarpa    ..Y..KE.L. ..A..T.... ....GA..V. .........I .........
Solanum lycopersicum   .T....E.L. .....T.... ....T...V. .......A.I ........I.
Sorghum bicolor        ..Y..KE.L. .....T.... ........V. ......... .........S
Spinacia oleracea_AAA  ........L. ......... ......... ......... .........
Suaeda salsa           ......... ......... ......... ......... .........
Suaeda liaotungensis   ......... ......... ......... ......... .........
Triticum aestivum      .C.LKKE... ..A..T.... .....V..V. ......... .........
Zea mays               .C.LR.E... .....T.... ........V. .......A.. .........
Zoysia tenuifolia      ..YA.KE... .....T.... ........V. ......... ........L.
Zoysia tenuifolia_BAD  ..YA.KE... .....T.... ........V. ......... ........L.

                              210        220        230        240        250
                       ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola      CLEFGEVCNE VGLPPGVLNI LTGLGPDAGA PIVSHPDIDK TAFTGSSTTG
Atriplex centralasiat  .......... .......... .......... ........V.. I......A..
Atriplex hortensis     .......... .......... .......... .......... V......A..
Atriplex micrantha     .......... .......... .......... ........V.. I......A..
Atriplex semibaccata   .......... .......... .......... .......... I......A..
Atriplex nummularia    .......... .......... .......... .......... I......A..
Atriplex prostrata     .......... .......... .......... .......... I......A..
Atriplex tatarica      .......... .......... .......... .........M I......A..
Amaranthus hypochondr  ...LA...R. .......... ......E..G .LAC...V.. V.....TA..
Arabidopsis thaliana   ...LADI.R. .........V ...F.SE... .LA...GV.. I.....FA..
Avicennia marina1      ...LA...M. .......... ......E... .L.T..HVA. IS....D...
Avicennia marina2      ...LAQ..K. ....A..... ......E... .LA...HV.. IT....GA..
Brassica napus         ...LADI.R. .......... .....TE... .LA...HV.. IV....TA..
Chorispora bungeana    ...LADI.R. .........V ...Y.AE..G .LA...GV.. I.....FA..
Chrysanthemum lavandu  ...L....K. ....A..... .......... .LAA...V.. I.L....A..
Halostachys caspica    .......... .......... .S........ .L.....V.. I......A..
Haloxylon ammodendron  .......... .......... .......... .L.....V.. V......A..
Helianthus annuus      ...L....R. ......I... V.....E... .LAA...V.. I......A..
Hordeum brevisubulatu  ...L.AI.E. I...S..... I.......... ..A...HV.. I.....TA..
Hordeum vulgare        ...L.AI.E. I...S..... I.......... ..A...HV.. I.....TA..
Jatropha curcas        ...LA...R. .......... ......E... .L....QV.. V.....TA..
Kalidium foliatum      ...LAD..R. .......... ......E... .LAC...V.. V.....TA..
Lycium barbarum        ...L..I.R. ......A... ...W..V.A. .LS...HV.. IS....VP..
Ophiopogon japonicus   .......... .......... .......... .......... V......A..
Oryza sativa           ...L.GI.A. I......... I....TE... .LA...HV.. I.....TE..
Oryza sativa_Indica    ...LAD..K. ....S..... V....SE... .LS...GV.. V.....YE..
Panax ginseng          ...L....K. ......I... ......E... .LA...HV.. I.....TA..
Populus trichocarpa    ...LA...R. .......... .....TE... .LA...HV.. V......A..
Solanum lycopersicum   S..L..I.R. ......A.S. .....HE..S .L.....V.. I.....GP..
Sorghum bicolor        ...L.AI.M. I......F.V I....LKLVL HYPHI.CGIR LLLL..TE..
Spinacia oleracea_AAA  .......... .......... .......... .L.....V.. I......A..
Suaeda salsa           .......... .......... .......... .L.....V.. V......A..
Suaeda liaotungensis   .......... .......... .......... .L.....V.. V......A..
Triticum aestivum      ...L.D..K. I...S..... V....HE... .LS....V.. V.....YA..
Zea mays               ...LADI.K. .......... V......... .LSA...V.. V.....FE..
Zoysia tenuifolia      ...L.AI.I. I......... I.....E... .LA...HV.. V.....TE..
Zoysia tenuifolia_BAD  ...L.AI.I. I......... I.....E... .LA...HV.. V.....TE..
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                                260        270        280        290        300
                       ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola      SKIMASAAQL VKPVTLELGG KSPVIMFED- IDIETAVEWT LFGVFWTNGQ
Atriplex centralasiat  .......... .......... .........- .ETVV..... ..........
Atriplex hortensis     .......... .......... .........- .......... ..........
Atriplex micrantha     .......... .......... .........- .......... ..........
Atriplex semibaccata   .......... .......... .........- .......... ..........
Atriplex nummularia    .......... .......... .........- .......... ..........
Atriplex prostrata     .......... .......... .........- .......... ..........
Atriplex tatarica      .......... .......... .........- .......... ..........
Amaranthus hypochondr  ..V.S..... .......... ...IVI...- V.LDK.A... A..C......
Arabidopsis thaliana   ..V.TA.... ....SM.... ...L.V.D.- V.LDK.A..A ...C......
Avicennia marina1      V...TA.... .......... ...IVV...- V.LD..A... ...C......
Avicennia marina2      ....TA.... .......... ...IVV...- V.LD..A... ...C......
Brassica napus         .N..T...K. ....S..... ...I.V.D.- VK.DK..... M..C......
Chorispora bungeana    ..V.TA.... ....SM.... ...L.V.D.- V.LDK.A..A ...C......
Chrysanthemum lavandu  ....TA...N .......... ...IVV.D.- V..DK..... ...C......
Halostachys caspica    ..V.G..... .......... ...I.V...- N.LDI.A..A I..C......
Haloxylon ammodendron  ..V..... .......... ...IVV...- V.LDV.A... I..C......
Helianthus annuus      ....TA...N .......... ...IVV.D.- V..DK....A ...C......
Hordeum brevisubulatu  KM..TA...M ....S..... ...LVI.D.V A..DK....A M..C.FNG.R
Hordeum vulgare        KT..TA...M ....S..... ...LVT.D.V A..DK....P ML.C.FNG..
Jatropha curcas        .R.......M ....SM.... ...IVV...- V.LDK.A... A..C......
Kalidium foliatum      ....S....M .......... ...ILV...- V.LDK.A..A A..C......
Lycium barbarum        T...TA.... .......... ...IVV.D.I DNLDI....A ...C.SNA..
Ophiopogon japonicus   .......... .......... ......S..- .......... ..........
Oryza sativa           KR..IT.S.M ....S..... ...L.V.DEV D-.DK....A M..C.ANA..
Oryza sativa_Indica    K.......PM ....S..... ...IVV.D.- V.V.K..... ...C......
Panax ginseng          ....TA...Q .......... ...ILV...- V.LDK.A... I..C......
Populus trichocarpa    .........M ....SM.... ...I.V...- V.LDK..... ...C......
Solanum lycopersicum   V...TA.... .......... ...IVV.D.I HNLD..... ...C......
Sorghum bicolor        KR..T....M ....S..... ...L.V.D.I R..DK..... M..ILPNA..
Spinacia oleracea_AAA  ..V..... .......... ...IVV...- V..DKV.... I..C......
Suaeda salsa           ..V.G..... .......... ...I.V...V V.LDV.A... I..........
Suaeda liaotungensis   ..V.G..... .......... ...I.V...V V.LDV.A... I..........
Triticum aestivum      Q...VA..PT .......... ...IVV.D.- V..DK..... ...C......
Zea mays               K....A..PM .......... ...IVV.D.- V..DK..... ...C......
Zoysia tenuifolia      KRV.TA...M ....S..... ...L.V.D.I DN.DN....A M..I.ANG..
Zoysia tenuifolia_BAD  KRV.TA...M ....S..... ...L.V.D.I DN.DN....A M..I.ANG..

                                310        320        330        340        350
                       ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola      ICSATSRLLV HESIAAEFVD RMVKWTKNIK ISDPFEEGCR LGPVISKGQY
Atriplex centralasiat  .......... .......... .......... .......... ..........
Atriplex hortensis     .......... .......... .......... .......... ..........
Atriplex micrantha     .......... .......... .......... .......... ..........
Atriplex semibaccata   .......... .......... .......... .......... ..........
Atriplex nummularia    .......... .......... .......... .......... ..........
Atriplex prostrata     .........L .......... .......... .......... ..........
Atriplex tatarica      .......... .......... .......... .......... ..........
Amaranthus hypochondr  .......... ........L. .L...C.... .......... ....V..S..
Arabidopsis thaliana   .......... .....S..IE KL...S.... ....M..... ....V.....
Avicennia marina1      .......... .....TT.LE KL...CEK.. ....L..... ...IV.RR..
Avicennia marina2      .......... .....TT.LE KL...CEK.. ....L..... ...I..R...
Brassica napus         .......... ..K..D..L. KL........ .......... ....V.....
Chorispora bungeana    .......... ..N..PQ.IE KL...S.... ....M..... ....V.Q...
Chrysanthemum lavandu  ........IL .....K..L. KL...A.... ....L..... ....V.S...
Halostachys caspica    .......... .......... .L....EK.. .......... ..........
Haloxylon ammodendron  .......... .......... KL...SEK.. .......... .......N..
Helianthus annuus      ........IL .....K..L. KL...A.... ....L..... ....V.A...
Hordeum brevisubulatu  V........L ..K...R.L. .L.E.V.... ....L..... ..S.......
Hordeum vulgare        V........L ..K..EP.L. .L.E.A.... ....L..... ..S.......
Jatropha curcas        .......... ..R..S..L. .L...C.... ....L..... ....V.G...
Kalidium foliatum      .......... ......D.L. .LL..C.... V.....D... .......A..
Lycium barbarum        V.......II Q....S..L. .LLL...... ....L..D.K ...IV.S...
Ophiopogon japonicus   .......... .......... .......... .......... ..........
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Oryza sativa            V........L ..K..KR.L. .L.A.A.S.. ....L..... ..S.V.E...
Oryza sativa_Indica     ........IL .KK..K..QE ...A.A.... V...L..... ....V.E...
Panax ginseng           .......... ........L. KLM..... ....L..... ....V.G...
Populus trichocarpa     .......... .....S..L. .L.....K.. .......... ....V.G...
Solanum lycopersicum    ........II Q.T..PQ.LA .LLE..... ....L..D.K ......R...
Sorghum bicolor         V...A....L ..KM.KK.L. .L.HGA.... V...L...G. ..S.V.E...
Spinacia oleracea_AAA   .......... .......... KL........ .......... ..........
Suaeda salsa            .......... .........E KL...S.K.. .......... ..........
Suaeda liaotungensis    .......... .........E KL...S.K.. .......... ..........
Triticum aestivum       .........I .KN..K.... ...A.S.... V...L..... ....V.E...
Zea mays                .......... .TK..KK.NE ...A.A.... V...L..... ....V.E...
Zoysia tenuifolia       V......I.. ..K..KQ.L. .L.A.A.H.. ....L..... ..S.V.E...
Zoysia tenuifolia_BAD   V......I.. ..K..KQ.L. .L.A.A.H.. ....L..... ..S.V.E...

                                  360        370        380        390        400
                        ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola       DKIMKFISTA KSEGATILCG GSRPEHLKKG YYIEPTIITD ITTSMQIWKE
Atriplex centralasiat   ....N..... .......... .......... .......... ..........
Atriplex hortensis      .......... .......... .......... .......... ..........
Atriplex micrantha      .......... .......... .......... .......... ..........
Atriplex semibaccata    ....N..... .......... .......... .......... ..........
Atriplex nummularia     .......... .......... .......... .......... ..........
Atriplex prostrata      .......... .......... .......... .......... ..........
Atriplex tatarica       ....N..... .......... .......... .......... ..........
Amaranthus hypochondr   E.VL..... .......... .......... ..V.....S. VS......R.
Arabidopsis thaliana    E..L..... ........H. .......E.. FF........ V.......R.
Avicennia marina1       E.V..Y.... .E........ .A.....E.. .FVQ..... VK......I.
Avicennia marina2       E.V..Y.... .E........ .A.....E.. .FVQ..... VK......I.
Brassica napus          ERVV..V.N. RK....V... .A..G..... .FV..A..SN V....E..RD
Chorispora bungeana     E..L..... ........H. ......QE.. FF........ V.......R.
Chrysanthemum lavandu   E.VL..VE.. R.......S. .Q..Q..... FF...A.... ......V.R.
Halostachys caspica     .....Y.... .......S.. .......... .......V.. .S......R.
Haloxylon ammodendron   .....Y.... ........F. .......... .F........ .S.......
Helianthus annuus       E.VL..VE.. .R....V.F. .K..Q..T.. F.M..A.... V.......RD
Hordeum brevisubulatu   E..K..... R.......H. .D..K..G.. FF........ VS......R.
Hordeum vulgare         EQ.K..... R.......H. .D..K..G.. FF.....N.G VS......R.
Jatropha curcas         ..VL..... ........S. .A..K..N.. FF......S. VN......R.
Kalidium foliatum       E.VL..... ......V... .......... .F......S. VS........
Lycium barbarum         E.VL....N. .N......Y. .E........ ..VQ..... VN...E....
Ophiopogon japonicus    .......... .......... .......... .......... ..........
Oryza sativa            Q.S....... RC......Y. .A..Q..ER. FFS......N VS......R.
Oryza sativa_Indica     E..KQ.V... ..Q.....T. .V..K..E.. F........ VD......R.
Panax ginseng           E.V.ES.... .K........ .A..K..E.. FF......S. V.......R.
Populus trichocarpa     E.VLE..A.. R.......S. .D..K.FT.. FFV..... V.......R.
Solanum lycopersicum    E..L..... .D......Y. .D........ ...Q..... VD...E....
Sorghum bicolor         E..K..... R.......Y. .A..Q...R. FFL..... VS......R.
Spinacia oleracea_AAA   .......... ........Y. .......... .......V.. .S........
Suaeda salsa            .....Y.... .......... .......... .F.....V.. ..........
Suaeda liaotungensis    .....Y.... .......... .......... .F.....V.. ..........
Triticum aestivum       E..K..VAN. ........T. .V..K..E.. FF........ .N...E..R.
Zea mays                E..K...LN. ........T. .V..A..E.. FF........ .....E..R.
Zoysia tenuifolia       E..K..... RN......Y. .A..Q..RR. FF........ VS......R.
Zoysia tenuifolia_BAD   E..K..... RN......Y. .A..Q..RR. FF........ VS......R.

                                  410        420        430        440        450
                        ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola       EVFGPVICVK TFSTEDEAIE LANDTEYGLA GAMFSKDLER CERVTKALEV
Atriplex centralasiat   .......... .......... .......... ..V..... ..........
Atriplex hortensis      .......... ..K....... .......... ..V..... ..........
Atriplex micrantha      ......V... ..K....... .......... ..V..... ..........
Atriplex semibaccata    .......... .......... .......... ..V..... ..........
Atriplex nummularia     .......... .......... .......... ..V..... ..........
Atriplex prostrata      .......... ..K....... .......... ..V..... ..........
Atriplex tatarica       .......... .......... .......... ..V..... ..........
Amaranthus hypochondr   ......L.Q. ..GS...... .....Q...G A.VL....D. ...I......
Arabidopsis thaliana    ......L... ..AS...... ....SH...G A.VI.N.T.. .D.ISE.F.A
Avicennia marina1       ......L... ..A.....V. .....H.... A.VI....D. ...MA..FQ.
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Avicennia marina2      ......LG.. ...K...PF. ....PH..W. A.VL.Q.... ...M...FQA
Brassica napus         ......L... .........Q ....SQ.... ..VL.N.... .D..S..F.A
Chorispora bungeana    ......L... ..GS...... ....SH...G A.VI.N.T.. .D..SQ.F.A
Chrysanthemum lavandu  ......L... ...S.E.... .....H...G S.VI.N..D. .D...R.FDA
Halostachys caspica    ......L... ...S...... .......... A.V.....D. ..........
Haloxylon ammodendron  ......L... .......... .......... A.VL....D. ..........
Helianthus annuus      ......L... .....Q.... .....H...G S.II.N.... .D..A..F.A
Hordeum brevisubulatu  .......... V.K..S..V. .....H.... .GVI.DN... ...IA.VIHS
Hordeum vulgare        .......... V.K..S..V. .....H.... .GVI.D.... ...IA.VIHS
Jatropha curcas        ......L... ...S...... .....H...G A.VI.N.... .D..S.SFRA
Kalidium foliatum      ......L... ..RSD...V. ....SQ...G S.VL..N.... ..K...E.QA
Lycium barbarum        ......L... ..K..E.... .....K.... S.IM...V.. ...F...FHS
Ophiopogon japonicus   .......... ..K....... .......... ..V....... ..........
Oryza sativa           .......... E.R..R..V. .....H.... ..VI.N.... ...IS..IQS
Oryza sativa_Indica    ......L... E....E.... .....H.... ..VL.G.R.. .Q.L.EEIDA
Panax ginseng          ......L... ..R....... .....R...G ..VI.N.... ........QS
Populus trichocarpa    ......L... .......... .....H...G A.VI.N.... .D.....FRA
Solanum lycopersicum   ......L... ..K..E.... .....KF..G A.IL...... ...F...FQS
Sorghum bicolor        .......... E.RR.S..V. .....Q.... ..VI.S.Q.. .RAIS...QS
Spinacia oleracea_AAA  ......L... ...S.....A .......... A.V..N.... ...I......
Suaeda salsa           ......L... .....E..L. .......... A.V....... ..........
Suaeda liaotungensis   ......L... .....E..L. .......... A.V....... ..........
Triticum aestivum      ......L... E....E.... .....H.... ..VI.G.R.. .Q.LAEEIDA
Zea mays               ......L... E......... .....Q.... ..VI.G.R.. .Q.LSEEIDA
Zoysia tenuifolia      ......V... E....S..V. .....H.... .GVI.N.P.I ...L...IQA
Zoysia tenuifolia_BAD  ...D..V... E....S..V. .....H.... .GVI.N.P.I ...L...IQA

                                460        470        480        490        500
                       ....|....| ....|....| ....|....| ....|....| ....|....|
Atriplex amnicola      GAVWVNCSQP CFVHAPWGGV KRSGFGRELG EWGIENYLNI KQVTSDIS-D
Atriplex centralasiat  .......... .......... .......... .......... ........-.
Atriplex hortensis     .......... .......... .......... .......... ........-.
Atriplex micrantha     .......... .......... .......... .......... ........-.
Atriplex semibaccata   .......... .......... .......... .......... ........-.
Atriplex nummularia    .......... .......... .......... .......... ........-.
Atriplex prostrata     .......... .......... .......... .......... ........-.
Atriplex tatarica      .......... .......... .......... .......... ........-.
Amaranthus hypochondr  .......... ..TQ.....T .......... .......... ....R.T.T.
Arabidopsis thaliana   .I..I..... ..TQ..... .......... ...LD...SV ....LYT.-N
Avicennia marina1      .......... ..YQ.....K .......... .R.LDI...V ....RYV.-S
Avicennia marina2      .I........ ..CQ.....K .......D.. ...LD....V ....RYV.-S
Brassica napus         .I........ ..CQ.....T .......... ...L....SV ....QY..-N
Chorispora bungeana    .I..I..... ..TQ..... .......... ...LD...SV ....LYT.-N
Chrysanthemum lavandu  .I........ ..CQ.....K .......... ...LD...SV ....RY..-N
Halostachys caspica    .......... ..C....... .......... .......... ........-.
Haloxylon ammodendron  .......... ..C....... .......... .......... ........-N
Helianthus annuus      .I........ ..SQ.....K .......... ...LD...SV ....RY..-N
Hordeum brevisubulatu  .I..I..... TL.Q.....N .......... ...L....SV ....RYCK-.
Hordeum vulgare        .I..K..... TL.Q.....N .......... ...L....SV ....RYCK-.
Jatropha curcas        .I..I..... ..CQ.....L .......... ...LD...SV ....QY..-N
Kalidium foliatum      .I.....P.. ..CQ.....S .......... ...L..... ....EY..-.
Lycium barbarum        .II.I..... T.HQL....K .......D.. ...L.K.... ....EYT.-.
Ophiopogon japonicus   .......... .......... .......... .......... ........-.
Oryza sativa           .I..I..... ...Q.....N .......... Q..LD...SV ....KYC.-.
Oryza sativa_Indica    .II....... ..CQ.....N .......... .G..D...SV ....EYA.-.
Panax ginseng          .I..I..... ..CQ.....K .......... ...LD...SV ....QYV.-.
Populus trichocarpa    .I..I..... ..CQ.....I .......... ...L....SV ....QY..-.
Solanum lycopersicum   .I..I..... ..WQP....K .......... ..SL..... ....QYVTP.
Sorghum bicolor        AID--..... ...Q.....N ..-M..... ...LD..MTV ....KYC.-.
Spinacia oleracea_AAA  .......... ...Q.....I .......... ....Q..... ....Q...-.
Suaeda salsa           .......... ..C....... .......... .......... ........-.
Suaeda liaotungensis   .......... ..C....... .......... .......... ........-.
Triticum aestivum      .CI....... ..CQ.....N .......... .G..D...S. ....EYT.-.
Zea mays               .II....... ..CQ.....N .......... .G..D...SV ....EY..-.
Zoysia tenuifolia      .II.I..... ...Q.....N .......... ...LD..MTV ....KYC.-.
Zoysia tenuifolia_BAD  .II.I..... ...Q.....N .......... ...LD..MTV ....KYC.-.
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                                 510
                        ....|....| ....
Atriplex amnicola       EPWGWYKSP/ ----
Atriplex centralasiat   ........./ ----
Atriplex hortensis      ........./ ----
Atriplex micrantha      ........./ ----
Atriplex semibaccata    ....R..../ ----
Atriplex nummularia     ........./ ----
Atriplex prostrata      ........./ ----
Atriplex tatarica       ........./ ----
Amaranthus hypochondr   ........./ ----
Arabidopsis thaliana    D........N /---
Avicennia marina1       ........./ ----
Avicennia marina2       ...D.....S -KL/
Brassica napus          .........S -KL/
Chorispora bungeana     .........S -A/-
Chrysanthemum lavandu   DA....TP.S PKL/
Halostachys caspica     ........./ ----
Haloxylon ammodendron   ........./ ----
Helianthus annuus       ......TP.S -KL/
Hordeum brevisubulatu   .LY...QR.S -KL/
Hordeum vulgare         .LY...QR.S -KL/
Jatropha curcas         ......Q..S -KL/
Kalidium foliatum       ........./ ----
Lycium barbarum         DA.AF....S -N/-
Ophiopogon japonicus    ........./ ----
Oryza sativa            ..Y...RP.S -KL/
Oryza sativa_Indica     .........S -KL/
Panax ginseng           ......Q..S -KL/
Populus trichocarpa     ......Q..A -KL/
Solanum lycopersicum    ...AF....S -KL/
Sorghum bicolor         ......QP.S -KL/
Spinacia oleracea_AAA   ........./ ----
Suaeda salsa            ........./- ----
Suaeda liaotungensis    ........./- ----
Triticum aestivum       A......A.A N/--
Zea mays                ......R..S -KL/
Zoysia tenuifolia       ......QP.S -KL/
Zoysia tenuifolia_BAD   ......QP.S -KL/
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Protein sequence alignment of the predicted saltbushes* BADHs deduced from cDNA in comparison with the reported GenBank amino acid sequences of plant CMO protein (*Amaranthus hypochondriacus*: AAB58165.1; *Arabidopsis thaliana*: AAM64944.1; *Atriplex centralasiatica*: AAM19159.1; *Atriplex hortensis*: CAA49425.1; *Atriplex micrantha*: ABM97658.1; *Atriplex prostrata*: AAP13999.1; *Atriplex tatarica*: ABQ18317.1; *Avicennia marina* 1: AF170094.1; *Avicennia marina* 2: BAB18544.1; *Brassica napus*: AAQ55493.1; *Chorispora bungeana*: AAV67891.2; *Chrysanthemum lavandulifolium*: AAY33872.1; *Halostachys caspica*: ABO45931.1; *Haloxylan ammodendron*: ACS96437.1; *Helianthus annus*: ACU65243.1; *Hordeum brevisubulatum*: AAS66641.1; *Hordeum vulgare*: BAA05466.1; *Jatropha curcas*: ABO69575.1; *Kalidium foliatum*: ABI95806.1; *Lycium barbarum*: ACQ99195.1; *Ophiopogon japonicus*: ABG34273.1; *Oryza sativa*: ABB83473.1; *Oryza sativa* Indica: ACF06149.1; *Panax ginseng*: AAQ76705.1; *Populus trichocharpa*: XP_002322147.1; *Solanum lycopersicum*: ACI43573.1; *Sorghum bicolor*: AAC49268.1; *Spinacia oleracea* : AAA34025.1; *Suaeda liaotungensis*: AAL33906.1; *Suaeda salsa*: ABG23669.1; *Triticum aestivum*: AAL05264.1; *Zea mays*: AAT70230.1; *Zoysia tenuifolia*: BAD34957.1). The alignments were created in ClustalW under the 'Accessory Application' tab in BioEdit. A dot (.) indicates the presence of conserved nucleotides at the position aligned with *Atriplex centralasiatica* BADH protein. A dash (-) indicates a gap introduced to align the sequences or represent a missing residue. The *italicized* "***M***" at the start of the sequence denotes the start codon. The "/" symbol denotes the stop codon at the end of the sequence.

Appendix IV: Percentage sequence identity of full-length predicted saltbush CMO proteins with all other reported putative full-length CMO proteins

	A thaliana	*A tricolor*	*A hortensis*	*A nummularia*	*A prostrata*	*B vulgaris*	*H ammodendron*	*H vulgare*	*L barbarum*	*O japonicus*	*O sativa*	*R communis*	*S europaea*	*S oleracea*	*S liaotungensis*	*S salsa*	*Z mays*	**A nummularia*	**A semibaccata*	**A amnicola*
A thaliana	-	24.9	28.1	27.6	27.7	25.5	26.5	23.9	25.7	28.2	25.3	27.8	26.5	28.3	27.7	27.7	26.3	27.8	28	44.1
A tricolor	24.9	-	29.7	29.6	29.4	30.9	28.5	20.7	28.3	30.2	23.4	25.8	52.4	30.3	52.8	52.8	23.4	28.7	29.4	70.7
A hortensis	28.1	29.7	-	97.1	97.2	30.2	28.5	23.9	27.7	47.1	24.3	26.4	29.4	47.3	30.3	30.3	24.9	96.5	96.5	97.9
A nummularia	27.6	29.6	97.1	-	96.2	29.8	28.5	24.3	27	47	24.5	26.7	29.8	47.3	30.2	30.2	24.8	96.7	98.8	98.1
A prostrata	27.7	29.4	97.2	96.2	-	30.6	28.8	24.1	27.4	46.9	24.5	26.4	29.1	47	30	30	25	95.5	95.5	95.6
B vulgaris	25.5	30.9	30.2	29.8	30.6	-	29.1	22.4	25.3	29.9	25.4	24.5	28.9	29.9	28.3	28.3	24.5	29.9	29.9	72.3
H ammodendron	26.5	28.5	28.5	28.5	28.8	29.1	-	24.8	26	29.8	25	25.8	28.5	29.9	29.7	29.7	23.9	28.5	29	73
H vulgare	23.9	20.7	23.9	24.3	24.1	22.4	24.8	-	23.3	24.7	30.5	24.8	23.6	24.9	23	23	28.4	24.2	24	42
L barbarum	25.7	28.3	27.7	27	27.4	25.3	26	23.3	-	27.4	22.9	24.6	26.7	27.4	25.8	25.8	23.3	27.4	27.2	42.8
O japonicus	28.2	30.2	47.1	47	46.9	29.9	29.8	24.7	27.4	-	26.1	25.5	29.4	99.3	31.5	31.5	26.1	46.8	46.9	82.6
O sativa	25.3	23.4	24.3	24.5	24.5	25.4	25	30.5	22.9	26.1	-	26.7	24.1	26.4	24.1	24.1	82.1	24.2	24.8	43.5
R communis	27.8	25.8	26.4	26.7	26.4	24.5	25.8	24.8	24.6	25.5	26.7	-	27.5	25.8	26.6	26.6	25.1	26.7	26.7	42.7
S europaea	26.5	52.4	29.4	29.8	29.1	28.9	28.5	23.6	26.7	29.4	24.1	27.5	-	29.2	82.8	82.8	25.1	29.4	29.4	73.9
S oleracea	28.3	30.3	47.3	47.3	47	29.9	29.9	24.9	27.4	99.3	26.4	25.8	29.2	-	31.3	31.3	26.2	47	47	
S liaotungensis	27.7	52.8	30.3	30.2	30	28.3	29.7	23	25.8	31.5	24.1	26.6	82.8	31.3	-	100	24.5	29.7	30	74
S salsa	27.7	52.8	30.3	30.2	30	28.3	29.7	23	25.8	31.5	24.1	26.6	82.8	31.3	100	-	24.5	29.7	30	74
Z mays	26.3	23.4	24.9	24.8	25	24.5	23.9	28.4	23.3	26.1	82.1	25.1	25.1	26.2	24.5	24.5	-	24.7	25	42.6
**A nummularia*	27.8	28.7	96.5	96.7	95.5	29.9	28.5	24.2	27.4	46.8	24.2	26.7	29.4	47	29.7	29.7	24.7	-	96.2	97.4
**A semibaccata*	28	29.4	96.5	98.8	95.5	29.9	29	24	27.2	46.9	24.8	26.7	29.4	47	30	30	25	96.2	-	98.4
**A amnicola*	44.1	70.7	97.9	98.1	95.6	72.3	73	42	42.8	82.6	4.35	42.7	73.9	74	74	74	42.6	97.4	98.4	-

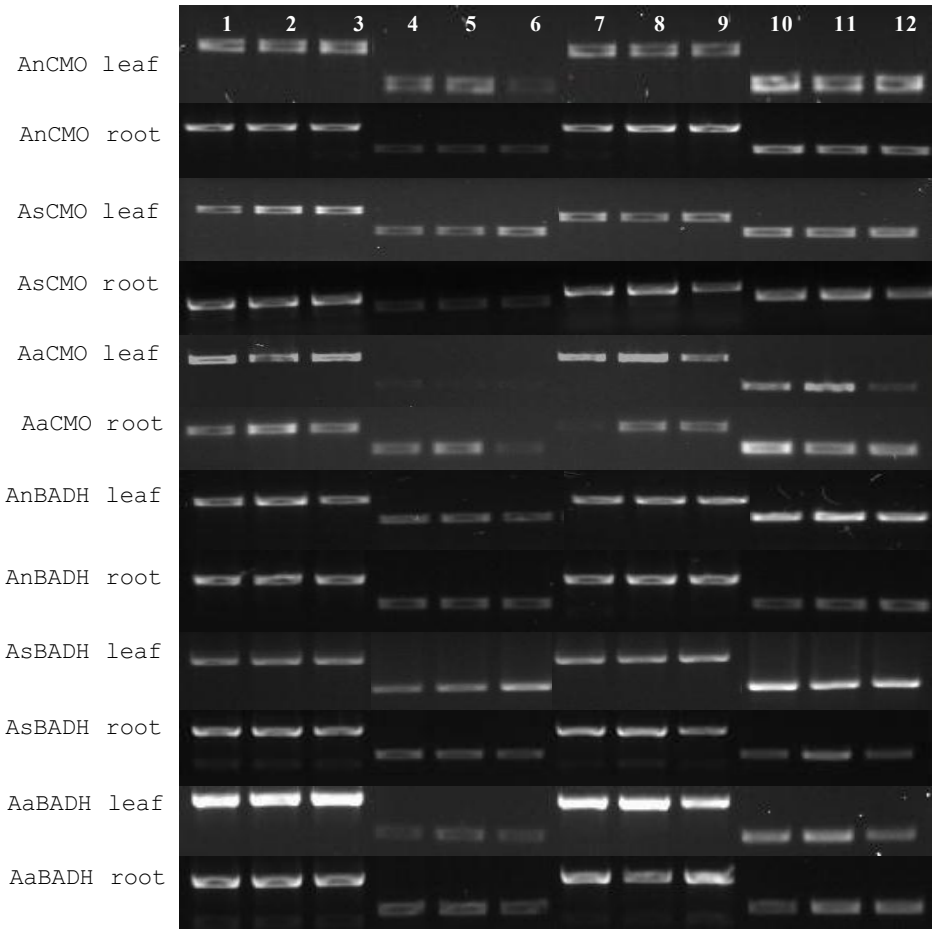
Sequence identities were calculated in BioEdit and are expressed in percentage. A dash (-) indicates comparison with the same sequence.

Appendix V: Percentage sequence identity of full length predicted saltbush BADH proteins with all other reported putative BADH proteins

	Atriplex amnicola	Atriplex_centralasiatica	Atriplex_hortensis	Atriplex_micrantha	Atriplex_semibaccata	Atriplex_nummularia	Atriplex_prostrata	Atriplex_tatarica	Amaranthus_hypochondriacus	Arabidopsis_thaliana	Avicennia_marina1	Avicennia_marina2	Brassica_napus	Chorispora_bungeana	Chrysanthemum_lavandulifolium	Halostachys_caspica	Haloxylon_ammodendron	Helianthus_annuus	Hordeum_brevisubulatum	Hordeum_vulgare	Jatropha_curcas	Kalidium_foliatum	Lycium_barbarum	Ophiopogon_japonicus	Oryza_sativa	Oryza_sativa_Indica	Panax_ginseng	Populus_trichocarpa	Solanum_lycopersicum	Sorghum_bicolor	Spinacia_oleracea_AAA34025.1	Suaeda_salsa	Suaeda_liaotungensis	Triticum_aestivum	Zea_mays	Zoysia_tenuifolia	Zoysia_tenuifolia_BAD34952.1|
Atriplex amnicola	ID	98	96	97.4	98.6	98.6	97.8	98.2	82.6	75	74	73.5	72.7	74.7	74.3	89.2	88.6	74.9	68.5	67.7	77.7	79.4	69.3	97.6	66.6	71.9	76.1	76.9	70.4	614	89.8	89.4	89.4	70.6	72	68.2	68
Atriplex_centralasiatica	98	ID	95.4	97.4	98.6	98.2	97.4	98.2	83	75.6	74	73.9	73.3	75.2	74.7	89.8	89.2	75.1	68.9	68.1	78.1	79.8	69.7	97	66.9	72.1	76.9	77.5	70.6	61.2	90.2	9	9	70.8	72	68.4	68.2
Atriplex_hortensis	96	95.4	ID	97.6	96	96.4	96.2	96	81.9	74.2	72.5	72.7	70.9	73.8	73.8	87.2	87.6	73.9	68.7	67.9	77.2	78.9	68.3	98	66.4	71.3	75.4	76.4	7	61.1	89.2	88	88	70.2	71.4	67.6	67.4
Atriplex_micrantha	97.4	97.4	97.6	ID	97.6	98	97.8	97.6	83.4	75.8	74.4	74.5	72.7	75.4	75.4	89.4	89.4	75.7	70.3	69.5	78.9	8	70.1	99.2	67.7	72.5	77.3	77.9	71.6	61.4	91.4	89.8	89.8	71.4	72.6	69.2	69
Atriplex_semibaccata	98.6	98.6	96	97.6	ID	98.8	98	98.8	82.8	75.4	74	73.9	73.1	75	74.7	89.4	88.6	75.1	68.9	68.1	77.9	79.6	69.5	97.6	66.7	72.1	76.7	77.3	70.6	61.4	9	89.4	89.4	70.8	72	68.2	68
Atriplex_nummularia	98.6	98.2	96.4	98	98.8	ID	98	98.8	83	75.2	73.8	74.1	72.9	74.9	74.9	89.4	89	75.3	69.1	68.3	78.1	79.4	69.1	98	66.9	71.7	76.3	77.3	70.6	61.4	90.4	89.8	89.8	70.6	71.8	68.4	68.2
Atriplex_prostrata	97.8	97.4	96.2	97.8	98	98	ID	97.6	82.6	75.2	73.8	73.5	72.3	75.2	74.9	88.6	87.8	75.1	69.5	68.7	77.5	79.4	69.7	97.8	67.1	71.9	76.3	76.7	70.8	61.6	90.2	88.6	88.6	70.6	71.8	68	67.8
Atriplex_tatarica	98.2	98.2	96	97.6	98.8	98.8	97.6	ID	82.8	75.2	73.4	73.7	72.7	74.9	74.5	89.4	88.8	75.3	68.9	68.1	77.7	79.8	69.1	97.6	66.7	72.1	76.9	76.9	70.4	61.4	90.4	89.8	89.8	70.8	71.8	68	67.8
Amaranthus_hypochondriacus	82.6	83	81.9	83.4	82.8	83	82.6	82.8	ID	79.4	76.2	78.1	77.1	78.9	78.8	83.6	84.8	79.5	7	69.2	81.7	87.6	71.2	83.2	67.8	73	80.9	79.9	72.6	62.1	83.8	83.6	83.6	71.4	73.1	69.2	69
Arabidopsis_thaliana	75	75.6	74.2	75.8	75.4	75.2	75.2	75.2	79.4	ID	74.8	77.5	79.1	95.4	77	75	75.2	78.5	70.5	69.3	82.9	77.4	70.7	75.2	70.9	76.1	80.1	82.3	70.2	63.4	76.2	76	76	74	75.2	71.9	71.7
Avicennia_marina1	74	74	72.5	74.4	74	73.8	73.8	73.4	76.2	74.8	ID	83.8	71.9	73.9	73.3	75	75.8	73.9	66	65.2	76.7	73.8	70.1	73.8	67.3	70.1	77.5	76.5	70.4	6	74.2	75	75	69.4	70.8	67.8	67.6
Avicennia_marina2	73.5	73.9	72.7	74.5	73.9	74.1	73.5	73.7	78.1	77.5	83.8	ID	77.3	77.5	77.2	75.1	74.9	77.9	68.1	67.7	79.9	76.3	71.2	73.9	68.9	74.3	81.1	79.1	72.6	608	74.1	76.3	76.3	70.1	73.4	70.4	70.2
Brassica_napus	72.7	73.3	70.9	72.7	73.1	72.9	72.3	72.7	77.1	79.1	71.9	77.3	ID	78.9	76	71.9	72.1	78.3	70.3	70.1	80.9	75.9	69.6	72.1	72.5	74.5	79.5	81.7	7	64.4	72.9	72.8	72.8	71.7	74.2	72.3	72.1
Chorispora_bungeana	74.7	75.2	73.8	75.4	75	74.9	75.2	74.9	78.9	95.4	73.9	77.5	78.9	ID	76	74.1	74.3	77.9	69.9	68.9	82.9	76.6	68.7	74.9	71.3	75.1	79.7	82.1	69.6	63.6	75	75.1	75.1	72.8	74.2	72.7	72.5
Chrysanthemum_lavandulifolium	74.3	74.7	73.8	75.4	74.7	74.9	74.9	74.5	78.8	77	73.3	77.2	76	76	ID	74.1	75	88.1	71	70.8	80.4	76.4	71.4	74.9	70.2	75.2	81.2	80.4	71.9	62.7	76.2	74.7	74.7	74.7	77	70.6	70.4
Halostachys_caspica	89.2	89.8	87.2	89.4	89.4	89.4	88.6	89.4	83.6	75	75	75.1	71.9	74.1	74.1	ID	92.2	74.3	66.7	65.8	76.9	80.6	69.1	88.8	65.6	69.9	76.3	75.5	7	586	88.4	93.4	93.4	69.4	70.4	66.8	66.6
Haloxylon_ammodendron	88.6	89.2	87.6	89.4	88.6	89	87.8	88.8	84.8	75.2	75.8	74.9	72.1	74.3	75	92.2	ID	74.5	67.5	66.6	78.3	80.8	68.1	89.2	65.4	70.7	77.1	76.3	70.8	586	89	93.8	93.8	70.4	71.4	67.2	67
Helianthus_annuus	74.9	75.1	73.9	75.7	75.1	75.3	75.1	75.3	79.5	78.5	73.9	77.9	78.3	77.9	88.1	74.3	74.5	ID	70.7	70.3	80.5	76.9	72.1	75.1	71.1	76.1	81.9	81.1	73.2	62.6	76.3	74.6	74.6	73.9	76.6	71.7	71.5
Hordeum_brevisubulatum	68.5	68.9	68.7	70.3	68.9	69.1	69.5	68.9	7	70.5	66	68.1	70.3	69.9	71	66.7	67.5	70.7	ID	96	73.3	69.1	67	69.9	81.6	72.1	73.3	72.1	66.3	71.5	69.5	67.1	67.1	70.6	70.9	81.6	81.4
Hordeum_vulgare	67.7	68.1	67.9	69.5	68.1	68.3	68.7	68.1	69.2	69.3	65.2	67.7	70.1	68.9	70.8	65.8	66.6	70.3	96	ID	72.5	67.7	65.8	69.1	8	71.3	72.3	71.3	65.5	70.7	68.9	66.6	66.6	69.4	70.1	8	79.8
Jatropha_curcas	77.7	78.1	77.2	78.9	77.9	78.1	77.5	77.7	81.7	82.9	76.7	79.9	80.9	82.9	80.4	76.9	78.3	80.5	73.3	72.5	ID	80.3	70.6	78.7	73.3	77.5	84.2	89.2	71.4	65.6	78.9	76.7	76.7	73.7	75.8	74.1	73.9
Kalidium_foliatum	79.4	79.8	78.9	8	79.6	79.4	79.4	79.8	87.6	77.4	73.8	76.3	75.9	76.6	76.4	80.6	80.8	76.9	69.1	67.7	80.3	ID	69.9	79.8	66.7	71.7	79.9	77.9	70.6	608	79.4	80.2	80.2	69.6	72.6	68	67.8
Lycium_barbarum	69.3	69.7	68.3	70.1	69.5	69.1	69.7	69.1	71.2	70.7	70.1	71.2	69.6	68.7	71.4	69.1	68.1	72.1	67	65.8	70.6	69.9	ID	69.5	65.4	66.6	73.7	71.2	82.7	596	69.9	68.7	68.7	66.6	67.4	68	67.8
Ophiopogon_japonicus	97.6	97	98	99.2	97.6	98	97.8	97.6	83.2	75.2	73.8	73.9	72.1	74.9	74.9	88.8	89.2	75.1	69.9	69.1	78.7	79.8	69.5	ID	67.3	72.3	76.7	77.7	71	61.4	90.8	89.6	89.6	71.2	72.4	68.8	68.6
Oryza_sativa	66.6	66.9	66.4	67.7	66.7	66.9	67.1	66.7	67.8	70.9	67.3	68.9	72.5	71.3	70.2	65.6	65.4	71.1	81.6	8	73.3	66.7	65.4	67.3	ID	73.1	73.5	72.7	65.1	74.1	67.9	66.4	66.4	70.2	71.9	85.7	85.6
Oryza_sativa_Indica	71.9	72.1	71.3	72.5	72.1	71.7	71.9	72.1	73	76.1	70.1	74.3	74.5	75.1	75.2	69.9	70.7	76.1	72.1	71.3	77.5	71.7	66.6	72.3	73.1	ID	76.5	76.5	67.9	66	70.9	71.4	71.4	86.9	88.3	74.9	74.7
Panax_ginseng	76.1	76.9	75.4	77.3	76.7	76.3	76.3	76.9	80.9	80.1	77.5	81.1	79.5	79.7	81.2	76.3	77.1	81.9	73.3	72.3	84.2	79.9	73.7	76.7	73.5	76.5	ID	84	74.8	66	77.5	77.1	77.1	73.1	75.8	74.3	74.1
Populus_trichocarpa	76.9	77.5	76.4	77.9	77.3	77.3	76.7	76.9	79.9	82.3	76.5	79.1	81.7	82.1	80.4	75.5	76	81.1	72.1	71.3	89.2	77.9	71.2	77.7	72.7	76.5	84	ID	71.8	64.6	78.1	75.9	75.9	72.9	74.2	73.9	73.7
Solanum_lycopersicum	70.4	70.6	7	71.6	70.6	70.6	70.8	70.4	72.6	70.2	70.4	72.6	7	69.6	71.9	7	70.8	73.2	66.3	65.5	71.4	70.6	82.7	71	65.1	67.9	74.8	71.8	ID	583	72.2	71.6	71.6	66.2	67.9	67.3	67.1
Sorghum_bicolor	61.4	61.2	61.1	61.4	61.4	61.4	61.6	61.4	62.1	63.4	6	60.8	64.4	63.6	62.7	586	586	62.6	71.5	70.7	65.6	608	596	61.4	74.1	66	66	64.6	583	ID	61.6	598	598	62.4	66	76.7	76.5
Spinacia_oleracea_AAA34025.1	89.8	90.2	89.2	91.4	9	90.4	90.2	90.4	83.8	76.2	74.2	74.1	72.9	75	76.2	88.4	89	76.3	69.5	68.9	78.9	79.4	69.9	90.8	67.9	70.9	77.5	78.1	72.2	61.6	ID	88.6	88.6	70.6	71.4	69.2	69
Suaeda_salsa	89.4	90	88	89.8	89.4	89.8	88.6	89.8	83.6	76	75	76.3	72.8	75.1	74.7	93.4	93.8	74.6	67.1	66.6	76.7	80.2	68.7	89.6	66.4	71.4	77.1	75.9	71.6	598	88.6	ID	1	71	72.5	68.2	68
Suaeda_liaotungensis	89.4	90	88	89.8	89.4	89.8	88.6	89.8	83.6	76	75	76.3	72.8	75.1	74.7	93.4	93.8	74.6	67.1	66.6	76.7	80.2	68.7	89.6	66.4	71.4	77.1	75.9	71.6	598	88.6	1	ID	71	72.5	68.2	68
Triticum_aestivum	70.6	70.8	70.2	71.4	70.8	70.6	70.6	70.8	71.4	74	69.4	70.1	71.7	72.8	74.7	69.4	70.4	73.9	70.6	69.4	73.7	69.6	66.6	71.2	70.2	86.9	73.1	72.9	66.2	62.4	70.6	71	71	ID	86.3	72	71.8
Zea_mays	72	72	71.4	72.6	72	71.8	71.8	71.8	73.1	75.2	70.8	73.4	74.2	74.2	77	70.4	71.4	76.6	70.9	70.1	75.8	72.6	67.4	72.4	71.9	88.3	75.8	74.2	67.9	66	71.4	72.5	72.5	86.3	ID	73.9	73.7
Zoysia_tenuifolia	68.2	68.4	67.6	69.2	68.2	68.4	68	68	69.2	71.9	67.8	70.4	72.3	72.7	70.6	66.8	67.2	71.7	81.6	8	74.1	68	68	68.8	85.7	74.9	74.3	73.9	67.3	76.7	69.2	68.2	68.2	72	73.9	ID	99.8
Zoysia_tenuifolia_BAD34952.1|	68	68.2	67.4	69	68	68.2	67.8	67.8	69	71.7	67.6	70.2	72.1	72.5	70.4	66.6	67	71.5	81.4	79.8	73.9	67.8	67.8	68.6	85.6	74.7	74.1	73.7	67.1	76.5	69	68	68	71.8	73.7	99.8	ID

Sequence identities were calculated in BioEdit and are expressed in percentage. A dash (-) indicates comparison with the same sequence.

Appendix VI: Semi-quantitative RT-PCR expression profiles of CMO and BADH mRNA from leaf and root tissues of *A. nummularia, A. semibaccata* and *A. amnicola* under salt-stress



Lanes 1, 2 and 3 denote actin cDNA amplified from control plants 1, 2 and 3. Lanes 4, 5 and 6 denote CMO/BADH cDNAs amplified from control plants 1, 2 and 3. Lanes 7, 8 and 9 correspond to actin cDNA amplified from 300mM salt-stressed plants 1, 2 and 3. Lanes 10, 11 and 12 correspond to CMO/BADH cDNA amplified from 300mM salt-stressed plants 1, 2 and 3. CMO expression levels were optimised at 20 amplification cycles; BADH was optimised at 25 cycles, and actin was amplified for 20 cycles for *CMO* analysis and 25 for BADH analysis.

Appendix VII: BLAST results confirming the identity of the partial *CMO* gene sequences from *Acacia pendula and Acacia victoriae*

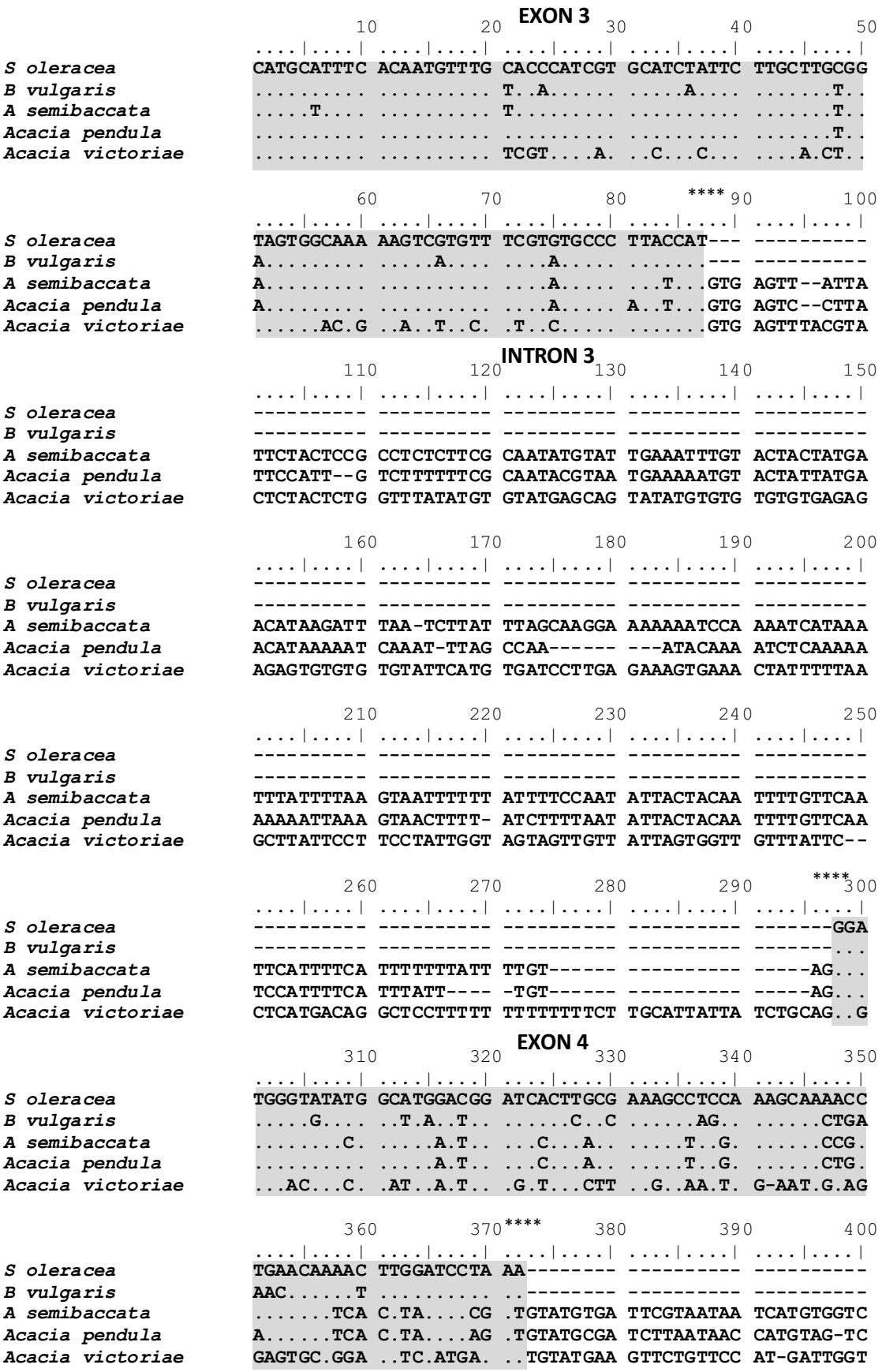
Accession	Description	Max score	Total score	Query coverage	Max identity
Acacia pendula					
AB112481.1	*Atriplex nummularia* AnCMO mRNA for choline monooxygenase, complete cds	327	984	43%	97%
AF270651.1	*Atriplex hortensis* choline monooxygenase (CMO) mRNA, complete cds	322	837	37%	97%
AY082068.1	*Atriplex prostrata* choline monooxygenase mRNA, complete cds; nuclear gene for chloroplast product	294	809	37%	98%
DQ645889.1	*Ophiopogon japonicus* choline monooxygenase (CMO) mRNA, complete cds	200	483	28%	94%
U85780.1	*Spinacia oleracea* choline monooxygenase precursor mRNA, nuclear gene encoding chloroplast protein, complete cds	200	483	28%	94%
Acacia victoriae					
NM_119135.4	*Arabidopsis thaliana* choline monooxygenase (AT4G29890) mRNA, complete cds	86.1	86.1	3%	93%
BT028917.1	*Arabidopsis thaliana* unknown protein (At4g29890) mRNA, complete cds	86.1	86.1	3%	93%
AY090377.1	*Arabidopsis thaliana*	86.1	86.1	3%	93%
AB093586.1	*Arabidopsis thaliana* CMO-like mRNA for choline monooxygenase, complete cds	86.1	86.1	3%	93%

Appendix VIII: Non-specific primer binding on *Acacia salicina* gDNA leading to amplification of unintended PCR product

```
>Consensus: 2 to 91: Frame 2 30 aa
IHAFHNVCLLVESLRHHFNHLVQRMHYPKP
```

A section of the translated sequence obtained from *Acacia salicina* gDNA. The underlined residues indicate the forward primer used to amplify partial *CMO* gene.

Appendix IX: Predicted partial CMO gene structures



INTRON 4

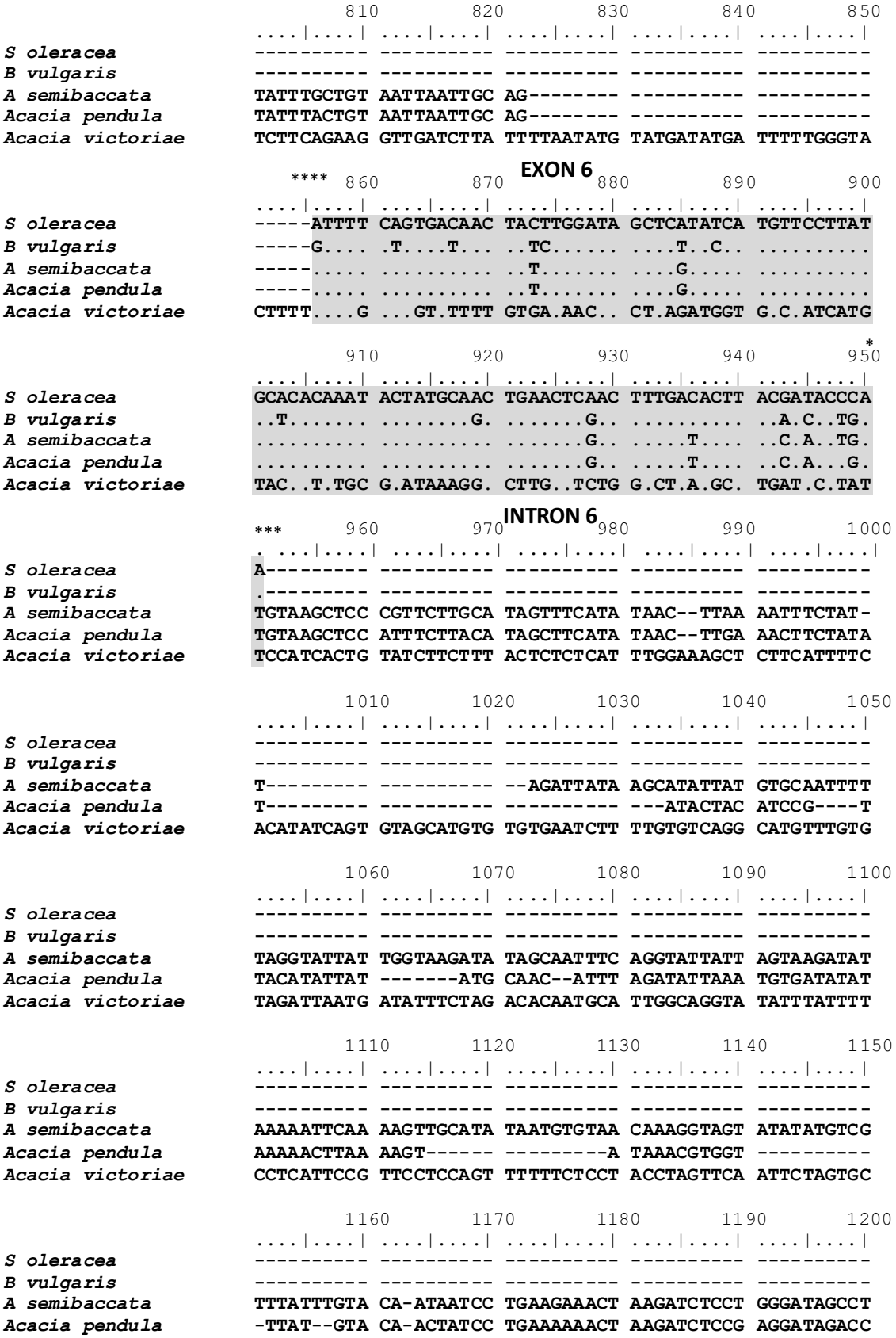
```
                    410        420        430        440        450
                    ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea          ---------- ---------- ---------- ---------- ----------
B vulgaris          ---------- ---------- ---------- ---------- ----------
A semibaccata       TTAATCACTT AATTAGTATC CAATTTTAAC AAATTTCTAT CAATTTCTAT
Acacia pendula      TTAATCAGTT AATTAGTATC CAATTTTAAC AAATTTCAAT CAATTTCTAT
Acacia victoriae    TTCTTGTA-A AACAAAATTC TGATCATAGT GGGAGAGTTT GGCTGATTTT

                                                        ****
                    460        470        480        490        500
                    ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea          ---------- ---------- ---------- --------GA ACTTGGGCTT
B vulgaris          ---------- ---------- ---------- --------.. ..........
A semibaccata       AAATTATTTG TAACTAAAAA TGGTGATTCA TGTTTTAG.. G.........
Acacia pendula      AATTTATTTG TAACTAAAAA TGGTGATTCA TGTTTTAG.. ..........
Acacia victoriae    TGTAACAATG GAGGA----- ---------- -----TAG.. CT.C......
```

EXON 5

```
                    510        520        530        540        550
                    ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea          GTACCCCTAA AAGTTGCAGT ATGGGGGCCG TTCGTTCTTA TCAGCTTGGA
B vulgaris          .C........ .........A ......C..A ...A...... ..........
A semibaccata       .....A.... .......... ......C..A ..TA.A.... ....T.....
Acacia pendula      .....A.... .......... G.....C..A ..TA.A..C. ....T.....
Acacia victoriae    A..T.AA.TG ....A..TA. T.....C..A ..T..A...C ...A.C..A.

                    560        570        580        590        600
                    ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea          CAGATCACTT GAAGAAGGTG GTGATGTTGG AACTGAGTGG CTTGGTACTT
B vulgaris          .C....T..A ..T.CTAA.. C......... ...A..... A......AA.
A semibaccata       .......AGC CGT....TA. ....C..... .T....A... .....C.G..
Acacia pendula      .......AGC CTT....CA. ....C..... .T....A... .......G..
Acacia victoriae    ..AGGAGGGC TTTCCTCAAA CA..A..C.A T.GCC.T..T G.G..A.GGG

                    610        620        630        640        650
                    ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea          CTGCTGAAGA TGTTAAG-GC CCATGCTTTT GATCCTTCAC TTCAATTCAT
B vulgaris          ....A..... .......-.. .......... ......AAT. .AA.G....C
A semibaccata       G......... .......-.. .......... ..C...AAT. ....G.....
Acacia pendula      G......... .......-.. .......... ..C...AAT. ....G.C...
Acacia victoriae    AATGGCTT.G .AGC.GTGCA GATATA..GA .CA.TAATGG AATTGA.TC.

                                                               *
                    660        670        680        690        700
                    ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea          TCACAGAAGT GAACTCCCAA TG-------- ------GAAT CTAATTGGAA
B vulgaris          C..T...... ...T...... ..-------- ------.... GC..C.....
A semibaccata       ...T..G... ...T.T.... .A-------- ------.... ..........
Acacia pendula      ...T..G... ...T...... .A-------- ------.... ..........
Acacia victoriae    ....TC.GT. ATCTCAG..G ACGCGAATAT ACAATT.... GC........
```

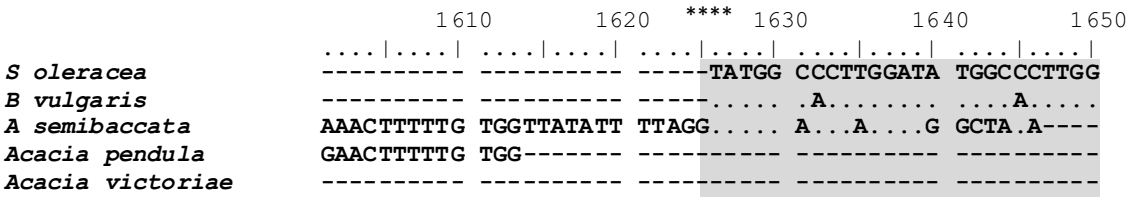
INTRON 5

```
                ***
                    710        720        730        740        750
                    ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea          G--------- ---------- ---------- ---------- ----------
B vulgaris          .--------- ---------- ---------- ---------- ----------
A semibaccata       .GTATATATT T--------- ----TAATAA ATTTGTTATT ATAAA-TT--
Acacia pendula      .GTATATTTT TAT------- -AATTTTGTT ATTTGTTAAT ATAAATTT--
Acacia victoriae    .GTATGGATT ATTTCAGAGA CTATCACCTT CACTCTTCTA GTAAGCTTAG

                    760        770        780        790        800
                    ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea          ---------- ---------- ---------- ---------- ----------
B vulgaris          ---------- ---------- ---------- ---------- ----------
A semibaccata       TTAAGAGCTT AATTA---TT ATTTGATAAA TAATCAAT-- TTAATGTTAT
Acacia pendula      TTTAGAGCTT AAGCA---TC ATATAATGAA CAATTGAT-- TTAATGATAT
Acacia victoriae    TTTATATCGT GAGCAGATTT ATCCTACAAG TGAAAAACCA TGGAATTTGT
```


EXON 6
INTRON 6
S oleracea
B vulgaris
A semibaccata
Acacia pendula
Acacia victoriae

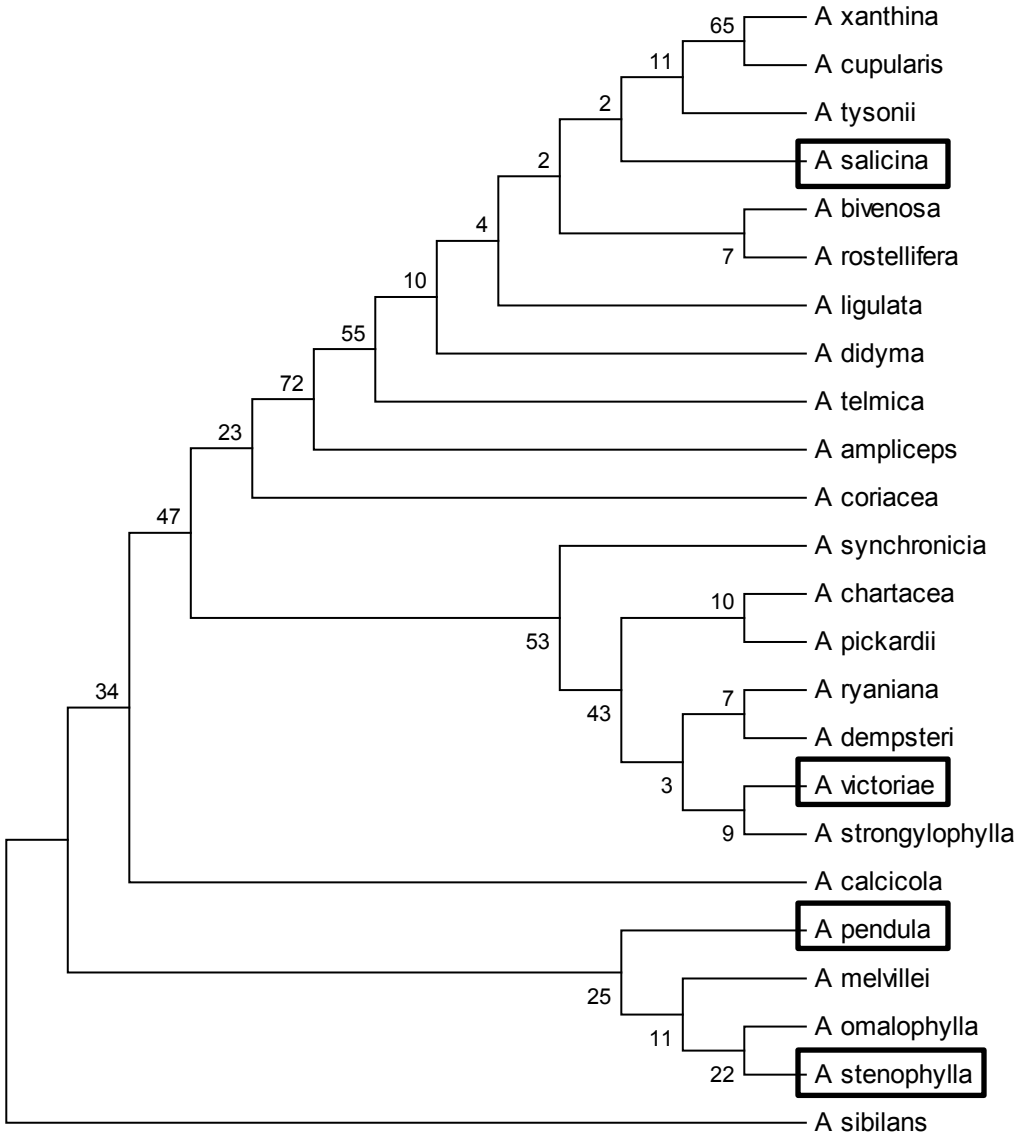
```
                        1210       1220       1230       1240       1250
                   ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea         ---------- ---------- ---------- ---------- ----------
B vulgaris         ---------- ---------- ---------- ---------- ----------
A semibaccata      ATGTGACTAA CTGTGGAAGA TTTCTTATTT ---CAAATTT CAGTTACTTT
Acacia pendula     GTGTGACTAA TTGTGTAAGA TCTTTTATTT ---CAAATTC AAGTAACTTA
Acacia victoriae   AGAGGCTTCC CTCCCAGGGA AAAAACAACT CCCAGAAGGA AGGTTTCCCC

                        1260       1270       1280       1290       1300
                   ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea         ---------- ---------- ---------- ---------- ----------
B vulgaris         ---------- ---------- ---------- ---------- ----------
A semibaccata      TATTTTTCTT TTTCTAAGTG CACCAAATAG CCACCAATTC TATAGTTAGA
Acacia pendula     GATTATT--- ---------- ---------- --------TC ---ATTTAGA
Acacia victoriae   TCCCCCCAAA CATCATTTTG GTACAGTAAT TAATGTAAAG AAAAGTATTT

                        1310       1320       1330       1340       1350
                   ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea         ---------- ---------- ---------- ---------- ----------
B vulgaris         ---------- ---------- ---------- ---------- ----------
A semibaccata      CGTATTATAC ATTTTTATGT TATGTACTTA TATCGACTAA TCATTAAATG
Acacia pendula     GGTGTTATAT ATTTTTATGT TAT------- ---CGACTAA TTATTAAATG
Acacia victoriae   ATAGCATAAA ATGCACCTGA TATTGGGCTT TCATTGTTCA TTGTAACCAA

                                                          ****
                        1360       1370       1380       1390       1400
                   ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea         ---------- ---------- ---------- ---------- -ATGATCGAA
B vulgaris         ---------- ---------- ---------- ---------- -........G
A semibaccata      TTTATATAAT TATATTT--- ---------- ---------A G...G.T.G.
Acacia pendula     TTTGTATAAT TAAATTT--- ---------- ---------A G...G.T.G.
Acacia victoriae   CTGTTTTTTT GGGTCCTAAG GGACTGCATT TAATGATGTA G...T.T...

                                   EXON 7
                        1410       1420       1430       1440       1450
                   ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea         AACGTTACAA TTCAAAGAGT GGAAGGAAGT TCAAACAAGC CTGATGGTTT
B vulgaris         ..ATG.GTG. .......... T.GTA.C... .......... .A.....A..
A semibaccata      ..T..C..G. .......G.. ..CT..G.C. ........-- ----......
Acacia pendula     ..T..C..G. .......G.. ..CT..G.C. ........-- ----......
Acacia victoriae   .GG..A.GC. .......TTG T...A.CG.C ...GTG..AA GGA.A.AGAG

                        1460       1470       1480       1490       1500
                   ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea         TGATAGAGTT GGAATTCAAG CATTCTATGC TTTCGCGTAT CCAAATTTCG
B vulgaris         .......C.. ....C.G... .......... ...TATT..C ..C..C..T.
A semibaccata      .A.....C.. ....C..... .C........ ...T..A..C ..T..C....
Acacia pendula     .A.....C.. ...TC..... .C.......T ...T..A..C ..T..C..T.
Acacia victoriae   CT..GATCGA CTTGGAA..A TTGCTATATA .GCTTTTGT. TGTCC.AA.T

                        1510 ****  1520       1530       1540       1550
                   ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea         -CTGTGGAA- -AGG------ ---------- ---------- ----------
B vulgaris         -........- -...------ ---------- ---------- ----------
A semibaccata      -........- -...TAAATT GGTTAACTCT TCCTACCTAT TCATATAATG
Acacia pendula     -.A......- -...TACATT GGTTAACTCT TCTTACCTTT TCATATAATG
Acacia victoriae   T.ATGAT..A T...TACTCG CTTGTTTTAG GTAATTATCA TGCAAAATAG

                                   INTRON 7
                        1560       1570       1580       1590       1600
                   ....|....| ....|....| ....|....| ....|....| ....|....|
S oleracea         ---------- ---------- ---------- ---------- ----------
B vulgaris         ---------- ---------- ---------- ---------- ----------
A semibaccata      GTGGAGTCAT TTAAACACTA TATGACAATG ACATTTTGGC TAATTTTTTG
Acacia pendula     GTGGAGTCAT TTTAACACTC TATGAAAATG ACATTTTGGC TAATTTTT-G
Acacia victoriae   TAGACTTTCT TAACTTTTGA TGTTTTCCAA A---------- ---------
```

EXON 8



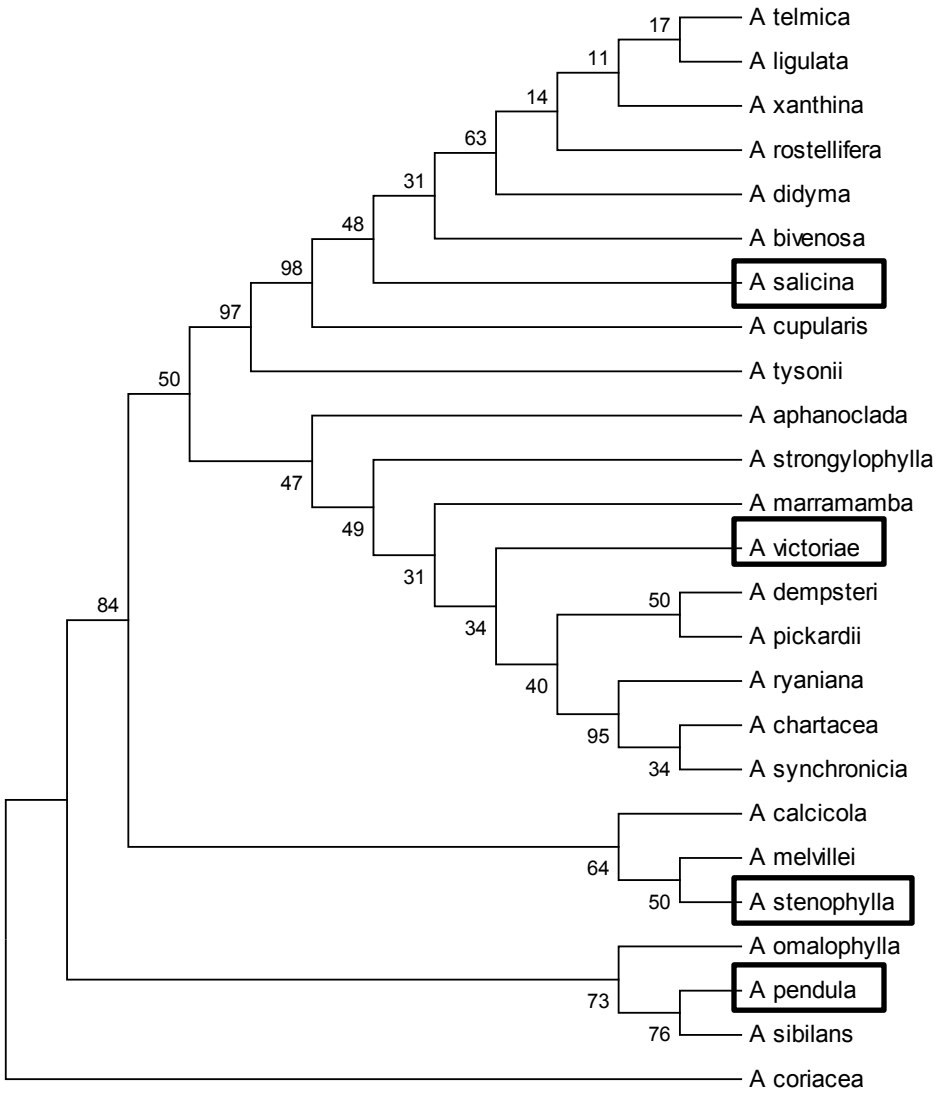
Sequence alignment of cloned, partial CMO genomic gene of *Acacia pendula* and *Acacia victoriae*. The alignments were created in ClustalW under the 'Accessory Application' tab in BioEdit. *Atriplex nummularia* CMO cds from GenBank (Accession no. AB112481.1) was also included. Exons are numbered and highligted in grey boxes. Introns are numbered and marked with a bold over line. Numbers are assigned based on the CMO gene structure of *Arabidopsis thaliana* gDNA gene (TAIR Accession no. AT4G29890.1) and cds (GenBank Accession no. NM_119135.4). Exon-intron/intron-exon junctions are marked with four asterisks (*) that depict the two start/end residues of introns and exons. A dot (.) indicates the presence of conserved nucleotides at the position aligned with *A. thaliana* CMO gene. A dash (-) indicates a gap introduced to align sequences or represent a missing nucleotide.

Appendix X: Evolutionary relationships of 24 taxa based on psb region



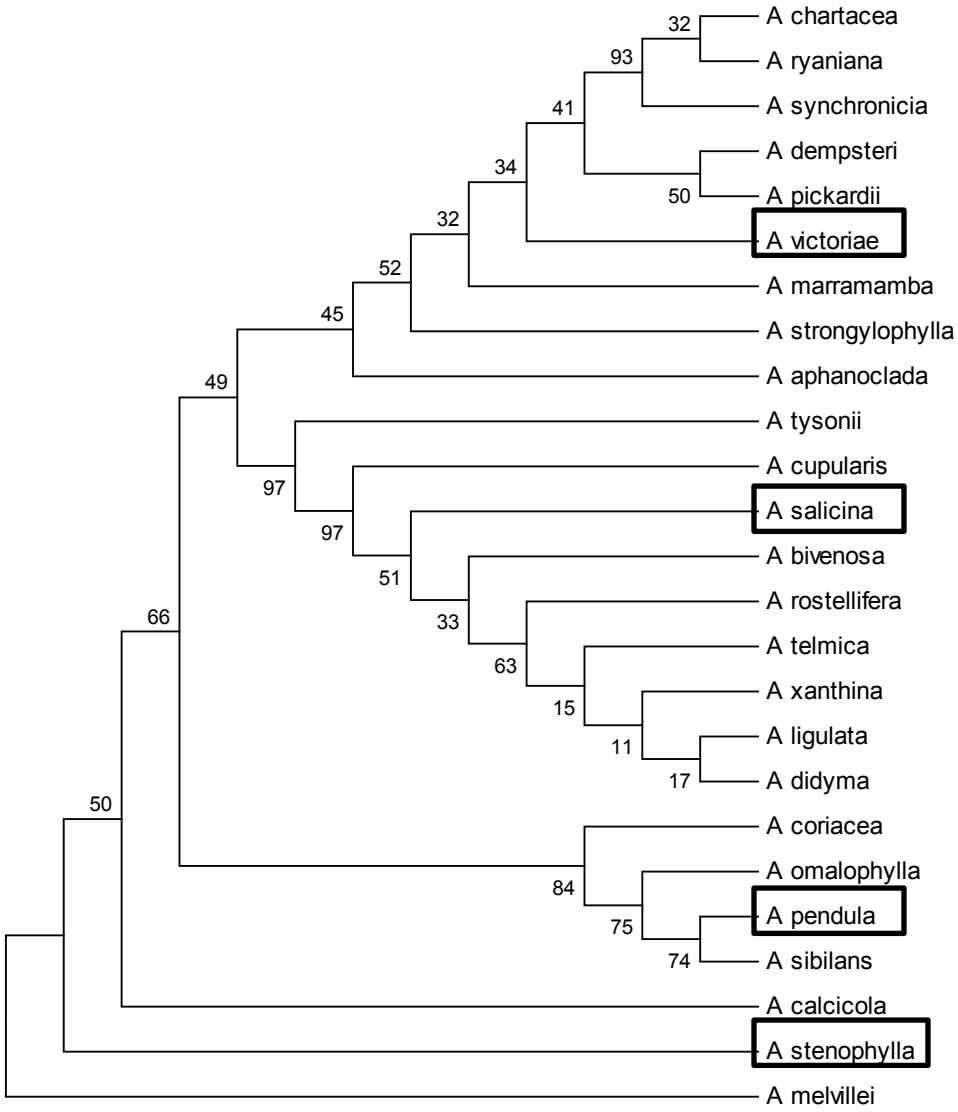
Bootstrap values with 1000 replicates (indicated as % of replicate trees) is shown next to each branch. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with random addition of sequences (10 replicates). All positions containing gaps or missing data were eliminated from the dataset (Complete Deletion option). Species in boxes are the four species currently in use for agroforestry in saline degraded lands at Bendigo, Victoria, Australia.

Appendix XI: Evolutionary relationships of 25 taxa based on rpl32-trnL region



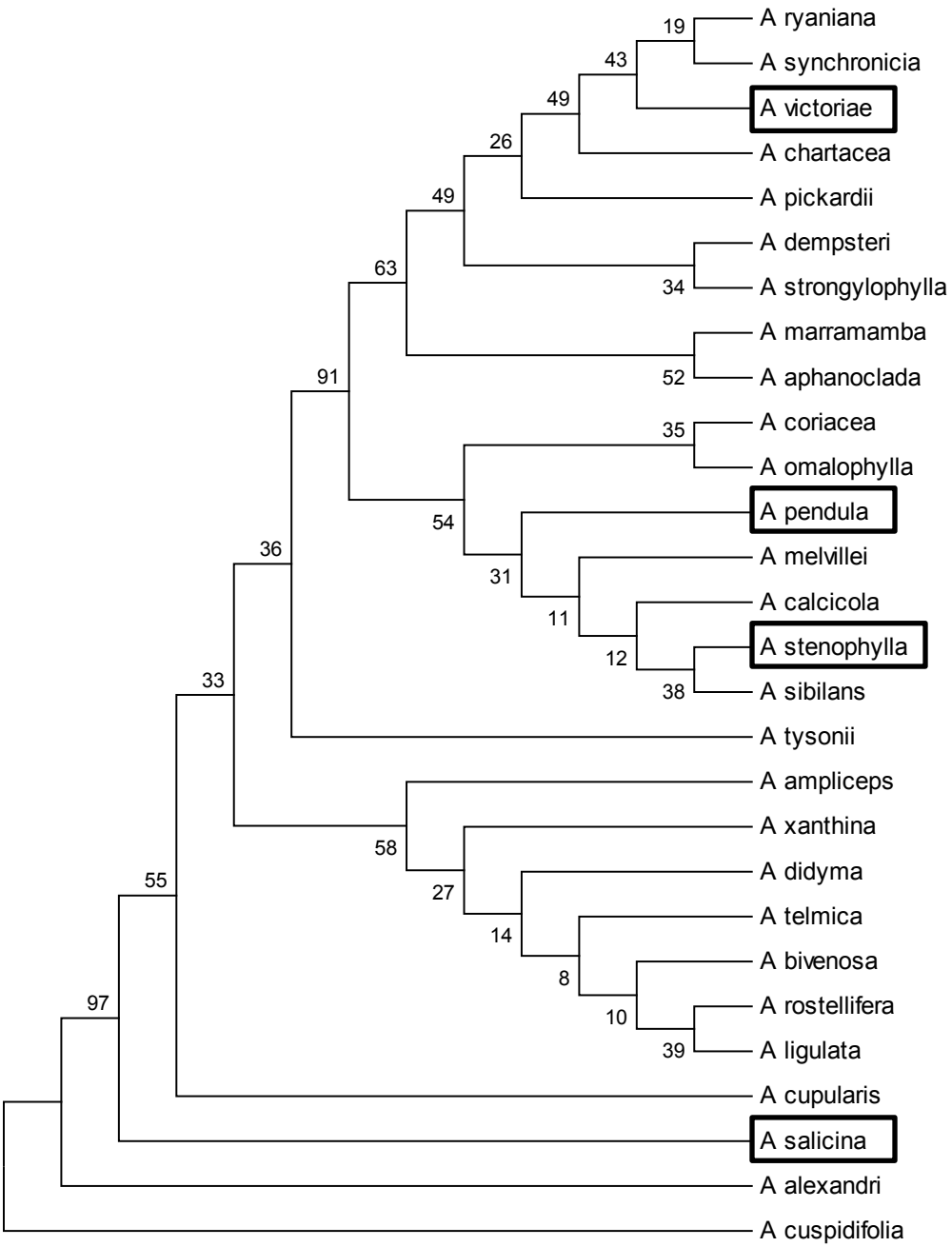
Bootstrap values with 1000 replicates (indicated as % of replicate trees) is shown next to each branch. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with random addition of sequences (10 replicates). All positions containing gaps or missing data were eliminated from the dataset (Complete Deletion option). Species in boxes are the four species currently in use for agroforestry in saline degraded lands at Bendigo, Victoria, Australia.

Appendix XII: Evolutionary relationships of 25 taxa based on tL-r32F(trnL-rpL32F) region



Bootstrap values with 1000 replicates (indicated as % of replicate trees) is shown next to each branch. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with random addition of sequences (10 replicates). All positions containing gaps or missing data were eliminated from the dataset (Complete Deletion option). Species in boxes are the four species currently in use for agroforestry in saline degraded lands at Bendigo, Victoria, Australia.

Appendix XIII: Evolutionary relationships of 28 taxa based on matk region



Bootstrap values with 1000 replicates (indicated as % of replicate trees) is shown next to each branch. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with random addition of sequences (10 replicates). All positions containing gaps or missing data were eliminated from the dataset (Complete Deletion option). Species in boxes are the four species currently in use for agroforestry in saline degraded lands at Bendigo, Victoria, Australia.