ISOLATION AND CHARACTERISATION OF A NOVEL LECTIN GENE, *ALLIUM TRIQUETRUM* AGGLUTIN, CONFERRING INSECTICIDAL PROPERTIES AGAINST *MYZUS PERSICAE*

Chenyi Yao

Thesis submitted for degree of Doctor of Philosophy

University of Southampton, UK

Faculty of Medicine, Health & Life Sciences

School of Biological Sciences

January 2009

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

SCHOOL OF BIOLOGICAL SCIENCES, UNIVERSITY OF SOUTHAMPTON

Thesis submitted for Doctor of Philosophy

ISOLATION AND CHARACTERISATION OF A NOVEL LECTIN GENE, *ALLIUM TRIQUETRUM* AGGLUTIN, CONFERRING INSECTICIDAL PROPERTIES AGAINST *MYZUS PERSICAE*

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Myzus persicae, more commonly known as green peach aphid, is an important pest in agriculture. Each year it causes great losses to agricultural and horticultural plants. It feeds on sap in the phloem, the main transportation route for plant nutrients. It also transmits more than 100 plant viruses, and as there are still no agrichemicals specifically against viruses, farmers often spray large amounts of pesticides to protect their crops.

A group of widely-distributed plant proteins, termed lectins, have been recently studied because of their insecticidal properties against aphids. Snowdrop lectin, *Galanthus nivalis* agglutinin (GNA) was the first isolated monocot mannose-binding lectin, conferring properties of resistance to sap-sucking or homopteran pests.

The aim of the project described in this thesis was to identify and express a novel lectin in *Arabidopsis thaliana* and to assess the performance of the green peach aphid, *Myzus persicae*, on both transgenic and wild type plants. A gene encoding a novel mannose-binding lectin was cloned from *Allium triquetrum* (wild garlic). The full-length cDNA of *Allium triquetrum* agglutinin (*ATA*) was 719 bp and contained a 522 bp open reading frame encoding a 173 amino acid polypepetide. Homology analysis showed that ATA has high similarity with other mannose-binding lectins and includes three putative mannose-binding subdomains, which suggests that ATA may also confer resistance against aphids. The pGreen 0029 vector and the 35S CaMV cassette were ligated to produce the expression construct.

An expression vector was engineered using the pGreen vector together with CaMV 35S promoter and the novel *ATA* gene was inserted. Transgenic *Arabidopsis thaliana* plants were subsequently generated using the *Agrobacterium*-mediated floral dip method. Six homozygous *ATA*-transformed lines and one empty vector-transformed control line were obtained using kanamycin selection. Several key growth parameters of the transgenic plants were assessed to demonstrate that expressing ATA causes few

phenotypic changes to the host plant. The transgenic plants were subsequently used for aphid bioassays, including choice and non-choice behaviour tests, Mean Relative Growth Rate test, fecundity test, and survival test. The aphid bioassays revealed that (1) ATA does not change the behaviour of aphids to choose a host; (2) Adults are more sensitive to ATA than nymphs; (3) ATA has significant detrimental effects on aphid fecundity.

The results described in the thesis show that the production of *Allium triquetrum* agglutinin in Arabidopsis confers partial resistance to the aphids, *Myzus persicae*. It is likely that this lectin in related Brassicaceae plants could be used, perhaps in conjunction with other pest-control measures in agriculture.

Key words: *Allium triquetrum* agglutinin, aphid bioassay, *Arabidopsis thaliana*, choice test, fecundity test, lectin, non-choice test, mean relative growth rate, *Myzus persicae*, pleiotropic effects, resistance against aphids, survival test.

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Declaration of authorship

I, **Chenyi Yao**, declare that this thesis entitled "Isolation and characterisation of a novel lectin gene, *Allium triquetrum* agglutin, conferring insecticidal properties against *Myzus persicae*" and all the work presented in it is my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University;
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
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- Where the thesis is based on work done by myself jointly with others I have made clear exactly what has been done by others and what I have contributed myself;
- None of this work has been published before submission.

Signed:………………………………………………………………………………..

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Acknowledgements

Abbreviations and definitions

AHA: *Arisaema heterophyllum* agglutinin

AKA: *Amorphophallus konjac* agglutinin

ANOVA: Analysis of variance

ATA: *Allium triquetrum* agglutinin

ASAL: *Allium sativum* leaf agglutinin

AUA: Allium ursinum agglutinin

BSA: Bovine serum albumin

Bt: *Bacillus thuringiensis*

CaMV: Cauliflower mosaic virus

cDNA: complementary Deoxy-ribonucleic acid

Con A: Concanavalin A

CPTI: Cowpea trypsin inhibitor

d.f.: Degree of freedom

DM: plant dry mass

DNA: Deoxy-ribonucleic acid

dNTP: deoxy-nucleoside triphosphate

E. coli: *Escherichia coli*

EDTA: Ethylene Diamine Tetra-acetic Acid

EPG: Electrical penetration graph

ERA: Ecological risk assessment

FT: Time taken to reach flowering (the appearance of the first flower bud)

GLM: General linear model

GM: Genetically modified

GNA: *Galanthus nivalis* agglutinin

GT: Germination time

JA: Jasmonic acid

K-S test: Kolmogorov-Smirnov normality test

K-W test: Kruskal-Wallis test

LB: Luria Bertani Broth

LSD: Least significant difference

N_L: The number of aphids settling on a leaf

MRGR: Mean relative growth rate

mRNA: Messenger Ribonucleic acid

MS: Murashige and Skoog

LN: Total leaf number when the first bud turns up

OPDA : 12-oxo-phytodienoic acid

ORF: Open reading frame

PAGE: Polyacrylamide gel electrophoresis

P_{AS}: The percentages of the surviving adults

P_C: The percentage of aphids that made a choice

PCR: Polymerase chain reaction

P_D: The percentage of aphids that chose D1201

PI: Protease inhibitor

PM: peritrophic matrix

P_{NS}: The percentages of the surviving nymphs

RACE: Rapid Amplification of cDNA Ends

RNA: Ribonucleic acid

RT PCR: Reverse transcription polymerase chain reaction

SA: Salicylic acid

SAP: Shrimp alkaline phosphatase

SDS: Sodium dodecyl sulphate

SE: Sieve element

SN: Seed number (Total number of seeds from one plant)

SM: Seed mass (average weight of seeds from one plant)

SSC: Saline-sodium citrate

SY: Seed yield (Total seed weight)

T_0: Untransformed plants

T_1: Primary transformed plants

T_2: Second generation transformants

T_3: Third generation transformants

T-DNA: Transfer DNA

TAE: Tris/acetate/EDTA

TE: Tris-EDTA

UPM: Universal Primer Mix

Chapter One

1 General Introduction

This thesis reports an investigation of some aspects of aphid and plant interactions. A group of plant proteins, called monocot mannose-binding lectins, are recently considered to have insecticidal effects against homopteran insects. Their toxicity is related to their exclusive binding specificity to mannose. This chapter reviews the life history of a homopteran insect, green peach aphid (*Myzus persicae*), its interaction with plants, and features of lectins.

1.1 Distribution and damage caused by the green peach aphid (Myzus persicae)

The family Aphididae comprises more than 4300 species, all of which are specialized to feed on phloem sap (Blackman and Eastop, 1994). Among them, the green peach aphid (*Myzus persicae*) is found throughout the world and has an exceptionally wide host range (Van Emden *et al.*, 1972). *M. persicae* feeds on hundreds of host plants in over 40 plant families. *M. persicae* is a phloem feeder and penetrates plant tissue by probing intercellularly through the epidermal and mesophyll cell layers with its stylet-like mouth parts to feed on photo assimilates translocating in the phloem sieve elements (Pollard, 1972). It is viewed as a pest due to its ability to transmit over 100 virus diseases of plants in about thirty different families including many major crops (Kennedy *et al.*, 1962). To make things worse, *M. persicae* can transmit viruses between different species due to its broad host spectrum. The mechanisms of virus transmission by aphids can be distinguished as circulative and non-circulative. Circulative viruses can be transported in the vectors' haemolymph and excreted in saliva while non-circulative viruses are only associated with the vectors' mouthparts and foregut (Ng and Perry, 2004). Although circulative viruses can be retained longer in *M. persicae*, the transmission of non-circulative viruses only requires brief peripheral cell penetrations.

1.2 The life cycle of Myzus persicae

Like other aphid species, *M. persicae*'s life cycle is unique for the alternation between asexual and sexual phases of reproduction, or holocyclic life cycle and anholocyclic life cycle (Figure 1.1).
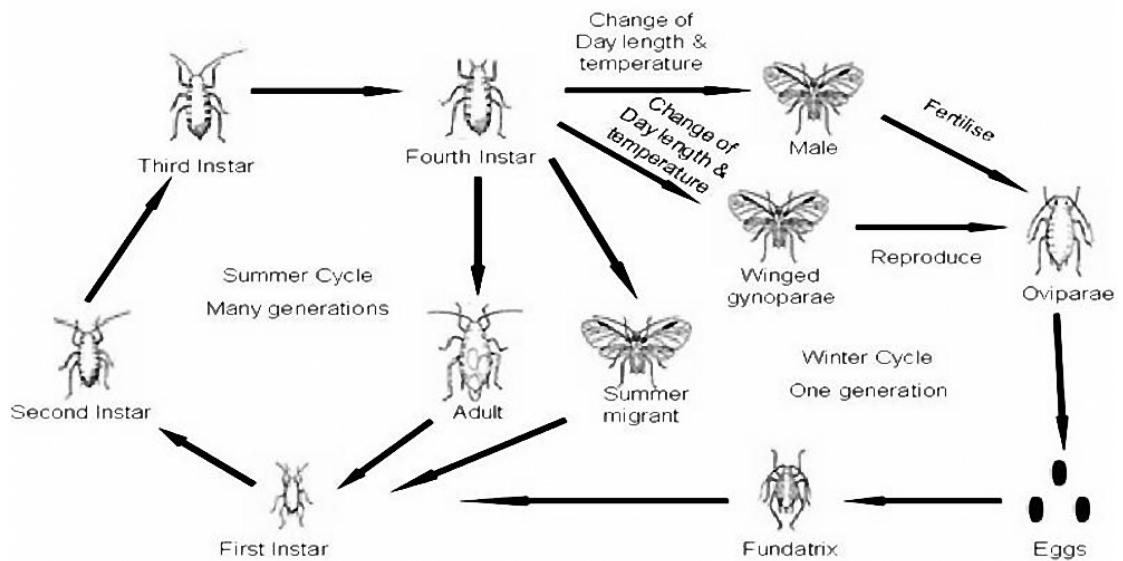
Figure 1.1 Life cycle of *M. persicae* (reproduced from http://bohart.ucdavis.edu)

The sexual phase only has one generation in winter while the asexual phase involves many generations throughout the summer. The sexual phase is triggered by the decrease of day length and temperature in a well defined time in autumn (Ward, 1934; Newton *et al*., 1953; Scholl and Daiber, 1958). The sexual phase starts with the production of winged gynoparae, which give birth to oviparae on and only on certain *Prunus* spp. and some related genera, known as primary hosts (Hille Ris Lambers, 1946). The male *M. persicae* are then attracted by the volatile sex pheromone of the oviparae and mate with them (Kennedy *et al*., 1963). Each fertilised ovipara can lay 4-13 eggs (Ward, 1934) and 40%-70% of the eggs eventually hatch (Ronnebeck, 1952). The emergence of fundatrices marks the beginning of the asexual phase. In the asexual phase, adult females can give birth to live nymphs without mating and a nymph can have embryos developing within them that also have embryos, causing the population of aphids to increase dramatically. The nymph needs to molt approximately four times before becoming an adult. The newly hatched fundatrices feed on the swelling buds of the primary host and produce about 50 to 60 young (Ward, 1934). Up to eight apterous generations of the fundatrices may occur on the primary host. However, with the approach of summer, the nutrient status in the primary host decreases (Van Emden *et al*., 1972), so in summer, alate migrants develop and migrate to secondary hosts including major vegetable crops in the families Solanaceae,

Chenopodiaceae, Compositae, Brassiceae, and Cucurbitaceae. In fact, the life cycle of *M. persicae* varies considerably, depending on the climates in different regions (Van Emden *et al.*, 1972). The critical low temperature for survival of apterae of *M. persicae* is 2°C (Adams, 1962) while the upper mean daily temperature for survival is about 32°C (Van der Plank, 1944).

1.3 The initial attack of Myzus persicae on plants

An aphid needs three steps to settle on a host plant: (1) landing; (2) testing of the plant surface and the outer plant tissue; and (3) penetration and evaluation of the final feeding tissue (Hille Ris Lambers, 1979; Klingauf, 1987). Some studies support the hypothesis that aphids locate their host plants by distinguishing different plant volatiles. Gynoparae and males of *Rhopalosiphum padi* L. were shown to be attracted by extracts of *Prunus padus*, their primary host (Pettersson, 1970). Similar behaviour was also found in *Brevicoryne brassicae* L. (Pettersson, 1973), *Aphis gossypii* (Pospisil, 1972), *Cryptomyzus korschelti* Bormer (Visser and Taanman, 1987), and *Cavariella aegopodii* Scopoli (Chapman *et al.*, 1981). However, such evidence has not been found in some polyphagous aphids, such as *M. persicae*. There is clear evidence that alate *M. persicae* alight on host and nonhost plants with equal frequency (Kennedy *et al.*, 1959, 1959a), but there are observations that *M. persicae* alatae avoid landing on certain plants or leaves (Kennedy *et al.*, 1959). Besides, visual stimuli are also involved in the process (Cartier, 1966). More alate *M. persicae* were caught in yellow traps over bare ground than over plots with plants (Moericke, 1957). Harrewijn (1990) reported that *M. persicae* started to insert its stylet shortly after its legs touched the cuticle whereas *Nasonnovia ribisnigri* hesitated much longer. This report might suggest that polyphagous aphids decide what to feed on by tasting. After aphids have landed on a potential host plant, they explore the leaf surface with sensillae on antennae to test the chemical nature of the surface and the outer tissue of the plants (Shambaugh *et al.*, 1978; Niemeyer, 1990). They also scan the surface of a plant with proboscises to detect the contours of veins, their preferred feeding sites (Tjallingii, 1978).

1.4 How do aphids feed on phloem?

Aphids probe into the plants with their mandibular and maxillary stylets (Dixon, 1998). On the plant surface, aphids secrete a small amount of gelling saliva (salivary flange) before stylet insertion (Tjallingii, 2006). Probing the leaf surface consists of short (about 1 to 5 min for *M. persicae*) repeated stylet insertion at different sites (Bradley, 1952). In general, the number of probes an aphid attempts is greater, and the probes are more prolonged, on host than on non-host tissue (Muller, 1965). On artificial diets, *M. persicae* probes longer on phago-stimulative diets (Mittler and Dadd, 1965). It used to be believed that aphids reach the phloem accidentally and the stylets follow both intracellular and intercellular routes (Kimmins, 1986). However, the work by Tjallingii and Hogen Esch (1993) with the electrical penetration graph (EPG) technique demonstrated that most cells along the stylet pathway are briefly punctured intracellularly, but the stylets are always withdrawn from the cells and then continue along the intercellular pathway. The stylets enter the plant epidermis starting at the border between two cells, following a pathway between the fibres of the secondary cell wall of one of these cells (Tjallingii, 2006). When aphids penetrate the plant tissue, they can secret salivary stylet sheaths making the flexible stylets rigid and easy to control (Pollard, 1973). The salivary sheathes remain in the plant after stylet withdrawal.

Although phloem sap is relatively nutrient-rich compared with many plant products, aphids still need to overcome two nutrient barriers when feeding on phloem: the low nitrogen concentration and the high sugar concentration (Douglas, 2006).

Low nitrogen concentration To grow, develop, and reproduce successfully, aphids require essential amino acids as do mammals, without which, it is impossible to produce proteins. If the concentration of just one of these essential amino acids is in short supply, protein synthesis and growth of aphids are constrained. The ratio of essential amino acids to non-essential amino acids in plant phloem sap is 1:4 - 1:20, which is considerably lower than the ratio of 1:1 in animal protein. Because of the inadequacy of phloem-derived amino acids, aphids manage to solve the problem endogenously. The endogenous source is symbiotic bacteria, *Buchnera* sp., which synthesize and provide these nutrients to the aphids (Douglas, 2003). Aphids with *Buchnera,* but not aposymbiotic aphids, can synthesize essential amino acids from dietary precursors such as sucrose and aspartate (Douglas, 1988; Febvay *et al*., 1995; Birkle *et al*., 2002). The most special feature of *Buchnera* is that they have retained genes coding for most enzymes in the biosynthesis pathways for essential amino acids, even though they have lost many other metabolic capabilities, including the capacity to synthesize most non-essential amino acids (Douglas,

1988). These studies suggest strongly that aphids have solved the problem of low nitrogen concentration in the phloem sap by their acquisition of essential amino acid-overproducing bacterial symbionts during evolution.

High sugar concentration The main compounds in phloem sap are sugars derived from photosynthetic carbon fixation. However, the high concentration of sugars in the phloem sap, up to and often exceeding 1 M sugar, is a great challenge to sap-feeders. The osmotic pressure of the phloem sap is 2-5 times greater than the osmotic pressure of the insect body fluids. Therefore, the obvious expected result of the continuous flow of fluid at high osmotic pressure into the gut is the transfer of water from the body fluids to the gut contents and osmotic collapse of the insect. However, this does not happen. It is certain that aphids excrete the excess sugar in the form of honeydew, the osmotic pressure of which is surprisingly similar to that of body fluids and is lower than the phloem sap (Douglas, 2006). The analysis of honeydew sugar composition shows that the honeydew derived by aphids reared on artificial diets with sucrose as the only sugar contains low levels of monosaccharides but high levels of oligosaccharides (Ashford *et al*., 2000). The high concentration of oligosaccharides helps to reduce the osmotic pressure because the osmotic pressure of solutions is determined by molality of sugars but not their mass. Aphids utilize sucrases in the gut or stomach, such as α-glucosidase, to convert sucrose to glucose and they may even use other enzymes to synthesize oligosaccharides (Ashford *et al*., 2000; Cristofoletti *et al*., 2003).

There is adequate evidence that aphids ingest food with the help of the osmotic pressure of plants. When the stylets of *Aphis fabae* were cut, liquid continued to exude for some time (Kennedy and Mittler, 1953). Similar exudation has been obtained from the severed stylets of *M. persicae* (Van Soest and De Meester-Manger Cats, 1956). This technique of gathering "stylet-sap" is also used in plant physiology research (Unwin, 1978; Pritchard, 1996; Zhu *et al*., 2005). However, the osmotic pressure is not always essential, because using artificial diet is a common method to rear aphids in the laboratory.

1.5 Phloem wounding responses and aphid salivation

Plants face a variety of physical, chemical, abiotic and biotic stresses. For example, injury caused by abiotic (wind, fire, drought, nutrition depletion) or biotic (stinging, browsing, chewing) agents. Sieve elements (SE) are particularly sensitive to injury and plants cope with wounds by SE sieve pore plugging (Will and Van Bel, 2006). There are two types of plugging: the long term plugging and the ready plugging. The long

term reaction is the production of callose, a 1,3-β-glucan with a few 1,6 branches (Aspinall and Kessler, 1957). Callose production is often observed around plasmodesmata induced by wounding (Stone and Clarke, 1992) or by microbial attack (Kauss, 1996; Donofrio and Delaney, 2001). However, it takes some time (several minutes) before such extensive callose structures are synthesized. Therefore, it is likely that some more rapid plugging mechanism is responsible for a ready response (a few seconds) to injury (Will and Van Bel, 2006). There are abundant different proteins found in transmission electron microscope (TEM) pictures of SEs (Evert, 1990). The occurrence of these macromolecules seems surprising, since mature SEs lack the capability for protein synthesis. However, proteins are the most likely candidates for the fast plugging event. Confocal laser scan microscope studies of damaged *Vicia faba* SEs confirmed this hypothesis. In response to injury inflicted by a micro-capillary tip, thick protein deposits readily appeared on the sieve plates and mass flow stopped immediately (Knoblauch and Van Bel, 1998). The protein plugs may be composed of dispersed forisomes and detached parietal proteins (Will and Van Bel, 2006). Ca^{2+} ions play an important role in both long term (King and Zeevaart, 1974) and ready plugging (Knoblauch *et al.*, 2001, 2003).

The feeding process of aphids apparently does not induce the above mentioned plugging reaction because aphids start ingesting from a SE within a few minutes after a puncture and may continue to do so for hours at least. Therefore, aphids must be able to inhibit the sieve wound response, or the punctured SEs would have similar plugging when wounded by a micro-capillary of a similar diameter (Tjallingii, 2006). Tjallingii and Hogen Esch (1993) suggest that the aphid saliva can prevent the clogging of proteins in the food canal.

1.6 Plant defence systems against aphids

Plant defence systems can be divided into two principal types, the physical defence systems and the chemical defence systems. Physical defence, also named structural defence, means some plant morphological characteristics helping to reduce aphid damage. For example, the undersides of the leaves of *Alnus incana* are covered with dense trichomes, which can reduce aphid survival and equip alder with resistance to colonization of *Pterocallis alni* (Gange, 1995). Chemical defence systems are more complex and can be divided into direct defence and indirect defence. Direct defence means the constitutive or induced production of insecticidal compounds by plants, such as protease inhibitors (PIs), xanthotoxin, furanocoumarin, and lectins. Plants can also use indirect defence mechanisms

to protect themselves against herbivorous insects. The major indirect defence mechanism is the emission of volatiles to attract natural enemies of insect pests.

1.6.1 Constitutive plant defence systems

Plants synthesize a wide range of secondary metabolites which are toxic to herbivores. The toxicity of hemlock, digitoxin and aconite to humans demonstrates how well natural products can defend plants, at least against mammalian herbivores (Wittstock and Gershenzon, 2002). However, a few plant toxic proteins do exhibit more or less insecticidal properties, such as lectins, protease inhibitors, α-amylase inhibitors, arcelins, canatoxin-like proteins, and ureases (Carlini and Grossi-de-Sá, 2002). Defense chemicals are costly for plants because of the resources consumed in their biosynthesis and their toxicity to the plant itself (Purrington, 2000). One way for plants to reduce the costs is developing induced defence systems. However, this strategy is sometimes risky because the initial attack may be too rapid for the induced defending systems to react (Wittstock and Gershenzon, 2002). Therefore, plants have developed different strategies to reduce the cost of constitutive defence.

(1) Some plants protect parts that are of high fitness value or under a high risk of attack by constitutive defence, whereas other parts are protected by the induced response. Zangerl and Rutledge (1996) found there are high constitutive levels of the toxic furanocoumarin and xanthotoxin in reproductive organs of wild parsnip which are frequently attacked by herbivores. In their experiments, artificial damage did not change the toxin level in the reproductive organs but did increase the toxin level in roots.

(2) "Paying for one and getting two" is another strategy to reduce the cost. Lectins are common and often abundant in plants (Murdock and Shade, 2002) and hundreds of different lectins have been identified. Van Damme *et al*. (1998) pointed out that lectins may be storage proteins for two reasons. First, lectins often occur most abundantly in seeds and vegetative storage tissue. Second, they accumulate during the growth and development or reproductive phase of the plant life cycle and are mobilized and utilized later. People used to regard lectins only as a storage form of protein for plant growth and development, but recent studies have indicated that they also help to defend plants against herbivores. *Galanthus nivalis* agglutinin (GNA) was the first monocot mannose-binding lectin (Van Damme *et al*., 1987) to be isolated. There is sufficient evidence to support that GNA is toxic to phloem sap-feeding insects (Powell *et al*., 1995; Gatehouse *et al*., 1996; Rao *et al*., 1998).

(3) Many defence compounds are toxic to the plant itself. To store toxins without poisoning themselves, plants store inactive precursors apart from activating enzymes (Wittstock and Gershenzon, 2002). For example, in Arabidopsis, sulphur-rich cells between the phloem and the endodermis of the flower stalk contain high concentrations of glucosinolates (Koroleva *et al*., 2000), while myrosinase is localised in adjacent phloem parenchyma cells (Andreasson *et al*., 2001). Upon tissue damage, the glucosinolates come into contact with myrosinase and are hydrolyzed irreversibly into an unstable aglycone, which rearranges into a variety of defending compounds, such as isothiocyanates and nitriles (Wittstock and Gershenzon, 2002).

1.6.2 Induced plant defence systems

Constitutive defence systems are usually costly to plants, and are species-specific. Therefore, induced defences are more economic and equip plants with protection against polyphagous insects (Gatehouse, 2002). The complexity of the responses of plants to wounding caused by insect feeding makes the study of induced defence systems more difficult. In the model plant Arabidopsis, the steady-state levels of over 700 mRNAs changed during defence responses in a microarray-based study (Schenk *et al*., 2000). Approximately 500 mRNAs have been estimated to constitute the insect-responsive transcriptome in tobacco (Hermsmeier *et al*., 2001). However, a straightforward cause and effect analysis of the factors involved in the production of defined insecticidal compounds or proteins can still be made (Gatehouse, 2002). Two signaling pathways identified as being important in induced plant defence mechanisms are those involving the plant hormone jasmonic acid (JA) (Karban and Baldwin, 1997; Dicke and Van Poecke, 2002), referred to as the jasmonate or octadecanoid pathway, and the salicylic acid pathway involving the plant hormone salicylic acid (SA) (Dicke and Van Poecke, 2002; Dempsey *et al*., 1999).

1.6.2.i The octadecanoid pathway

The octadecanoid pathway plays a key role in gene and metabolic regulation, responses to wounding and abiotic stress, reproduction, defence against insects and pathogens and possibly in communication (Liechti and Farmer, 2002). Jasmonic acid (JA) is a terminal product of the octadecanoid pathway. Several intermediates in the biosynthetic pathway of JA and some derivatives of JA also have biological activities

(Devoto and Turner, 2003). A breakthrough for understanding jasmonate-signalled responses was made by Stintzi *et al.* (2002), who found 12-oxo-phytodienoic acid (OPDA, a precursor of JA) can induce a broad-spectrum of resistance in the absence of JA. Farmer and Ryan (1992) managed to establish a causal link between wounding (caused by insect herbivores), the formation of JAs, and the induction of genes for protease inhibitors that deter insect feeding. Wounding increases the endogenous levels of JAs in many plant species, and exogenous application of JA can stimulate production of defense compounds (Staswick and Lehman, 1999). Ellis *et al.* (2002, 2002a) demonstrated that the Arabidopsis mutant *cev1,* which has a constitutive production of JA and ethylene showed enhanced resistance to *M. persicae.*

1.6.2.ii The salicylic acid (SA) pathway

Using grafting techniques, Vernooij *et al.* (1994) performed a classical experiment with transgenic tobacco. Their work proved that SA could not induce systematic acquired resistance but is essential in defence response. The salicylic acid pathway has proved to be important in protection of plants against many pathogens (Dempsey *et al.*, 1999). A study with Arabidopsis showed that herbivory can induce an increase in the endogenous levels of SA and activate SA-response genes, but no evidence suggests that this has a direct negative effect on herbivore performance (Bi *et al.*, 1997; Moran and Thompson, 2001). Genes have been identified in Chinese cabbage – *Brassica rapa* subsp. pekinensis that are induced by SA but not by Methyl-JA (Lee and Cho, 2003; Park, *et al.*, 2003). However, there is no evidence that they are induced by insect feeding.

1.6.3 Indirect defence systems

Some plant species can produce volatiles to attract the natural enemies of herbivores in response to insect feeding. There is evidence to suggest that natural enemies can use plant volatiles to locate host/prey (Dicke, 1999). It was suggested that naïve females of the parasitic wasp, *Aphelinus ervi,* showed strong oriented flight responses to aphid-infested plants and to aphid-damaged plants from which the aphids had been removed (Guerrieri *et al.,* 1993; Du *et al.,* 1996). Furthermore, the responsiveness of some aphid parasitoid species to aphid-induced plant volatiles can increase if the parasitoid is allowed previous experience of that specific plant/host complex (Reed *et al.*, 1995; Storeck *et al.*, 2000). A recent report by Girling and colleagues (2006) also suggested that naïve

Diaeretiella rapae, reared in *M. persicae* on *B. rapa* subsp. pekinensis, were significantly attracted to volatiles from Arabidopsis infested with *M. persicae* over undamaged plants.

The octadecanoid pathway also plays an important role in the indirect defence pathway. Van Poecke and Dicke (2002) measured responses of the parasitoid wasp *Cotesia rubecula* to *Pieris rapae* reared on a transgenic Arabidopsis (S-12), which has no wound-induced elevation of JA, and wild type Arabidopsis (Col-0). Their research demonstrated that caterpillar-infested S-12 plants were less attractive than caterpillar-infested wild type plants. However, caterpillar-infested S-12 plants still attracted more parasitoids than non infested plants.

As mentioned above, aphid feeding does result in the up-regulation of SA-responsive genes in Arabidopsis, although this does not affect aphid performance. However, Ozawa *et al*. (2000) suggested a signaling role for SA in indirect defences: treatment of lima bean plants with methyl-SA, a derivative of SA, induced the emission of several volatiles.

It is obvious that jasmonates play a central role in the whole plant defence system. Gatehouse (2002) summarises functions of jasmonates (Figure 1.2) in his review about plant defence against insect herbivores.
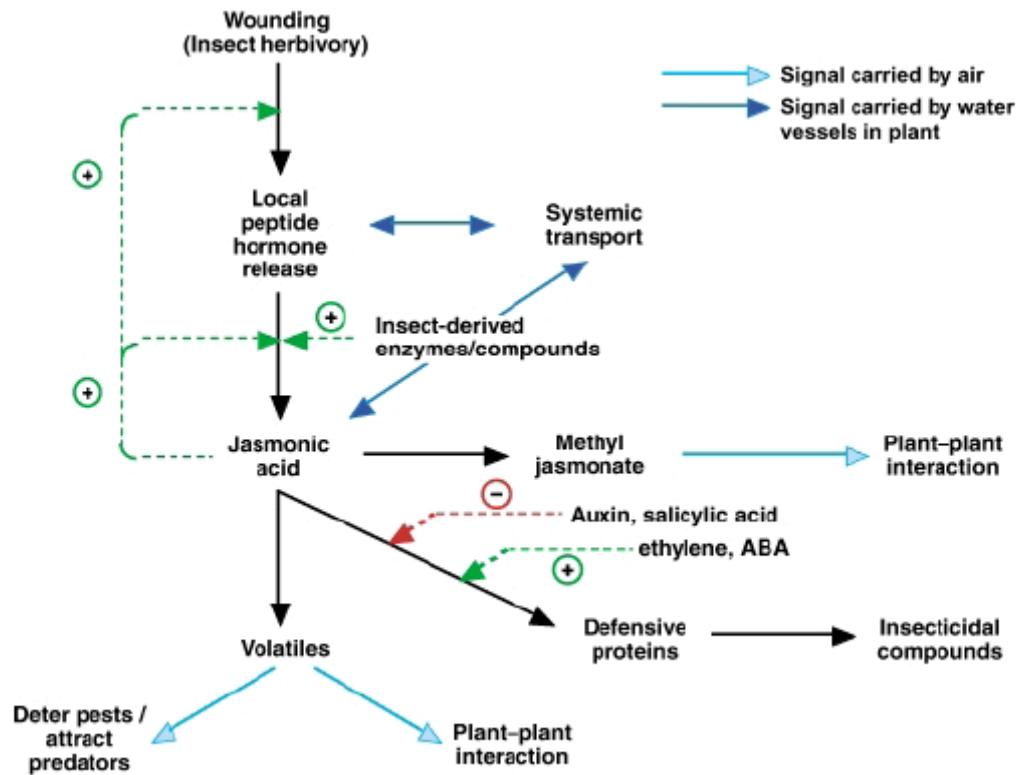
Figure 1.2 Overview of the plant wounding response, and signaling molecules which can modulate it (Taken from Gatehouse, 2002)**.** Black solid arrows indicate a "leads to" relation, either locally or in response to transmitted signals. Dark blue solid arrows indicate systemic signals within the plant; light blue solid arrows indicate signals transmitted by volatiles. Green dashed arrows indicate a positive modulation; red dashed arrows indicate a negative modulation. The solid arrow from "jasmonic acid" to "volatiles" indicates that jasmonic acid biosynthesis also leads to production of green leaf volatiles, and that jasmonic acid stimulates the synthesis of other volatiles such as terpenoids.

1.7 Techniques applied to control aphids

Hitherto, the major method applied to control aphids is using insecticides. We cannot deny the contribution of insecticides in agriculture, but the shortcomings of insecticides are also significant. Therefore, the effort of seeking for alternative techniques never stops.

1.7.1 Breeding plants resistant to aphids

The most basic breeding method is for farmers to choose the healthiest seeds for next year's cultivation. The researchers then consciously choose some varieties with better phenotypes for agriculture. With the flow of time, scientists have developed different methods for breeding.

1.7.1.i Crossing or hybridization

Nature always eliminates the plants that are particularly susceptible to pests or diseases, so most of the existing plants have some kind of resistance to certain pests and diseases. This is the source of resistance for hybridization. Such sources maybe present in existing or old varieties, in wild forms of the same species, in closely related species, or even in different genera (Russell, 1978). The briefest steps for hybridization are: (1) selecting desirable plants in an open-pollinated population; (2) selfing these plants through several generations to produce homozygous inbred lines; (3) crossing chosen lines (Allard, 1964). Breeders have screened out a few strains of different crops which have resistance to aphids. Painter and Pathak (1962) found a maize hybrid (Kansas 1859) that has resistance to corn leaf aphid (*Rhopalosiphum maidis*). This strain has made a significant contribution to the control of corn leaf aphid in USA. Leo, a raspberry strain, which was developed from a cross between an aphid-resistant species, *Rubus occidentalis*, and a cultivated red raspberry, carries the A_{10} gene for resistance to all four of the main *Amphorophora idaei* biotypes in UK and has been commercialised (Keep, 1975). In addition, similar work was also carried out on Brassica crops (Palmer, 1960; Dunn and Kempton, 1969).

1.7.1.ii Somaclonal variation

Sacristan and Melchers (1969) first observed that tobacco regenerated after a long period of subculture presented morphological variations. After cytological tests, they found the variants were aneuploid. Heinz and Mee (1971) reported a high frequency of genetic changes in sugarcane regenerated plants, and some of the variants showed some better traits. Larkin and Scowcroft (1981) named this phenomenon somaclonal variation. Many somaclonal variants have been isolated from different crops, including potato (Shepard, 1980), rice (Oono, 1985), and wheat (Larkin *et al.*, 1984). Therefore, somaclonal variation is a significant source of genetic variation for breeding. Zemetra *et al.* (1993) regenerated five plants from 100 Russian wheat aphid phytotoxin treated wheat calli and found that R2 and R3 generations have resistance to the aphid.

1.7.1.iii Protoplast fusion or somatic hybridization breeding

Hanstein (1880) first proposed the term "protoplast" and defined it as the soft substance containing a nucleus and surrounded by a plasma membrane. Power *et al.* (1970) first began to research into protoplast fusion. Based on protoplast culture, Carlson *et al.* (1972) applied the $NaNO_3$ method to induce the fusion of protoplasts from different tobacco strains and first obtained somatic hybridization. In 1973, Keller and Melchers (1973) achieved protoplast fusion in high Ca^{2+} and high pH conditions. Melchers *et al.* (1978) used the same method to hybridize potato and tomato protoplasts together. This report suggested that protoplast fusion could solve the problem of sexual incompatibility in traditional hybridization. By using somatic hybridization, scientists have produced various disease resistant varieties (Valkonen *et al.*, 1994; Gerdemann-Knorck *et al.*, 1995). Although this is a potential strategy for breeding aphid-resistance species, few reports on this field are available.

1.7.2 Biocontrol strategies

The use of natural enemies for the biological control of insect pests has become an integral part of many pest management programs. Two major natural enemies, namely, parasitoids and predators, are used in horticulture and agriculture. Compared with open fields, glasshouses are closed ecosystems, so it is relatively easy to use natural enemies in glasshouse environments than in field environments (Van Lenteren and Woets, 1988). Methods for controlling insect pests using natural enemies in glasshouses have been

developed for more than 40 years (Hirose, 2006) and quite a few natural enemies have been commercialized, such as *Aphidius colemani* (parasitoid)*, Aphidius ervi* (parasitoid)*, Aphelinus abdominalis* (parasitoid)*, Aphidoletes aphidimyza* (predator)*, Chrysoperla rufilabris* (predator)*,* and *Hippodamia convergens* (predator)*.* The application of natural enemies commercially is relatively infrequent in agricultural systems (Collier and Van Steenwyk, 2004). Collier and Van Steenwyk reviewed 31 worldwide records, including unsuccessful cases, of using natural enemies in an open field situation. However, all the fours cases of aphids they reviewed were unsuccessful. Six criteria for the evaluation of natural enemies to be used as success biological control agents need to be satisfied: (1) Internal synchronization with the host; (2) climate adaptation; (3) lack of negative effects against biological control; (4) existence of good culture methods; (5) high reproductive potential and killing rate; and (6) good searching efficiency (Van Lenteren, 1986; Van Lenteren and Woets, 1988; and Van Lenteren and Manzaroli, 1999). Therefore, the effect of applying natural enemies is sometimes only marginal.

1.7.3 Genetically modified (GM) plants

The system of isolating and manipulating single genes through recombinant DNA technology (Watson, 1987) and the method to insert a specific foreign gene into plants (Chilton, 1983) enabled scientists to generate transgenic plants. In the past 20 or more years, scientists have applied this technology to modify plants to combat insects. Transgenic plants expressing *Bacillus thuringiensis* endotoxin are, after herbicide resistant crops, the second most important commercially used GM crops (O'Callaghan, 2005). Other genes used include protease inhibitor (*PI*) genes, the *Mi* gene, and lectin genes.

1.7.3.i Bacillus thuringiensis (Bt) genes

When talking about the use of genetically modified plants that are resistant to insect pests, *Bt* must be mentioned. *Bt* was first named by Ernst Berliner in 1915 (http://www.bt.ucsd.edu/bt_history.html), but it was not until the 1950s that serious attempts were made to use it as a biological pesticide (Hall, 1964). *Bt* can produce the Cry protein in the form of the protoxin which is proteolytically activated upon ingestion (Hofte and Whiteley, 1989). Crystal (Cry) proteins can bind to specific sites in the midgut cells of susceptible insects, and ion-selective channels in the cell membrane (English and Slatin, 1992), breaking the cells' osmotic balance, therefore lysing the cells and killing the insects.

The *Bt* genes have been widely introduced into different plants, including cotton (Cousins *et al.*, 1991), maize (Hill *et al.*, 1995), rice (Nayak *et al.*, 1997), chickpea (Sanyal *et al.*, 2005), oil seed rape (King,1990). Currently, four *Bt* genes (*Cry1Ab, Cry1Ac, Cry2Ab, Cry9C*) are commercially used in maize and cotton for their proved safety and effectiveness (Shelton *et al.*, 2002). Some studies have observed that products of *Bt* transgenic plants may have potential toxicity to parasitoids or other non-target organisms (Losey *et al.*, 1999; Prutz *et al.*, 2004; Liu *et al.*, 2005). However, all these studies were under laboratory conditions, so they were "snap-shots" and "worst-case scenarios", which might lead to incorrect conclusions (Poppy, 2000). Another problem is that most of the species chosen for risk assessment were those easily maintained in laboratory conditions, which cannot give the whole picture of the conditions in which the *Bt*- transformed crops are grown (O'Callaghan *et al.*, 2005).

There is another toxin from *Bacillus* worth a mention, vegetative insecticidal protein (VIP) (Estruch *et al.* 1996, Schnepf *et al.* 1998). VIPs obtained their name because they are produced at the vegetative growth stage of *Bacillus*. Unlike Cry proteins, VIPs are secreted as soluble proteins rather than as crystals. A member of the VIPs, namely VIP3A from *B. thuringiensis* exhibits very broad spectrum activities against lepidopterans and exerts its toxic effect on insect midgut epithelial cells in a manner that is histopathologically similar to that of Cry proteins (Yu *et al.*, 1997)

1.7.3.ii Protease inhibitor genes

Some insects use digestive proteases to obtain essential amino acids they need for their growth, development, and reproduction (Murdock & Shade, 2002). Lipke *et al.* (1954) first found that a preparation of protease inhibitor from soybean could inhibit the digestive proteolytic activity from the red flour beetle. This report gave the evidence that a plant protease inhibitor could inhibit insect proteases and constrain their development. Afterwards, different protease inhibitor genes were isolated and transformed into plants (Hilder *et al.*, 1987; Hao and Ao, 1997; Delledonne *et al.*, 2001).

Tobacco transformed with a cowpea trypsin inhibitor (*CPTI*) gene is the first successful demonstration of transgenic plants conferring resistance to insects (Hilder *et al.*, 1987). Tobacco (*Nicotiana tabacum*) plants expressing high levels of CPTI in their leaves (2.5-9.6μg of *CPTI*/mg of soluble leaf protein) caused increased mortality (up to 50%) of tobacco budworm larvae (*Heliothis virescens*) feeding on the plants and stunted the growth of the surviving larvae as well (Murdock and Shade, 2002).

However, the usefulness of transgenic plants expressing protease inhibitors can be very marginal. Larvae of *Helicoverpa armigera* grew normally on transgenic tobacco expressing Kunitz trypsin inhibitor (SBTI), despite the accumulation of the inhibitor in the plant leaves and despite its ability to inhibit *H. armigera* gut proteolytic activity (Nandi *et al.*, 1999). Moreover, transgenic potato expressing *CPTI* reduced the growth of the tomato moth larvae by 45%, but there was no reduction in leaf damage (Gatehouse *et al.*, 1997), probably because the insects compensated for inadequate protein digestion by consuming increased amounts of leaf tissue. Besides, insect midgut contains an estimated 1020 different proteases (Bown *et al.* 1997) which are differentially regulated and all cannot be inhibited by plant's PIs (Broadway, 1997). Insects are also able to minimize the impact of protease inhibitors by altering their protease complement, or by partial or complete degradation of the inhibitor proteins (Bolter and Jongsma, 1995; Girard *et al.*, 1998; 1998a; Giri *et al.*, 1998).

1.7.3.iii The Mi gene from tomato

Smith (1944) introduced resistance to root knot nematode (*Meloidogyne* spp.) into tomato by an embryo culture method, from a wild tomato species *L. peruvianum*. This kind of resistance is mediated by a single, dominant gene called *Mi*, which was isolated in 1998 by positional cloning (Kaloshian *et al.*, 1998; Milligan *et al.*, 1998). Rossi *et al.* (1998) found that the *Mi* gene could also be expressed in leaves where it confers resistance to potato aphid, *Macrosiphum euphorbiae*. Unfortunately, the mechanism of resistance mediated by the *Mi* gene is not known. Kaloshian and colleagues (1998) showed that *Mi*-mediated aphid resistance is regulated by the development stage of plants. A similar report was also published on the resistance to whitefly (Martinez de Ilarduya *et al.*, 2004), and another study pointed out that *Mi*-mediated aphid resistance develops with plant age but is not correlated with the *Mi*-transcript levels (Goggin *et al.*, 2004). The resistance against insects of different plants transformed with the same *Mi* genes also varies. For example, the potato cyst nematode resistance gene *Hero*, bears strong similarity to *Mi-1*.2, conferred resistance when transferred to susceptible cultivated tomato, but it failed to confer nematode resistance in potato (Sobczak *et al.*, 2005).

1.7.3.iv Lectin genes

Plant lectins are proteins with at least one noncatalytic domain that binds reversibly to specific mono- or oligosaccharides (Van Damme *et al.*, 1998). Lectins have been studied for more than 100 years and they have the longest scientific history of all plant proteins (Van Damme *et al.*, 1998). The first lectin was described and isolated by Peter Hermann Stillmark in 1888 in the University of Dorpat (now Tartu, Estonia) (reviewed by Franz, 1988). Elfstrand (1898) first introduced the term "Blutkörperchenagglutinin" (hemagglutinin) as a common name for all plant proteins that cause clumping of cells. In the 1940s, Boyd and Karl O. Renkonen found that a crude extract from lima bean, *Phaseolus limensis*, and tufted vetch, *Vicia cracca* showed agglutinating specificity for different blood types. Consequently, Boyd and Shapleigh (1954) decided to name them *lectins*, from the Latin, *legere*, to pick up or choose. It is obvious that the term "lectin" was introduced to highlight the selective agglutination behavior of hemagglutinins, but, ironically, it was later applied to all the proteins that have agglutinating activity and has become a synonym of agglutinin and hemagglutinin (Van Damme *et al.*, 1998). Nowadays, lectin is the name most commonly used.

Lectins are abundant in plants. Typical legume lectins usually account for 1 to 10% of the total soluble seed protein (Van Damme *et al.*, 1998). In the bark of several legume trees, the content of lectins can reach 20 to 50% of the total soluble protein (Nsimba Lubaki and Peumans, 1986). A mannose-binding lectin of about 13 kDa makes up 75% of the protein in the nectar of leek (*Allium porrum*) flowers (Peumans *et al.*, 1997). So far, detailed sequence information for more than 100 plant lectin genes is available. These lectins can be divided into seven families based on their sequence and origin, namely, the legume lectins, the monocot mannose-binding lectins, the chitin-binding lectins, the type 2 ribosome inactivation proteins (RIP), the jacalin-related lectins, the amaranthin lectins, and the *Curcurbitaceae* phloem lectins (Van Damme *et al.*, 1998). Scientists used to regard lectins only as storage proteins for plant growth and development. Recent studies indicated that they also help to defend plants against herbivores and pathogens. With the exception of the *Curcurbitaceae* phloem lectins, all the other six lectin families have been associated with defence against insects. There is a report describing the insecticidal effect of jacalin towards potato leafhopper (Habibi *et al.*, 1993). However, Van Damme *et al.* (1998) suggested that jacalin-related lectins, the type 2 RIPs, and amaranthin lectins may only play the role of protecting seeds from seed predators. Similar to jacalin-related lectins, all the studies with the more famous chitin-binding lectins in artificial diets only revealed that they are toxic to chewing insects (Murdock *et al.*, 1990; Huesing *et al.*, 1991; Czapla and

Lang, 1990; Harper *et al.*, 1998). Most of the studies reporting the anti-aphid properties of lectins actually concentrate on the remaining two lectin families, the legume lectins and the monocot mannose binding lectins. Therefore, these two families are discussed in detail.

Legume lectins These constitute a specific category of lectins based not only on their source but also on other characteristics. Thus, all the "legume lectins" are exclusively isolated from *Leguminoseae*, but not all the lectins isolated from legume species are in the category of legume lectins. All legume lectins are built up of subunits of about 30 kDa and the unique feature of legume lectins is the presence of divalent cations at specific metal-binding sites, which are essential for the carbohydrate-binding activity (Van Damme *et al.*, 1998). The legume lectins are synthesized on the endoplasmic reticulum (ER) as preproteins and subsequently enter the secretory pathway. After the removal of the signal peptide, the polypeptide is transported into storage protein vacuoles for further processing of the backbone and N-linked glycan chains (Van Driessche, 1988). One thing worth mentioning is that most of the pioneering work in the field of biochemistry, physiology, and molecular biology of plant lectins has been done with legume lectins. For example, Concanavalin A (Con A) from Jack bean was the first plant lectin to be purified and crystallized (Summer and Howell, 1936). The same lectin was the first lectin whose primary structure and three-dimensional structure was resolved (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). The first plant lectin gene was isolated from soybean seeds (Vodkin *et al.*, 1983). In recent years, the anti-insect property of Con A was studied using both artificial diets and transgenic plants (Rahbe and Febvay, 1993; Rahbe *et al.*, 1995; Gatehouse *et al*, 1999; Sauvion *et al.*, 2004) and showed some inspiring results. But the toxicity of Con A to higher mammals may limit the use of this potential aphid-resistance lectin.

Interestingly, another group of potential insect-resistant proteins, the α-amylase inhibitors, with an anti-nutritional property, are legume lectin-related proteins. The α-amylase inhibitors share high sequence similarity to the polypeptide of phytohemagglutinin from common bean, but molecular modeling showed that they lack two loops important for the formation of the sugar-binding site of legume lectins (Rougė *et al.*, 1993). Although lacking the sugar-binding ability, the α-amylase inhibitors that widely occur in cereals (Abe *et al.*, 1993; Feng *et al.*, 1996; Y; Franco *et al.*, 2000) and legumes (Marshall and Laude, 1975; Ishimoto, 1996) can inhibit the activity of α-amylase and impair the nutrition of the insects. The α-amylase catalyzes the initial hydrolysis of α-1,4-linked sugar polymers, such as starch and glycan, into shorter oligosaccharides, an important step

towards transforming sugar polymers into single units that can be assimilated by insects. Therefore, α-amylase is crucial for many insects that feed exclusively on seed products during larval and/or adult life (Carlini and Grossi-de-Sā, 2002).

Monocot mannose-binding lectins Monocot mannose-binding lectins are structurally and evolutionarily unrelated to the mannose/glucose specific legume lectins (Van Damme *et al*, 1998). They are exclusively found in six different monocot families, Alliaceae, Amaryllidaeae, Araceae, Bromeliacaea, Liliaceae, and Ochidacaea and exclusively bind to mannose. Like legume lectins, the monocot mannose-binding lectins are also synthesized on the ER as preproteins (Van Damme and Peumans, 1988). After cotranslational removal of the signal peptide, the proprotein undergoes proteolytic modification during or after its transportation from the ER to their final destination, probably the vacuole or a vacuole-derived organelle (Van Damme *et al*., 1998). A typical mannose-binding motif is composed of Gln, Asp, Asn, Val, and Tyr. In *Galanthus nivalis* agglutinin (GNA), each subunit contains three of this kind of motif. When binding a single mannose, the binding of Asp and Asn with O2, Gln with O3, and Tyr with O4 form a network of four hydrogen bonds (Figure 1.3). The hydrophobic residue Val interacts with C3 and C4 of mannose through hydrophobic interactions (Hester *et al*., 1995). However, the carbohydrate-binding sites of mannose-binding lectins preferentially accommodate oligomannosyl residues rather than single mannose molecules. The binding of oligomannosides involves the assistance of a neighbouring motif in the same or different subunit (Hester and Wright, 1996). The preferential binding of oligomannosides reinforces the conclusion that monocot mannose-binding lectins play a defensive role in plants, because these oligosaccharide structures are typical constituents of insect glycoproteins. Moreover, the fact that some of these lectins accumulate in the phloem provides further support to the defensive role against sap-sucking insects. For example, the most abundant protein in the phloem exudate of flowering stalks of leek is a typical monocot mannose-binding lectin (Peumans *et al*., 1997). Based on this assumption, great efforts have been made on cloning new monocot mannose-binding lectin genes, incorporating lectin in artificial diets, and expressing lectin ectopically to study the anti-aphid property of this family of lectins. So far, a few lectins, such as GNA and *Allium sativum* leaf agglutinin (ASAL) have been commonly regarded as potential anti-aphid toxins.

Various hypotheses of how lectins play their anti-insect roles have been proposed. The well known ones are, binding of carbohydrate moieties associated with the

membrane of chemosensory sensilla (Murdock and Shade, 2002), binding of digestive enzymes (Peumans and Van Damme, 1995a and 1995b), disturbing the peritrophic matrix (PM) (Harper *et al*., 1998), and the binding of the receptors on the luminal layers of the gut section (Bandyopadhyay *et al*., 2001). Insects bear many sensilla on antennae, tarsi, tips of feet, and mouthparts, some of which are used in food recognition. Lectins may bind to the membranes of the sensillae and block food chemical signals from reaching their actual receptor proteins (Reviewed by Sharon and Lis, 2004). When European corn borer larvae were fed on wheat germ agglutinin, the single-layer inerratic peritrophic matrix (PM) in the midgut was observed to become a mass of convoluted form, giving rise to bacterial penetration (Harper *et al*., 1998). Powell's immunohistochemical and developmental studies as well as ultrastructural studies demonstrated that binding of GNA to the midgut epithelial cells of brown plant-hoppers could cause disruption of microvilli and abnormalities in these epithelial cells (Powell *et al*., 1998). Con A has been proved to induce the swelling of epithelial cells of the midgut of pea aphid (Sauvion *et al*., 2004). Recent ligand blot analyses have also identified receptors in various sap-sucking insects (Majumder *et al*., 2004; Dutta *et al*., 2005).
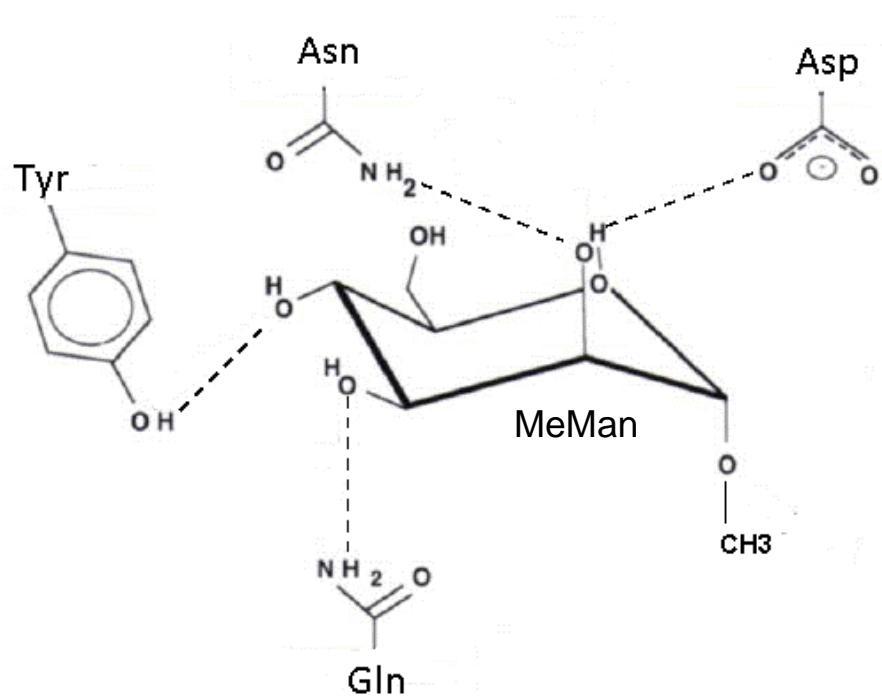
Figure 1.3 Structure of a typical mannose-binding site showing the network of four hydrogen bonds (dotted lines) connecting a methyl-α-mannoside residue (MeMan) (Reproduced from Van Damme *et al*., 1998)

1.8 Aims and objectives

1.8.1 Aims

The aim of this study can be summarized as identifying a candidate gene that confers resistance to *Myzus persicae*. *M. persicae* is an important pest in agriculture and horticulture because of its wide host range, rapid population growth, and ability to transmit viral disease. The traditional and existing measures are not ideal for controlling *M. persicae*. Genetic modification is a potential solution to controlling *M. persicae*. The monocot mannose-binding lectins are currently the most promising insecticidal agents for transgenic use against sap-sucking pests. Therefore, this project will be focused on identifying a novel lectin gene and assessing its anti-aphid-resistance properties.

1.8.2 Objectives

(1) To identify a novel lectin gene from an appropriate plant species;

(2) To predict the function of the novel lectin gene by analyzing the sequence;

(3) To construct an expression vector for transforming the novel lectin gene into Arabidopsis;

(4) To isolate at least six homozygous lines containing the novel lectin;

(5) To evaluate the unintended effects of expressing the novel lectin gene on Arabidopsis;

(6) To assess the insecticidal effects of the novel lectin gene in a *Myzus persicae*/Arabidopsis model system.

1.9 Chapter Summary

To fulfill the aims and objectives of this project, the work was divided into three parts and thus the thesis is divided into five chapters.

Chapter One – rationale behind the project giving an overview of the importance of *Myzus persicae* and the potential measures to control this pest.

Chapter Two – the isolation and sequence determination of the novel lectin gene *Allium triquetrum* agglutinin (*ATA*) gene from *Allium triquetrum*; prediction of the function of ATA based on analysis of the *ATA* sequence; designing and constructing the expression vector.

Chapter Three – Segregation patterns of the six ATA transformed Arabidopsis lines; using PCR, RT PCR, and western blot analysis to assess the status of *ATA* in Arabidopsis; experimental data gained from the analysis to characterize the growth parameters of the transgenic plants.

Chapter Four – Aphid bioassays illustrating the effects of ATA on the behaviour, development, fecundity, and survival of *M. persicae*.

Chapter Five – General discussion and conclusions of the implications of the study as a whole and the potential of ATA and other monocot mannose-binding lectins as anti-aphid proteins.

Chapter Two

2 Cloning of a lectin gene from *Allium triquetrum* and construction of an expression vector

2.1 Introduction

Myzus persicae is a world-wide pest which feeds on plant phloem and transmits many plant viral diseases as well. This is an important issue as up until now, there has been no effective chemical against viral diseases. *M. persicae* can reproduce asexually, (parthenogenesis), and therefore the population expands rapidly. The excreted honeydew causes further problems by promoting the growth of sooty mould, which also reduces the crop quality.

Agronomic crops transformed with the *Bacillus thuringiensis* (*Bt*) gene are the most successful commercialized transgenic crops with anti-insect traits. However, Bt is not useful in controlling homopteran pests. In recent years, a group of plant proteins, namely the monocot mannose-binding lectins, have been associated with resistance to sap-sucking insects. *Galanthus nivalis* agglutinin (GNA) was the first isolated monocot mannose-binding lectin (Van Damme *et al*., 1987). The study with GNA and some other plant lectins in artificial diet experiments indicated that these lectins were toxic to some sap-feeding pests (Powell *et al*., 1995). GNA has been transformed into tobacco (Hilder *et al*., 1995), potato (Gatehouse *et al*., 1997), wheat (Stoger *et al*., 1999), and rice (Rao *et al*., 1998) and has proved to be effective in controlling sap-feeding pests. In 1995, the three dimensional structure revealed by X-ray crystallographic analysis was published (Hester *et al*., 1995). The analysis demonstrated that GNA is a tetramer of identical subunits forming a central 16 Å wide solvent channel. Each subunit can potentially accommodate three mannose residues (12 per tetramer). The high affinity binding depends on auxiliary contacts with neighbouring molecules of GNA; therefore the tetramer structure may explain the relatively strong biological activity of GNA. Some other lectin genes also have been cloned from monocot plants and transformed into various plants. The *Amaranthus caudatus* agglutinin (*ACA*) gene (Guo *et al*., 2004), the *Zephyranthes candida* agglutinin (*ZCA*) gene (Pang *et al*., 2004), and the *Pinellia ternata* agglutinin (*PTA*) gene (Yao *et al.*, 2003) were cloned and transformed into tobacco and led to different levels of aphid resistance. Recently, *Allium sativum* leaf agglutinin (*ASAL*) (Smeets *et al*., 1997) has been

expressed in Indian mustard (Saha *et al.*, 2006) and tobacco (Sadeghi *et al.*, 2007) providing resistance to mustard aphid and cotton leafworm, repectively. All the monocot mannose-binding lectin genes that have been cloned so far are exclusively from six plant families, Alliaceae, Amaryllidaceae , Araceae, Bromeliaceae, Liliaceae, and Ochidacaea.

   Bt-transformed crops are not only successful cases of commercialized novel plant biotechniques, but also provide valuable experience for future transgenic research. Since the application of Bt, insects have evolved different levels of resistance (Tabashnik, 1994; Perez and Shelton, 1997; Ferre and Van Rie, 2002). Thus, different *Bt* genes have been isolated to cope with the reduced sensitivity of the insects to Bt. If lectin genes are to be commercialized, more candidate genes will provide not only wider choices but also more chances to reduce the risk of insect resistance.

   The occurrence of monocot mannose-binding lectins is best documented for Amaryllidaceae, Alliacae, and Orchidacae. It is very likely that most species of these three families contain lectins (Van Damme *et al.*, 1998). *Allium triquetrum*, Alliaceae, also known as three-cornered leek or wild garlic, is a British native specimen. Its height is up to 45 cm and it is in leaf from February to July, flowering from April to June. The scented white flowers are hermaphrodite and are pollinated by insects. Interestingly, few pests have been observed feeding on *A. triquetrum*; therefore, *A. triquetrum* is a potential source of useful lectin genes.

   Scientists have developed different strategies for cloning plant genes in the past decades. (1) Functional cloning is based on information about the function of a known protein that is responsible for a disease or phenotype. It usually requires the purification of the protein of interest, which is time consuming, and therefore, this approach has limited application. (2) Another well known method is positional cloning, which aims to localize determinants of a phenotype in the DNA sequence prior to determining their actual functions by linkage analysis (Monaco, 1994). This method also takes great effort in crossing parental plants and waiting for offspring. (3) Feldmann and Marks (1987) established a novel seed transformation method to obtain large numbers of T-DNA transformed Arabidopsis lines to identify important genes. T-DNA can act as a mutagen when it integrates into the plant genome; therefore the T-DNA can be used as a tag to label the corresponding gene(s) of a phenotype. (4) During the 1980s, Fedoroff and colleagues (1983) cloned the Maize *Ac* (*Activator*) and *Ds* (*Dissociator*) transposable elements, which led to the establishment of transposon-tagging cloning methods. Transposons which translocate in the plant genome, can also serve as mutagens and tags for cloning. These two methods are mainly applied in cloning important genes with easily identifiable

phenotypes. (5) Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from an mRNA template between a defined internal site and sequences at either the 3' or the 5' -end of the mRNA (Frohman *et al*., 1988). It can be used to clone plant genes, but it requires some known sequence in the genes of interest. A conserved domain appears in the known lectin genes, and considering the simplicity and efficiency of RACE, it was decided to use this technique to clone a lectin gene from *A. triquetrum*.

For this project, the pGreen 0029 plasmid was selected as the backbone of the binary vector (Hellens *et al*., 2000). The pGreen system has several advantages such as a relatively small size for a binary vector and a fairly high copy number, which greatly facilitates the cloning process. The 35S CaMV promoter was selected to drive the expression of *ATA* because it is a strong promoter and drives constitutive expression in the whole plant.

2.2 Material and methods

2.2.1 Plant material

Allium triquetrum (wild garlic, three-cornered leek) is a commercially available horticultural specimen. The plants were harvested from a local garden (Southampton, UK).

Arabidopsis Columbia-0 (referred to as Arabidopsis in the following text) was grown on sterilized compost consisting of equal volume of Levington F2S growth media, John Innes No2 Compost, and Vermiculite. 4-8 seeds were sown in each 4 inch square pot. The pots were covered with cling film to keep the humidity high and moved to 4°C to break the dormancy of the seeds. After 4 days, the pots were transferred to climate controlled rooms (20±2°C, L16: D8). After the second pair of true leaves emerged, each healthy seedling was transferred to a new pot.

2.2.2 RNA isolation from plant material

Total RNA was extracted using a Qiagen RNeasy Plant Kit (Qiagen, Germany) according to the manufacturer's instructions. Essentially, freshly harvested plant tissue was ground in liquid nitrogen with a pestle and mortar and approximately 100mg of the sample was used. The powered sample was immediately resuspended in a lysis buffer RLT or RLC. In most cases, RLT is used due to the greater cell disruption and denaturation properties of guanidine isothiocyanate (GITC), which is the difference between RLT and RLC. However, depending on the amount and type of secondary metabolites in some tissues, such as milky endosperm of maize, GITC can cause solidification of the sample, making extraction of RNA impossible. Therefore, buffer RLC can be used in these cases. The RNA is then bound to a silica gel membrane by passing the lysate through a column. Contaminants are washed away with various solutions and the RNA is eluted in water.

2.2.3 Elimination of genomic DNA from RNA preparations

Elimination of genomic DNA from RNA was performed with rDNase I (Ambion, CA, USA). The treatment was carried out under the manufacturer's instruction with slight modifications. Up to 10μg RNA was used in a 20μl reaction containing 2μl 10×DNase buffer, 1ul (2U) rDNase. The reaction mixture was incubated at 37°C for 20-30 min. 2μl DNase inactivation reagent was added to the reaction mixture and incubated at room

temperature for 2 min. The microfuge tube was centrifuged at 10,000×*g* for 1.5 min and the RNA was transferred to a fresh microfuge tube.

2.2.4 Agarose gel electrophoresis of nucleic acids

Agarose gels were prepared and electrophoresis carried out according to Sambrook *et al*., (1989). 0.8-1.2% (w/v) agarose (Duchefa, Suffolk, UK) was dissolved in 1× TAE buffer by bringing to the boil in a microwave oven. After the gel was cooled to below 50ºC, ethidium bromide was added to 1µg/ml. The gel was poured into a gel mould, into which the well comb was placed and left to set at room temperature.

DNA samples were prepared in 1× orange G loading dye before being loaded into wells of the gel. PCR products produced with Bioline Biomix Red (containing loading dye) were directly loaded on gels.

RNA samples were prepared in 1× RNase free Orange G loading dye. The flask, electrophoresis tank, gel mould, and well comb were all pretreated by RNaseZap (Ambion, Austin, USA) to remove RNase.

Electrophoresis was carried out in 1x TAE and run at 10-15 Vcm^{-1} until the desired amount of separation of the DNA fragments was achieved.

The nucleic acid was visualised using an Alpha Imager^{TM} 1220 (Alpha Innotech, supplied by GRI, Braintree, UK), the size of the fragments were estimated by comparison with a 1kb DNA ladder (Gibco BRL, Cheshire, UK).

2.2.5 The Polymerase Chain Reaction (PCR)

Either high fidelity PCR kit (Promega, Southampton, UK) or Biomix (Bioline, London, UK) was used for each reaction. Final concentrations of template used were: 10-20ng genomic DNA/1-5ng plasmid DNA/1µl of a 5ml culture grown for 1.5-2 hr at 37°C (*Escherichia coli*) or 4-6 hr at 28°C (*Agrobacterium*). Biomix method: 1× Biomix (contains DNA polymerase, buffer, dNTPs), 500nM of each primer. The general PCR program was preceded by a 2-min denaturation step (94°C). Subsequently each cycle has a 15-30 sec denaturiation period (94°C), followed by a 15-30 sec annealing period. The annealing temperature was estimated by the general Equation 2.1.

Tm = 2(Σ As and Ts in primer) + 4(Σ Cs and Gs in primer)
Annealing temperature = Tm – (5~10°C)   (Equation 2.1)

This was refined through trial and error. The annealing step was followed by an extension phase (1 min per Kb of DNA to be amplified, 72°C). This cycle was repeated 30-40 times. The final cycle was followed by a five-minute extension period (72°C) and a 12°C hold.

2.2.6 The 3' Rapid Amplification of cDNA Ends (RACE) of the Allium triquetrum Agglutinin (ATA) Gene

Initially, a solution containing 5μg of total RNA was made up to 10μl with RNase-free water. The RNA was heated at 72°C to denature for 5 min, rapidly transferred to ice, and cooled for 2 min. Afterwards, the mRNA was reverse transcribed with Invitrogen Reverse transcription (RT) kit (Invitrogen, Paisley, UK). The RT reaction was carried out in a total volume of 20μl containing: 10μl RNA, 4μl 5× RT buffer, 2μl 0.1 M DTT (Dithiothreitol), 2μl 5mM dNTP, 1μl RNase OUT, 1μl 500ng/ul CY AP primer. The reaction was warmed to 42°C prior to the addition of 1μl SuperScript II Reverse Transcriptase. The reaction was incubated at 42°C for 1 hr and then heat inactivated at 75°C for 10 min. To amplify specific cDNA, PCR was carried out in a total volume of 20μl with CY SPEC AG1 or CY DEG AG1 primer with the CY AUAP primer, (which was designed according to the adaptor tail). PCR was performed under the following conditions: 94°C for 2 min, (94 °C for 20 sec, 50°C for 20 sec, 72°C for 1 min)×35 cycles, 72°C for 5 min. The PCR products were gel purified and cloned into the pGEM-T Vector (Promega, Southampton, UK) in accordance with the manufacturer's instruction and transformed into *E. coli* DH5α for sequencing.

2.2.7 Isolation of DNA from agarose gels

DNA fragments (100bp to 10kb), separated by agarose gel electrophoresis, were excised under ultraviolet light with a razor blade. The DNA was purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Southampton, UK) in accordance with the manufacturer's instructions.

2.2.8 Transformation of Escherichia coli DH5α by heat shock

One 50μl vial of One Shot® competent *E. coli* DH5α cells (Invitrogen, Paisley, UK) was thawed on ice for each transformation. 1 to 5μl plasmid was mixed with the

competent cells by tapping gently. The vial was incubated on ice for 30 min. The vial was moved to a 42°C water bath and incubated for 30 to 45 sec. The vial was then quickly removed from the 42°C water bath and placed on ice. 250μl SOC medium (at room temperature) was added to the vial. The cells were allowed to recover by shaking at 37°C for 1 hr at 225 rpm. 20μl to 200μl of the transformation vial was spread on LB agar plates containing appropriate antibiotics. The plates were incubated at 37°C overnight and colonies were selected for further analysis.

2.2.9 Screening of transformed bacteria

200μl of liquid LB was inoculated with the bacteria from the colony that was able to grow on the LB agar plates containing appropriate antibiotics and incubated at 37°C (28°C for *Agrobacterium*) for 1.5-2 hr (4-6 hr for *Agrobacterium*) with agitation at 225 rpm. 2μl of the culture was than used for PCR screening. The positive colonies verified by PCR were selected for further analysis

2.2.10 Extraction of plasmid DNA from Escherichia coli

A single colony from a freshly streaked selective plate was picked to inoculate a starter culture in 5 ml LB medium containing the appropriate selective antibiotic. The culture was incubated for 16 hr at 37˚C with vigorous shaking (approx. 225 rpm). The bacterial cells were harvested by centrifugation at 6,000×*g* for 15 min. Plasmid DNA was extracted from the bacterial pellet with a Qiagen Mini plasmid kit (Qiagen, West Sussex, UK).

2.2.11 Purification of DNA with kit

DNA from PCR, endonuclease digestion, or similar must be cleaned before subsequent experiments. The DNA was purified using a microCLEAN kit (Microzone, West Sussex, UK) in accordance with the manufacturer's instructions.

2.2.12 Purification of DNA and RNA by ethanol salt precipitation

Ethanol salt precipitation was mainly applied to purify and concentrate RNA samples and sometimes for DNA purification. Small volumes of nucleic acid were made

up to 20μl with distilled water. 1μl (20μg/μl)of glycogen (not always necessary), 2.5 volumes of 100% ethanol and 1/10 volume of 3M sodium acetate pH 5.4 was added to one volume of nucleic acid solution. Samples were mixed by pipetting or tapping and incubated at -20ºC for at least 1 hr. The nucleic acid was pelleted by centrifugation at 13,000×g for 15 min. The supernatant was discarded and salts removed by washing with 70% (v/v) ethanol. The nucleic acid pellet was either air dried or dried in a heating block at 50ºC and resuspended in an appropriate volume of water or 1×TE buffer.

2.2.13 Sequencing

Sequencing analysis was performed on a CEQ 8800 sequencer (Beckman Coulter, Buckinghamshire, UK). Sequencing reactions were carried out in a total volume of 20μl containing 200~500 ng plasmid DNA, 2μl 1.6μM primer, 8.0μl Terminator Ready Reaction Mix. The thermal cycle program was (96ºC for 20 sec, 50ºC for 20 sec, 60ºC for 4 min) × 30 cycles. The product was then ethanol precipitated and resuspended in 10μl formamide for sequencing analysis.

2.2.14 The 5' Rapid Amplification of cDNA Ends with BD SMART RACE cDNA Amplification Kit

The 5' RACE was carried out using the BD SMART RACE kit (BD Biosciences, Oxford, UK) according to the manufacturer's instructions. The mRNA was first purified from total RNA with the Promega PolyAT tract mRNA Isolation System (Promega, Southampton, UK) according to the manufacturer's instructions before the mRNA was reverse transcribed to cDNA. 50ng mRNA was heated with 1μl 5'-CDS primer, and 1μl BD SMART II A oligo at 70ºC for 2 min, then cooled on ice for 2 min. Thereafter, 2μl 5× First-strand buffer, 1μl DTT (20mM), 1μl dNTP Mix (10 mM), and 1μl BD PowerScript Reverse Transcriptase were added to the previous mixture and incubated at 42ºC for 1.5 hr. Finally, the reaction was diluted with 20μl TE buffer. PCR was utilized to amplify specific cDNA, PCR products were gel purified and cloned into the pGEM-T Vector (Promega, Southampton, UK) in accordance with the manufacturer's instructions and transformed into *E. coli* DH5α for sequencing.

2.2.15 Detection and characterisation of full length cDNA

The sequences of the 3' end and the 5' end were aligned together to get the full-length cDNA sequence with the program Contig Express in Vector NTI 9.0 (Invitrogen, Paisley. UK). The homology analysis of the full length cDNA and the deduced amino acid sequence were performed with BlastN (nucleotide-nucleotide BLAST) and BlastP (protein-protein BLAST) on NCBI (www.ncbi.nlm.nih.gov).

2.2.16 Molecular evolution analysis of Allium triquetrum agglutinin

Phylogenetic analysis was performed by aligning sequences of deduced amino acid sequence of ATA and mannose binding lectins from other plant species belonging to the families Araceae, Iridaceae, Amaryllidaceae, Bromeliaceae, Liliaceae and Orchidaceae using the online service of ClustalW 2 (www.ebi.ac.uk/Tools/clustalw2/index.html). The phylogenetic tree was constructed by the neighbor-joining (NJ) method with Phylip (http://evolution.genetics.washington.edu/phylip.html) using default parameters.

2.2.17 Restriction Endonuclease Digestion

All digests with restriction endonucleases were carried out in the digestion buffer provided by the manufacturers. The buffers generally contain different concentrations of Tris-HCl, $MgCl_2$, NaCl, KCl, DTT. Digests were normally arranged such that the DNA was at a concentration of 0.2μg/μl and were incubated to give a 2-4 fold over digestion with respect to the specific activity of a particular restriction enzyme at 37˚C. When digesting with two different enzymes, the DNA was cut in a buffer which provides best percent activity for both enzymes. The digestion was heated at 65˚C for 15 min to inactivate the restriction endonucleases before further experiments.

2.2.18 Ligation of DNA fragments

Insert and vector DNA were prepared by restriction enzyme digestion, fractionation on an agarose gel and resuspension in 1×TE buffer. If the vector DNA possessed ends which were capable of self ligation then it was normally phosphatased (see 2.2.19), prior to gel purification. The amount of vector used for a given amount of insert was calculated with Equation 2.2:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of} \frac{\text{insert}}{\text{vector}} = \text{ng of insert} \qquad \text{(Equation 2.2)}$$

To keep DNA concentration as high as required, sometimes it was necessary to mix appropriate amounts of purified vector and insert and then ethanol precipitated. The ligation was carried out in a volume of 10μl containing precipitated DNA, 1μl (400 Units) T4 DNA ligase (New England Biolabs, MA, USA), 1μl 10×T4 DNA ligase buffer. The DNA was ligated at 16ºC overnight then transformed into *E. coli* DH5α for screening.

2.2.19 Phosphatase treatment of vector DNA

To prevent the self ligation of vector DNA, the terminal 5' phosphates can be removed using Antarctic Shrimp Phosphatase (ASP) (New England Biolabs, MA, USA). Up to 1μg vector DNA was restriction digested using normal procedures, then ethanol precipitated. The DNA pellet was recovered by centrifugation, and air dried. The reaction was performed in a 10μl volume containing 1 μl (5 units) of ASP, 1 μl 10× Reaction Buffer, and 8 μl water. As vector DNA was subsequently purified by fractionation on an agarose gel it was not necessary to inactivate or remove the phosphatase.

2.2.20 Preparation of electro-competent *Agrobacterium tumefaciens* GV3101

20ml LB medium in a 50ml screw-capped centrifuge tube was inoculated with *A. tumefaciens* GV3101 from a fresh (< 2 weeks) plate. The culture was incubated at 28ºC with shaking at 225 rpm until the culture had obviously grown, usually after 1-2 days. The culture was chilled on ice for 5 min and then centrifuged at 4ºC at 4000×*g* for 10 min. The supernatant was removed. The pellet was gently resuspended in 5ml ice cold 10% (v/v) sterilized glycerol to wash away any residual salts. The 10% (v/v) glycerol wash was

repeated for two more times. After the final wash, the bacterial pellet was resuspended in 200 to 400μl 10% (v/v) glycerol. The resuspension was stored at -70ºC in aliquots of 40μl.

2.2.21 Transformation of Agrobacterium tumefaciens GV3101

Up to 1μg plasmid DNA and 40μl competent cells were placed in an ice-cold 0.2 cm cuvette and electroporated with 1.25kv/0.1cm for about 5.0~5.5 ms in a Bio-Rad Gene pulser (Bio-Rad, Herts, UK). The cells were diluted into 0.5ml LB medium and incubated at 28°C with shaking for 4 hours before plating onto LB Agar containing appropriate antibiotics. Colony screening was carried out by PCR and putative transformants were confirmed by sequencing.

2.2.22 Plasmid extraction from Agrobacterium tumefaciens

The extraction was performed with a Qiagen Mini plasmid kit (Qiagen, West Sussex, UK) with modifications to the manufacturer's instruction. 5ml culture of *Agrobacterium* was fully grown in LB containing appropriate antibiotics. Cells were harvested by centrifugation at $4000 \times g$ for 10 min and resuspended in 150μl solution P1 (from the plasmid kit). The cell suspension was transferred to a 1.5ml tube and 300μl solution P2 was added. The mixture was incubated at room temperature for 10 min prior to the addition of 45μl alkaline-phenol (water saturated phenol: 0.2M NaOH = 1:2). After vigorous mixing, the solution became very viscous. 225μl solution P3 was added, mixed by inversion several times; placed at -20°C for 1 hr or more. After centrifugation at full speed for 3 min, the supernatant was transferred to a fresh tube, mixed with 500μl isopropanol by inversion and left on ice for 10 min. The tube was centrifuged at full speed for 10 min and the supernatant discarded. The pellet was washed with 70% (v/v) ethanol and air dried before resuspending in 20μl 1×TE.

2.3 Results

2.3.1 Choice Test between Allium triquetrum and Arabidopsis with Myzus persicae

A brief test was performed to see the choice of *M. persicae* between Arabidopsis and *A. triquetrum*. At day 0, one Chinese cabbage (*Brassica rapa* subsp. pekinensis) plant infested with *M. persicae* was placed between three healthy Arabidopsis plants and one pot of *A. triquetrum* plants (Figure 2.1). Figure 2.2 and 2.3 show the infestation status of the plants and the population of *M. persicae* on the plants after 14 and 21 days.

At 14 days, there were almost no aphids on the *A. triquetrum* plants (Figure 2.2). In contrast, numerous aphids were observed on the bolts of each Arabidopsis. Both *A. triquetrum* and Arabidopsis were still healthy at this stage. However, accumulation of anthocyanin (a purple colour) was observed on the stems of Arabidopsis, which is usually a sign of stress and senescence.

At 21 days, there were almost no aphids on the *A. triquetrum* plants (Figure 2.3). In contrast, although not many aphids were observed on Arabidopsis, the plants showed obvious senescence, such as yellow wilting on the leaf edge. However, the *A. triquetrum* was still healthy. All these observations suggest *M. persicae* do not feed on *A.triquetrum* under these conditions, which is possibly because of lectin(s) in the plant.

Figure 2.1 The infestation status and aphid population on Day 0. The infested Chinese cabbage was placed in the middle of three healthy Arabidopsis and one pot of *A. triquetrum* plants. All three plant species have contact with each other to ensure that the aphids can move freely between the plants.

Figure 2.2 The infestation status and aphid population on Day 14. *A. triquetrum* plants (top half). Arabidopsis plants (Bottom half).

Figure 2.3 The infestation status and aphid population on Day 21. *A. triquetrum* plants (top half). Arabidopsis plants (Bottom half).

2.3.2 The cloning of full length cDNA of Allium triquetrum agglutinin (ATA)

As a monocot plant, *A. triquetrum* has potential resistance to *M. persicae* (Figure 2.1, 2.2, and 2.3). Therefore, it is a potential source of novel lectin genes. For this reason, the technique called rapid amplification of cDNA ends (RACE) was attempted to clone a lectin gene with potential to confer resistance against aphids.

2.3.2.i RNA isolation

Garden grown-plants were collected and transported to the lab for further processing. Plants were washed and dissected before total RNA was extracted and analysed by gel electrophoresis (Figure 2.4). Different RNA extraction buffers (RLT and RLC) were used in accordance with manufacturer's instructions to compensate for different extraction efficiencies from starchy plant tissues. Figure 2.4 shows less RNA was yielded from root tissue probably because root tissue is harder to grind and root cells are harder to disrupt. Less RNA was yielded by using RLC than RLT, indicating that RLC is not necessary for RNA extraction from *A. triquetrum* root tissue. Guadinine isothiocyanate in RLT does not appear to interact with any secondary metabolites in *A. triquetrum* root tissue.
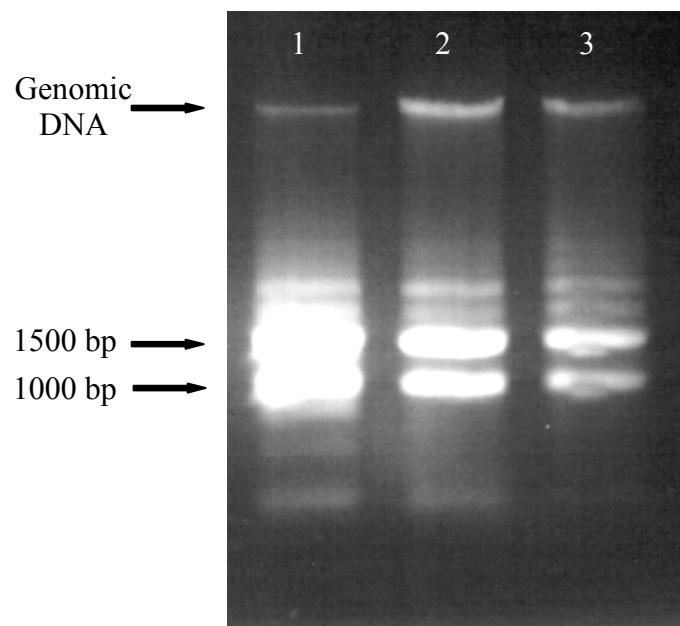
Figure 2.4 Electrophoresis of total RNA extracted from *Allium triquetrum.* Lane1: *A. triquetrum* leaf. Lane 2: *A. triquetrum* bulb/root extracted using RLT buffer. Lane 3: *A. triquetrum* bulb/root extracted using RLC buffer.

2.3.2.ii The 3' rapid amplification of cDNA ends (RACE) of lectin gene(s)

After RNA was isolated, the mRNA was reverse transcribed to cDNA with CY AP primer. The CY AP primer is essentially an oligo dT sequence linked to a specific adaptor tail at the 3' end to allow subsequent PCR amplification. The PCR of the 3' end segment of an agglutinin gene (Figure 2.5) was achieved with both the CY SPEC AG1 primer and the CY DEG AG1 primer. Both the CY SPEC AG1 and CY DEG AG1 primers were designed based on a conserved motif MNDDCNL, which is found in most reported lectins. Since some amino acids are encoded by several codons, the CY SPEC AG1 was designed according to just one set of codons while CY DEG AG1 was a mixture of all the sets of codons. The results showed that both primers are able to amplify distinct products. The CY SPEC AG1 primer produced stronger bands than CY DEG AG1 primer, which indicates that more products were amplified. This may due to a higher concentration of the single specific primer sequence as the degenerate primer consists of multiple primers, each at lower concentrations.

In Figure 2.5, the products in lane 1 and lane 2 look similar; however, there are two differences between lane 1 and lane 2. Firstly, there is a faint 350bp band in lane 1 which does not appear in lane 2. Secondly, the intensity ratio of the bands for each tissue type varied suggesting that genes identified are likely to be differentially expressed. The presence of two bands might suggest two different lectins in *A. triquetrum*. By comparing with the literature (Smeets *et al*., 1997), the 500bp fragment which appears to be expressed more in leaf was probably a leaf-specific lectin for its much higher expression in the leaf tissue, and therefore it was chosen for further study because leaf specific lectins usually confer properties of insect resistance. The 500bp PCR product of leaf cDNA was cloned into a pGem-T vector and transformed into *E. coli* DH5α. Twelve colonies of *A. triquetrum* leaf cDNA were selected for initial screening by PCR prior to sequencing analysis. A 478 bp fragment which had significant homology to several other published plant agglutinin genes was revealed by sequencing
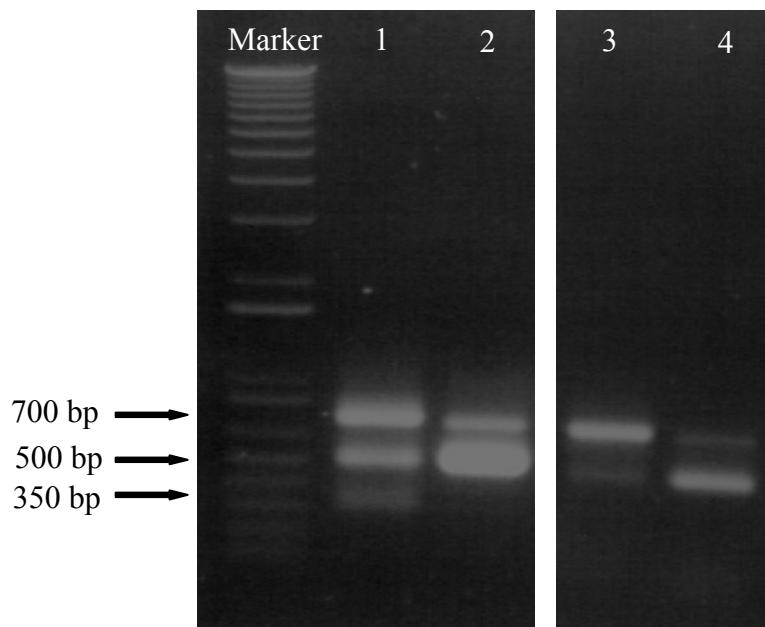
Figure 2.5 Electrophoresis of products of 3' RACE of the potential lectin gene(s) in *Allium triquetrum.* Lanes 1 and 2 were PCR carried out with the CY SPEC AG1 primer. Lane 1: *A. triquetrum* bulb/root cDNA; Lane 2: *A. triquetrum* leaf cDNA. Lanes 3 and 4 were PCR carried out with the CY DEG AG1 primer. Lane 3: *A. triquetrum* bulb/root cDNA; Lane 4: *A. triquetrum* leaf cDNA.
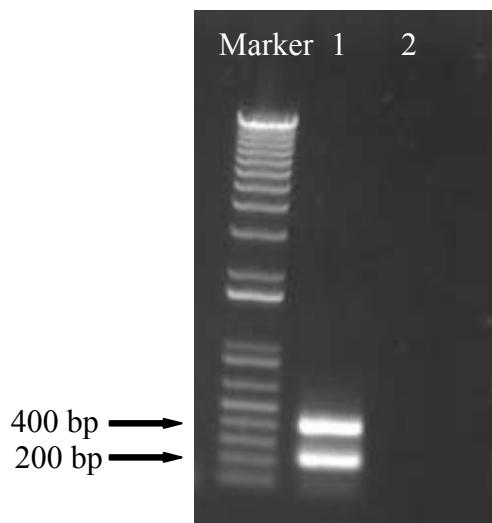
Figure 2.6 Electrophoresis of products of 5' RACE of the potential lectin gene(s) in *Allium triquetrum.* Lane 1: RACE products amplified with leaf cDNA; Lane 2: RACE products amplified with bulb/root cDNA.

2.3.2.iii The 5' rapid amplification of cDNA ends of the putative leaf-specific lectin gene

To identify the 5' cDNA corresponding to the 3' cDNA of the *A. triquetrum* cDNA already cloned, 5' RACE was employed and the results are shown in Figure 2.6. The 5' RACE amplified products of about 400 bp and about 200 bp from leaf cDNA but nothing from bulb/root cDNA. Both fragments from leaf cDNA were cloned into a pGem-T easy vector, and transformed into *E. coli* DH5α prior to screening by PCR. Plasmid DNA was extracted from positive colonies for sequencing analysis. The sequencing identified a 367 bp fragment with homology to lectins, and overlapping with the previous 3' cDNA.

2.3.2.iv Full length cDNA of the putative leaf-specific lectin gene

A clear homologous overlap between the 5' and 3' end fragment was revealed by an analysis with Vector NTI 9.0. The full sequence of the putative *A. triquetrum* agglutinin gene was obtained by assembling two segments together with Vector NTI 9.0. The cDNA contained a 522 bp open reading frame with three possible start codons at positions 1, 11, and 27 of the deduced amino acid sequence. Translation starting from the first ATG codon reveals a 174 amino acid sequence with a calculated molecular mass of 18369 Da (Figure 2.7).

2.3.3 Sequence analysis of the deduced amino acid sequence

Bioinformatics methods were applied to predict the class and function of the possible novel leaf-specific gene (Figure 2.7) before testing in transgenic Arabidopsis.

2.3.3.i Basic local alignment and search tool (BLAST)

BLAST searching detected a putative conserved domain in the deduced amino acid sequence and the BLAST program predicts the putative leaf-specific gene is a bulb-type mannose-specific lectin (B-lectin). The domain contains a three-fold internal repeat (beta-prism architecture). The consensus sequence motif QXDXNXVXY (Figure 2.8) is involved in α-D-mannose recognition. All the evidence suggests that the newly cloned gene is a new lectin gene. Consequently, it is named as the *Allium triquetrum* agglutinin gene (*ATA*).

A BlastP comparison was performed with the deduced amino acid sequence of ATA and other mannose-binding lectins from Alliaceae and *Galanthus nivalis* agglutinin (GNA) (Figure2.8).

The BlastP comparison reveals that ATA shares high sequence similarity with other lectins from the Alliaceae family as well as GNA. The percentage identity ranged from 73% to 81%, which reinforces the conclusion that ATA is a new lectin. The alignment of mature GNA with ATA supports the conclusion that the cleavage site of the N-terminal signal peptide predicted by SignalP 3.0 Server is correct (Figure 2.7). The result also reveals that ATA has a higher similarity to those leaf-specific lectins that are from the Alliaceae family than other lectins.

```
001 AAGCAGT

008 GGTACAACGCAGAGTACGCGGGACACAGAAACATACAATTGCTAAAAAAACAAAACA

065 ATGGCCTATTCAGTAACTTGTAAACTAATAATGGTATGCACAGTAGGCGCAATACTA

001  M   A   Y   S   V   T   C   K   L   I   M   V   C   T   V   G   A   I   L

122 AGCGTTCTAACGGCAACCTGCATGGGCAGAAACATACTTCTGAACGGAGAAGGGCTG

020  S   V   L   T   A   T   C   M   G   R   N   I   L   L   N   G   E   G   L

179 TACGCGGGCCAGTCATTGGAGGAAGGGCCCTACAGACTCGCAATGCAGGATGACTGC

039  Y   A   G   Q   S   L   E   E   G   P   Y   R   L   A   M   Q   D   D   C

236 AACCTCGTGCTCTATGATGAGTACAGCAGACCTGTTTGGGCCTCCAACACGGGCGTA

058  N   L   V   L   Y   D   E   Y   S   R   P   V   W   A   S   N   T   G   V

293 ACGGGCCGCAACGGGTGCAGGGCTGTGATGCAGGCCGATGGAAATTTCGTGGTTTAT

077  T   G   R   N   G   C   R   A   V   M   Q   A   D   G   N   F   V   V   Y

350 GACTCAAACAGCCGCGCTGTTTGGGCTAGCAACAGTAGAAAAGGTAACGGGAATTAC

096  D   S   N   S   R   A   V   W   A   S   N   S   R   K   G   N   G   N   Y

407 ATTTTGGTGCTCCAGAAAGATAGGAATGCTGTTATTTATGGAAGTGATATATGGTCC

115  I   L   V   L   Q   K   D   R   N   A   V   I   Y   G   S   D   I   W   S

464 ACGGGTACCTACAGAAGAGGTGTGGGTGGATCCGTTGTTACCGCCATGAACGGCACC

134  T   G   T   Y   R   R   G   V   G   G   S   V   V   T   A   M   N   G   T

521 GTTGATGCAGGCTTTGCTGTGAAGAATGTGACTACGGCTGCCGTGGGAGATGTTGCT

153  V   D   A   G   F   A   V   K   N   V   T   T   A   A   V   G   D   V   A

578 ATTGCTTGAATTTGGAAATGAGTTTTAATTTATGAATAAAGTGTGTATGAGGGTCTC

172  I   A   *

635 ATCATGTCTCTTCTGCATGTGTATGGTTTCCTTTATTTATCTTATGAATCTACCCTT

692 GTTTTCACTATCAAAAAAAAAAAAAAAA
```

Figure 2.7 The full-length cDNA and deduced amino acid sequence. Start and stop codons are underlined and the stop codon is *underlined italics*. The positions of the

conserved motifs are shown in **bold**. The position of the Allium tri R2 primer is shown in ***bold italics***. The position of the AATAAA box is framed. The arrowhead indicates a possible processing site for the cleavage of the signal peptide predicted with SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/).

```
AUAL    MAISVNCKIIMVCAVGTILSILTPTSMGRNILLNGEGLYAGQSLEEGSYK
ATA     MAYSVTCKLIMVCTVGAILSVLTATCMGRNILLNGEGLYAGQSLEEGPYR
AUAG1          MVCVVGTILS--T-TCMGRNILGNEEGLYADQSLEEGPYK
AUAG2   MAISVTCNIIMLCALGTILS--T-TCVGRNLLGNEEGLYADQSLEEGPYK
AUAG0          MVCAVGTILS--T-TCMGRNILGNEQALYADQSLDEGPYK
ASAL    MGRTTSSPKAMMRIATVAAIL-TIASTCMARNLLTNGEGLYAGQSLDVEQYK
GNA                              DNILYSGETLSTGEFLNYGSFV

AUAL    LIMQDDCNLVLF-EYSTQVWASNTGVSGRNGCRAVMQADGNFVVYD-SNS
ATA     LAMQDDCNLVLYDEYSRPVWASNTGVTGRNGCRAVMQADGNFVVYD-SNS
AUAG1   LIMQKDCNLVLF-EHSTVVWATKTGVTGRNGCRAVMQSDGNFVVHD-SNG
AUAG2   LIMQKDCNLVLF-EHSTVVWATKTGVTGRNGCKAVMQADGNFVVHD-SNG
AUAG0   LIMQKDCNLVLF-EHNTVRWATKTGSLGHYECYALMQSDGNFVVHN-RNG
ASAL    FIMQDDCNLVLY-EYSTPIWASNTGVTGKNGCRAVMQRDGNFVVYD-VNG
GNA     FIMQEDCNLVLYDVDK-PIWATNTGGLSRS-CFLSMQTDGNLVVYNPSNK

AUAL    RAVWASQSRRGNGNYIILALQEDRNVVIYGTDIWSTGTYRRGVGGTVVTV
ATA     RAVWASNSRKGNGNYILVLQKDRNAVIYGSDIWSTGTYRRGVGGSVVTA
AUAG1   RIVWASNSRKGNGNYILVLRENGNVVIYGSDIWSTGTHTRDVKGVIVTA
AUAG2   RIVWASNSIKGNGNYILVLRNNGNVVIYGSDIWSTGTHIRGVKGAIVTA
AUAG0   AIVWASNSRRGNGEYVLVLRENGNVVIYGSDIWSTGTHIKGVIVTAVAG
ASAL    RPVWASNSVRGNGNYILLLQEDRNVVIYGSDIWSTGTYRRSVGGAVVMA
GNA     PI-WASNTGGQNGNYVCILQKDRNVVIYGTDRWATGTHT-G

AUAL    INGTVDAGSGMENVTATAA
ATA     MNGTVDAGFAVKNVTTAAVGDVAIA
AUAG1   MNGTIDAGFGMKNSAAVAGKGGRGGEAASAA
AUAG2   MNGTINTGFRMDNSTTVVGIGGVGKVASAA
AUAG0   LGGGV
ASAL    MNGTVDGGSVIGPVVVNQNVTAAIRKVGSGAA
GNA
```

Figure 2.8 Multiple alignment of the amino acid sequence of ATA with those of mannose-binding lectins from Alliaceae. The alignment was performed with the online service of ClustalW2 by using the published mannose-binding lectin sequences from Alliaceae, AUAL (*Allium ursinum*), AUAG0 (*Allium ursinum*), AUAG1(*Allium ursinum*), AUAG2 (*Allium ursinum*), ASAL (*Allium sativum*) (Smeets *et al.,* 1997) and GNA (RCSB No#: 1MSA). Gaps are introduced for optimal alignment and maximum similarity between all compared sequences. The identical amino acids among all the aligned sequences are shown in red background and the identical amino acids with ATA are shown in blue background.

2.3.3.ii Molecular evolution analysis

The amino acid sequences of various monocot mannose-binding lectins were used to construct a consensus phylogenetic tree (Phylip 3.67) (Figure 2.9). The mannose-binding lectin sequences were downloaded from NCBI, and the references are listed in Table 2.1.

According to the phylogenetic tree shown in Figure 2.9, all the *Allium* lectins are in the same branch and grouped in three clusters. All the bulb lectins, AUAG0, AUAG1, and AUAG2 are grouped in the same cluster.The leaf-specific lectins, ATA and AUAL, are grouped in the same cluster. The leaf specific lectin ASAL is in a cluster alone suggesting it is relatively far from other lectins.

All the evidence so far supports the conclusion that ATA is a leaf-specific lectin, so the structure of an ATA subunit should be similar to that of GNA. Therefore, three subdomains of ATA can be predicted based on comparison with the reported subdomain of GNA (Hester *et al.*, 1995). Figure 2.10 shows the sizes of the subdomains are from 35 to 39 amino acids. Since the three mannose-binding motifs in ATA are identical and the three subdomains show high sequence similarity, it is of interest to look at the relationship between different subdomains. Figure 2.11 reveals that subdomains 1 and 3 are more closely related to each other than to subdomain 2.

Table 2.1 Lectins used for molecular evolution analysis

Lectin name	Source	Plant family	Reference
CAA	*Crinum asiaticum*	Amaryllidaceae	AAO59506
GNA	*Galanthus nivalis*	Amaryllidaceae	RCSB No#: 1MSA
HTA	*Hippeastrum sp. tetrameric*	Amaryllidaceae	AAA33362
ZGA	*Zephyranthes grandiflora*	Amaryllidaceae	AAP37975
ZCA	*Zephyranthes candida*	Amaryllidaceae	AAM94385
ASAL	*Allium sativum*	Alliaceae	ASU58947
AUAL	*Allium ursinum*	Alliaceae	AUU68531
AUAG0	*Allium ursinum*	Alliaceae	L14783
AUAG1	*Allium ursinum*	Alliaceae	L14783
AUAG2	*Allium ursinum*	Alliaceae	L14785
ATA	*Allium triquetrum*	Alliaceae	ABA00714
AKA	*Amorphophallus konjac*	Araceae	AAP22169
AHA	*Arisaema heterophyllum*	Araceae	AAP50524
ALA	*Arisaema lobatum*	Araceae	AAS60304
AMA	*Arum maculatum*	Araceae	AAC48997
CEA	*Colocasia esculenta*	Araceae	BAA03722
PPA	*Pinellia pedatisecta*	Araceae	AAR27793
PTA	*Pinellia ternata*	Araceae	AAO20876
TDA	*Typhonium divaricatum*	Araceae	AAQ55289
ACA	*Ananas comosus*	Bromeliaceae	AAM28277
CVA	*Crocus vernus*	Iridaceae	AAG10402
EHA	*Epipactis helleborine*	Orchidaceae	AAC48927
PMA	*Polygonatum multiflorum*	Ruscaceae	AAC49413
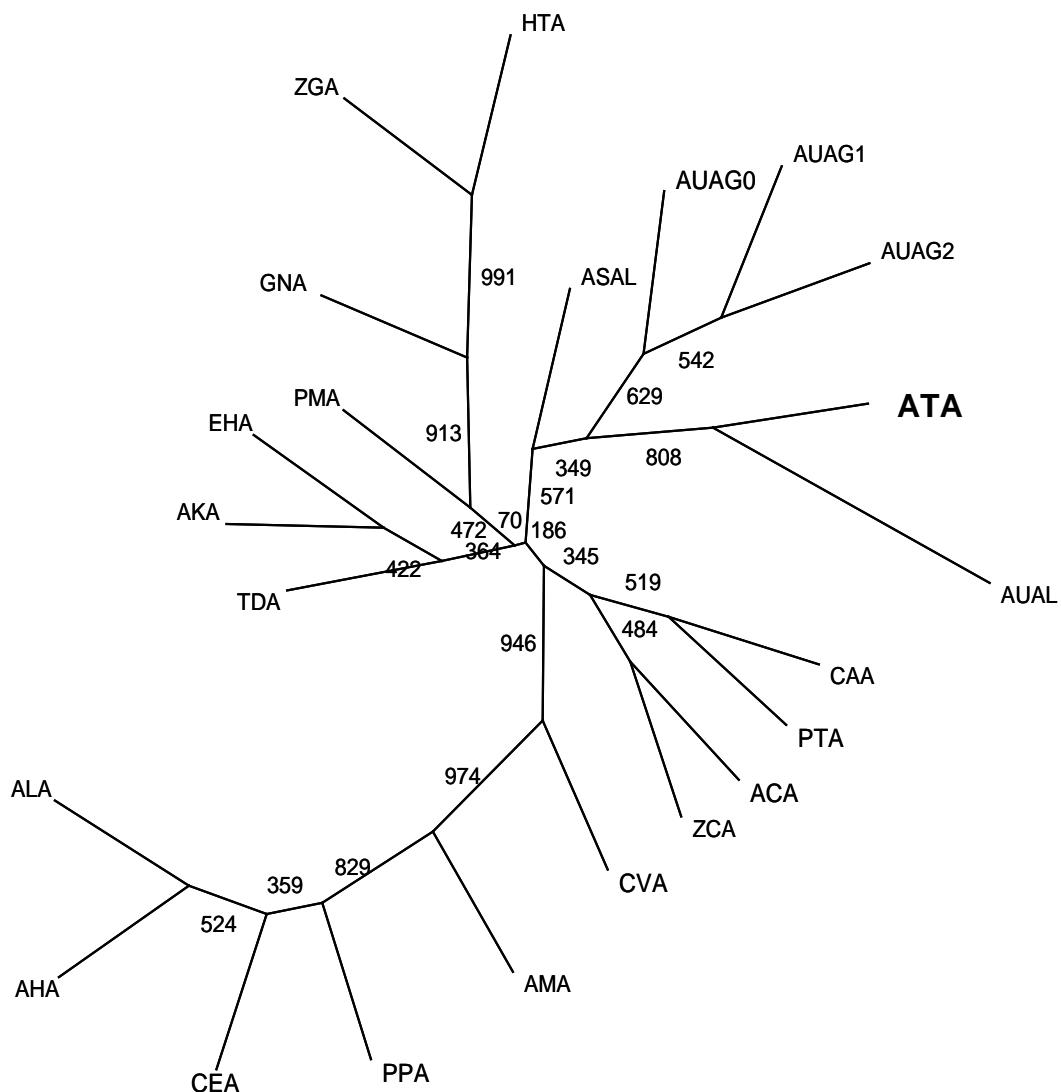
Figure 2.9 The consensus phylogenetic tree of mannose-binding lectins from the Araceae, Alliaceae, Irideceae, Amaryllidaceae, Bromeliaceae, and Orchidaceae families. The numbers on the branches indicate the number of times the partition of the species into the two sets which are separated by that branch occurred among the trees, out of 1000 trees. ATA is highlighted in **bold.**

Subdomain I

```
     29    34   112                               140
ATA  RNILLN     GNYILVLQKDRNAVIYGSDIWSTGTYRRG
GNA  DNILYS     GNYVCILQKDRNVVIYGTDRWATGTHT-G
     1     6    82                               109
```

Subdomain II

```
     35    40   79                                  111
ATA  GEGLYA     RNGCRAVMQADGNFVVYD-SNSRAVWASNSRKGN

GNA  GETLST     RS-CFLSMQTDGNLVVYNPSNKPI-WASNTGGQN
     7     12   50                                  81
```

Subdomain III

```
     41    46   47                                  78
ATA  GQSLEE     GPYRLAMQDDCNLVLYDEYSRPVWASNTGVTG

GNA  GEFLNY     GSFVFIMQEDCNLVLYD-VDKPIWATNTGGLS
     13    18   19                                  49
```

Figure 2.10 Prediction of the subdomains in ATA by aligning with GNA. Gaps are introduced for optimal alignment and maximum similarity between all compared sequences. The identical amino acids among all the aligned sequences are shown in red background.
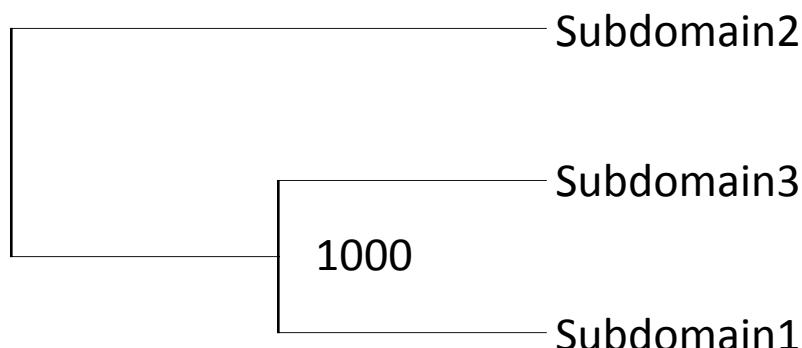
Figure 2.11 The consensus phylogenetic tree of the three subdomains in ATA. The numbers on the branches indicate the number of times the partition of the species into the two sets which are separated by that branch occurred among the trees, out of 1000 trees.

2.3.3.iii Structure prediction of Allium triquetrum agglutinin

The previous purified leaf specific lectin from Alliaceae have the molecular weight of about 12kDa. It is very likely that ATA has a similar molecular weight. However, the 522 bp open reading frame of *ATA* should encode a 173 amino acid sequence, which should be around 18 kDa. This means there was a 6 kDa difference between the predicted molecular weight from the open reading frame and the observed mature ATA. The molecular weight of the predicted N-terminal signal peptide is about 2.9 kDa, indicating additional cleavage is required. Comparison of the ATA sequence with that of *Galanthus nivalis* agglutinin (GNA) revealed that a C-terminal peptide is further cleaved during the post-translational modification of ATA. Because the X-ray structure of GNA has been resolved, it is possible to predict the 3-D structure of ATA. The alignment of the ATA precursor and the mature GNA revealed the amino acid sequence of the mature ATA (Figure 2.12). A prediction of secondary (Figure 2.12) and tertiary structure (Figure 2.13 and 2.14) of both GNA and ATA was performed with the online service provided by Swiss Model, Expasy.

In Figure 2.13, the predicted tertiary structure produced for GNA was very similar to the published X-ray diffraction structure. The only difference was that the online tool failed to predict β-strand 12 (β12). The same problem also occurred in the secondary structure prediction of ATA and GNA (Figure 2.12). This was possibly due to the limitations of the online tools. However, the overall prediction showed that the online tool was precise in predicting the tertiary structure of lectins.

In Figure 2.14, the online prediction tool again failed to predict β12 in ATA monomer, however, the sequence alignment of ATA and GNA (Figure 2.12) revealed that the existence of this β-strand was very likely. The overall predicted structure of ATA was very similar to the X-ray diffraction structure of GNA. The residue orientations and positions of three mannose-binding sites in both lectins are exactly the same, but there is one minor difference worth mentioning. The conformation of loop A in predicted ATA is concave while the counterpart in GNA is protruding. Since loop A' and B' in GNA are involved in the stabilization of quaternary structure (a tetramer in GNA), therefore the conformation difference may affect the quaternary structure of ATA.

```
ATA 001    MAYSVTCKLIMVCTVGAILSVLTATCMGRNILLNGEGLYAGQSLEEGPY
GNA 001                                DNILYSGETLSTGEFLNYGSF
                                        β1    β2      β3


ATA 050    RLAMQDDCNLVLYDEYSRPVWASNTGVTGRNGCRAVMQADGNFVVYD-S
GNA 022    VFIMQEDCNLVLYDVDK-PIWATNTGGLSRS-CFLSMQTDGNLVVYNPS
              β4           β5        β6            β7          β8


ATA 098    NSRAVWASNSRKGNGNYILVLQKDRNAVIYGSDIWSTGTYRRGVGGSVV
GNA 068    NKPI-WASNTGGQNGNYVCILQKDRNVVIYGTDRWATGTHT-G
               β9            β10         β11      β12
ATA 147    TAMNGTVDAGFAVKNVTTAAVGDVAIA
```

Figure 2.12 Alignment and secondary structure prediction of the ATA precursor and secondary structure of mature GNA from its X-ray structure. The letters in red indicate identical amino acids in the two sequences. Hyphens are gaps generated in the alignment. The letters in *italics* are the mannose-binding sites in GNA. The predicted β-strands in ATA precursor are highlighted in green background. The β-strands in mature GNA are highlighted in yellow background. The positions of the predicted and actual β-strands in ATA and GNA are described as β1 to β12.
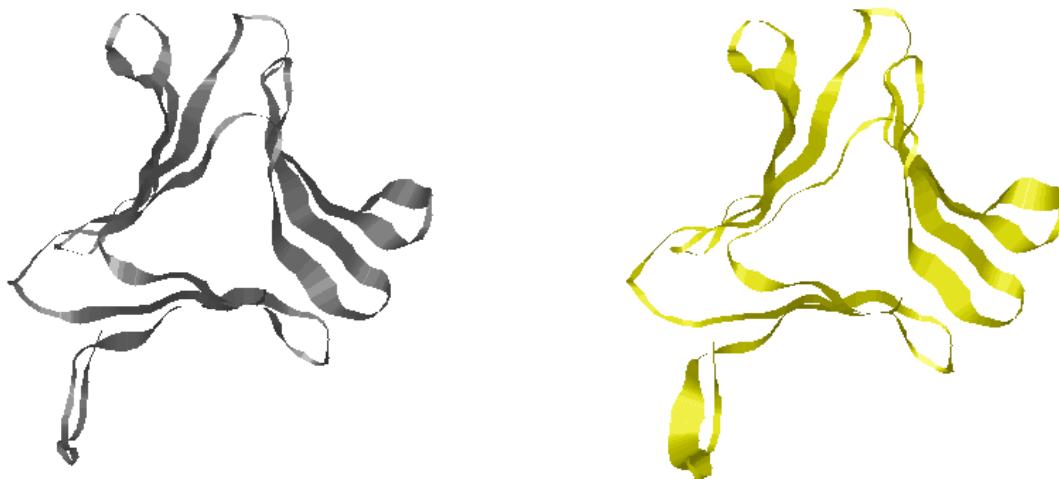
Figure 2.13 Comparison of the predicted tertiary structure (left, grey) and the X-ray diffraction structure of the GNA monomer (right, golden).
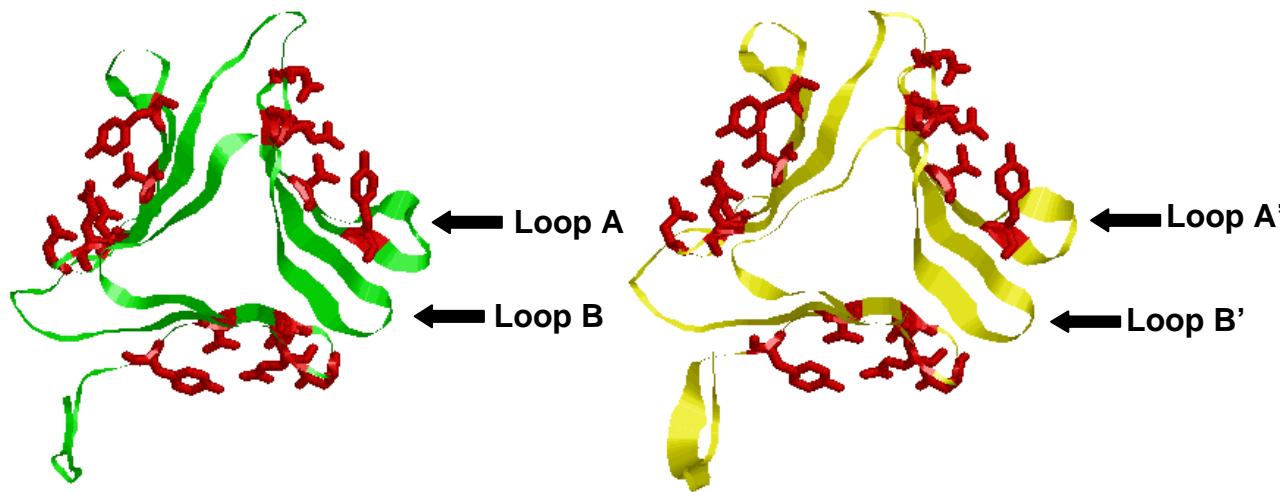
Figure 2.14 Comparison of the predicted tertiary structure of ATA (left green) and X-ray diffraction structure of GNA monomer (left golden). The residues involved in mannose-binding in each subdomain are highlighted with red sticks. Loop A and B and Loop A' and B' indicate the possible structures involved in the formation of quaternary structure in ATA and GNA, respectively.

2.3.4 Construction of an Allium triquetrum agglutinin expression vector

The aim was to produce a vector that drives over-expression of the novel lectin gene in all parts of the plant. To this end, the pGreen plasmid was chosen (Hellens *et al*., 2000) and the 35S promoter was selected to drive its expression.

2.3.4.i Ligation of the 35S-CaMV cassette and pGreen 0029

The 35S cassette consists of the 35S promoter, a short multiple-cloning site, and a *nos* terminator in a plasmid vector. Based upon the multiple-cloning sites in the 35S cassette (Figure 2.15) and the T-DNA of pGreen 0029 (Figure 2.16), two PCR primers were designed to insert the 35S-CaMV cassette into pGreen 0029. The first 35S Sal F primer adding a *Sal* I site and the second 35S Stu R adding a *Stu* I site.

The Tli high fidelity DNA polymerase (Promega, Southampton, UK) was used to generate a 35S promoter with desired endonuclease cleavage sites by incorporating the 35S Sal F and the 35S Stu R primers. Both the pGreen 0029 and 35S cassette were digested by *Sal* I and *Stu* I, ligated and transformed into *E. coli* DH5α. Positive colonies were selected by PCR screening. Plasmid DNA was extracted from four positive colonies for sequencing analysis. All the sequences were the same but slightly different from the 35S sequence provided by the pGreen website (www.pgreen.ac.uk), which suggested that the sequence

on the pGreen website might be wrong. But these differences are all in the multi-cloning region, so the function of the 35S-CaMV cassette should not be affected. Therefore, the empty expression vector pGreen 0029-35S was constructed.
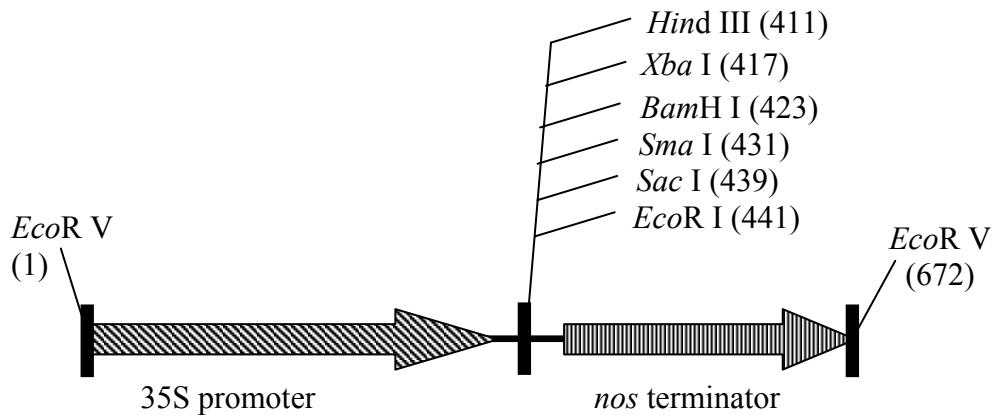
Figure 2.15 the 35S-CaMV cassette. The 35S-CaMV cassette consists of a 35S promoter and *nos* terminator. There is a short multiple-cloning site between the promoter and terminator.
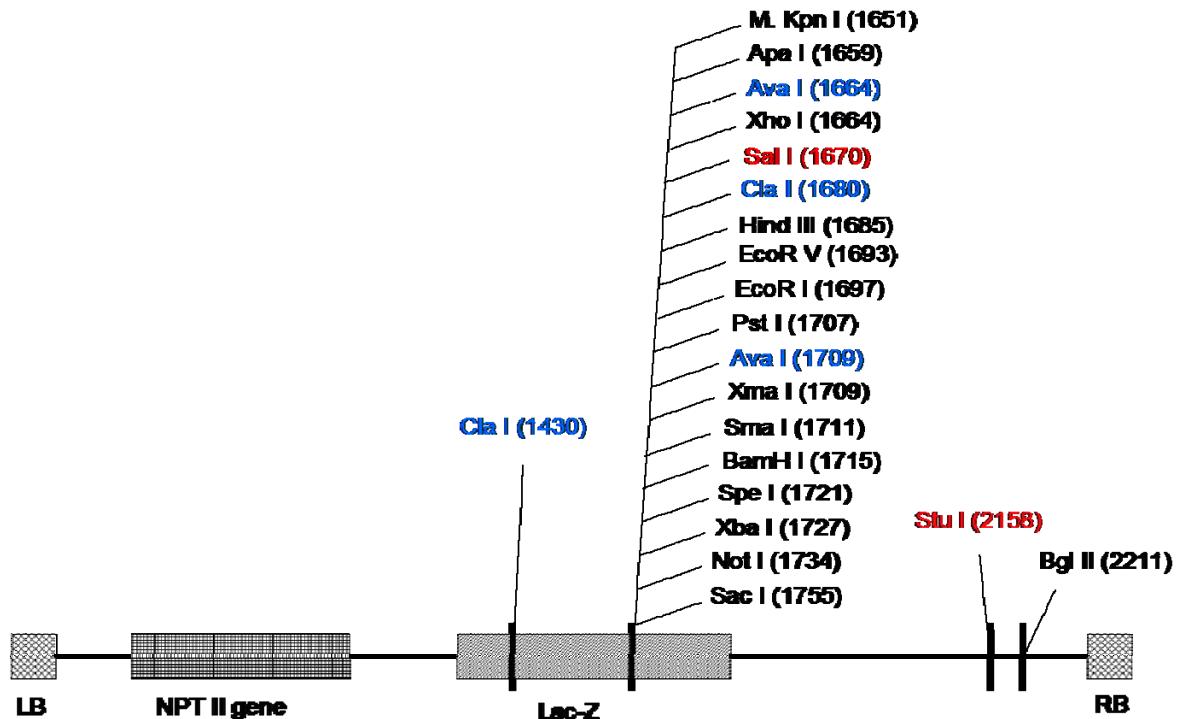
Figure 2.16 The T-DNA of pGreen 0029. The restriction enzyme sites in black are unique in pGreen 0029. LB= Left Border; RB= Right Border; *NPT II* gene= Neomycin phosphotransferase II gene. The cleavage sites in **red** show the ligation positions of the 35S-CaMV cassette in pGreen

2.3.4.ii Insertion of ATA into the pGreen 0029-35S vector

The Tli high fidelity DNA polymerase (Promega, Southampton, UK) was used to generate a single DNA fragment containing the entire open reading frame (ORF) of *ATA* with the desired endonuclease cleavage sites using primers Allium ORF F and Allium ORF R. Both *ATA* and the pGreen 0029-35S vector were digested with *Xba* I and *Sac* I, ligated, and transformed into DH5α. Positive colonies were selected by PCR screening. Plasmid DNA was extracted from positive colonies. Six colonies were selected for plasmid extraction and digested with *Sac* I and *Xba* I (Figure 2.17) to verify the quality of ligation.

In Figure 2.14, faint but clear products are visible in lanes 2, 3, 5, and 6. It is possible that lanes 1 and 4 also have products but they are less clear and this may simply result from less plasmid DNA being present. Sequencing of the plasmid DNA from colonies 2, 3 5, and 6 also indicated there were no mutations in these plasmids.
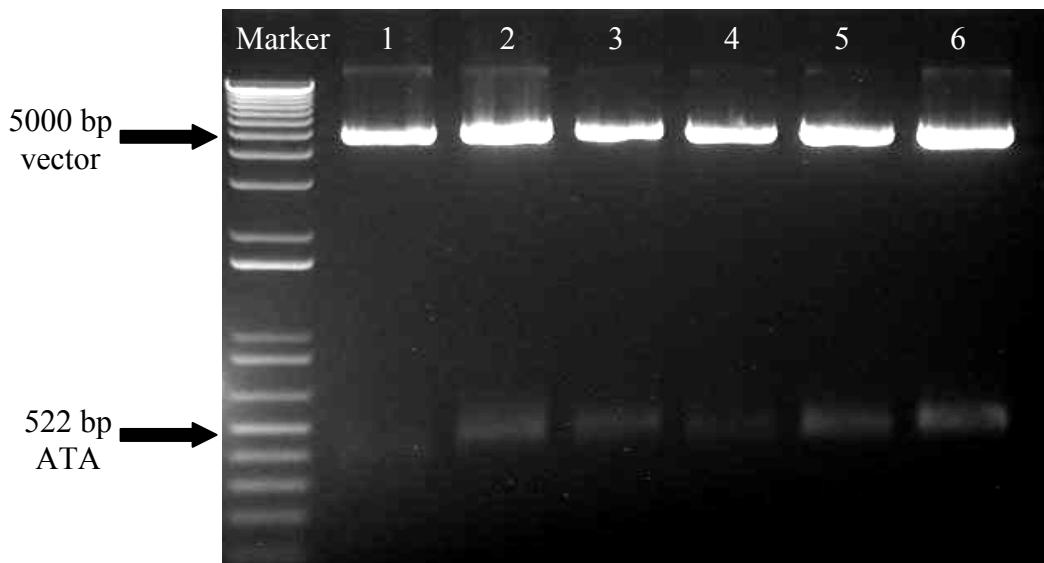
Figure 2.17 The endonuclease digestion products from putative expression constructs pGreen 0029-35S+ATA. Lane 1 to 6 are plasmid DNA from colony 1-6 of putative pGreen 0029-35S+*ATA* expressing constructs digested with *Sac* I and *Xba* I to specifically release the ATA of ORF fragment.

2.3.4.iii Transformation of pGreen 0029-35S+ATA into Agrobacterium tumefaciens GV3101

The pGreen 0029-35S+ATA plasmid was introduced into *A. tumefaciens* strain GV3101 by electroporation. Because the pGreen plasmid lacks the replication origin to replicate in *A. tumefaciens*, it requires the assistance of a helper plasmid pSoup, which is

reported to carry tetracycline resistance. At the beginning, attempts were made to transform pSoup into *A. tumefaciens* first prior to the transformation of pGreen, because one plasmid is always easier to transform into hosts than two. However, no positive colonies were observed four days after the pSoup-transformed *A. tumefaciens* were spread on LB media containing tetracycline. The transformation was repeated several times but without success. One possible explanation is that the pSoup in our lab does not carry the resistance to tetracycline. To solve the problem, an attempt was made to transform both pSoup and pGreen together into *A. tumefaciens*. Because pGreen cannot replicate without pSoup, the selection media only need to contain selection pressure for pGreen. Four days after the transformation, a few colonies were observed on the plates. Some colonies were relatively big and some were small. Therefore, three small colonies (S1 to S3) and three big colonies (B4 to B6) were chosen for PCR screening (Figure 2.18).

In Figure 2.15, PCR screening showed that colonies 1, 4 and 6 carried the ATA fragment. However, there appeared to be no correlation between the earlier colony size and plasmid construct present. Plasmid DNA was extracted from colony B4 and transformed into *E. coli* DH5α. Seven putatively transformed *E. coli* DH5α colonies were selected for PCR screening (Figure 2.19). Plasmid DNA was extracted from colony in Lane 1 for sequencing analysis. The sequencing result verified that the putatively transformed *A. tumefaciens* colony B4 carries pGreen 0029-35S+*ATA*.
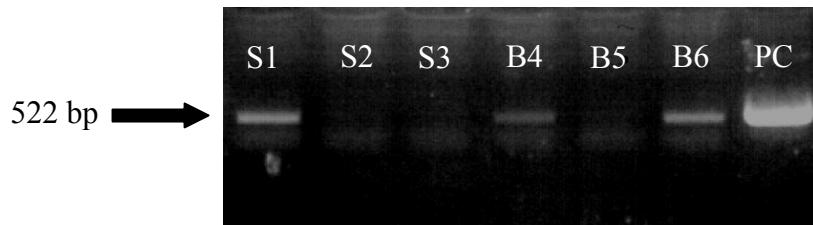
Figure 2.18 Electrophoresis of PCR products for screening of pGreen 0029-35S+ATA transformed GV3101 with Allium ORF F primer and Allium ORF R primers. PC, positive control, pGreen 0029-35S+*ATA*.
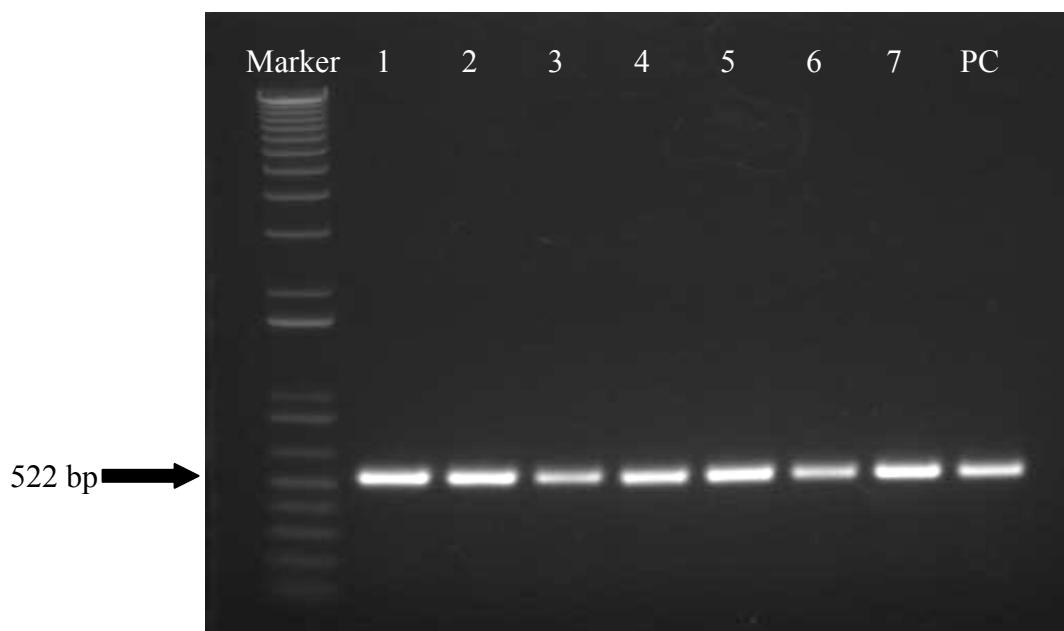
Figure 2.19 Electrophoresis of PCR products for the screening of *Escherichia coli* DH5α colonies transformed with plasmid DNA from putative pGreen 0029-35S+*ATA* transformed *A. tumefaciens* GV3101 with Allium ORF F primer and AlliumORF R primer. Lane 1 to 7: DNA from the seven selected colonies. PC, positive control, pGreen 0029-35S+*ATA*.

2.3.4.iv Transformation of pGreen 0029-35S into Agrobaterium tumefaciens GV3101

The empty expressing vector pGreen 0029-35S was transformed into *A. tumefaciens* GV3101, which will be used to establish a transgenic Arabidopsis line carrying the T-DNA without the *ATA* gene as a control. The pGreen 0029-35S was introduced into *A. tumefaciens* by electroporation. Seven colonies were analysed by PCR (Figure 2.20), however, only colony 7 contained the desired construct and therefore was reserved for future transformation of Arabidopsis.
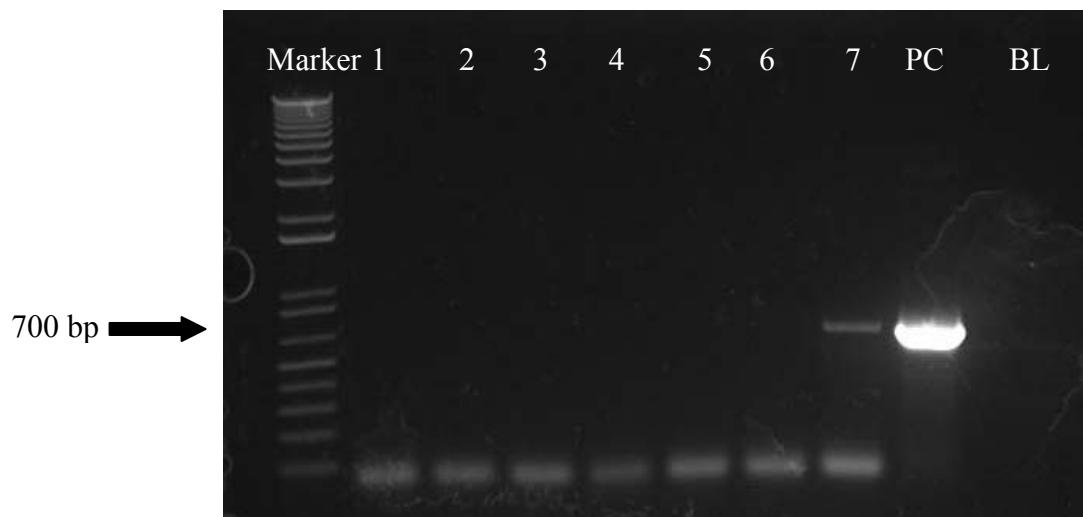
Figure 2.20 Electrophoresis of PCR products for screening of *A. tumefaciens* GV3101 after transformation with pGreen 0029-35S with the 35S Sal F and 35S Stu R primers. Lanes 1 to 7 are seven colonies (colony 1 to 7) selected for PCR analysis. PC, positive control, pGreen 0029-35S+*ATA*. BL, blank reaction in which DNA template was replaced with H_2O.

2.4 Discussion

Allium triquetrum is a member of the Alliacae family. In nature, aphids are rarely observed attacking its leaves and tubers. In a choice test of *Myzus persicae* between *A. triquetrum* and Arabidopsis, after 14 days, the Arabidopsis plants were heavily infested with *M. persicae* while there were almost no aphids on *A. triquetrum*. After 21 days, the Arabidopis plants showed obvious signs of senescence because of aphid infestation while there were still almost no aphids on *A. triquetrum*. *M. persicae* may prefer Arabidopsis which is also in the Brassicaceae famil, like Chinese cabbage, but since *A. triquetrum* does not show obvious physical defence, suggesting that *A. triquetrum* has some intrinsic mechanism to protect itself from attack by *M. persicae* and this could be due to the presence of defensive proteins, such as lectins, protease inhibitors, and amylase inhibitors.

A gene encoding a novel monocot mannose-binding lectin from *Allium triquetrum* (*ATA*) has been cloned, which encodes a protein with high similarity to many mannose-binding lectins. The 719 bp cDNA contains a 522 bp open reading frame (ORF) the size of which is very similar to those of lectin genes from other Alliaceae species (Figure 2.8). This open reading frame encodes a 174 aa sequence. Downstream of the ORF, an AATAAA box was identified 34 bp away from the stop codon. The AATAAA box is believed to be essential for the addition of the polyA tail. By using the online tool Signalp 3.0, a 28aa signal peptide was predicted, which also agrees with those reported mannose-binding lectin from Alliaceae and Amaryllidaceae speices (Van Damme, 1991, 1992, 1993a, 1993b, 1996). The N-region is possibly 8aa long (MAYSVTCK); the H-region is possibly 14aa long (LIMVCTVGAILSVL); and the C-region is possibly 5aa long (TATCM). The N-terminus signal peptide contains a high percentage of hydrophobic amino acid residues (53.57%), suggesting it is a secretory signal peptide, which is in accordance with the previous description of biosynthesis of the monocot mannose-binding lectins (Van Damme *et al*., 1998).

Like most mannose-binding lectins from Alliaceae and Amaryllidaceae species (Van Damme *et al*., 1991, 1992, 1993, 1993a, 1996), three mannose binding motifs (QXDXNXVXY) were identified in *ATA*. The amino acid residues represented by "X" in ATA are slightly different from those in GNA. In motif I, valine is replaced with alanine. In motif II, threonine is replaced with alanine and leucine is replaced with phenylalanine. In motif III, glutamic acid is replaced with aspartic acid. But these differences are unlikely to affect the binding ability of mannose. By comparing the DNA sequences of the motifs of GNA and ATA, these differences of amino acid residues are due to single base pair mutations. Only the replacement of threonine with alanine in motif II is due to a two base

pair mutation. Besides, all these mutations are transitions except the replacement of glutamic acid with aspartic acid in motif I (from G to T). Transition mutations are easier than transversion mutations, which means the mannose-binding motifs are well protected in *A. triquetrum*. In other words, the mannose-binding ability of ATA is still important for *A. triquetrum*. Because the mannose-binding ability of this lectin family must be responsible for their anti-aphid properties, it is logical to conclude that *A. triquetrum* does utilize ATA as a defense protein.

Three phylogenetic trees were built with aligned amino acid sequences of each motif with a variety of mannose-binding lectins from different families (data not shown). The toplogy of these trees for the three motifs are different, indicating that the evolution rates of the three motifs in the different plant species are different. Van Damme *et al*. (1998) proposed that the formation of a subunit is the result of the duplication of one primary subdomain. The different topology of the three trees may also suggest that the order of the duplications vary in different plant species. Focusing on the evolutionary relationship between the subdomains of ATA (Figure 2.11), it appears that subdomain II is the primary subdomain. The closer relationship between subdomain I and III suggests that the formation of these two subdomains occured at a similar time (Figure 2.21).
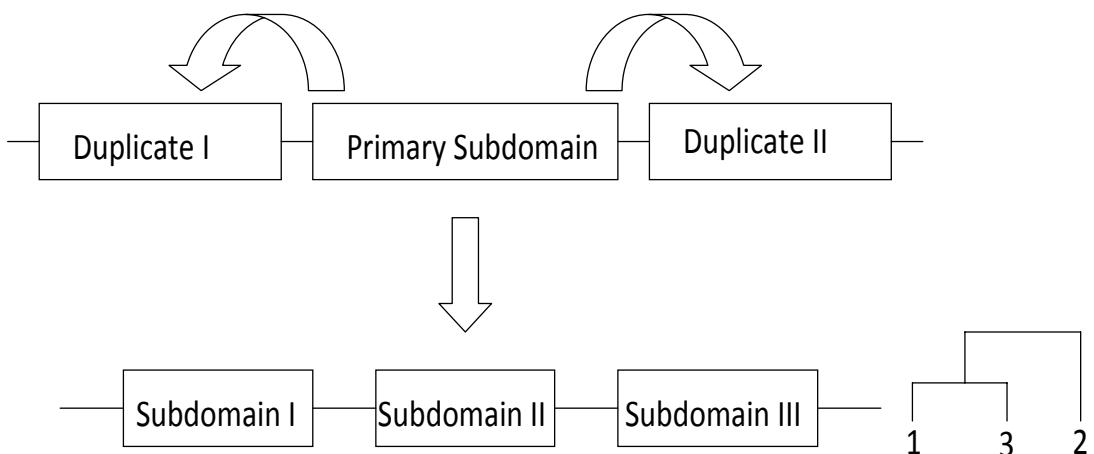
Figure 2.21 Model of the molecular evolution of the three subdomains of ATA. The primary subdomain duplicated itself twice to form the other two subdomains. The duplications with the primary subdomain together form the present structure of ATA.

The phylogenetic tree built with lectins from Alliaceae, Amaryllidaceae, Araceae, Bromeliaceae, Iridaceae, Orchidaceae, and Ruscaceae shows all the lectins from Alliaceae, including ATA, sit in the same cluster (Figure 2.9), which suggests that all the lectins in the Alliacae originated from a common ancestor. The tree also shows that the garlic leaf

lectin (ASAL) emerged earlier than the ramson leaf lectins (AUAL) and ATA. After that, AUAL and ATA diverged from the bulb lectins. During evolution, most mutations in the DNA sequence do not result in a functional change of the protein, but the alignment of ATA with other Alliaceae lectins revealed that some lectins' mannose-binding motifs are slightly different from the standard form (QXDXNXVXY). So far, three similar lectins have been identified from *A. ursinum*, two Bulb-lectins (B-lectins) and one leaf-specific lectin, and all of these three lectins show some loss of mannose-binding motifs. Together with information provided by the phylogentic tree (Figure 2.9), it can be assumed that the lectins in *A. ursinum* and *A. triquetrum* have a common ancestor; after the leaf lectins and the bulb lectins diverged, all the lectins in *A.ursinum* have lost some mannose-binding ability, which is possibly because *A. ursinum* no longer requires this ability. It is very likely that the loss of the mannose-binding motifs is a major issue in separating ATA and AUAL during evolution.

The high sequence similarity between ATA, ASAL, and GNA imply that the structure and function of these three lectins may be very similar. 11 β-strands were predicted in the secondary structure of the ATA precursor and the positions of these predicted β-strands can be matched to their counterparts in the mature GNA (Figure 2.12). Although the programme only predicted 11 β-strands in both the ATA precursor and GNA (Figure 2.12) whereas there are actually 12 β-strands in mature GNA, the sequence of β-strand 12 (β12) of the mature GNA shows high sequence similarity to its counterpart in the ATA precursor (Figure 2.12). Therefore, it is likely that there are also 12 β-strands in ATA. β12 is important in forming the tertiary structure of GNA (Hester *et al.*, 1995) because the β12 from one subunit crosses over into the other subunit and inserts as the fourth strand into the β sheet of subdomain I to form a hybrid β sheet, which helps to assemble two monomers into a dimer. The homogeneous tetramer of GNA consists of two dimers, stablised by hydrophobic contacts between loops, involving residues 16-20 (loop A) and 34-38 (loop B) (Figure 2.14). The counterpart of loop A in the ATA precursor has low sequence similarity and lower hydrophobic residue content than GNA, while the counterpart of loop B has high sequence similarity and similar hydrophobic residue contents as GNA. This evidence suggests there may not be enough hydrophobic contact between the dimers of ATA to construct a tetramer, which reinforces the conclusion from the phylogenetic study. As with many other monocot lectins that have been modeled using the X-ray coordinates of GNA (Barre *et al.*, 1996), the modeled ATA exhibits an overall three-dimensional structure very similar to that of GNA, despite some discrepancies, such as deletions or insertions of a few residues along the amino acid sequence. The three anti-

parallel strands of β-sheets of each subunit form an almost closed structure (Figure 2.22); thus creating a very rigid and strong structure that can partly explain the high stability of monocot lectins in adverse environments.

β-sheet 3   β-sheet 2

β-sheet 1
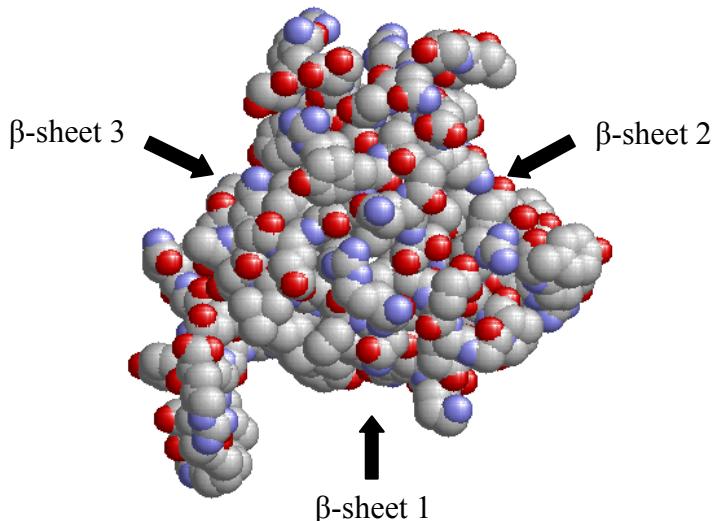
Figure 2.22 The space filling form of the predicted structure of ATA. The arrows indicate the positions of the β-sheets. (Produced with Raswin V2.7.3.1)

It has been hypothesised that the more subunits a lectin has, the better affinity for its substrate. The reason is that high affinity binding is dependent on auxiliary contacts from neighbouring lectin molecules (Hester *et al*., 1995). However, although it is reasonable to assume that the affinity binding ability of monocot mannose-binding lectins should have some relationship to their toxicity, it does not necessarily mean that a tetramer is more toxic than a dimer. In fact, for Bt toxins, it has been shown that the strength of toxin binding can correlate inversely with toxicity (Wolfersberger, 1990). Similarly, a recent study with a hybrid lectin, a fusion of garlic and onion lectins, indicated that there was no direct correlation between the mannose-binding capacity and efficiency of insect toxicity (Hossain *et al*., 2006). Moreover, studies with phytohemagglutinin showed that there was differential lectin binding between the two hemipteran species investigated, suggesting differential distribution of carbohydrates in the membrane of their digestive tracts (Habibi *et al*., 1998; 2000). Therefore, it is possible that a dimer is more toxic to some insects than a tetramer because the distribution of mannose or glycoproteins in the digestive systems of different insects varies.

Usually, leaf-specific lectins from onion, leek, shallot, garlic have better agglutinating ability than B-lectin. In the case of lectins from garlic, ASAL is only a moderately abundant protein (a few % of the total soluble protein), but it has been shown that ASAL is 500 times more active as a lectin compared with ASA II (up to 50% of the

total soluble protein in the bulb of garlic) (Smeets *et al*., 1997). The result of a BLAST searching suggests that ATA is Bulb-lectin, but, the phylogenetic analysis shows that ATA is more likely to be a leaf specific lectin because it is closer to AUAL and ASAL, which is in accordance to the assumption made in the gene cloning stage. But unlike AUAL, ATA has three complete mannose-binding motifs, so it can be assumed that ATA has a better affinity for mannose and higher biological activites than AUAL. Based on the fact that the leaf-specific lectins from Alliaceae are all homodimers (Smeets *et al*., 1997, 1997a), it is likely that ATA should also be a homodimer.

In both 3' and 5' RACE, more than one band was observed, which means that there are several different lectins in *A. triquetrum*. In 3' RACE, the 700bp fragment appeared both in bulb/root and leaf tissue and is likely to encode a heterodimer Bulb-lectin (B-lectin) because of its size (Van Damme *et al*., 1998). But B-lectins are thought to be storage proteins only. The 350bp fragment that appeared only in bulb/root tissue probably encodes another B-lectin. In 5'RACE, no product was obtained from bulb/root cDNA, which reinforces the assumption that the 500bp fragment chosen in 3' RACE belongs to a leaf-specific lectin. However, two fragments were observed in leaf cDNA; the one of about 400bp contains a 378bp sequence that can be perfectly aligned with 3'end fragment; whilst the sequence released from the 200bp fragment cannot be aligned with 3' end fragment.

In recent years, there has been an increasing interest in the potential use of lectins in integrated pest management via transgenic plants. Several lectins show insecticidal activity, and some of these lectins originated from edible plants or traditional herbal medicines, such as *A. sativum* (Van Damme *et al*., 1991; 1992; Smeets *et al*., 1997) and *Pinellia tenata* (Yao *et al*., 2003). Using lectins from an edible plant may be less controversial than the use of a transgene from a poisonous plant. As *A.triquetrum* is also called wild garlic, it is consumed by the people in Europe who are fond of wild food both cooked and uncooked. Therefore, the lectin from *A.triquetrum* described in the present study should be safe to higher mammals. In fact, there has been no report to show that lectins from Alliaceae species are harmful to humans and other mammals (Sadeghi *et al*., 2008).

2.5 Conclusions

So far, a gene coding for a novel monocot leaf-specific lectin, *Allium triquetrum* agglutinin (ATA), has been identified in *A. triquetrum*. Each subunit of ATA contains three intact mannose-binding motifs, which may play an important role in the plant defence system. The molecular modeling reveals ATA has a similar three-dimensional structure to most other monocot mannose-binding lectins, including *Galanthus nivalis* agglutinin (GNA). However, the molecular modelling is based on the assumption that ATA has similar molecular weight to GNA, so the result cannot be fully acceptable unless the actual size of ATA is revealed by western blot. It is also likely to be relatively safe for humans because it is used as a food material occasionally. Therefore, ATA is a good candidate lectin worth further investigation into its anti-aphid properties. To this end, the 522bp ORF has been inserted into the expression vector pGreen 0029-35S. The expression vector has been transformed into *Agrobacterium tumefaciens* GV3101 and was used to generate transgenic Arabidopsis.

Chapter Three

3 Plant transformation and characterization

3.1 Introduction

Chapter 2 described how a novel lectin gene, *Allium triquetrum agglutinin* (*ATA*) gene, was cloned. The analysis of its DNA sequence and deduced amino acid sequence suggests that this novel lectin gene could confer resistance to homopteran insects. Ectopic expression of lectins has been proved to be an effective way of assessing the insecticidal properties of these proteins (Nagadhara *et al*., 2003; Yao *et al*., 2003; Sadeghi *et al*., 2007). Besides, it is also of interest to see whether the novel lectin gene can be precisely expressed ectopically. Arabidopsis has been a model plant for more than sixty years. Its short life cycle, high yield of seeds, and relatively small genome make it suitable for various research systems (Somerville and Koornneef, 2002). This chapter describes how the *ATA* gene was transformed into Arabidopsis. The integration and expression of the "foreign" gene was characterized by PCR, R/T PCR, and western blotting. With the actual size of the mature ATA revealed by western blotting, the molecular modelling of the tertiary structure of ATA can be confirmed.

Two plant transformation methods have been most commonly applied, namely particle bombardment and *Agrobacterium tumefaciens*-mediated transformation. The particle bombardment technique is simple and straight-forward, but it usually introduces multiple copies of the transgene into plants and the transformation efficiency is not as high as *Agrobacterium tumefaciens*-mediated transformation. In nature, *Agrobacterium tumefaciens* infects its host by transferring a well defined DNA segment from its tumour-inducing (Ti) plasmid to the host genome (Gelvin, 2000). The transferred DNA (T-DNA) which is defined by two 25 base pair repeats at each end. The T-DNA is transferred from *A. tumefaciens* to the host cell and imported with the help of several bacterial virulence proteins (Vir) into the host nucleus as a single-stranded form (Tinland, 1996). The transformation process starts with the bacterium-plant attachment (Tzfira and Citovsky, 2006). Once the VirA sensory protein perceives certain host signals (Turk *et al*., 1994), autophosphorylation of the VirA protein and subsequent transphosphorylation of the VirG protein result in the activation of *vir* gene transcription. By nicking the plasmid at the T-DNA borders, the VirD1 and the VirD2 together produce a single stranded T-DNA (T-

strand) (Filichkin & Gelvin, 1993). Following nicking, the VirD2 molecule covalently attaches to the 5' end of the resulting DNA molecule, which are together, called the immature T-complex (Ward & Bames, 1988). With the guidance of the VirD2 (Gelvin, 2000), the T-strand enters the host cell via a VirB/D4 type IV secretion system (Christie, 2004). Once inside the host cell cytoplasm, the entire T-strand is coated with numerous VirE2 molecules, altogether called the mature T-complex (Pansegrau *et al*., 1993; Abu-Arish *et al*., 2004). Indeed, both the VirD2 (Ballas & Citovsky, 1997) and the VirE2 (Tzfira *et al*., 2001) have been shown to interact with host proteins for their nuclear import in host cells. The hypothesis that the T-complex uses the plant cytoskeleton, probably radial microtubules, as a track to move towards the nuclear pores is a new proposal based on the data obtained in an animal cell system (Christie, 2004). Inside the nucleus, the T-complex targets the integration site by utilizing the affinity of the plant VirE2-interacting protein 1, and perhaps other transcription factors (Mysore, 2000).

Various techniques have been applied to improve plant traits, but plant transformation has caused the biggest public concern. Most of this concern focuses on the unexpected effects of integrated DNA on the host plant genome. For example, increased lignin content has been observed in maize transformed with *Bt* toxin gene (Poerschmann *et al*., 2005). Such unexpected effects have also been observed in transgenic trees. Aspen transformed with the sucrose phosphate synthase gene had higher concentrations of salicin, tremuloidin, condensed tannins and nitrogen, and lower concentrations of coumaric acid and four flavonoids. The same transformed line even showed better resistance to leaf beetle (Hjaltén *et al*., 2007). The unexpected effects can be divided into two types:

 (1) The influence of the transgene on the functioning of the surrounding sequences (insertion effect). Recent work by Li *et al*. (2006) suggested that the T-DNA insertions were not randomly distributed in the Arabidopsis genome and that the favored targets for T-DNA integration were transcription initiation and polyadenylation site regions in the genes. Nevertheless, theoretically, the T-DNA can insert into any position in the plant genome. If the transgene happens to insert into some key genes controlling the expression of several other genes, a few phenotypic characters may change.

 (2) The production of the transgenic protein can establish new metabolic sinks for certain amino acids or unspecified substrates for relevant enzymes (Tagashira *et al*., 2005) (expression effect). Levels of transgene expression in plants are generally unpredictable and vary between independent transformants and variability is usually explained by differences in transgene copy number (Stockhous *et al*., 1987; Hobbs *et al*., 1990) and/ or integration sites (Jones *et al*., 1985; Eckes *et al*., 1985; Prols and Meyer,

1992). Usually a higher expression level of lectins means better resistance to pests, but the high expression of a foreign gene may bring physiological costs to the host plant (Herms and Mattson, 1992).

Due to the unexpected effects of transformation, it is important to evaluate the growth performance of different transgenic lines to test whether ATA is toxic to Arabidopsis metabolism or whether the transgene insertion causes severe abnormal phenotypic changes. In the work described in this chapter, six *ATA*-transformed Arabidopsis lines and one empty vector Arabidopsis line were produced using the *A. tumefaciens*-mediated floral dipping method and homozygous lines were established. Growth parameters, representative of important agronomic traits, were selected, based on observations in the homozygote screening stage and on traits measured in previous research to evaluate the fitness of each transgenic line. These traits include germination time (variation observed in the homozygote screening), flowering time (Bernier *et al*., 1993; Jarillo *et al*., 2008), plant dry mass at the flowering stage (Kronberg *et al*., 2007; Xu *et al*., 2008), and seed production (Jackson *et al*., 2004; Cipollini; 2007), including seed yield and seed mass.

3.2 Material and Methods

3.2.1 Plant material

Arabidopsis Columbia-0 (referred to as Arabidopsis in the following text) was grown on sterilized compost consisting of equal volume of Levington F2S growth media, John Innes No2 Compost, and Vermiculite. 4-8 seeds were sown in each 4 inch square pot. The pots were covered with cling film to keep the humidity high and moved to 4°C to break the dormancy of the seeds. After 4 days, the pots were transferred to climate controlled rooms (20±2°C, L16: D8). After the second pair of true leaves emerged, each healthy seedling was transferred to a new pot.

3.2.2 Arabidopsis transformation

5ml LB medium containing antibiotics was inoculated with *Agrobacterium tumefaciens* GV3101 containing plasmid and incubated at 28°C for 1~2 days with vigorous agitation. 1 ml of this culture was added to 200ml LB medium and incubated again with vigorous agitation for another 2 days. The bacteria were pelleted by centrifugation and resuspension in 200ml 0.5 × Murashige and Skoog (MS) with 5% (w/v) sucrose solution. The detergent Silwet L-77 was added to a concentration of 0.02% (v/v) and mixed well before dipping. The plants with 15-20 cm high bolts were used for transformation. The whole of the above-ground parts of the plant were dipped in the *Agrobacterium* suspension for 2~3 min, with gentle agitation. The dipped plants were placed in plastic sleeves for 24 hr to maintain high humidity. The plants were then rinsed with tap water to wash away the sucrose to prevent the growth of fungus. The dipping was repeated with a fresh bacterial suspension after 7 days.

3.2.3 Surface sterilization of Arabidopsis seeds

About 0.1ml seeds were placed in a 1.5ml microcentrifuge tube and washed in 1ml 70% (v/v) ethanol for no more than 1 min, the ethanol removed and the seeds briefly rinsed with 1ml sterilized distilled water. The seeds were then washed with 1ml 15% (v/v) Domestos bleach (Domestos, UK) for 20 minutes with agitation. After rinsing with 1ml sterilized distilled water five times, the seeds were resuspended in 1ml sterilized water.

3.2.4 Kanamycin selection of transformed plants

The pGreen vector confers kanamycin resistance (Chapter 2), so using a sterilized Pasteur pipette, surface sterilized seeds were plated on $0.5 \times$ MS (Duchefa, Haarlem, Netherlands) media containing 40μg/ml kanamycin. The plates were sealed with Parafilm tape and incubated in a plant tissue culture room (L16:D8, $22 \pm 2°C$). After about ten days, transformed plants were visible as green seedlings with long roots, while untransformed seedlings were yellow with short roots.

3.2.5 Genomic DNA extraction from Arabidopsis (kit method)

Genomic DNA extraction from Arabidopsis was performed with Microzone DNAMITE plant kit (Microzone, West Sussex, UK). 1 cm^2 of the leaf material was put into a 1.5ml microcentrifuge tube. 200μl lysis solution LA (from the kit) was added and the plant material was ground with a pestle. 20μl PA solution was added to precipitate the protein. The mixture was centrifuged and the supernatant was transferred into a fresh tube containing 100μl of DNA precipitation Solution CA. After mixing, the DNA was recovered by centrifugation and washed with 500μl 70% (v/v) ethanol. DNA was dissolved in 20μl $1 \times$ TE buffer.

3.2.6 Reverse transcription PCR analysis of transgenic Arabidopsis

Total RNA was extracted using a Qiagen RNeasy Plant Kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. Approximately 1.5μl RNA was treated with DNase (Ambion, Warrington, UK) and 4μl of DNase treated RNA was used for reverse transcription (RT) to generate cDNA with Promega ImProm-11 RT System (Promega, Southampton, UK). PCR analysis of the cDNA was done in a total volume of 10μl containing: 5μl 2×Bioline, 0.5μl 35S F primer, 0.5μl 35S R primer, 1μl cDNA, 3μl dH$_2$O; the following conditions were used for PCR: 94ºC for 2 min, (94 ºC for 20 sec, 50ºC for 20 sec, 72ºC for 1 min) $\times$ 35 cycles, 72ºC for 5 min. PCR products were analysed in a 0.8% (w/v) agarose gel.

3.2.7 Genetic analysis of segregation of the transgene in T_1 progenies

About 200 seeds from each T_1 plant were plated on 0.5 × MS media containing 40 µg/ml kanamycin. After 10 days, the seedlings were analyzed and their segregation ratios calculated. The number of green seedlings and bleached seedlings were recorded to determine the segregation pattern of T_2 plants. The Chi-square (χ^2) statistical test was used to determine how well a set of segregation data fits to predicted segregation rates. With one degree of freedom, if the calculated $\chi_c^2 < \chi_{0.95}^2 = 3.84$, the hypothesis that the results are reasonable for 3:1 segregation can be accepted. The χ_c^2 values were calculated with Equation 3.1.

With one degree of freedom, $\chi_c^2 = \sum \dfrac{\left(\left|Observed - Expected\right| - 0.5\right)^2}{Expected}$ (Equation 3.1).

3.2.8 Protein extraction from Arabidopsis

The EZ method developed by Martinez-Garcia *et al*. (1999) was slightly modified to extract protein from Arabidopsis. 8-10 mature Arabidopsis leaves were ground in liquid nitrogen with a pestle and mortar. The powder was transferred to a 50ml tube, to which 200µl buffer E was added. The tube was then incubated at 4°C to let the powder gradually thaw and mix with buffer E. After 2 hr, the tube was briefly vortexed and warmed to room temperature to resolubilize the SDS, centrifuged at 10,000 × g for 10 min and the supernatant was saved. The supernatant was diluted with 1/10 volume of buffer Z for SDS polyacrylamide gel electrophoresis and western blotting.

3.2.9 Analysis of proteins by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was carried out essentially according to Laemmli (1970). Discontinuous vertical slab gels were poured and run using a Hoefer dual cooled slab unit SE 250 (Hoefer, MA, USA). Gels were normally 1.5 mm thick and 80 mm long. The components of these gels were mixed as described in Table 3.1.

Protein samples to be analysed were denatured by heating in a heating block at 100°C for 3 min. Gels were run at a fixed current of 60 mA in 1×protein gel running buffer until the bromophenol marker dye reached the bottom of the gel. Separated proteins were visualized by immersing the gel in Commassie brilliant blue stain for 2-3 hr at room

temperature, followed by subsequent removal of excess dye by prolonged incubation in Destain solution.

Table 3.1 Components of Discontinuous SDS Polyacrylamide Gels

GEL TYPE		SEPARATING GEL			STACKING GEL
GEL PERCENTAGE		10%	12.5%	15%	5%
30% (w/v) Acrylamide, 0.8% (w/v) Bis-acrylamide	ml	5.00	6.25	7.5	0.50
QH$_2$O	ml	5.00	3.75	2.5	2.15
1.25 M Tris-HCl pH 8.8	ml	4.50	4.50	4.50	
1.25 M Tris-HCl pH6.8	ml				0.30
10% (w/v) SDS	ml	0.15	0.15	0.15	0.03
TEMED	ml	0.015	0.015	0.015	0.0025
10% (w/v) Ammonium Persulphate	ml	0.15	0.15	0.15	0.025

3.2.10 Western blotting

Western blotting was based on the method described by Towbin *et al* (1979). The initial protocol involved transfer of proteins to pre-cut PVDF membranes (Amersham Pharmacia, Little Chalfont, UK) using a BioRad Trans-blot cell (BioRad, Herts, UK). The assembled electroblot apparatus was run in 1× western blot buffer at a constant current of 100 mA for 2 hr. The degree of transfer of proteins was determined by observing the transfer of Kaleidoscope protein standards (BioRad, Herts, UK).

After transfer of proteins, membranes were air dried to let the protein fix on the membrane. The membranes were wetted with 5% Tween-20 and briefly washed twice with 1× TBS and then incubated for 6-8 hr at 4ºC in blocking buffer. This was carried out in order to saturate all the binding capacity of the membrane, necessary to reduce non-specific background at a later stage.

Membranes were subjected to a two-step immunological reaction. Firstly, membranes were placed in plastic containers with primary antibody (Appendix 2) diluted in blocking buffer (1:1000) and incubated overnight at 4°C. After incubation, the membranes were washed twice in 50 ml TBST for 10 min each, then washed twice in 50ml 1×blocking buffer for 10 min each at room temperature with gentle agitation. The following steps were performed with BM Chemiluminescence Western Blotting kit (Roche Diagnostics, Mannheim, Germany). The membranes were incubated with peroxidase-

labeled secondary antibody diluted in blocking buffer (40mU/ml) at room temperature for 1 hr. The membranes were then washed four times in 50ml TBST for 15 min each.

For detection, the membranes were transferred into plastic bags and 2 ml of detection reagent was added to the membranes. The bags were immediately sealed with electrically heated plastic bag sealer after eliminating any air bubbles. The membranes were inserted into a film cassette, protein side up, after incubation in the detection reagent for 1 min. In a dark room, a sheet of a Kodak film was placed onto the membranes and exposed for 10 to 60 sec with the cassette closed. The films were developed with X-Ograph X-2 automatic x-ray film processor (X-Ograph Ltd, Tetbury, Gloucestershire, UK).

3.2.11 Molecular modeling to reveal the potential 3-D structure of ATA

Secondary and tertiary structure predictions of ATA were performed with ExPASy Proteomics tools (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1).

The figures of 3-D structure were produced with RasWin V2.7.3.1. (http://www.umass.edu/microbio/rasmol/getras.htm).

3.2.12 Analysis of germination time and flowering time of Arabidopsis

When homozygous lines were identified, the variation in germination time and flowering time were observed. Therefore, a test of germination time and flowering time in a highly controlled environment was necessary. The method of measuring flowering time is described in Weigel and Glazebrook's Arabidopsis laboratory manual (2002). The same method was slightly modified to suit measurements of germination time. The measurements were performed in both short day length photoperiod (10 hr light/14 hr dark) and long day length photoperiod (16 hr light/8 hr dark). Seeds were sown in 4 inch square pots and incubated in a cold room for 4 days to break the dormancy. Eight plants of each line were set up for testing. Germination time (GT) was defined as the numbers of days before cotyledons are visible above the soil surface. Flowering time was described by two parameters. First, the number of days between germination and the appearance of the first flower bud were recorded as FT. Second, the total leaf numbers when the first flower bud appears were recorded as LN. All experiments were repeated once in each of the two different photoperiods after one week. All the data were first tested for normality with Kolmogorov-Smirnov (K-S) test first. The GT test data were not normally distributed and

could not be transformed. Therefore, the data of GT test were processed with a non-parametric Kruskal Wallis test (Minitab 15, Minitab Inc, PA. USA). The data of FT and LN were normally distributed and processed with Analysis of Variance (ANOVA) (SPSS 15.0, IL, USA). Once the ANOVA shows there was a significant difference between lines, a Least Significant Difference (LSD) test was performed to reveal the FT and LN of which lines were different from others (SPSS 15.0, IL, USA). The interaction plot was produced with Minitab 15.0 (PA, USA).

3.2.13 Analysis of plant dry mass

After the flowering times were recorded, the above ground parts of Arabidopsis were harvested and placed in a 20cm×20cm paper bag. To remove error caused by variable water contents, the plants were dried at 60°C for at least 3 days to constant weight, which was measured with a five decimal place balance (Model R180D, Sartorius research). The normality of the data was checked by K-S test (Minitab 15.0) before analysis. The data were processed with two-way ANOVA (SPSS 15.0). When the GLM ANOVA was significantly different, a LSD test was performed to reveal the dry mass (DM) of which lines were different from others (SPSS 15.0). The interaction plot was produced with Minitab 15.0.

3.2.14 Analysis of average seed mass and seed yield

In these analyses, a new method was developed to gather all the seeds produced. A 3 ½ inch saucer with an 3/8 inch hole in the middle was placed on the top of 3 ½ inch round pot filled with soil. A few seeds were sown into the holes and 8 pots of each line were set up for seed mass and seed yield analysis. Once the first pair of true leaves emerged, the healthiest seedling was kept in each pot. The seeds were first incubated at 4°C for 4 days and grown in 12 hr day /12 hr dark at 22°C. When the first flower bud appeared, the plants were wrapped with plastic sleeves to prevent crossing and losing seeds. All seed from a single plant were harvested when the plant was mature and the last siliques produced on the inflorescence had elongated, turned brown, dried, and were ready to dehisce. Mature plants were cut at the crown and crushed by hand to release seeds from all remaining siliques. These hand-harvested seeds were combined with those caught in the saucer, sieved to remove plant debris, and dried at room temperature for two weeks (Kronberg *et al.*, 2007). The total seeds from a single plant were weighed by a five decimal

place balance for seed yield (SY) analysis. The total seed numbers were counted with Image J (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). Average seed mass (SM) was determined by dividing the total seed weight with the total seed number. The data of average seed mass and seed yield analysis were tested for normality by K-S test (Minitab 15.0) before processing by one-way ANOVA (SPSS 15.0). When the ANOVA test showed a significant difference between lines, a LSD test was performed to reveal the SY and SM of which lines were different from others (SPSS 15.0)

3.3 Results

3.3.1 Production of T_1-generation transformed Arabidopsis

Agrobacterium tumefaciens GV3101 carrying the expression vector pGreen-35S+*ATA* was used to transform Arabidopsis. Six independent T_1-generation transformed plants (C1101-C6101) were identified by selection on kanamycin (Figure 3.1). A line of GV3101 carrying the empty expression vector pGreen-35S was also used to establish transgenic Arabidopsis lines without the *ATA* gene. Three independent T_1-generation transformed plants (D1101-D3101) were identified by selection on kanamycin. The empty vector transformed line was produced to serve as a good negative control in future aphid bioassay.

All the transgenic plants were given a code, which is composed of one letter and four numbers, for example, C1101. All the plants transformed with the pGreen-35S+*ATA* vector start with letter "C" and all the plants transformed with the pGreen-35S empty vector start with letter "D". The first number is the line number indicating each plant's T_1 parental line. The second number is the generation number. The last two numbers were used to distinguish each individual in one generation from the same parental line.
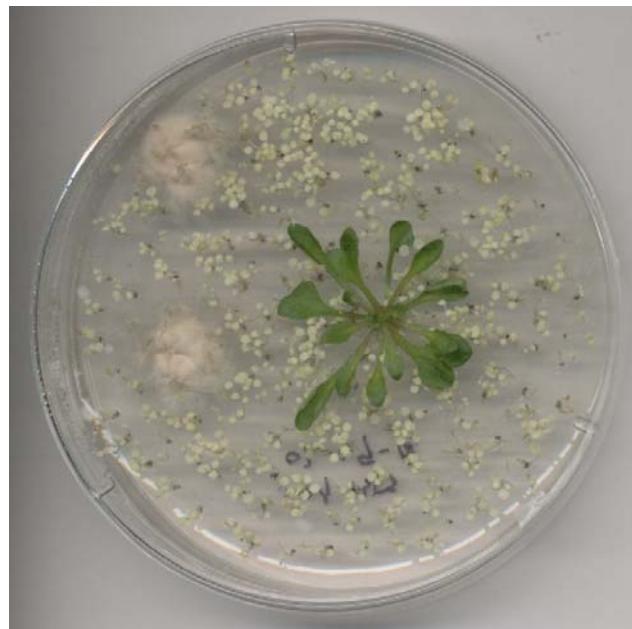
Figure 3.1 Selection of transformed plants on medium containing kanamycin (40μl/ml). After growth on kanamycin, most seedlings were bleached, and only about 0.01% to 0.1% were green healthy seedlings with long roots.

3.3.2 Detecting integration of T-DNA into the Arabidopsis genome

PCR was used to demonstrate the integration of T-DNA into the plant genome. Genomic DNA was extracted from C1101-C6101 and PCR was performed with 35S F and 35S R primer. All the DNA extracts from the *ATA*-transformed lines showed products of the same size as the positive control, indicating that the foreign lectin gene had been successfully integrated into the Arabidopsis genome (Figure 3.2).

Genomic DNA was also extracted from D1101-D3101 and PCR was performed with 35S Sal F and 35S Stu R primers (Figure 3.3). D1101 has a band of same size as the positive control. D2101 has a band of same size as the *ATA* control III (lane 7). D3101 has no visible PCR product. The results indicated that only D1101 is a real pGreen 0029-35S transformant. D2101 is more like a pGreen-35S+ATA transformant, which can be explained as contamination during harvesting or plating. D3101 is a pseudo transformant.
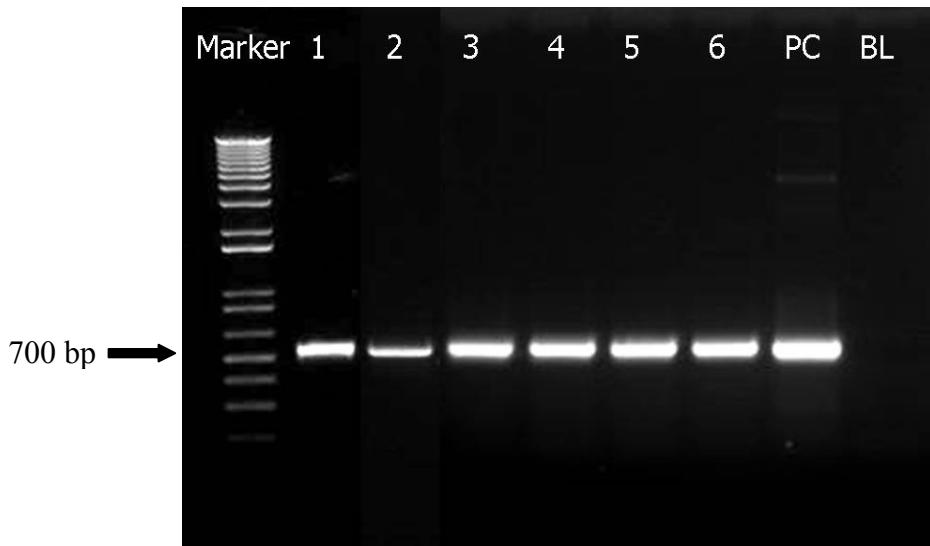
Figure 3.2 Electrophoresis of PCR products with DNA from *ATA*-transformed Arabidopsis. The PCR were performed with 35S F and 35S R primers. Lanes 1-6 were six independent *ATA*- transformed Arabidopsis lines (C1101-C6101). PC, positive control, pGreen 0029-35S+*ATA*. BL, blank reaction in which DNA template was replaced with H_2O.
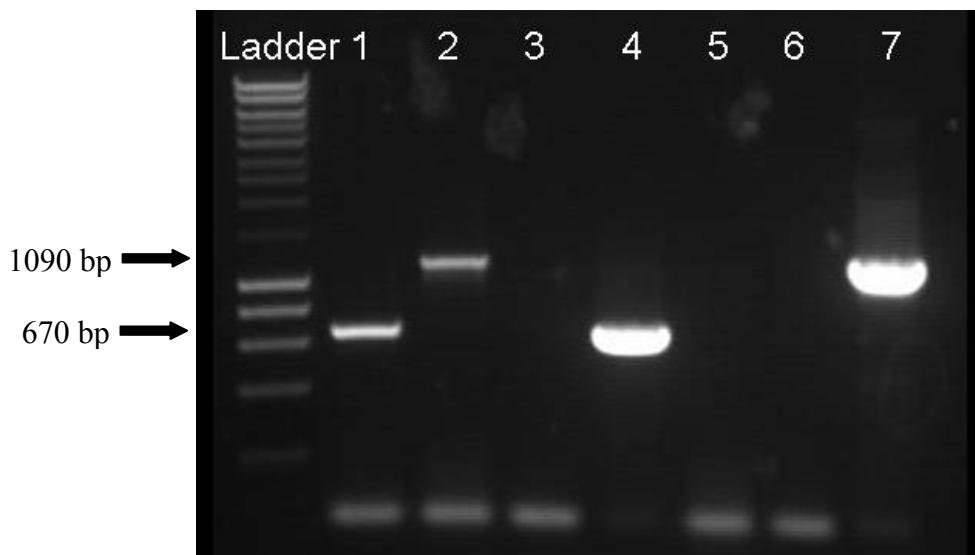
Figure 3.3 Electrophoresis of PCR products with DNA from Arabidopsis transformed with pGreen 0029-35S. Lanes 1 to 3 were three transformed plants (D1101-D3101). Lane 4 was pGreen 0029-35S plasmid DNA as a positive control. Lane 5 was wild type Arabidopsis DNA as a negative control I. In lane 6, the DNA template was replaced with H_2O as a negative control II. Lane 7 was pGreen 0029-35S+*ATA* plasmid DNA as a control III.

3.3.3 Transcription of the Allium triquetrum agglutinin gene in transformed Arabidopsis

Reverse transcription (RT) PCR was used to demonstrate transcription of ATA in transformed plants. RNA was extracted from C1101-C6101 and RT PCR was performed with ORF F and ORF R primers (Figures 3.4, 3.5, and 3.6). Since the D1101 line could grow on kanamycin selection media and contains no *ATA*, there was no need to perform a RT PCR.

In Figure 3.4, there was no band in the RNA preparation that had been treated with DNase whereas lanes 1 and 2 had the same size as the positive control (PC). The results suggested that the RNA preparation was contaminated with genomic DNA (gDNA) while the DNase treated RNA preparation was free of gDNA. Therefore, the band in lane 2 shows that *ATA* has been expressed in line C1101 at the RNA level.

In Figure 3.5, the PCR products of RNA preparations and cDNA of each plant had the same size as the PC. There was a faint band of the same size as the PC in lane 3 and 6 while there was no band in lane 9. The results showed that the RNA preparations were contaminated by gDNA and the DNase treatment removed most of the gDNA from RNA of C2101 and C3101 and all the gDNA from RNA of C4101. Because the bands in

lane 3 and 6 were much fainter than the counterparts in lane 1 and 4, the PCR product in lane 2 and 5 was mostly from cDNA instead of gDNA. Therefore, the bands in lanes 2, 5, and 8 showed that *ATA* has been expressed in lines C2101 to C4101 at the RNA level.

In Figure 3.6, there was a band of the same size as the PC in the first two lanes of each plant respectively. In addition, there was a faint band of same size as the PC in lane 6 whereas there was no band in lane 3. The results showed that the RNA preparations were contaminated by gDNA and the DNase treatment removed most of the gDNA from RNA preparations of C6101 and all the gDNA from RNA of C5101. Because the band in 6 was much fainter than the counterpart in lane 4, the PCR product in 5 was mostly from cDNA instead of gDNA. Therefore, the bands in lanes 2 and 5 showed that *ATA* has been expressed in lines C5101 and C6101 at the RNA level.
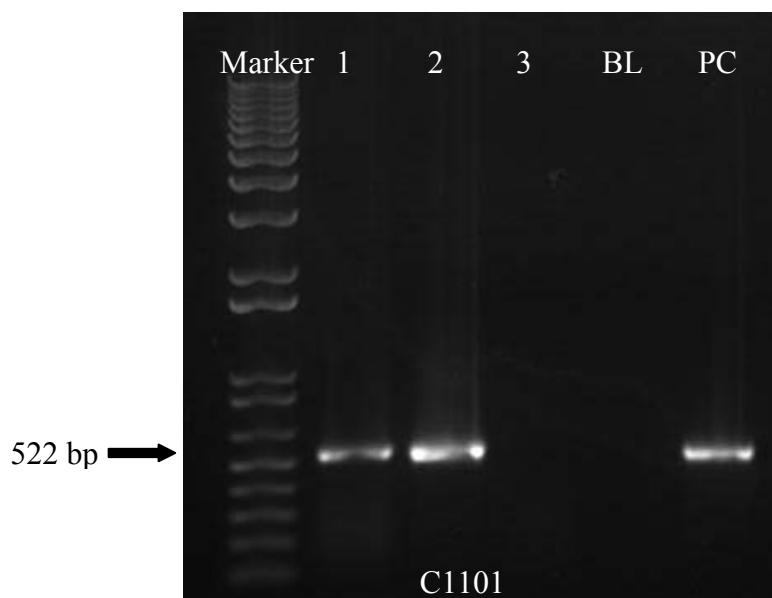
Figure 3.4 Electrophoresis of RT PCR products with RNA from C1101 *ATA*-transformed Arabidopsis. From lane 1 to 3, RNA preparation, cDNA from RT reaction, and DNase-treated RNA preparation were used as template respectively. BL, blank reaction in which DNA template was replaced with H_2O. PC, positive control, pGreen 0029-35S+*ATA*.
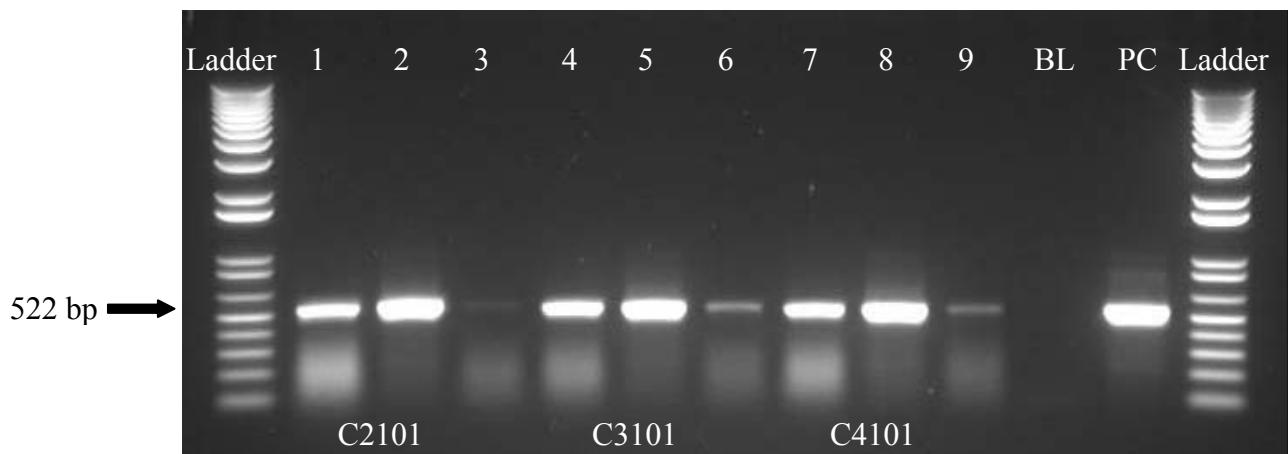
Figure 3.5 Electrophoresis of RT PCR products with RNA from C2101 to C4101
ATA- **transformed Arabidopsis.** Groups of three lanes represent samples from individual plants (Lane 1-3: C2101; Lane 4-6: C3101; Lane 7-9: C4101). The templates used in the first lanes of each plant were RNA preparations. The templates used in the second lanes of each plant were cDNA from the RT reaction. The templates used in the third lanes of each plant were DNase-treated RNA preparations. BL, negative control, in which water was used to replace the template. PC, positive control, pGreen 0029-35S+*ATA*.
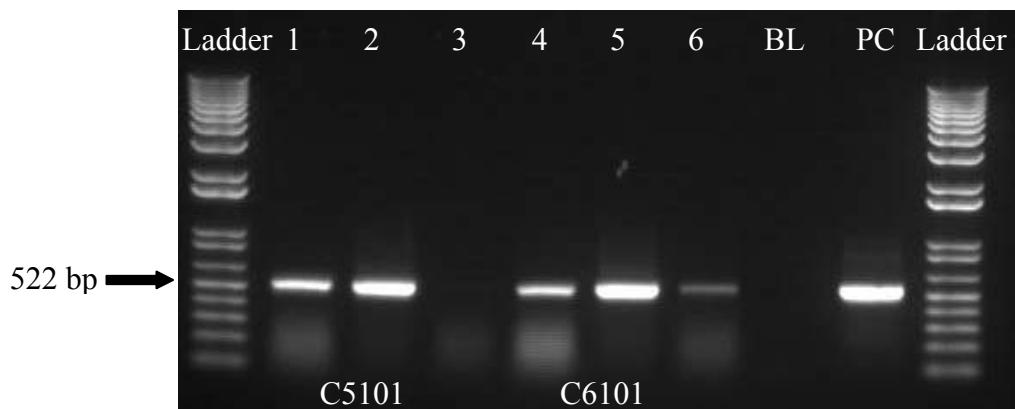
Figure 3.6 Electrophoresis of RT PCR products with RNA from C5101 to C6101
ATA- **transformed Arabidopsis.** Groups of three lanes represent samples from individual plants (Lane 1-3: C5101; Lane 4-6: C6101). The templates used in the first lanes of each plant were RNA preparations. The templates used in the second lanes of each plant were cDNA from the RT reaction. The templates used in the third lanes of each plant were DNase-treated RNA preparations. BL, negative control, in which water was used to replace the template. PC, positive control, pGreen 0029-35S+*ATA*.

3.3.4 Characterization of segregation pattern in T_2-generation of plants

After PCR and RT PCR analysis, C1101-C6101 and D1101 were grown to maturity to obtain T_2-generation seeds. About 200 T_2-generation seeds were grown on kanamycin selection media and green and bleached seedlings were counted in order to determine the segregation pattern, which was characterized with a χ^2 test (Table 3.2). According to Mendel's law, the copy numbers of T-DNA can be determined based on the segregation pattern. If a T_1-generation plant carries a single copy of the T-DNA, the phenotype of the T_2 offspring will show a clear 3:1 segregation ratio. If a T_1-generation plant carries two copies of T-DNA, the segregation ratio of the phenotype of the T_2 offspring will be between 3:1 to 15:1, depending on whether the two copies of T-DNA are linked or not, and on the locations of the T-DNA. Table 3.2 suggests that plant C1101, C2101, C5101, C6101, and D1101 are likely to have only one copy of the *ATA* transgene (or multiple copy at one locus) in their genome whereas plant C3101 and C4101 may contain more than one copy.

Table 3.2 Segregation analysis of T_2-generation seeds. Transformed seedlings remained green while those untransformed were bleached white. Non-germinated seeds were not included. The significance level at P=0.05 for a χ^2 value with one degree of freedom = 3.84 (significant values are shown in **bold**).

	Segregation	Goodness of fit χ^2	
T_1 plant	Green: Bleached	3:1	15:1
C1101	134 : 56	1.80	**170.95**
C2101	155 : 41	1.53	**69.50**
C3101	27 : 1	**5.76**	0.038
C4101	104 : 13	**11.31**	**3.93**
C5101	59 : 26	1.13	**81.83**
C6101	54 : 14	0.49	**21.47**
D1101	113:36	0.00045	**5851**

3.3.5 Identification of homozygous lines in T_3 -generation seeds

10 to 20 kanamycin resistant T_2-generation seedlings were randomly chosen and transferred to soil to produce T_3 seed to establish homozygous lines. 80 T_3-generation seeds of each line were grown on kanamycin. The percentage of the healthy (green) seedlings was used to identify homozygous lines (Table 3.3 – Table 3.9). If the geminated seedlings from one single line are all healthy, that line is homozygous for the transgene (or for one of the transgenes if multiple copies are present).

Table 3.3 suggests that many lines showed a rough 3:1 phenotype ratio and the phenotype ratios of most of the lines were between 1:1 and 3:1. However, except for one un-germinated seed, all the seedlings from C1212 were green, which indicates it is a homozygous line. Therefore, the seeds from C1212 were selected for further experiments.

Table 3.4 suggests that many of the lines showed a rough 3:1 phenotype ratio, whereas a few lines showed some different ratios, such as 4:1 and 5:1. However, except for three un-germinated seeds, all the seedlings in C2212 were green, which means it is a homozygous line. Therefore, the seeds from C2212 were selected for further experiments.

Table 3.5 suggests that only two lines showed a rough 3:1 phenotype ratio. For many more lines, except for a few un-germinated seeds, all the seedlings were green. The fact that more than 1/4 of the lines are not segregating for kanamycin resistance reinforced that assumption that line C3101 contains more than one copy of the transgene. The seeds from C3202 were selected for further experiments because of its high germination rate.

Table 3.6 suggests that most of lines showed a phenotype ratio between 20:1 and 3:1. In four lines, C4205, C4206, C4207, and C4214, except for a few un-germinated seeds, all the seedlings were green, which means these four lines are homozygotes. The seeds from C4214 were selected for further experiments because of its high germination rate

Table 3.7 suggests that except line C5206, all the geminated seedlings of other lines showed a rough 3:1 phenotype ratio. For line C5206, except for six un-germinated seeds, all the other seedlings were green, which means it is a homozygous line. Therefore, the seeds from C5206 were selected for further experiments.

Table 3.8 suggests that most of the lines showed a 3:1 phenotype ratio. A few lines showed a 2:1 ratio. Only two seeds geminated in line C6203 were green, so it was not used for aphid bioassay even though both these two seedlings were green. For line C6207, except for two un-germinated seeds, all the seedlings were green, which means it is a homozygous line. Therefore, the seeds from C6207 were selected for further experiments.

Table 3.9 suggests that most of the lines showed a rough 3:1 phenotype ratio. However, for three lines, D1201, D1204, and D1206, except for a few un-germinated seeds,

all the seedlings were green, which means these three lines are homozygotes. The seeds from D1201 were selected for further experiments because of its high germination rate .

Thus, the T_3 -generation seeds of C1212, C2212, C3202, C4214, C5206, C6207, and D1201 were used in further experiments. For convenience, the line names of their parental lines were used when describing plants grown from these T_3-generation seeds in further experiments.

Table 3.3 Homozygote screening in T_3-generation of C1101

T_2 Plant	Green	Bleached	Non-germinating
C1201	36	44	0
C1202	59	21	0
C1203	50	30	0
C1204	41	39	0
C1205	58	22	0
C1206	48	32	0
C1207	65	15	0
C1208	46	34	0
C1209	57	23	0
C1210	62	18	0
C1211	56	24	0
*C1212	79	0	1

The line marked with the asterisk was chosen for further experiments

Table 3.4 Homozygote screening in T$_3$-generation of C2101

Plant Code	Green	Bleached	Non-germinating
C2201	58	22	0
C2202	60	13	7
C2203	54	26	0
C2204	67	8	5
C2205	65	14	1
C2206	63	16	1
C2207	61	12	7
C2208	54	21	5
C2209	49	19	12
C2210	61	16	3
C2211	71	9	0
*C2212	77	0	3
C2213	58	22	0
C2214	75	4	1
C2215	58	21	1
C2216	68	10	2
C2217	66	5	9
C2218	60	16	4
C2219	70	9	1

The line marked with the asterisk was chosen for further experiments

Table 3.5 Homozygote screening in T$_3$-generation of C3101

Plant Code	Green	Bleached	Non-germinating
C3201	60	16	4
*C3202	76	0	4
C3203	65	0	15
C3204	57	0	23
C3205	71	0	9
C3206	62	0	18
C3207	61	0	19
C3208	64	0	16
C3209	75	0	5
C3210	51	2	27
C3211	56	0	24
C3212	56	0	24
C3213	49	23	8
C3214	69	0	11

The line marked with the asterisk was chosen for further experiments

Table 3.6 Homozygote screening in T$_3$-generation of C4101

Plant Code	Green	Bleached	Non-germinating
C4201	58	16	6
C4202	62	2	16
C4203	62	7	11
C4204	60	11	9
C4205	41	0	39
C4206	51	0	29
C4207	56	0	24
C4208	55	6	19
C4209	50	10	20
C4210	48	2	30
C4211	57	6	17
C4212	44	12	22
C4213	41	4	35
*C4214	64	0	16
C4215	44	5	21
C4216	43	12	25
C4217	32	4	44
C4218	32	5	43

The line marked with the asterisk was chosen for further experiments

Table 3.7 Homozygote screening in T$_3$-generation of C5101

Plant Code	Green	Bleached	Non-germinating
C5201	58	19	3
C5202	52	26	2
C5203	57	21	3
C5204	52	25	3
C5205	62	17	1
*C5206	74	0	6
C5207	49	21	10
C5208	57	21	3
C5209	63	17	0
C5210	59	20	1

The line marked with the asterisk was chosen for further experiments

Table 3.8 Homozygote screening in T$_3$-generation of C6101

Plant Code	Green	Bleached	Non-germinating
C6201	30	15	35
C6202	27	13	40
C6203	2	0	78
C6204	33	13	34
C6205	45	24	11
C6206	54	20	6
*C6207	78	0	2
C6208	47	17	16
C6209	42	12	26
C6210	39	18	23

The line marked with the asterisk was chosen for further experiments

Table 3.9 Homozygote screening in T$_3$-generation of D1101

Plant Code	Green	Bleached	Non-germinator
*D1201	74	0	6
D1202	59	17	4
D1203	52	24	4
D1204	72	0	8
D1205	59	17	14
D1206	76	0	4
D1207	54	22	4
D1208	56	20	4
D1209	63	16	3
D1210	58	12	10
D1211	47	15	18
D1212	60	16	4
D1213	59	19	2
D1214	62	15	3
D1215	40	8	32

The line marked with the asterisk was chosen for further experiments

3.3.6 Translation of the Allium triquetrum agglutinin mRNA in plants

Western blotting was used to demonstrate the expression of ATA in the homozygous lines. Protein was extracted from the six homozygous *ATA*-transformed lines, empty vector transformed line (D1201), wild type Col-0, and *A. triquetrum*. The serum of the last bleed was used to detect the existence of ATA in the total protein extracts (Figure 3.7) and the serum of pre-immune blood was used as a negative control (Figure 3.8).

Figure 3.7 demonstrates that all the bands detected in the six *ATA* transformed lines had the same size as the positive control (PC) indicating the presence of a 12kDa protein as expected if the *ATA* is expressed. There was also a band of about 100 kDa detected in the six *ATA*-transformed lines and the empty vector transformed line, D1201. However, this band is absent from protein from either wild type Col-0 or the PC.

Figure 3.8 shows no band was detected in all the total protein of different lines when probed with the pre-immune serum.
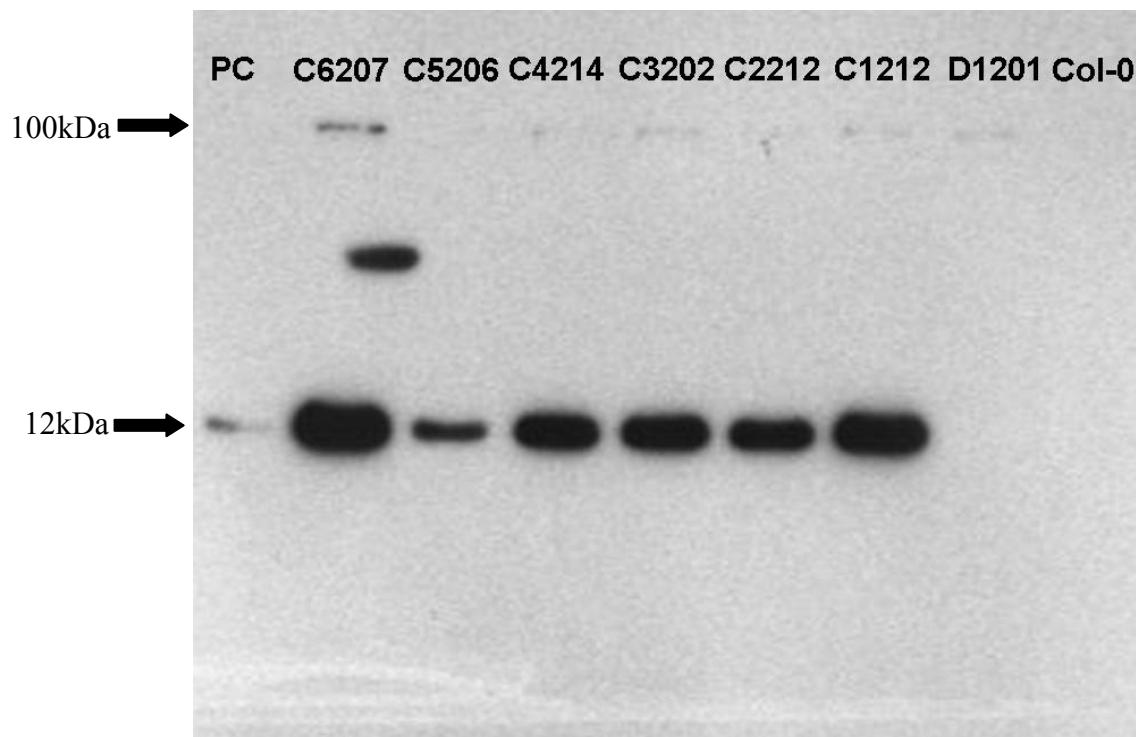
Figure 3.7 Western blots showing expression of *ATA* in six homozygous lines (C1212-C6207) using serum from the last bleed. PC, positive control, proteins from *A. triquetrum*. Negative controls, protein from D1201 and wild type Col-0.

Figure 3.8 Western blots of expression of ATA in six homozygous lines (C1212-C6207) with serum of pre-immune bleed. PC, positive control, proteins from *A. triquetrum*. Negative controls, protein from D1201 and wild type Col-0.

3.3.8 Analysis of the germination time of the homozygous lines

The germination time (GT) analysis was performed in both short (L:10/D:14) and long (L:16/D:8) photoperiods. The Kolmogorov-Smirnov (K-S) normality test indicated that the data from GT analysis were not normally distributed and the data transformation was not useful. The non-parametric Kruskal-Wallis (K-W) test suggested that the data from the two replicates were statistically identical. Therefore, the data from the two replicates were pooled and processed with K-W Test (Figure 3.9 and 3.10).

In long photoperiods (Figure 3.9), there was a significant difference of GT between different lines (H = 95.86, d.f. = 7, P < 0.001). Therefore, a least significant difference (LSD) test was performed to reveal the GT of which lines were different from others. The results showed that the GT of D1201, C2212, and C6207 were significantly shorter than that of the wild type Col-0. However, it took a longer time for C3202, C4214, and C5206 to germinate than the wild type Col-0.

In short photoperiods (Figure 3.12), there was a significant difference of GT between lines (H = 92.12, DF = 7, P < 0.001). Therefore, a LSD test was performed to reveal the GT of which lines were different from others. The results showed that the GT of C1212, C2212, and C6207 were significantly shorter than that of the wild type Col-0. However, it took a longer time for D1201, C3202, C4214, and C5206 to germinate than the wild type Col-0.
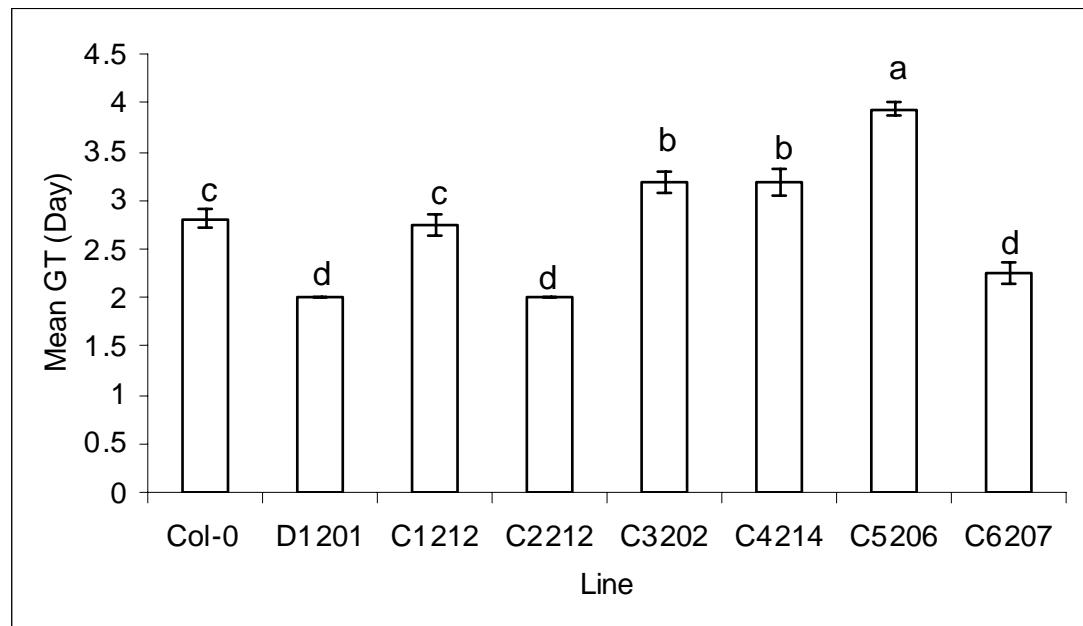
Figure 3.9 Germination times of eight lines grown in long photoperiods. The X axis indicates different line tested and the Y axis indicates the mean GT for each line. Error bars = Stand Error (SE). The letters above each data bar indicates the result of the LSD test.
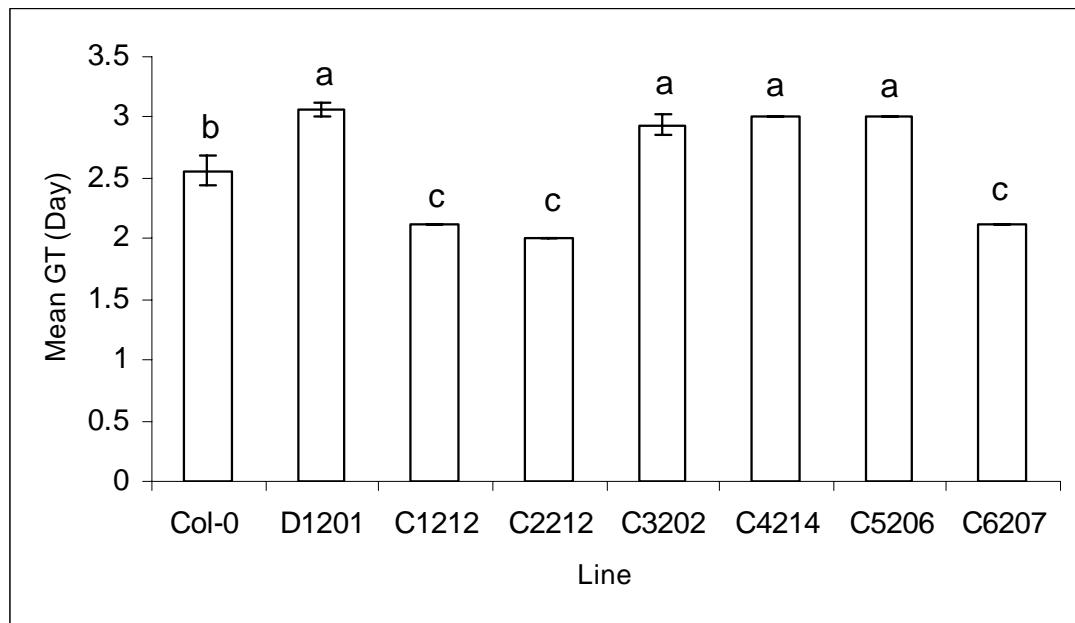
Figure 3.10 Germination times of eight lines grown in short photoperiods. The X axis indicates different line tested and the Y axis indicates the mean GT for each line. Error bars = SE. The letters above each data bar indicates the result of the LSD test.

3.3.9 Analysis of the time taken to reach flowering (FT) of the homozygous lines

The K-S normality test showed that the FT values were normally distributed. The two-way Analysis of Variance (ANOVA) was applied to compare the FT of different lines grown in short (Figure 3.11) and long (Figure 3.13) photoperiods.

In short photoperiods (Figure 3.11), there was a significant difference in FT between different lines ($F_{7,112} = 9.97$, P<0.001), but there was also a significant difference in FT between the replicates of each line ($F_{1,112} = 34.06$, P < 0.001), and there was no significant interaction between the two factors ($F_{7,112} = 0.76$, P = 0.619). The LSD test showed, except for C3202, that the FT of all the other lines were significantly shorter than the wild type Col-0. Since there was a significant difference between the replicates, an interaction plot (Figure 3.12) was produced to illustrate the way in which the FT depended on two factors.

In short photoperiods (Figure 3.12), the interaction plot showed that the FT of all the lines in the second replicate were all shorter and therefore the shorter FT in the second replicate was possibly due to of an unknown system difference.

In long photoperiods (Figure 3.13), there was a significant difference in FT between different lines ($F_{7,112} = 5.08$, P<0.001). However, there was also a significant

difference in FT between the replicates ($F_{1,112}$ = 9.21, P = 0.003) and there was no significant interaction between the two factors ($F_{7,112}$ = 0.07, P = 0.999). The LSD test showed except for line C1212 and C3202, that all the other lines have the same FT as the wild type Col-0. Since there was significant difference between the replicates, an interaction plot (Figure 3.14) was produced to illustrate the way in which the FT depended on two factors.

In long photoperiods (Figure 3.14), the interaction plot showed the FT of all the lines in the second replicate were all longer and therefore the longer FT in the second replicate was possibly because of an unknown system difference.

To analyse the interaction of the different photoperiods and the different lines, the mean FT under both photoperiods were plotted (Figure 3.15). A correlation test showed there was no correlation (p = 0.135) between the FT during growth in the two photoperiods. The patterns of the FT in the two different photoperiods appear different, which was supported by the correlation test.
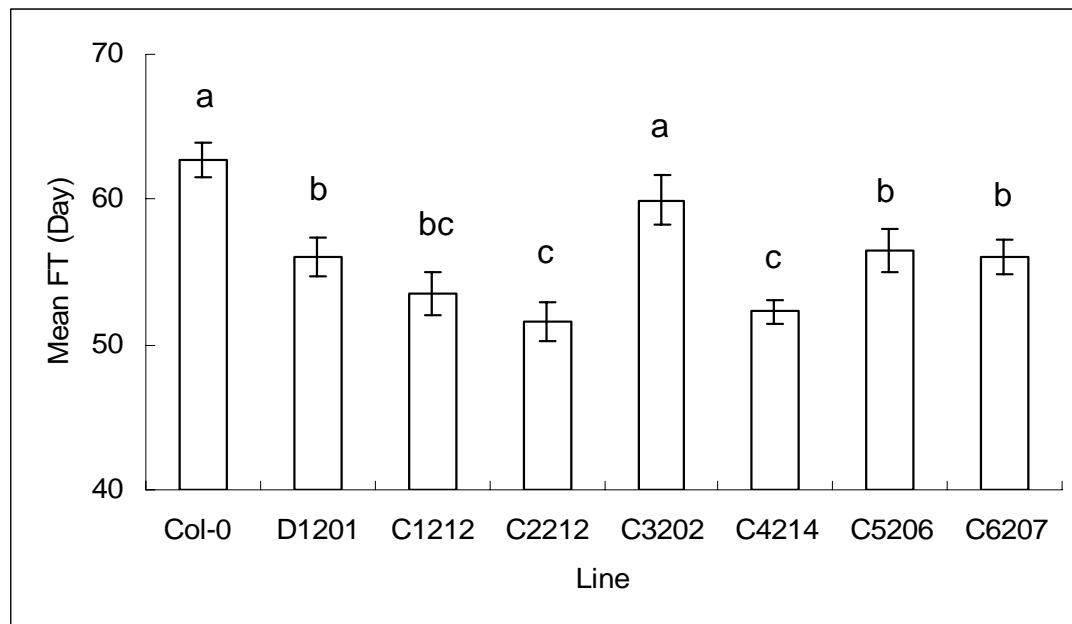
Figure 3.11 The mean time taken to reach flowering (FT) for different lines grown in short photoperiods. The X axis indicates the different lines tested and the Y axis indicates the mean FT for each line. Error bars = SE. The letters above each data bar indicate the result of the LSD test.
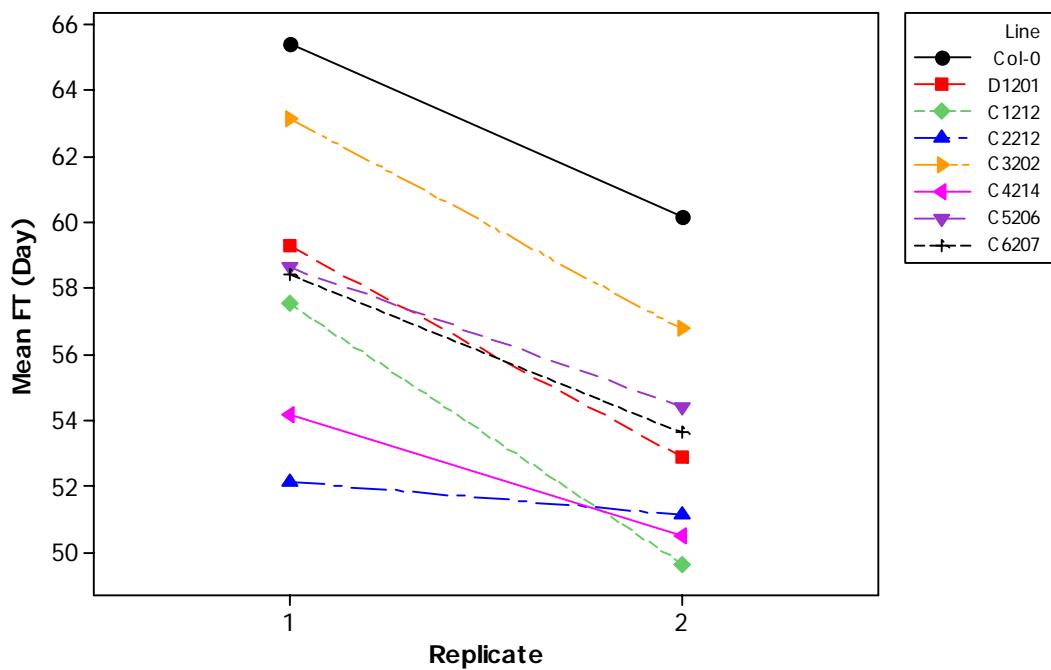
Figure 3.12 Interaction plot for the mean time taken to reach flowering (FT) with two factors: replicates and lines grown in short photoperiods. The X axis indicates two replicates tested and the Y axis indicates the mean FT for each line.
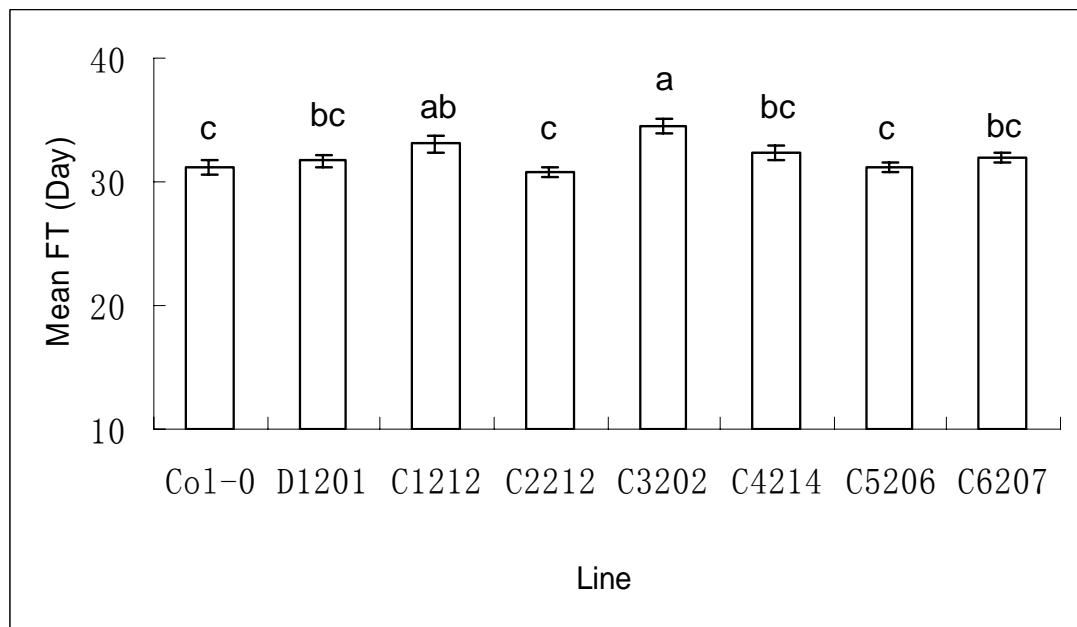
Figure 3.13 The mean time taken to reach flowering (FT) for different lines grown in long photoperiods. The X axis indicates the different lines tested and the Y axis indicates FT for each line. Error bars = SE. The letters above each data bar indicate the result of the LSD test.
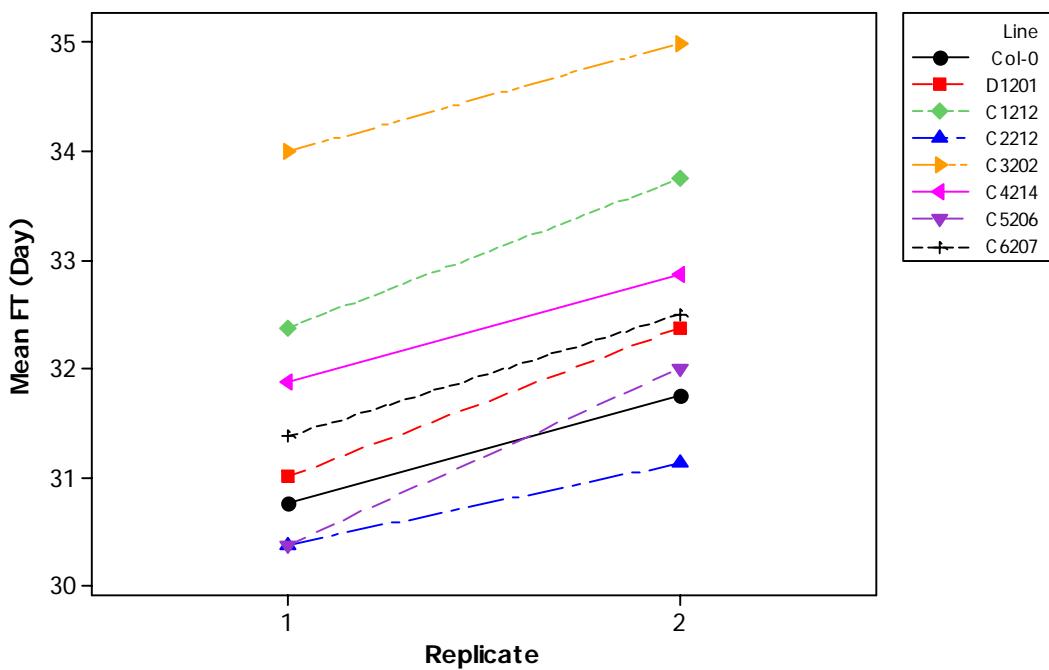
Figure 3.14 Interaction plot for the mean time taken to reach flowering (FT) with two factors: replicates and lines in long photoperiods. The X axis indicates two replications tested and the Y axis indicates the mean FT for each line.
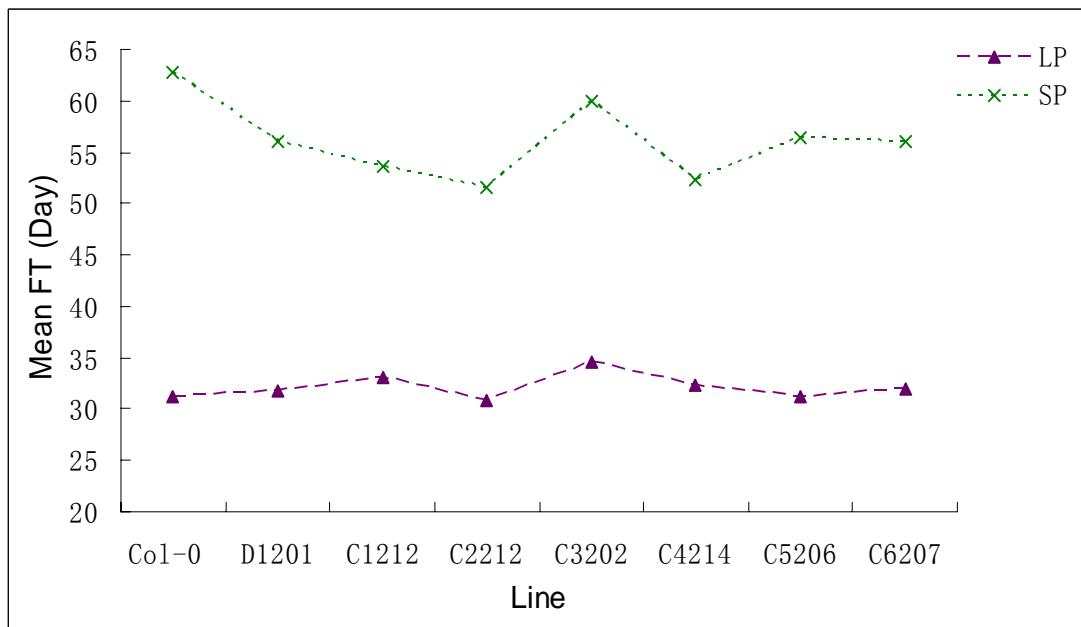
Figure 3.15 Mean time taken to reach flowering (FT) of eight lines grown in both photoperiods. The X axis indicates the lines tested and the Y axis indicates the mean FT for each line. LP, long photoperiods; SP, short photoperiods. The data points for the same photoperiods were joined to reveal the general pattern of FT grown in a certain photoperiods.

3.3.10 Analysis of the total leaf number (LN) at flowering stage of the homozygous lines

The K-S normality test shows the data is normally distributed. Two-way ANOVA was applied to compare the total leaf numbers (LN) of the different lines at flowering under short (Figure 3.16) and long (Figure 3.18) photoperiods.

In short photoperiods (Figure 3.16), there was a significant difference of LN between different lines ($F_{7,112}$ = 40.86, P<0.001). But there was also a significant difference of LN between the two replicates ($F_{1,112}$ = 10.91, P = 0.001) and the interaction between the two factors was not significant ($F_{7,112}$ = 1.58, P = 0.150). The LSD test showed the LN of line C1212 was significantly higher than that of wild type Col-0; and the LN of line D1201, C3202, C4214, and C5206 are significantly lower than that of wild type Col-0; whilst the LN of line C2212, C6207 were the same as that of the wild type Col-0. Because there was a significant difference between the two replicates, an interaction plot (Figure 3.17) was produced to demonstrate the way in which the LN depended on two factors.

In short photoperiods (Figure 3.17), the interaction plot showed that the LN of all the lines in the second replicate were all lower than the first replicate and therefore was possibly due to an unknown system difference in two replicates. Despite the variation between the two replicates, the rank order of each line was similar suggesting genuine differences in LN by flowering.

In long photoperiods (Figure 3.18), there was a significant difference of LN between different lines ($F_{7,112}$ = 28.57, P<0.001). However, there was also a significant difference in LN between the two replicates ($F_{1,112}$ = 10.22, P = 0.002) and there was no significant interaction between the two factors ($F_{7,112}$ = 0.93, P = 0.483). The LSD test showed the LN of line C3202, C4214, and C5206 were significantly lower than that of the wild type Col-0, whereas the other lines had the same LN as the wild type Col-0. Because there was a significant difference between the two replicates, an interaction plot (Figure 3.19) was produced to illustrate the way in which the LN depended on two factors.

In long photoperiods (Figure 3.19), the interaction plot showed the FT of all the lines in the second replicate were all longer than the first replicate and therefore was possibly due to an unknown system difference in two replicates. Despite the variation between the two replicates, the rank order of each line was similar suggesting genuine differences in LN by flowering.

To analyse the interaction of different photoperiods and different lines, the mean LN of eight lines in both photoperiods was plotted (Figure 3.20). A correlation test showed there was a correlation (p < 0.001) between the LN of the eight lines in the two

photoperiods. The pattern of LN in the two different photoperiods looks similar, which is supported by the correlation test.
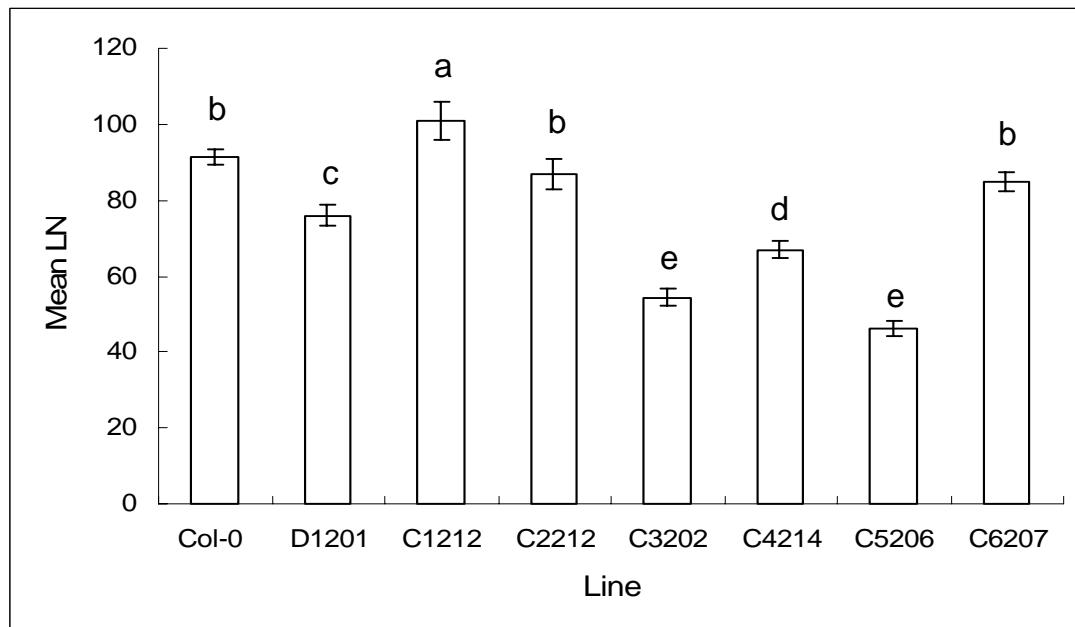
Figure 3.16 Mean total leaf numbers (LN) at flowering time of different lines grown in short photoperiods. The X axis indicates the different lines tested and the Y axis indicates the mean LN for each line. Error bars = SE. The letters above each data bar indicate the result of the LSD test.
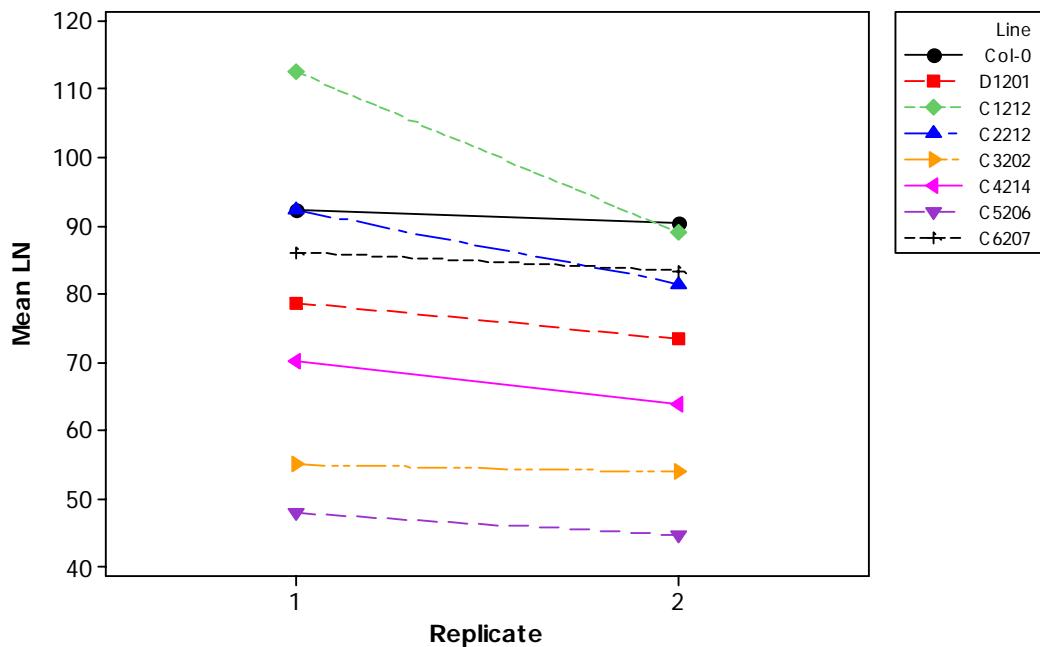
Figure 3.17 Interaction plot for mean total leaf number (LN) with two factors: replicates and lines in short photoperiods. The X axis indicates two replicates tested and the Y axis indicates the mean LN for each line.
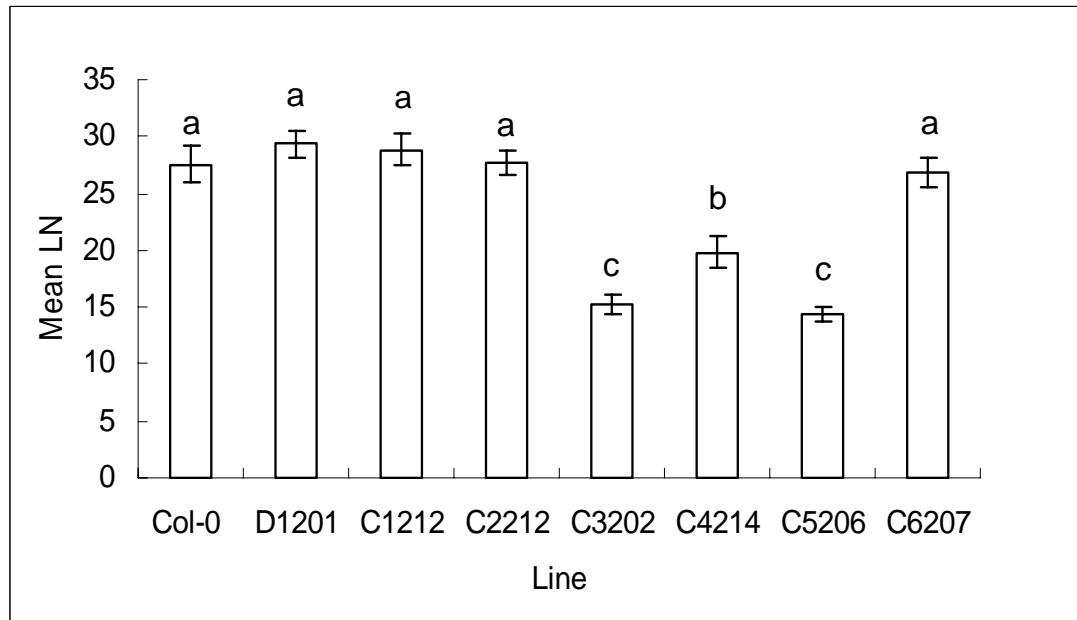
Figure 3.18 Mean total leaf numbers (LN) at flowering in different lines in long photoperiods. The X axis indicates the different lines tested and the Y axis indicates the mean LN for each line. Error bars = SE. The letters above each data bar indicate the result of the LSD test.
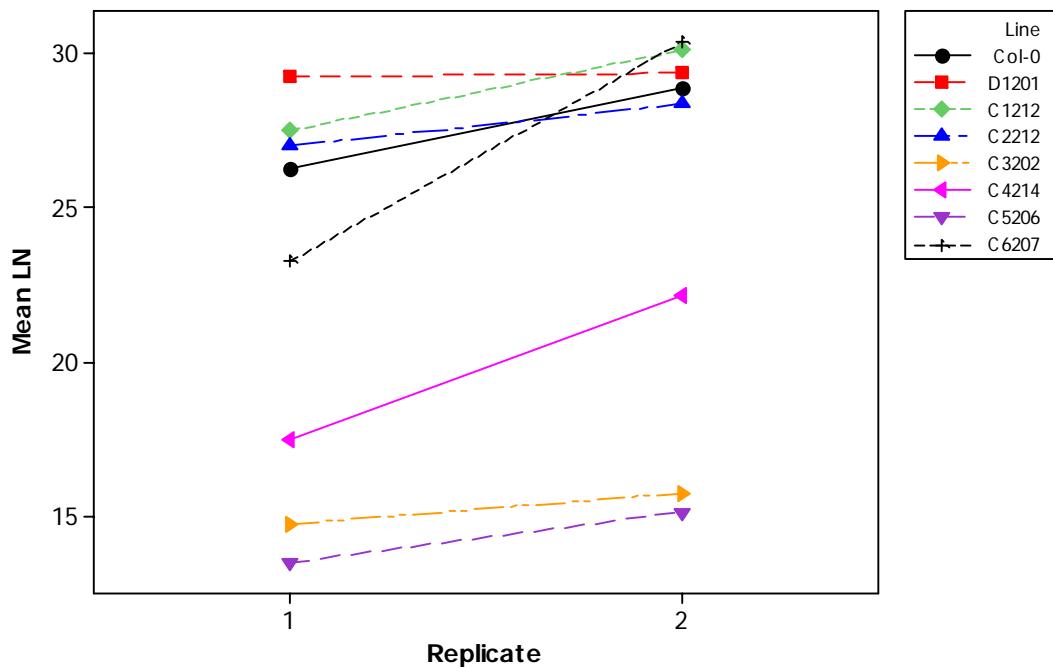
Figure 3.19 Interaction plot for mean total leaf number (LN) with two factors: replicates and lines in long photoperiods. The X axis indicates two replicates tested and the Y axis indicates the mean LN for each line.
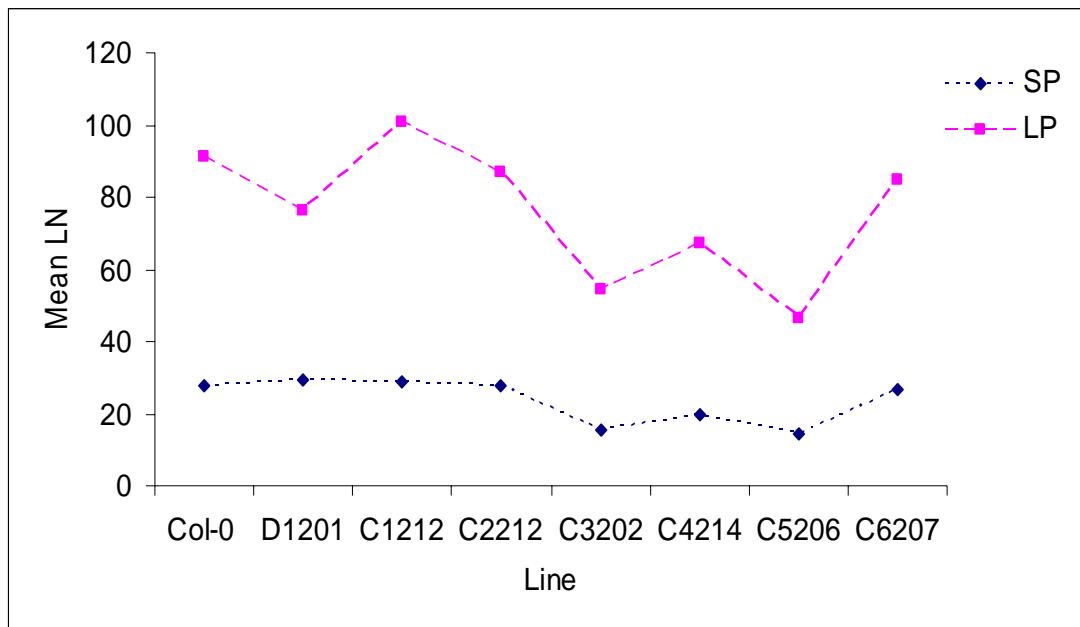
Figure 3.20 Mean total leaf number (LN) of eight lines grown in both photoperiods.
The X axis indicates different line tested and the Y axis indicates the mean LN for each
line. LP indicates long photoperiod and SP indicates short photoperiod. The data points for
the same photoperiods were joined to reveal the general pattern of LN grown in a certain
photoperiods.

3.3.11 Analysis of Dry Mass (DM) of the homozygous lines

The K-S normality test shows the DM data in the long photoperiod were not
normally distributed. Therefore, a square root transformation was applied to make the data
normally distributed. Two-way ANOVA was applied to compare the DM values in the
short photoperiods (Figure 3.21) and the square root transformed DM values in the long
photoperiods (Figure 3.23).

In short photoperiods (Figure 3.21), there was a significant difference of DM
between the eight lines ($F_{7,112} = 21.22$, $p < 0.001$) and between the replicates ($F_{1,112} = 24.54$,
$p < 0.001$). There was no significant interaction between lines and replicates ($F_{7,112} = 1.78$,
$p = 0.097$). The LSD test showed that the DM of line C2212, C3202, C4214, and C5206
were significantly lower than that of the wild type Col-0, while the other lines have the
same DM as the wild type Col-0. Because there was a significant difference between the
two replicates, an interaction plot (Figure 3.22) was produced to illustrate the way in which
the DM depends on two factors.

In short photoperiods (Figure 3.22), the interaction plot showed except for the
wild type Col-0 and C6207, the DM of all the other lines in the second replicate showed an

obvious decline compared with the first replicate. The curves represent the wild type Col-0 and C6207 are level, meaning the unknown factor(s) which caused the change in other lines failed to affect these two lines. However, considering the deviation of the sample and the results of the two-way ANOVA (Figure 3.21), the unknown factor(s) might also have impact on the wild type Col-0 and C6007, but it was not shown in these observations. However, it is clear that the rank order was fairly similar between replicates and therefore the lines did have different dry masses.

In long photoperiods (Figure 3.23), there was a significant difference in the DM between the eight lines ($F_{7,112} = 11.23$, $p < 0.001$), and there was no significant difference of DM between two replicates ($F_{1,112} = 2.99$, $p = 0.087$). There was no significant interaction between lines and replicates ($F_{7,112} = 0.26$, $p = 0.968$). The LSD test showed only the DM of lines C3202 and C5206 were lower than that of Col-0 whereas the other lines had the same DM as the wild type Col-0. Although there was no statistically significant difference of DM between two replicates, the p value is quite close to 0.05, an interaction plot of the two factors was still produced (Figure 3.24).

In long photoperiods condition (Figure 3.24), the DM of the second replicate was higher compared with that of the first replicate. However, the rank order was similar between replicates and therefore it is likely the lines did have different dry masses.

The figures of the mean LN and mean DM showed similar patterns in short and long photoperiods (Figure 3.16, 3.18, 3.21, 3.23), and therefore, correlation tests were performed to reveal the relationships between LN and DM. The correlation test shows there was a significant correlation between LN and DM in both short (Figure 3.25) and long (Figure 3.26) photoperiods ($p < 0.001$).
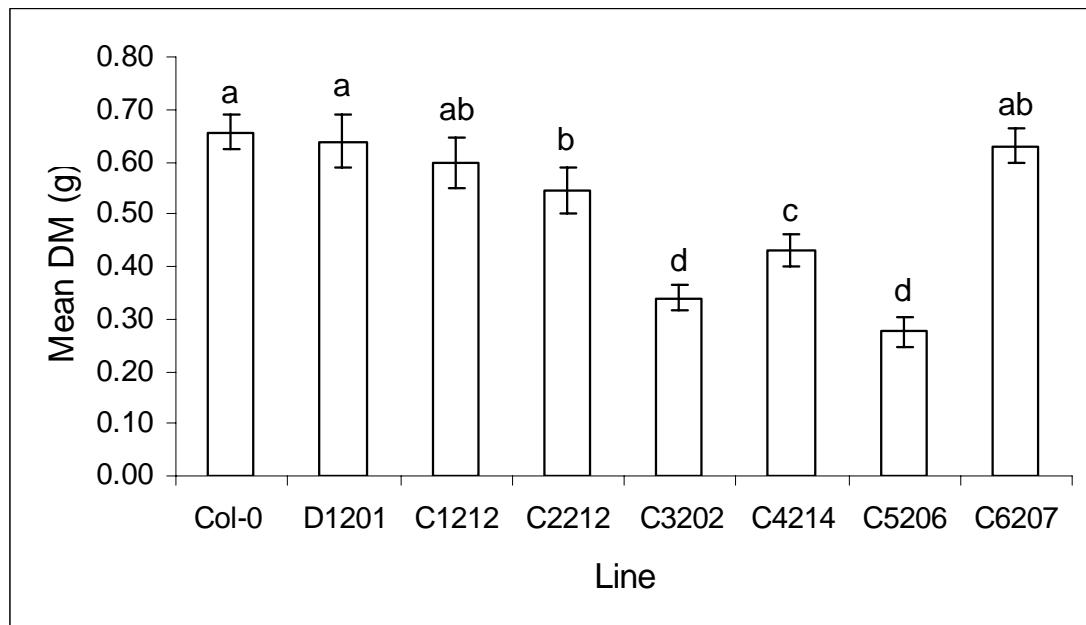
Figure 3.21 The mean dry mass (DM) of different lines grown in short photoperiods.
The X axis indicates the different lines tested and the Y axis indicates the mean DM for
each line. Error bars = SE. The letters above each data bar indicate the result of the LSD
test.
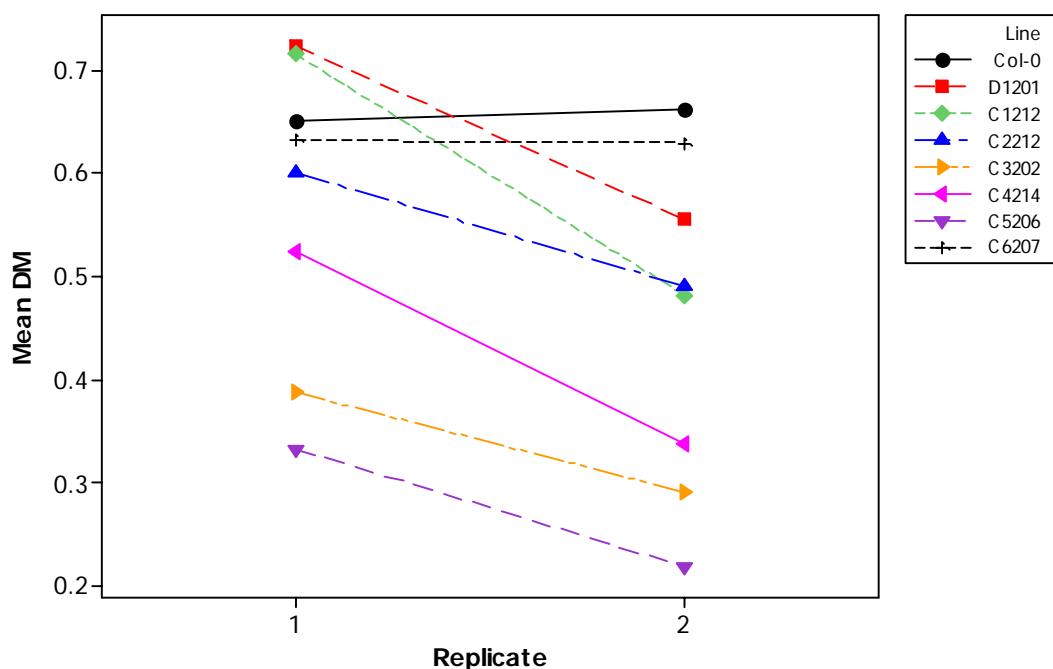
**Figure 3.22 Interaction plot for mean dry mass (DM) with two effects: replicates and
lines in short photoperiods.** The X axis indicates two replicates tested and the Y axis
indicates the mean DM for each line.
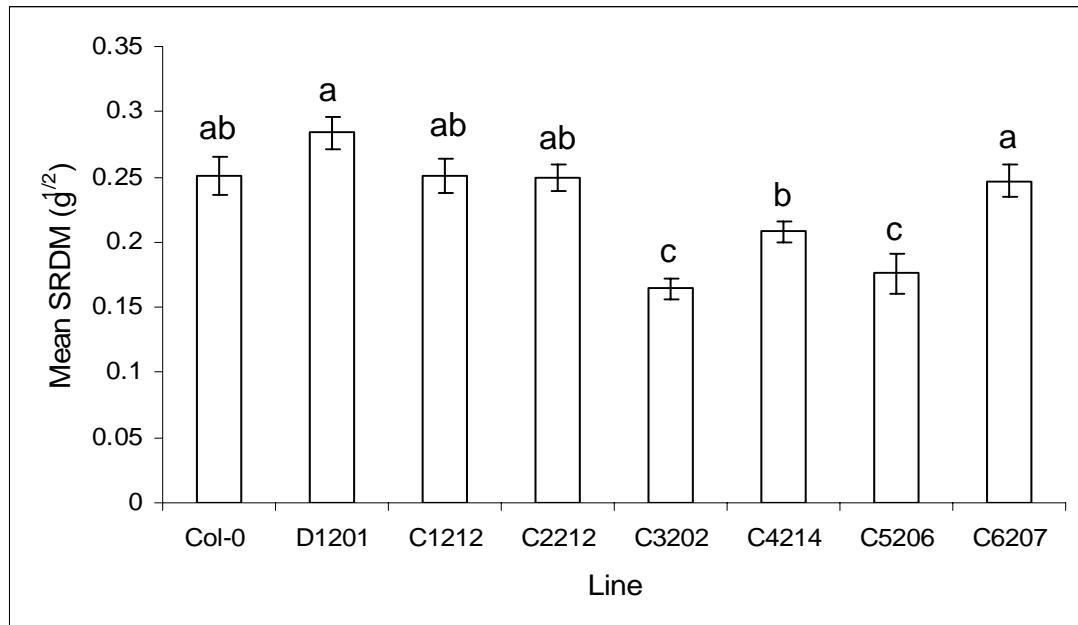
Figure 3.23 The square root transformed mean dry mass (SRDM) of different lines grown in long photoperiods. The X axis indicates the different lines tested and the Y axis indicates the mean SRDM for each line. Error bars = SE. The letters above each data bar indicate the result of the LSD test.
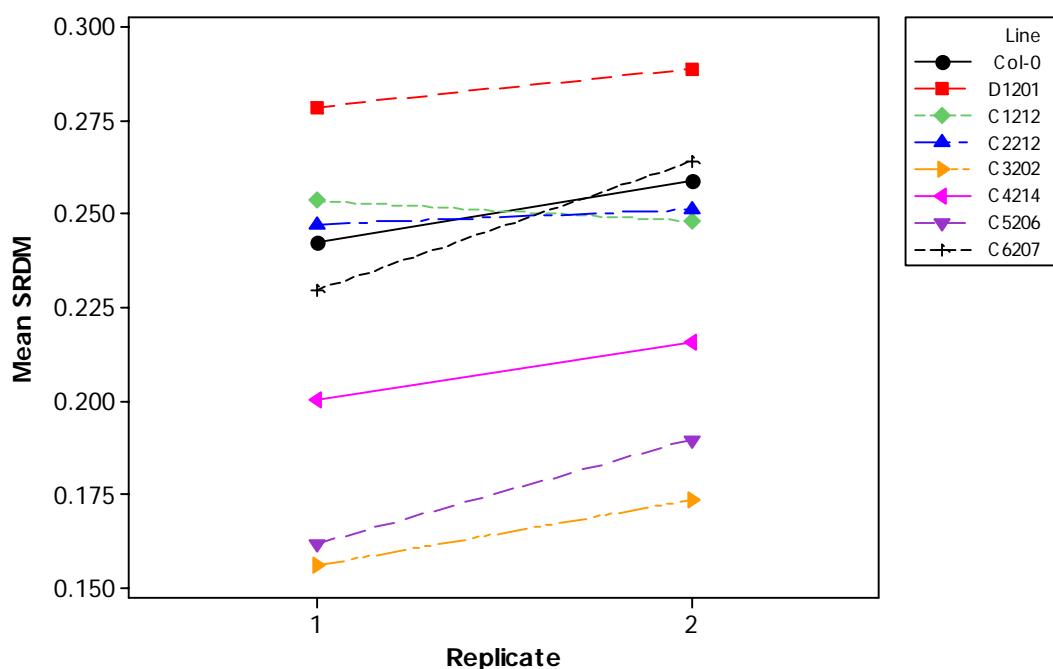
Figure 3.24 Interaction plot for the square root transformed dry mass (SRDM) with two effects: replicates and lines in long photoperiods. The X axis indicates two replicates tested and the Y axis indicates the mean SRDM for each line.
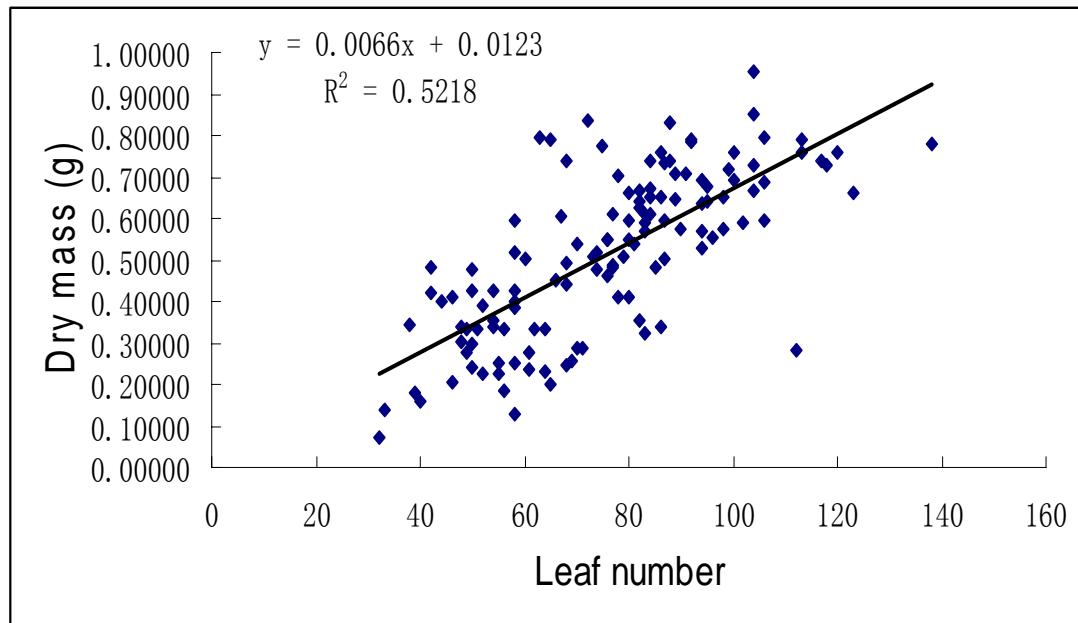
Figure 3.25 Correlation between the total leaf number (LN) and the dry mass (DM) of plants grown in short photoperiods. The X axis indicates the LN of the tested plants and the Y axis indicates the DM of the tested plants.
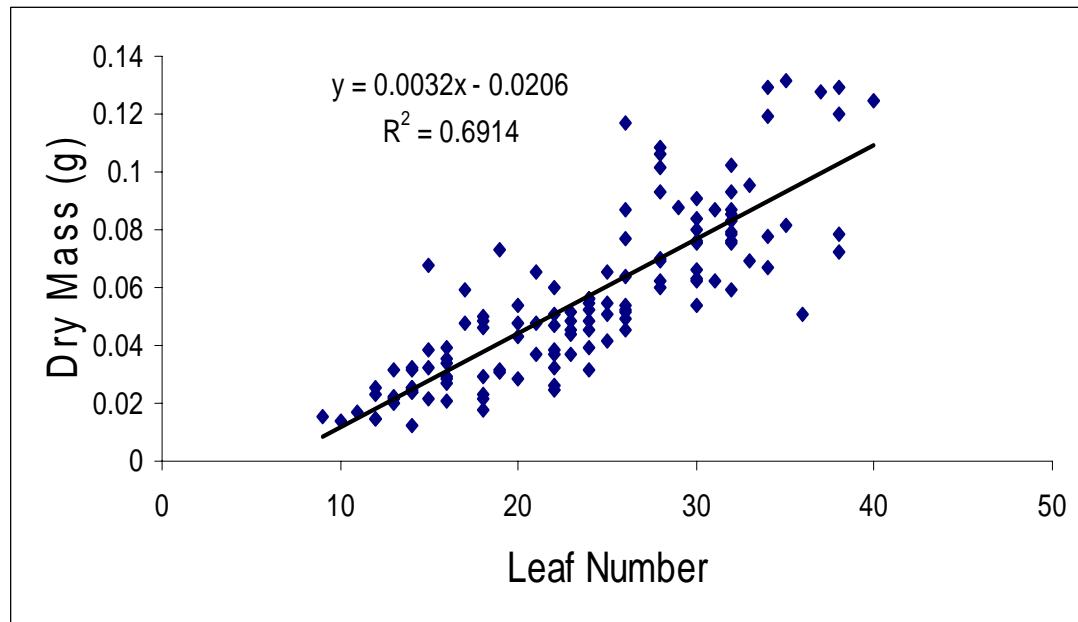
Figure 3.26 Correlation between the total leaf number (LN) and the dry mass (DM) of plants grown in long photoperiods. The X axis indicates the LN of the tested plants and the Y axis indicates the DM of the tested plants.

3.3.12 Analysis of Seed yield (SY) and seed mass (SM) of the homozygous lines

Total seeds were harvested from eight plants of each line. Both total SY and SM were measured. The K–S normality test showed the data were normally distributed. One way ANOVA was applied to compare the SY (Figure 3.27) and SM (Figure 3.28) of the different lines.

There was a significant difference in the SY between the eight lines (Figure 3.27), (F = 9.31, d.f. = 7, p < 0.001). The LSD test showed that, except for line C6207, the SY for all the other lines were significantly lower than the wild type Col-0.

There was a significant difference in the SM between the eight lines (Figure 3.28), (F = 4.35, d.f. = 7, p = 0.001). The results showed that the SM for lines D1201, C3202, and C5206 were lower than that of the wild type Col-0 whereas all the other lines had the same SM as the wild type Col-0.

Because there was not much difference between the SM of different lines, a correlation test was performed between the SY and the seed number (SN) (Figure 3.29). The correlation test showed there was a significant correlation between SN and SY, (F = 902.75, p < 0.001).
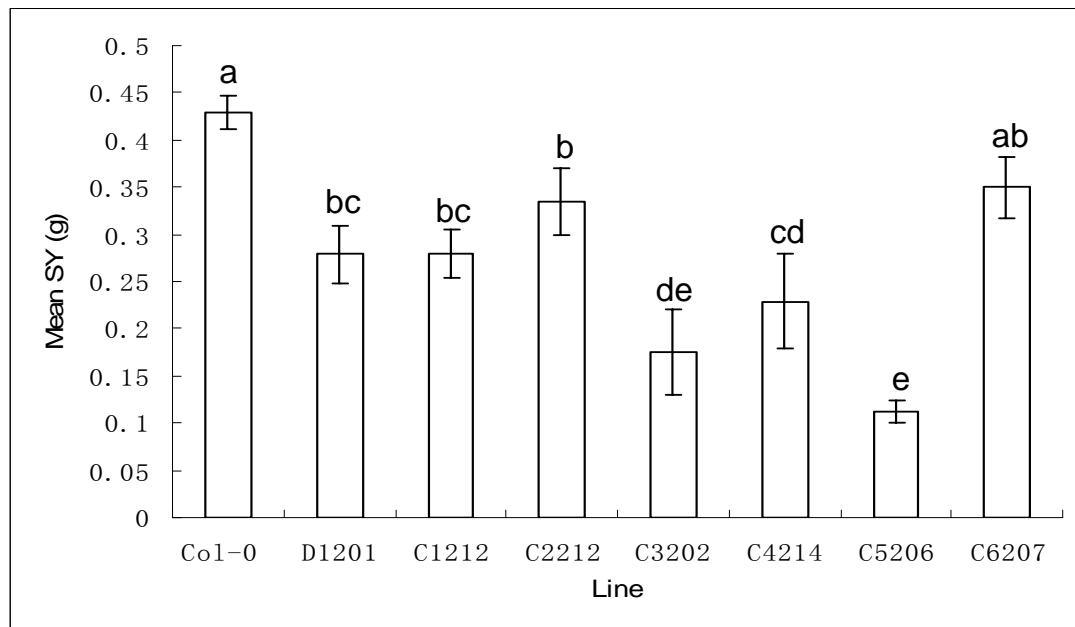
Figure 3.27 Mean seed yield (SY) for different lines. The X axis indicates the different lines tested and the Y axis indicates the mean SY for each line. Error bars = SE. The letters above each data bar indicate the result of the LSD test.
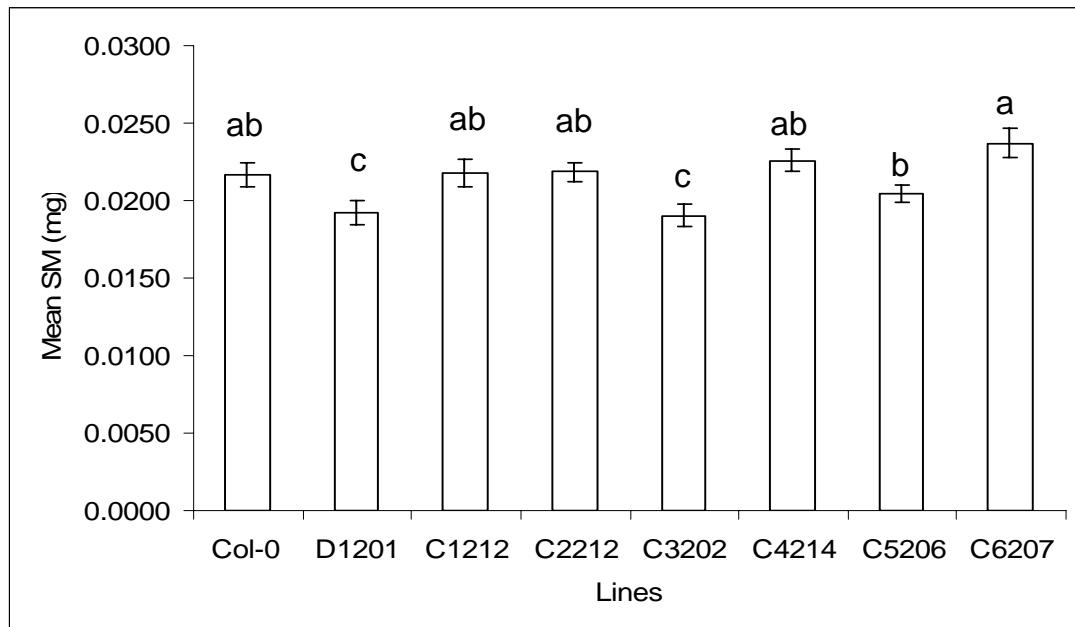
Figure 3.28 Mean seed mass (SM) for different lines. The X axis indicates the different lines tested and the Y axis indicates the mean SM for each line. Error bars = SE. The letters above each data bar indicates the result of the LSD Test.
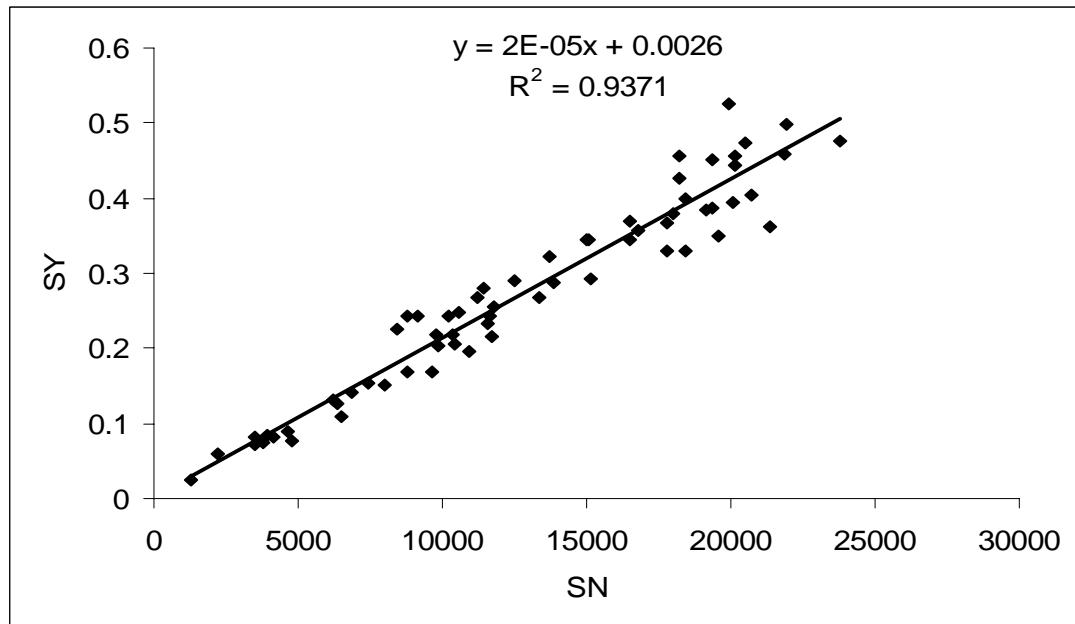
Figure 3.29 Correlation between seed yield (SY) and seed number (SN) of the tested plants. The X axis indicates the SN of each tested plant and the Y axis indicates SY of each tested plant.

3.4 Discussion

3.4.1 Transformation of Arabidopsis

In the present study, the *Allium triquetrum* agglutinin gene (*ATA*) has been transformed into Arabidopsis by *Agrobacterium*-mediated floral dipping transformation. Using the method, six transformed lines were screened out by kanamycin selection. The integration of *ATA* into the genome of Arabidopsis T_1 heterozygotes was verified by PCR. Transcription of the *ATA* gene in plants was verified by reverse transcription (RT) PCR. PCR and RT PCR result proved that the *ATA* gene has successfully integrated into the Arabidopsis genome and been transcribed. However, the substance that works on aphids is the protein – plant lectin. Therefore, a western blot analysis was essential to see whether the mRNA was translated to protein. The western blotting was performed on T_3 homozygotes and the results showed that the six ATA transformed lines contain a 12 kDa band which was also detected in protein extracts of *Allium triquetrum*. This 12 kDa band was not detected in either wild type Arabidopsis (Col-0) or the empty vector transformed line (D1201) suggesting that it represented the mature ATA. The size of ATA is in accordance with most of the reported monocot mannose-binding lectins (Van Damme *et al.*, 1998). The same sizes of ATA expressed in Arabidopsis and in *Allium triquetrum* also indicate that the posttranslational modification of ATA has been successfully achieved when expressed in Arabidopsis. The result of the western blot supported the assumption made in Chapter 2 that ATA would have similar size as ASAL and GNA and also confirmed that the molecular modelling of ATA based on GNA was reliable. Oddly, an additional band of about 100 kDa was detected in all the transformed lines including (D1201. However, this band was not observed in protein extracts of wild type Arabidopsis or *Allium triquetrum*, indicating that this band was not due to the proteomic background of Arabidopsis or *Allium triquetrum*. It seems that the only explanation is that the extra protein band is a result of the transformation. However, there was low similarity between the oligo-peptide antigen (QSLEEGPYRLAMQDDC) and the sequence of translated six open reading frames of the plasmids, including pGreen, pSoup, and Ti plasmid, in *Agrobacterium* (result not shown). Less than five continuous amino acids were identical, showing the extra band is also not related to the plasmids. Although it is obvious that the extra 100 kDa band is related to the transformation process, the source of this protein band cannot be specified.

It is likely that there are multiple lectin genes in *Allium triquetrum* (Chapter 2). The fact that only one protein band of around 12 kDa was detected in the western blotting

suggests that either there is relatively low sequence similarity at the N-terminus between different lectins in *A. triquetrum* or the different lectins in *A. triquetrum* have very similar molecular weight.

3.4.2 Unexpected effects of ATA on transgenic Arabidopsis

T-DNA insertion mediated by *Agrobacterium tumefaciens* is widely used as a mutagen in Arabidopsis research, and has lead to the isolation and characterization of quite a few genes that play various roles in the plant life cycle (Parinov and Sundaresan, 2000; Deineko *et al*., 2007). In addition, environmentally-induced maternal effects on offspring phenotypes are well known in plants (Roach and Wulff, 1987; Stratton, 1989; Mazer, 1992). Even small differences in the environment in which parental (even grandparental) plants are raised can result in large phenotypic variations among their progeny with respect to both size and performance (Miao *et al*., 1991; Wulff and Bazzaz, 1992; Curtis *et al*., 1994; Mazer and Gorchov, 1996). Therefore, the phenotype of transgenic plants can be a blended impact of the T-DNA insertion and the maternal effect caused by the floral dipping transformation process. In the germination time analysis, wild type Col-0, C2212, C3202, C4214, and C6207 showed relatively similar germination times when grown in both short and long photoperiods, while the other three lines were significantly different (Figures 3.9 and 3.10). It has previously been shown that day length does not affect the gene expression pattern in seeds at the germination stage (Cadman *et al*., 2006). Interestingly, except for D1201, all the other lines showed more or less delayed germination in long photoperiods, which suggests that the delay resulted from other factors, such as slightly different light intensity and temperature in different growth rooms. Therefore, the distinct growth pattern of D1212 must be due to the impact of the T-DNA insertion in a gene(s) related to germination. Holdsworth and coworkers (2008) found that α-amanitin (a transcription inhibitor) failed to inhibit seed germination, but cycloheximide (a translational inhibitor) inhibited the seed germination. However, they also pointed out new transcripts must be synthesized during imbibition to enhance germination vigour. In our study, all lines selected for testing had germination rates of at least 90% (Table 3.3 - 3.9), so the significantly delayed germination of D3202, C4214, and C5206 probably resulted from disturbance of the synthesis of the new transcripts helping to enhance germination.

Flowering represents the transition from the vegetative to the reproductive phase. The analysis of the time taken to reach flowering (FT) for all the transgenic lines showed

they still followed the character of long day plants. The FT of all the eight lines were very similar when grown in long photoperiods (LP) (Figure 3.13). By contrast, the FT varied much more when grown in short photoperiods (SP) (Figure 3.11). In these conditions, the FT of all the transgenic lines was significantly shorter than wild type Col-0. There are two possible explanations for this magnified difference of FT in SP condition. First, the nearly doubled FT under SP may also double the variation. Second, early flowering is usually related to a stress response (Grime, 1989), so as the life span is longer, the transgenic effects, including the accumulation of the expressed product of the foreign gene or the distorted host genome, is more obvious. The mean FT in the SP condition is 1.75 fold longer than that in the LP condition. However, the standard deviation of FT in the SP condition is 2.64 fold greater than that in the LP condition, meaning the huge difference of FT between the wild type and transgenic lines in the SP condition did not come only from the magnified variation effect. In wild type plants, a good correlation was observed between the number of days to flowering and the total number of leaves produced, so flowering time is sometimes also defined in terms of leaf number (Koornneef *et al*., 1991). Except for D1201, the total leaf number (LN) of the other lines showed the same pattern in both the SP and LP conditions (Figure 3.20). Together with the FT result, it can be concluded that all the lines took a similar time to reach flowering in the LP condition. The LN of C3202, C4214, and C5206 were significantly fewer than that of the wild type Col-0, but because these three lines exhibited the same or longer FT as the wild type Col-0, it is more likely that the development of leaves was retarded, rather than indicating any changing of the flowering time. Similarly, in the SP condition, all the transgenic lines took less time to reach flowering than the Col-0 and C3202, which exhibited a most similar FT to the wild type Col-0. However, C3202, C4214, and C5206 still produced significantly fewer leaves than other lines. In this research, FT is a better indicator of plant flowering than LN. The LN is better considered as a plant fitness parameter. Flowering is controlled by complex and highly regulated pathways (McClung, 2006) which allow plants to modulate their developmental programs to maximize adaptation to environmental cues (Jarillo *et al*., 2008). Therefore, the altered FT of the transgenic plants in the LP condition is likely to be of benefit to themselves. Mutations in genes that are not primarily involved in the control of flowering time can still affect flowering (Weigel and Glazebrook, 2002). Because almost all the transgenic lines showed earlier flowering in SP, it is more likely that the genes regulating flowering have not been directly affected.

The term growth not only means the displacement velocity of material points (Gandar, 1983) but also refers to an increase in dry mass (DM). There is an obvious

correlation between leaf number and plant dry mass (Figure 3.25 and 3.26), which also supports the conclusion that LN can be regarded as mainly a fitness parameter. When high concentrations of DL-beta-amino-n-butanoic acid (BABA) and benzothiadiazole (BTH) were applied to induce a defence response in Arabidopsis, the relative growth rates of both young (3-4 weeks) and old (6-7 weeks) plants were reduced significantly (Van Hulten, 2006). It is reasonable to conclude that the constitutive expression of ATA will cost more to the host. However, the expression of ATA does not always reduce the dry mass of transformed Arabidopsis. C1212, C2212, and C6207 exhibited similar LN and DM as Col-0, indicating the stunted phenotype of C3202, C4214, and C5206 are due to alteration of host gene function.

Seed yield and seed mass are two important parameters used to describe plant fitness (Jackson *et al*., 2004; Cipollini; 2007). Seed yield usually shows a positive correlation with dry mass of the inflorescence and the number of florets at anthesis and a negative correlation with plant biomass and the number of tillers in monocots. Similarly, seed mass is often positively correlated with seed viability, seed germination rate, seedling vigor, seedling survival, carpel mass at anthesis, and rate of grain filling (Gupta *et al*, 2006). Seed development depends on interactions between the genotype and the environment of the plant that bears the seeds. The experiments described here showed that all the transgenic lines produced fewer seeds than the wild type Col-0 (Figure 3.27), indicating that the transgene somewhat diminishes seed production. Therefore, the cost of expressing *ATA* is more likely to be reduced production of seeds rather than a decrease in whole plant dry mass. Usually, the insertion of T-DNA alone should not lead to extra costs to the host plants (Bergelson *et al*., 1996; El Ouakfaoui and Miki, 2005), but the seed yield of D1201 (empty vector) is about 30% less than the wild type Col-0 indicating that the T-DNA itself in D1201 disturbed some crucial genes related to seed production in Arabidopsis. Although the seed yield and plant dry mass measurements were performed in different photoperiods (because of technical problem with climate control rooms), the seed yield showed a similar pattern as the plant dry mass. Line C3202, C4214, and C5206 produced fewer seeds than the other lines. Another observation is the standard deviations (SD) in the experiments with each of the transgenic lines were higher than that with the wild type Col-0, which suggests that the transgenic effect is not stable. C3202 and C4214 showed even higher SD values than the other transgenic lines. In the segregation analysis, the χ^2 test has revealed that these two lines are possibly multi-copy transformed lines (Chapter 2). This implies that the instability may be related to copy number. There is usually a negative correlation between individual seed mass and seed number in nature.

Although the transgenic lines produced fewer seeds than the wild type Col-0, the seed mass was fairly constant. Besides, the SD of all the lines was also constant. The result of the seed production shows that, in *ATA*-transformed plants, seed yield is more easily affected than is the seed mass. This is likely to be a trade off between offspring quantity and quality.

3.5 Conclusions

The *Allium triquetrum* agglutinin gene (*ATA*) has been successfully expressed in Arabidopsis. Multiple copies of transgenes usually cause more obvious changes to host plants (Wenck *et al*., 1997). The same phenomenon has been observed in the present study. The putative multiple-copy transformed C3202 and C4214 showed a few severe abnormal phenotypic characteristics, such as earlier flowering, fewer rosette leaves, and lower seed production. Other lines suffered less from transformation, but they still exhibited some difference from the wild type Col-0. Most of the abnormalities of the transgenic plants were due to the insertion effect of the transgene. However, it is possible to avoid this kind of insertion effects because it has been proved that the insertion of T-DNA alone can cause very little impact on the plants (Bergelson *et al*., 1996; Ouakfaoui and Miki, 2005). The expression of *ATA* can more or less reduce seed production. Proteins are important components of Arabidopsis seeds, so the low seed yield is possibly a balance of costs and benefits of a new trait (Heil and Baldwin, 2002; Jackson *et al*., 2004). However, it should always be born in mind that changes to host plants are not specific to genetic engineering, as it is also quite common in conventional breeding.

The expression of ATA in Arabidopsis provides the opportunity of assessing its insecticidal properties in the future, as described in Chapter 4.

Chapter Four

4 Aphid bioassays with Arabidopsis transformed with *Allium triquetrum* agglutinin gene

4.1 Introduction

Genetic engineering offers new possibilities for crop protection. *Bacillus thuringiensis* (*Bt*) tobacco expressing Cry protein was the first reported insect-resistant transgenic plant (Vaeck *et al*., 1987). However, Bt cannot control certain classes of insects, such as *Homopterans* (Schuler *et al*., 1998). The major pests in this class are the cabbage aphid (*Brevicoryne brassicae*), green peach aphid (*Myzus persicae*), mustard aphid *(lipaphy erysimi*), rice brown plant hopper (*Nilaparrvata lugens*), and green leafhopper (*Nephotettix* spp).

So far, the main method applied to control sap-feeding pests is still using vast amounts of chemicals (Pimentel *et al*., 2005). However, there are several major problems in the intensive and wide spread use of systemic and contact pesticides: (1) Insects can evolve resistance to pesticides (Devonshire *et al*., 1998); (2) The synthetic chemicals can cause health and environmental risks (Pimentel *et al*, 2005); (3) No chemical can effectively control virus diseases vectored by these insects; (4) Indiscriminate usage of pesticides, especially during the early crop stages, causes outbreaks of sap-feeding insects (Hadfield, 1993). Despite the extensive use of pesticides, it has been estimated that 37% of all crop production is lost worldwide to pests and diseases, with at least 13% directly due to insects (Gatehouse and Gatehouse, 1998).

Since the first plant lectin was described in the 1950s (Boyd and Shapleigh, 1954), numerous plant lectins have been isolated. Although many plant lectins are able to bind simple sugars such as glucose, mannose, or galactose, they have a much higher affinity for oligosaccharides, which are not common or totally absent in plants. Besides, the marked stability of lectins under unfavourable conditions has also been noticed. They are stable over a wide pH range, able to withstand heat, even resistant to animal and insect proteases. Because of all the above features, scientists gradually reached a common point of view that plant lectins have a defensive function against various target organisms, including mammals, insects, and plant pathogens (Peumans & Van Damme, 1995). The first transgenic plants expressing a lectin from pea were generated in 1990 and they

showed enhanced resistance to insect pests (Boulter *et al*., 1990). *Galanthus nivalis* agglutinin (GNA), the first isolated monocot mannose-binding lectin (Van Damme *et al*., 1987), was reported to be effective against pea aphid, *Acrythosiphin pisum* Harris (Rahbe *et al*., 1995), the green peach aphid, *Myzus persicae* Sulzer (Hilder *et al*., 1995; Gatehouse *et al*., 1996; Sauvion *et al*., 1996), the foxglove aphid, *Aulacorthum solani* Kaltenbach (Down *et al*., 1996), and the grain aphid, *Sitobion avenae* Fabricius (Stoger *et al*., 1999).

The ability of lectins to bind sugars is reasonably linked with their insecticidal properties. The possible working mechanisms of lectins involve, binding of carbohydrate moieties associated with the membrane of chemosensory sensilla, binding of digestive enzymes, disturbing the peritrophic matrix (PM), and the binding of the receptors on the luminal layers of the gut section (Chapter 1). The last hypothesis is most likely in explaining the resistance mechanism against sap-feeding pests for the following reasons: (1) Lectin-conditioned aphids can still feed on both transgenic plants and artificial diets (Hilder *et al*., 1995; Sauvion *et al*., 2004), which rules out the possibility of the blocking effect of chemosensory sensilla by the lectin binding; (2) Sap-feeding pests are known to feed on free amino acids from plant sap and obtain the essential amino acids from the symbionts, and therefore do not have to depend on digestive enzymes; (3) PM helps to protect insects from mechanical damage by food particles; sap-feeding insects feed on liquid phloem sap and therefore do not have PM; (4) The luminal side of the gut is covered with potential binding sites for dietary lectins. When the protein extracts of brush border membrane vesicles of mustard aphid and red cotton bug were challenged with anti-*Allium sativum* leaf agglutinin (ASAL) antisera, a 55kDa protein band and a 45kDa protein band were detected respectively (Bandyopadhyay *et al*., 2001). Moreover, *Galanthus nivalis* agglutinin (GNA) was also recovered from the fat body, the ovarioles and throughout the hemolymph of the rice planthopper after feeding on artificial diets containing GNA (Powell *et al*., 1998).

Chapter 2 describes recent isolation of a gene from *Allium triquetrum* leaf encoding a new mannose-binding lectin, *Allium triquetrum* agglutinin (ATA) (Chapter 2). It has high sequence similarity to *GNA* and *ASAL* and three mannose-binding motifs have been revealed in this novel lectin (Chapter 2). Therefore, this lectin possibly has anti-aphid properties. To assess this, *ATA* has been introduced into Arabidopsis and expressed ectopically. Six independent *ATA*-transformed homozygous lines (C1212, C2212, C3202, C4214, C5206, and C6207) and one empty vector-transformed control line, D1201, (Chapter 3) were isolated for a series of bioassays to look at the effect of ATA on aphid behaviour and fitness. The aphid *M. persicae* Sulzer was selected because it has been used

successfully with the model plant Arabidopsis in previous researches into insect-plant interactions (Beale *et al*., 2006; Girling *et al*., 2006). Besides, *M. persicae* is generally less sensitive to mannose-bind lectins than other specialist feeders (Rahbe *et al*., 1995; Sauvion *et al*., 1996). In the present chapter, the selected bioassays include non-choice and choice behaviour tests, Mean Relative Growth Rate test (MRGR) (Adams and Van Emden 1972), nymph survival test, and fecundity test. These bioassays were selected to address the following questions: (1) Does the expression of *ATA* change the traits of Arabidopsis as a host plant and (2) can aphids detect the presence of ATA in the transgenic plants? (3) Does the presence of ATA affect the nutrition aphids can obtain and (4) the population growth rate?

4.2 Material and Methods

4.2.1 Plants and insects

The Arabidopsis plants for aphid bioassays were grown on sterilized compost consisting of equal volumes of Levington F2S growth media, John Innes No2 Compost, and Vermiculite in a climate controlled growth room ($20\pm2°C$, L12:D12).

A clone of *Myzus persicae* (obtained from Imperial College, UK), green peach aphid, was reared on Arabidopsis in an insectary ($20\pm2°C$; L16:D8).

4.2.2 Choice Behaviour test

The aim of this test is to see how aphids behave when given a choice between the empty vector-transformed control line (D1201) and other lines (wild type Col-0 and *ATA*-transformed lines). 16 plants of the wild type Col-0 and each *ATA*-transformed line and 128 D1201 plants (8-9 week old) were used in this test. The experiment was performed in 16 batches, each of which contained one plant from each line. Two mature, fully developed whole rosette leaves were excised from each Arabidopsis plant with a pair of scissors. One D1201 leaf and one wild type or *ATA*-transformed leaf were placed in the centre of a 9 cm Petri dish (Sterling, Staffs, UK) lined with 2 layers of moistened filter paper (Millipore, Hertfordshire, UK), which helped to maintain the quality of the excised leaves and to seal the gaps of the Petri dishes. The distance between the two leaves was approximately 4-5cm. 10 late instar apterous aphids were starved for 1 hr before being placed in the middle of the two leaves. The numbers of aphids on the control leaves (D1201), on the wild type or transgenic leaves, and those failing to make a choice (wandering around, dead, or missing) were recorded after 30 min, 2 hr, and 24 hr. Before the data were processed, they were treated in the following ways. The total numbers of aphids that made a choice in each Petri dish was divided by 10 to get the percentage of aphids that made a choice (P_C). The P_C values were tested for normality with Kolmogorov-Smirnov (K-S) test. The K-S test showed that the data were not normally distributed and various transformations did not work, and therefore the original untransformed P_C values were analysed with the Friedman test (Minitab 15.0, Minitab Inc, PA, USA). Similarly, the number of aphids in each Petri dish that settled on D1201 was divided by the total number of all the aphids that made a choice to get the percentage of aphids that chose D1201 (P_D) and the same procedure used to process P_C values was followed.

4.2.3 Non-choice Behaviour test

The aim of this test is to investigate the behaviour of *M. persicae* in the non-choice circumstances. 16 plants of each line (8-9 week old) were used in this test. The experiment was performed in 16 batches, each of which contained one plant from each line. Two mature, fully developed whole rosette leaves were excised from each Arabidopsis plant by a pair of scissors. Each leaf was placed in the centre of a 9 cm Petri dish lined with 2 layers of moistened filter paper. 10 late instar apterous aphids were starved for 1 hr before being placed on the leaves. The number of aphids settling on each leaf (N_L) was recorded every 15 minutes for a total of 6 times (90 minutes in total) and again after 24 hr. The K-S normality test showed the data were not normally distributed and various transformations did not work, and therefore, the original untransformed N_L values were analysed with the Friedman test (Minitab 15.0).

4.2.4 Mean relative growth rate (MRGR) and nymph survival test

The MRGR bioassay was first developed by Adams and Van Emden (1972) to measure the performance of individual aphids. In the present study, two or three Arabidopsis leaves were placed in a 9 cm Petri dish lined with two layers of moistened filter paper. About 20 adult aphids were placed on the leaves and checked every 12 hours for neonates. Six neonates less than 12 hours old were weighed as a group (W_0) before placed on a mature, fully developed rosette leaf of 8-9 week Arabidopsis plants and constrained with foam clip cages as described in Figure 4.1. The numbers of survived neonates were recorded on day 3 and day 6. The aphids surviving on each plant were weighed on day 6 (W_D). The MRGR was calculated with W_0 and W_D with Equation 4.1.

$$\text{MRGR} = \frac{\lg \overline{W_0} - \lg \overline{W_D}}{D} \qquad \text{(Equation 4.1)}$$

$\overline{W_0}$ and $\overline{W_D}$ were calculated by dividing W_0 and W_D with the start aphid number, respectively.

The K-S normality test was performed on MRGR before processing. The MRGR of aphids on different line were compared with two-way ANOVA (Minitab 15.0,). The interaction plot was produced with Mintab 15.0.

The numbers of nymphs surviving on each plant were transformed into percentages (P_{NS}) and the data was processed with 2-sample t-test, as this test can be used to compare percentages drawn from two independent samples. To achieve this, the 2-proportion function in Minitab 15.0 was used.

A
- A layer of sponge sheet
- A layer of camping mat

B
- Fine mesh
- Opening part of a 15ml falcon tube

C 2mm thick polythene plate

500μl mircofuge tube

Bamboo skewer

Steel wire

Hair clip
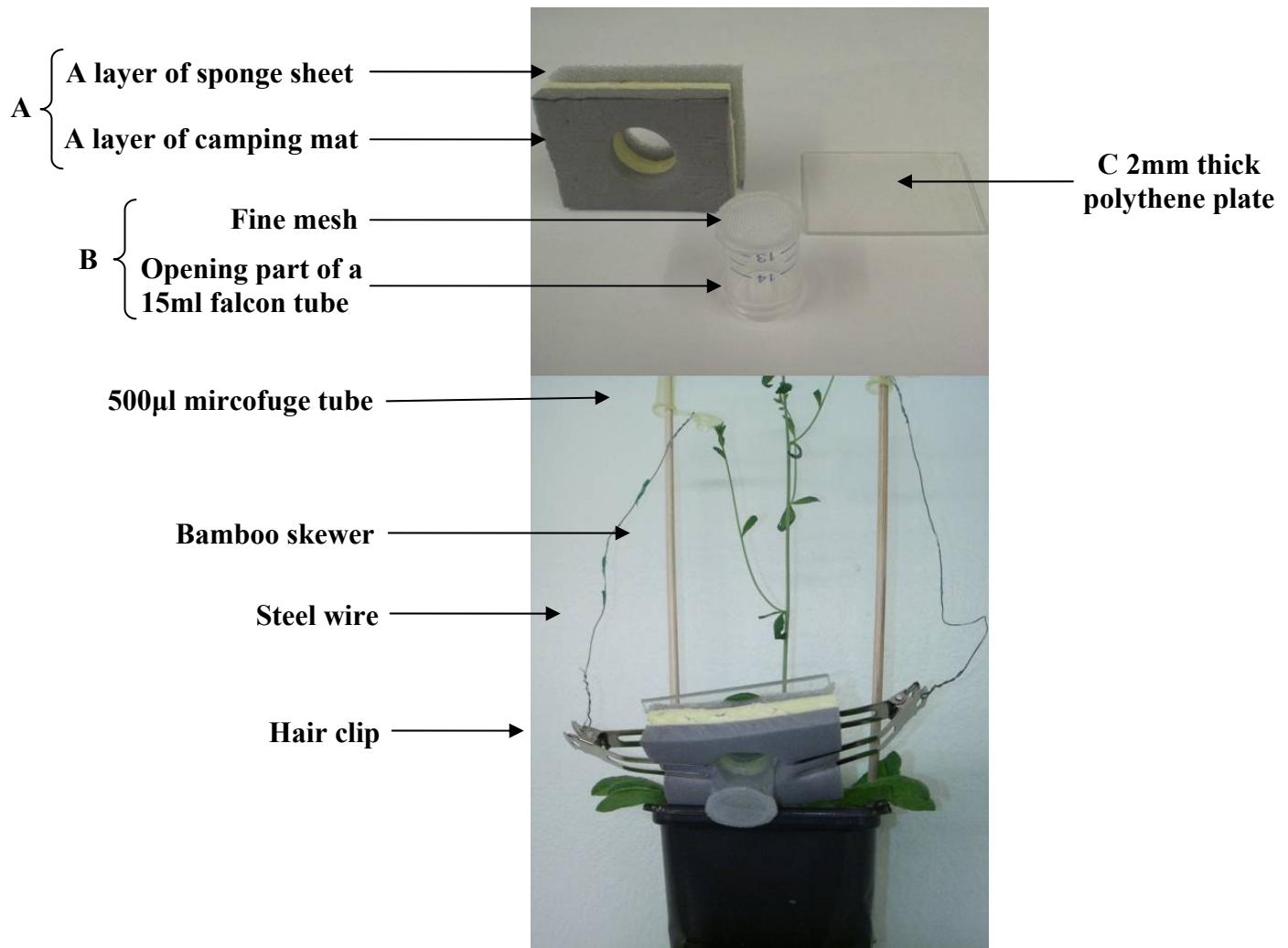
Figure 4.1 Graphical description of the sponge clip cage. The upper part of the Figure demonstrates the basic components of a foam clip cage. The foam cage is composed of three parts. Part A is a 5cm×3.5cm foam complex consisting of a piece of camping mat and a piece of sponge sheet (obtained in local shops), which were attached to each other by double-sided sello tape. A 1.5cm cork borer was used to drill the hole in the centre of the foam complex. 2-2.5 cm pipe was cut from a 15ml falcon tube and a piece of fine mesh was stuck to the opposite end of the screw end with plastic glue to generate part B. Part C is a piece of 5cm×3.5cm×2mm (width×height×thickness) polyethylene plate. The bottom part shows how the foam clip cage is attached to the plant. Two hair clips were used to seal each foam clip cage and the weight of the cages were supported by a simple device consisting of steel wires, bamboo skewers, and 500μl microfuge tubes.

4.2.5 Fecundity test

Several mature wild type Arabidopsis (Col-0) leaves were placed in a 9 cm Petri dish lined with two layers of moistened filter paper. Adult aphids (1st generation) were placed on the leaves and checked every 12 hours for neonates (2nd generation). The 2nd generation neonates were transferred onto fresh leaves in a separate Petri dish. The 2nd generation neonates were inspected twice a day. Once any 3rd generation neonates were perceived, the 2nd generation were used for fecundity tests. One 2nd generation adult aphid was placed on the reverse side of a mature rosette leaf of a 8-9 week old (in the first replicate in Group S, the plants used were 10-11 week old due to a Arabidopsis plant pathogen infection to the plant of right age) and constrained with foam clip cages (Figure 4.1).

The numbers of newly-produced neonates were recorded 5 times every other day and the newly-produced neonates were removed after counting each time. In this assay, 8 plants of each line were used in each experiment and the experiments were performed three times. The first analysis of aphid reproduction performance was to compare the reproduction pattern of aphids on different *ATA*-transformed lines. To this end, at each time point, the numbers of neonates reproduced by the still surviving adults were compared. There are two reasons to take out the data from the aphids which died during this experiment. Firstly, if the zero reproduction is due to the death of the adults, the data points are meaningless. Secondly, a few lines caused quite high mortality to aphids, so taking out those zero data points helped to make the remaining data points normally distributed. After the data were tested for normality with the K-S test, the data were processed with repeated measures ANOVA (SPSS 15.0, IL, USA), because repeated measures ANOVA can analyze groups of related dependent variables that represent different measurements of the same attribute. The second analysis of aphid reproduction performance was the intrinsic rate of increase, r_m, calculated according to the simplified but robust method of Wyatt & White (1977):

$$r_m = 0.738(\ln Md)/d \qquad \text{(Equation 4.2)}$$

Where d is the whole experimental period, Md is any effective fecundity achieved in the whole experimental period, regardless of whether the adult survived throughout the experiment or not, and 0.738 is an empirically determined constant for aphids (Wyatt & White, 1977).

The r_m values were tested for normality with K-S test prior to being processed. The K-S test shows that the r_m values were not normally distributed and various transformations failed to normalize the data. Therefore, a non-parametric K-W (Minitab

15.0) test was applied to analyse the data. The percentages of the surviving adults (P_{AS}) at the end of the fecundity test were compared with 2 sample t-test (Minitab 15.0).

4.2.6 Analysis of the relationship between Allium triquetrum agglutinin concentration, plant age, and resistance against aphids

In the previous bioassays, it was noticed that older plants appeared to have better resistance to *M. persicae*. A series of experiments were designed to address this observation. Firstly, protein assay and western blotting were performed to evaluate the lectin concentration of plants at different ages (4 and 8 week old plants). Secondly, a fecundity test of aphids, which is the same as described above, was performed with plants of different ages (4 and 8 week old plants).

4.2.6.i Protein Assay

The Bio-Rad DC Protein Assay is a colorimetric assay for protein concentration which is similar to the Lowry (1951) assay. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. There are two steps involved in colour development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent with the copper-treated protein.

The protein assay was performed in 1/4 of the volume suggested in the instruction manual; therefore all the reagents used were reduced to 1/4 of the original volume and the protein assay carried out in accordance with the manufacturer's instructions. A standard curve was prepared in the same buffer as the sample each time the assay was performed. The absorbance was read at 750nm. Because of the sap in the leaves, the volume of the protein extracts was about twice as much as that of buffer E used. Besides, the protein extracts were diluted ten times for the protein assay. Therefore, the actual concentration of the compositions of buffer E in the protein sample used for protein assay was 1/20 of the original concentration. In order to achieve reliable results, the standards were prepared in 1/20 × buffer E. Seven dilutions (Table 4.1) of 2mg/ml BSA was used to produce the standard curve.

Table 4.1 Concentration of seven dilutions of 2mg/ml BSA standards

Volume of 2mg/ml BSA	0	2	4	8	12	16	20
Concentration of standards (mg/ml)	0	0.16	0.32	0.64	0.96	1.28	1.6

4.2.6.ii Western blotting

SDS-PAGE and western blotting were performed as described in Chapter 3. Because two pieces of SDS-PAGE gels were required, the two gels were electrophoresed at the same time in the same tank. The blotting was also performed in the same conditions to eliminate any system bias.

4.2.6.iii Plant age-fecundity correlation test

Eight 4-week old and eight 8-week old plants from lines wild type Col-0, D1201, C1212, and C6207 were used in the test. The experimental setup was the same as described in 4.2.5. The reproduction patterns were analyzed with repeated measures ANOVA (SPSS 15.0). Because there are two levels of plant age and four levels of plant type, there are eight different combinations of the factors. To compare the difference of r_m values caused by different lines and plant ages, each possible combination was viewed as a treatment and the r_m were compared with one-way ANOVA (SPSS 15.0).

4.3 Result

4.3.1 Choice behaviour test

To simplify the test procedure, all the eight plant lines for testing were divided into two groups. The two multi-copy transformed lines, C3202 and C4214, were allocated into Group M, which stands for multi-copy group and the remaining lines were allocated into Group S, which stands for single-copy group. Both wild type Col-0 and the empty vector-transformed line, D1201, were used as controls in each group.

The choice behaviour test was performed as described in 4.2.2. In order to see whether the presence of the *ATA*-transformed leaves influences the ability of aphids to locate the host, the total percentage of aphids that made a choice (P_C) were analysed. Various transformation methods were applied to normalize P_C values, but all failed. Thus, a non-parametric Friedman test was applied to compare the P_C values at each measuring time point (Figure 4.2 (Group M) and 4.3 (Group S)). Friedman test is a nonparametric analysis of a randomized block experiment, and thus provides an alternative to the Two-way ANOVA. The lines were set as the factor and the16 batches were set as a block effect.

In Group M (Figure 4.2), at each time point, there was no significant difference in the P_C values in different choice scenarios (Table 4.2). However, there was an ascending trend from 30 min to 2 hr and a descending trend from 2 hr to 24 hr, and therefore a Kruskal-Wallis (K-W) test was applied to compare the P_C values in all choice scenarios at different measuring points. The K-W test showed there was a significant difference in P_C values at the three measuring time points (H = 15.23, d.f = 2, P < 0.001).

In Group S (Figure 4.3), at each time point, there was no significant difference in the P_C values in different choice scenarios (Table 4.3). However, there was an ascending trend from 30 min to 2hr and an obvious descending trend from 2 hr to 24 hr, and therefore a K-W test was applied to compare the P_C values in all choice scenarios at different measuring point. The K-W test showed there was a significant difference in P_C values at the three measuring time point (H = 24.70, d.f. = 2, P < 0.001).

After the P_C values were compared, the percentage of aphids that chose the control line D1201 (P_D) at each time point in each choice scenario were compared with the Friedman test (Figure 4.4 (Group M) and 4.5 (Group S)).

In Group M (Figure 4.4), at each time point, there was no significant difference in the P_D values in different choice scenarios (Table 4.4). The K-W test shows there was no significant difference in P_D values at the three measuring time point (H = 4.24, d.f. = 2, p = 0.120).

In Group S (Figure 4.5), at each time point, there was no significant difference in the P_D values in different choice scenarios (Table 4.5). The K-W test showed there was no significant difference in P_D values at the three measuring time point (H = 1.77, d.f. = 2, p = 0.413).

Table 4.2 Friedman test of percentages of aphids that made a choice (P_C) in Group M

Time	S value	d.f.	p
30 min	0.33	2	0.849
2 hr	1.10	2	0.576
24 hr	0.14	2	0.934

Table 4.3 Friedman test of percentages of aphids that made a choice (P_C) in Group S

Time	S value	d.f.	p
30 min	3.45	4	0.485
2 hr	2.67	4	0.615
24 hr	2.34	4	0.673

Table 4.4 Friedman test of percentages of aphids that chose D1201 (P_D) in Group M

Time	S value	d.f.	p
30 min	1.56	2	0.459
2 hr	2.63	2	0.269
24 hr	1.17	2	0.556

Table 4.5 Friedman test of percentages of aphids that chose D1201 (P_D) in Group S

Time	S value	d.f.	p
30 min	3.62	4	0.460
2 hr	2.09	4	0.720
24 hr	2.27	4	0.686
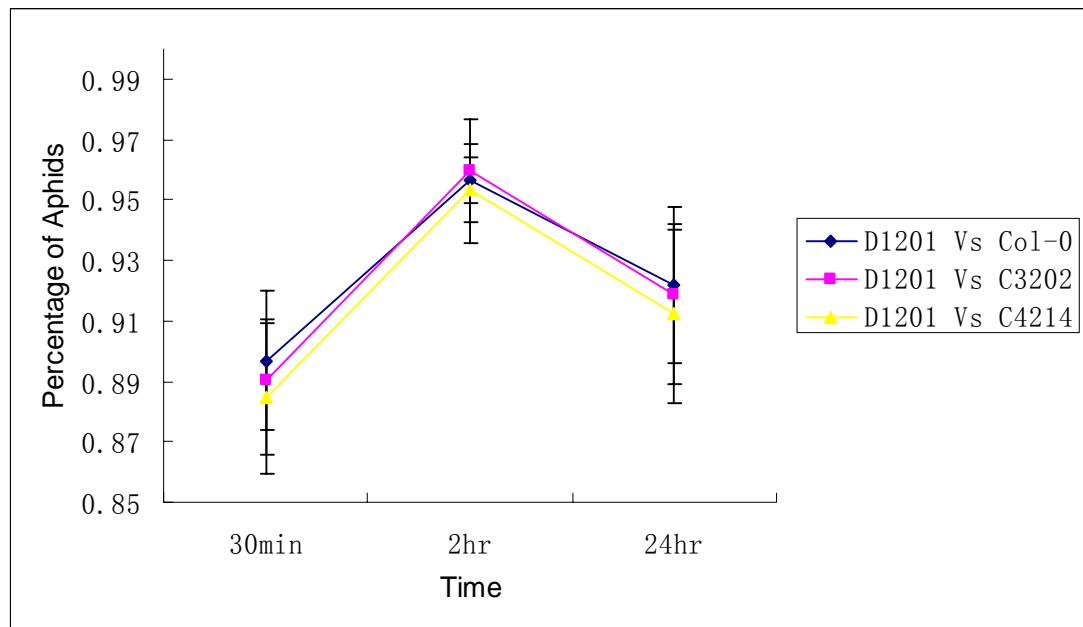
Figure 4.2 Percentages of aphids that made a choice at different time point in different choice scenarios (Group M). The X axis indicates different measuring time points and the Y axis indicates the percentage of aphids that made a choice. Error bars = SE.
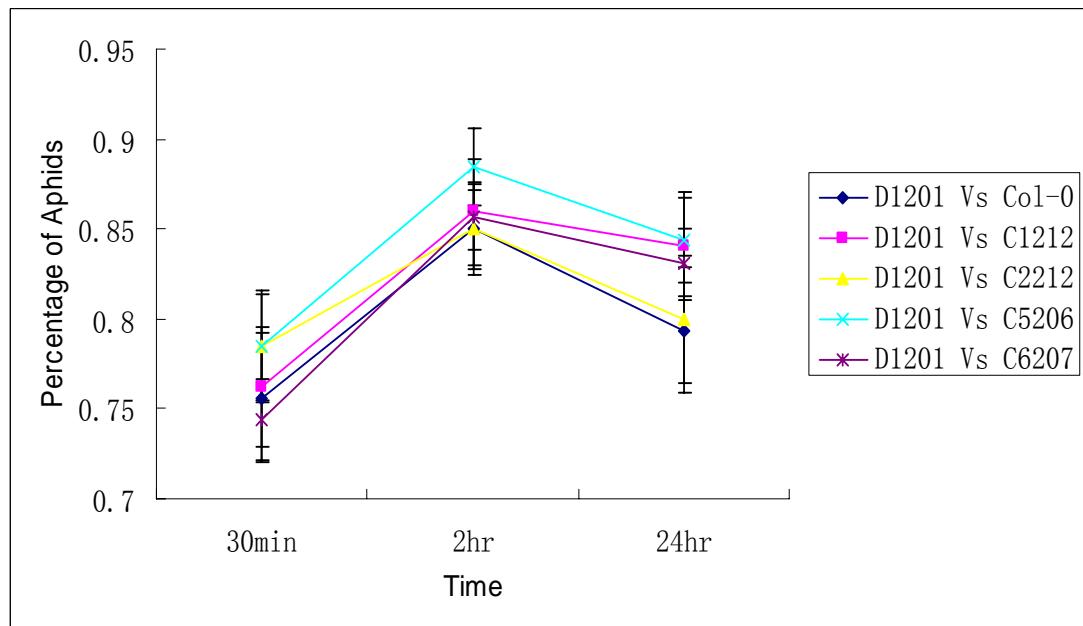
Figure 4.3 Percentages of aphids that made a choice at different time point in different choice scenarios (Group S). The X axis indicates different measuring time points and the Y axis indicates the percentage of aphids that made a choice. Error bars = SE.
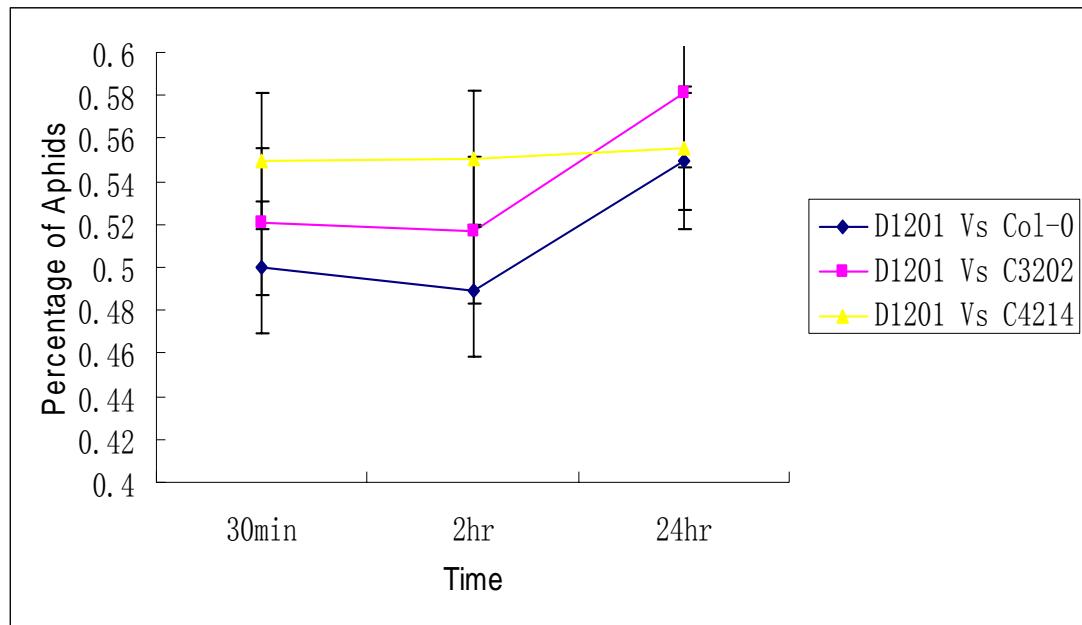
Figure 4.4 Percentages of aphids that chose D1201 at different time point in different choice scenario (Group M). The X axis indicates different measuring time points and the Y axis indicates the proportion of aphids that chose D1201. Error bars = SE.
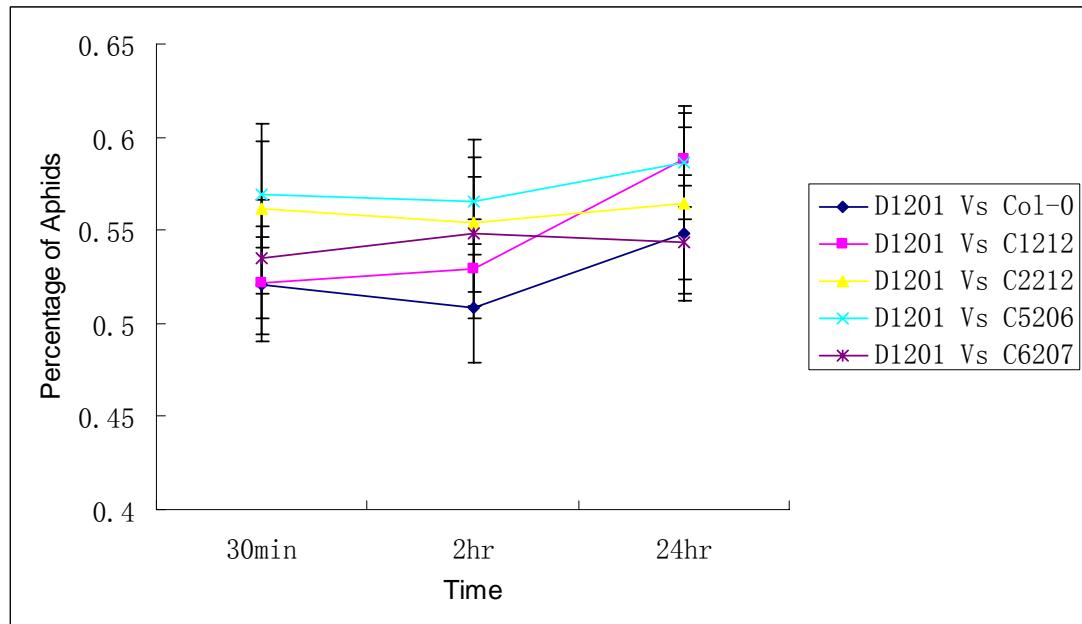
Figure 4.5 Percentages of aphids that chose D1201 at different time point in different choice scenario (Group S). The X axis indicates different measuring time points and the Y axis indicates the proportion of aphids that chose D1201. Error bars = SE.

4.3.2 Non-choice behaviour test

In agriculture, usually only a single crop is grown in a vast field at one time. Therefore, it is important to look at aphid behaviour under a non-choice scenario. The non-choice behaviour was carried out as described in 4.2.3. The numbers of aphids that stayed on the leaves (N_L) were not normally distributed and the transformation did not work. Therefore, the non-parametric Friedman test was applied to compare the N_L values of different lines at each time point. The line was set as factor and the 16 batches were set as block effect. A K-W test was used to analyze total numbers of aphids settled on any leaves at different time points (Figure 4.6 (Group M) and 4.7 (Group S)).

In Group M (Figure 4.6), there was a significant difference between N_L values on different lines at 15 min and 24 hr while there was no difference at other measuring time point (Table 4.6). The total numbers of aphids settled on any leaves in the first 90 min stayed constant, but it declined after 24 hours, (H = 70.00, d.f. = 6, p < 0.001).

In Group S (Figure 4.7), there was no significant difference between numbers of aphids on different lines in the first 90 min but there was a significant difference after 24 hr (Table 4.7). The total numbers of aphids settled on any leaves in the first 90 min stayed constant, but it declined after 24 hours, (H = 178.64, d.f. = 6, p < 0.001).

Table 4.6 Friedman test on numbers of aphids settled on leaves (Group M). The significant differences are highlighted in **bold**.

Time	S value	d.f.	p
15 min	**12.88**	**3**	**0.005**
30 min	7.21	3	0.065
45 min	7.64	3	0.054
60 min	5.10	3	0.165
75 min	2.63	3	0.452
90 min	5.12	3	0.164
24 hr	**9.53**	**3**	**0.023**

Table 4.7 Friedman test on numbers of aphids settled on leaves (Group S). The significant difference is highlighted in **bold**.

Time	S value	d.f.	p
15 min	4.71	5	0.453
30 min	3.61	5	0.606
45 min	1.32	5	0.932
60 min	1.90	5	0.863
75 min	3.53	5	0.619
90 min	2.48	5	0.780
24 hr	**18.81**	**5**	**0.002**
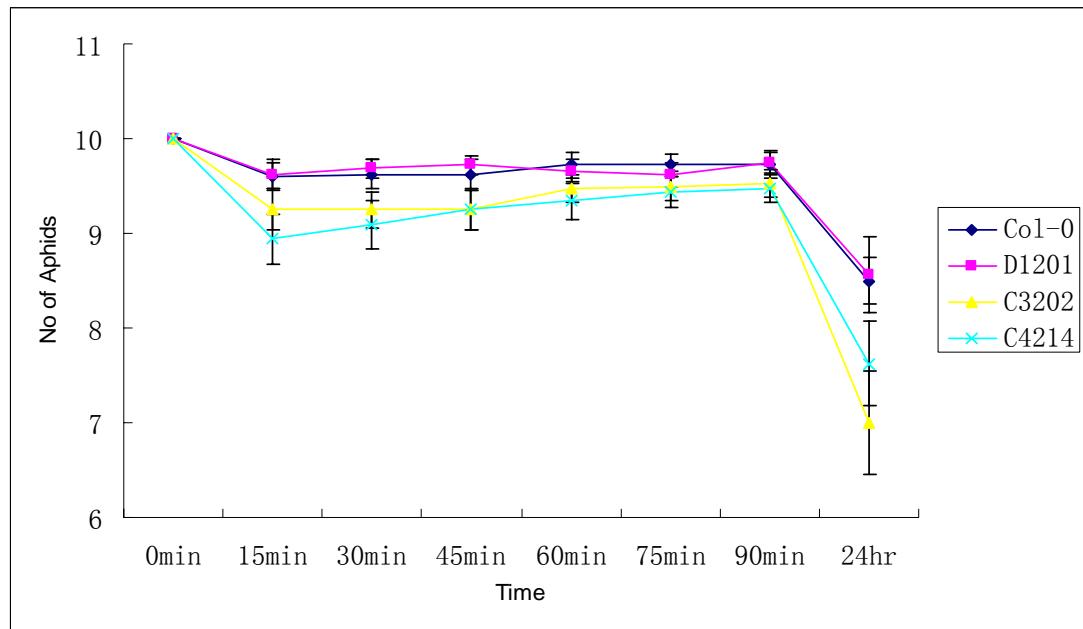
Figure 4.6 Numbers of aphids settled on leaves at different time point (Group M). The X axis indicates different measuring time points and the Y axis indicates the number of aphids that stayed on the leaves. Error bars = SE.
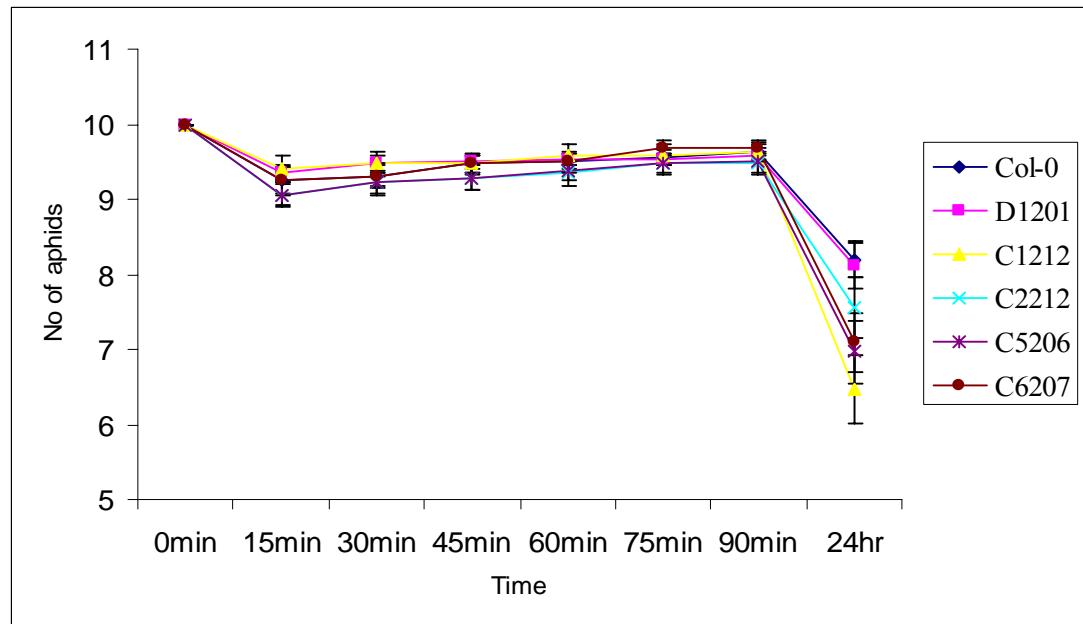
Figure 4.7 Numbers of aphids settled on leaves at different time point (Group S). The X axis indicates different measuring time points and the Y axis indicates the number of aphids that stayed on the leaves. Error bars = SE.

4.3.3 Mean Relative Growth Rate (MRGR) test

The MRGR test was performed as described in 4.2.4. The Kolmogorov-Smirnov normality test shows the calculated MRGR values were subject to normal distribution. Therefore, two-way ANOVA was applied to analyse the MRGR values. The line was set as a fixed factor and replicates were set as a random factor (Figure 4.8 (Group M) and 4.9 (Group S)).

In Group M (Figure 4.8), there was a significant difference between lines, ($F_{3, 84}$ = 6.39, p = 0.024). There was a significant difference between replicates, ($F_{2, 84}$ = 13.70, p < 0.006). There was no interaction between two effects, $F_{6, 84}$ = 0.649, p = 0.690. A least significant difference (LSD) test showed that nymphs on D1201 had the highest MRGR values and those on C3202 had the same MRGR values as those on D1201. Nymphs on wild type Col-0 and C4214 had lower MRGR values than those on D1201.

In Group S (Figure 4.9), there was no significant difference in MRGR values between lines, ($F_{5, 125}$ = 1.44, p = 0.291). There was a significant difference in MRGR values between replicates, ($F_{2, 125}$ = 32.41, p < 0.001). There was no interaction between two effects, $F_{6, 84}$ = 1.04, p = 0.417. A LSD test showed that nymphs on wild type Col-0 and D1201 had higher MRGR values than those on C2212 whereas the nymphs on the other *ATA*-transformed lines had the same MRGR values as those on the control lines.

Since significant differences in MRGR values of the nymphs on the same line in different replicates were detected in both groups, interaction plots were produced to reveal the source of the difference (Figure 4.10 (Group M) and 4.11 (Group S)).

In Group M, the interaction plot (Figure 4.10) showed that the MRGR values of nymphs in the three replicates were different from each other. The MRGR values in replicate 1 were the lowest and those in replicate 3 were the highest. However, the rank order of different lines was very similar in the three replicates suggesting there was some real difference between the lines.

In Group S, the interaction plot (Figure 4.11) showed that the MRGR values of nymphs in the three replicates were different from each other. The MRGR values in replicate 1 were the highest and those in replicate 2 were the lowest. However, the rank order of different lines was very similar in the three replicates suggesting there was some real difference between the lines.
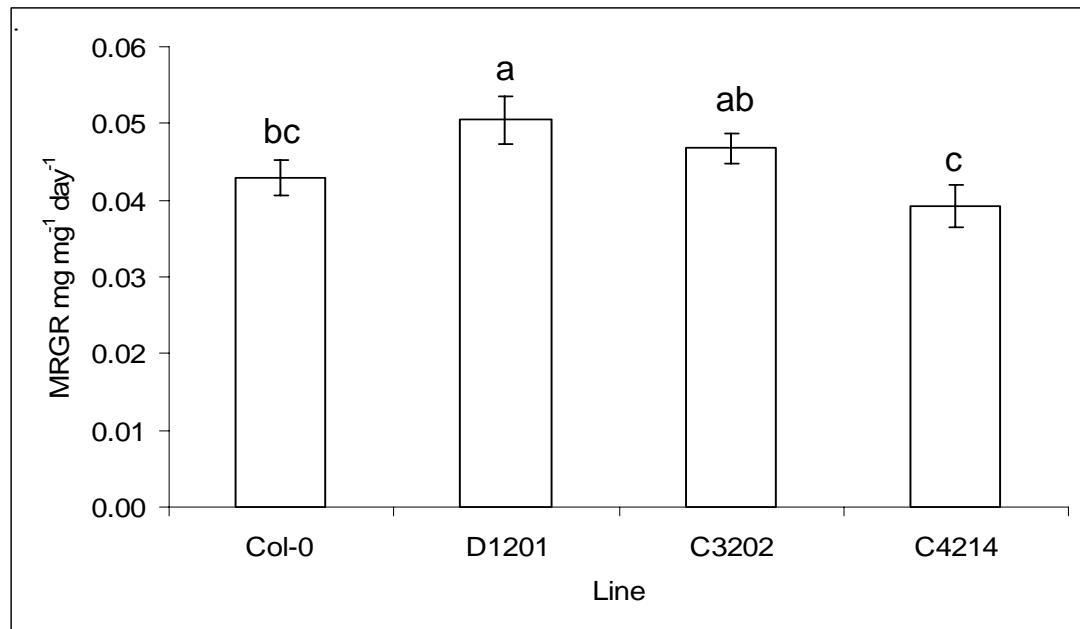
Figure 4.8 MRGR values of nymphs on different lines (Group M). The X axis indicates different tested lines and the Y axis indicates the MRGR of the aphids. Error bars = SE. The letters above the data bars indicates the results of the least significant difference (LSD) test.
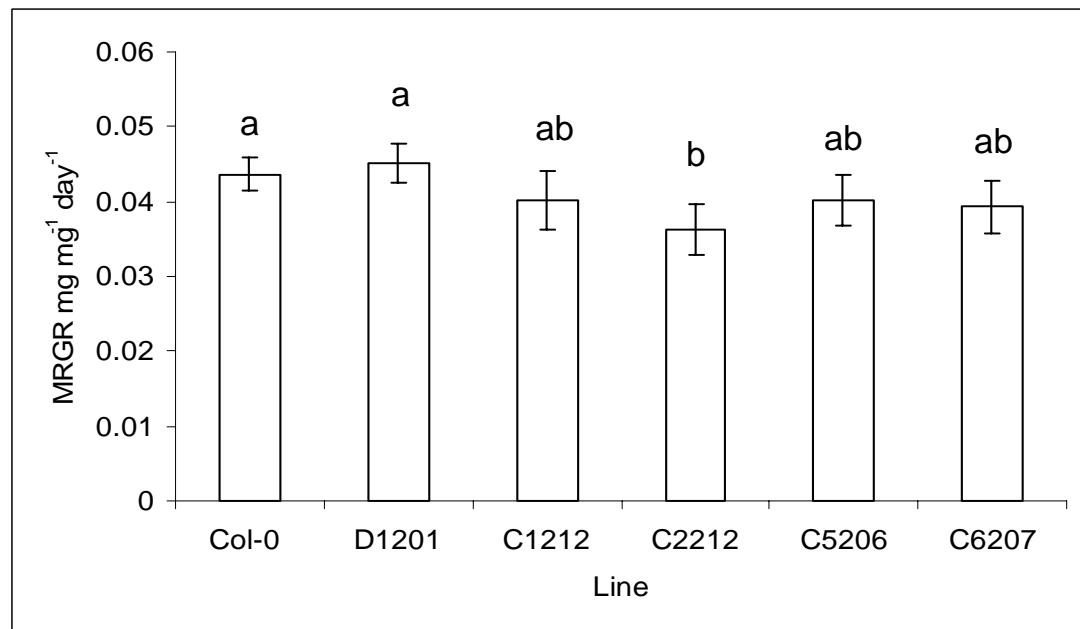
Figure 4.9 MRGR values of nymphs on different lines (Group S). The X axis indicates different tested lines and the Y axis indicates the MRGR of aphids. Error bars = SE. The letters above the data bars indicates the results of the LSD test.
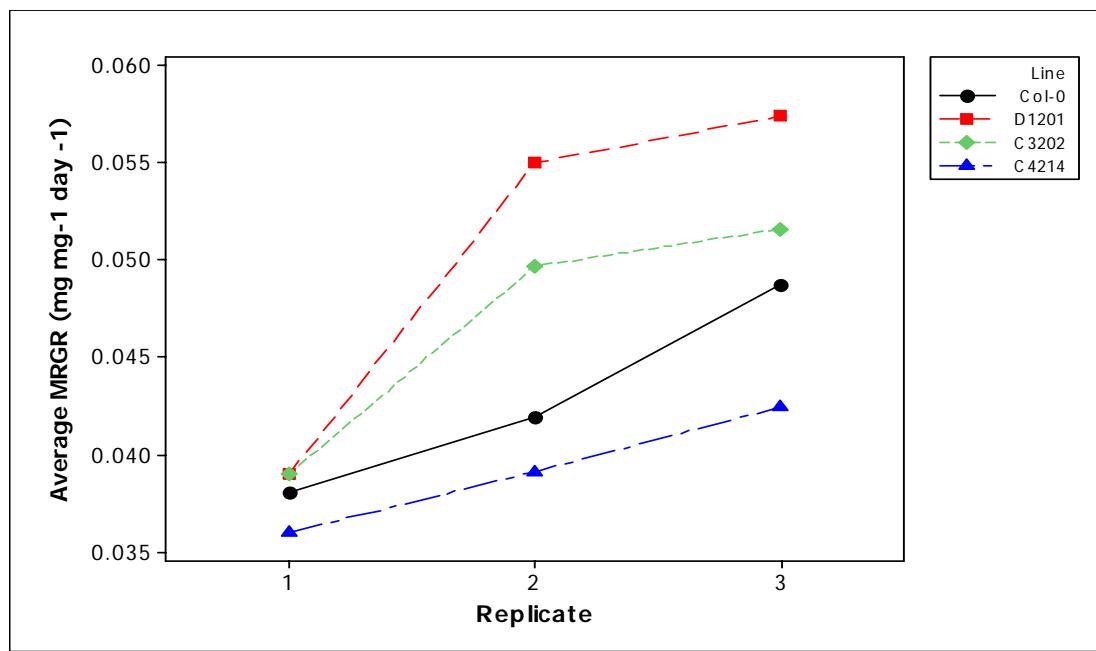
Figure 4.10 Interaction plot of the two factors in MRGR test: lines and replicates (Group M). The X axis indicates three replicates tested and the Y axis indicates the mean MRGR of aphids on each line.
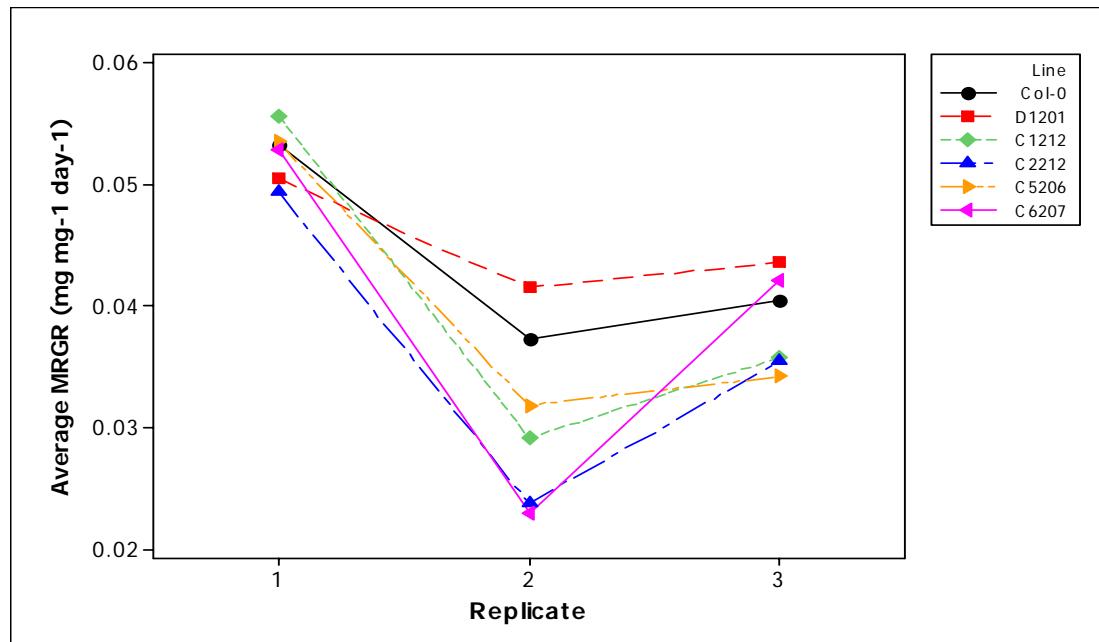
Figure 4.11 Interaction plot of the two factors in MRGR test: lines and replicates (Group S). The X axis indicates three replicates tested and the Y axis indicates the mean MRGR of aphids on each line.

4.3.4 Nymph survival test

When the MRGR test was performed, the percentages of surviving nymphs (P_{NS}) were also recorded as described in 4.2.4. 2 sample t-test was applied to compare both the P_{NS} of nymphs on different lines at each measuring time point and the P_{NS} values of nymphs on each line at different time points (Figure 4.12 (Group M) and 4.13 (Group S)).

In Group M (Figure 4.12), there was no significant difference between the P_{NS} values of nymphs on control lines and those on the *ATA*-transformed lines after 3 days (Table 4.8). After 6 days, still no significant difference was observed between wild type Col-0 and the *ATA*-transformed lines, but a significant difference was detected between D1201 and the *ATA*-transformed lines (Table 4.9). Except for line C3202, no significant difference of surviving nymphs was observed between Day 3 and Day 6 (Table 4.10).

In Group S (Figure 4.13), there was no significant difference between the P_{NS} values of nymphs on control lines and those on the *ATA*-transformed lines after 3 days (Table 4.11). After 6 days, a significant difference was observed between the P_{NS} values of nymphs on wild type Col-0 and C5206 whereas no significant difference was detected between the two control lines and other *ATA*-transformed lines (Table 4.12). When comparing the P_{NS} values of nymphs on each line between different time points, the P_{NS} values of nymphs after 6 days were all significantly lower than those after 3 days (Table 4.13).

Table 4.8 2 sample t-test of the percentage of surviving nymphs on different lines after 3 days (Group M).

Line	Z value	p
Col-0 Vs D1201	1.69	0.092
Col-0 Vs C3202	0.51	0.608
Col-0 Vs C4214	-0.24	0.812
D1201 Vs C3202	-1.25	0.211
D1201 Vs C4214	-1.88	0.061

Table 4.9 2 sample t-test of the percentage of surviving nymphs on different lines after 6 days (Group M). The significant differences are highlighted in **bold**.

Line	Z value	p
Col-0 Vs D1201	1.67	0.094
Col-0 Vs C3202	-0.51	0.477
Col-0 Vs C4214	-0.51	0.477
D1201 Vs C3202	**-2.31**	**0.021**
D1201 Vs C4214	**-2.31**	**0.021**

Table 4.10 2 sample t-test of the percentage of surviving nymphs between day 3 and day 6 (Group M). The significant difference is highlighted in **bold**.

Line	Z value	p
Col-0	-1.45	0.148
D1201	-1.48	0.139
C3202	**-2.52**	**0.012**
C4214	-2.31	0.058

Table 4.11 2 sample t-test of the percentage of surviving nymphs on different lines after 3 days (Group S).

Line	Z value	p
Col-0 Vs D1201	-1.70	0.089
Col-0 Vs C1212	-1.30	0.193
Col-0 Vs C2212	-1.30	0.193
Col-0 Vs C5206	-0.31	0.759
Col-0 Vs C6207	-1.08	0.280
D1201 Vs C1212	0.44	0.658
D1201 Vs C2212	0.44	0.658
D1201 Vs C5206	1.43	0.152
D1201 Vs C6207	0.68	0.498

Table 4.12 2 sample t-test of the percentage of surviving nymphs on different lines after 6 days (Group S). The significant difference is highlighted in **bold**.

Line	Z value	p
Col-0 Vs D1201	-0.65	0.513
Col-0 Vs C1212	-1.40	0.160
Col-0 Vs C2212	-1.82	0.068
Col-0 Vs C5206	**-1.96**	**0.05**
Col-0 Vs C6207	-1.26	0.208
D1201 Vs C1212	-0.76	0.448
D1201 Vs C2212	-1.19	0.235
D1201 Vs C5206	-1.33	0.185
D1201 Vs C6207	-0.61	0.540

Table 4.13 2 sample t-test of the percentage of surviving nymphs between day 3 and day 6 (Group S). The significant differences are highlighted in **bold**.

Line	Z value	p
Col-0	**-2.91**	**0.004**
D1201	**-2.10**	**0.036**
C1212	**-3.14**	**0.002**
C2212	**-3.49**	**<0.001**
C5206	**-4.16**	**<0.001**
C6207	**-3.19**	**0.001**
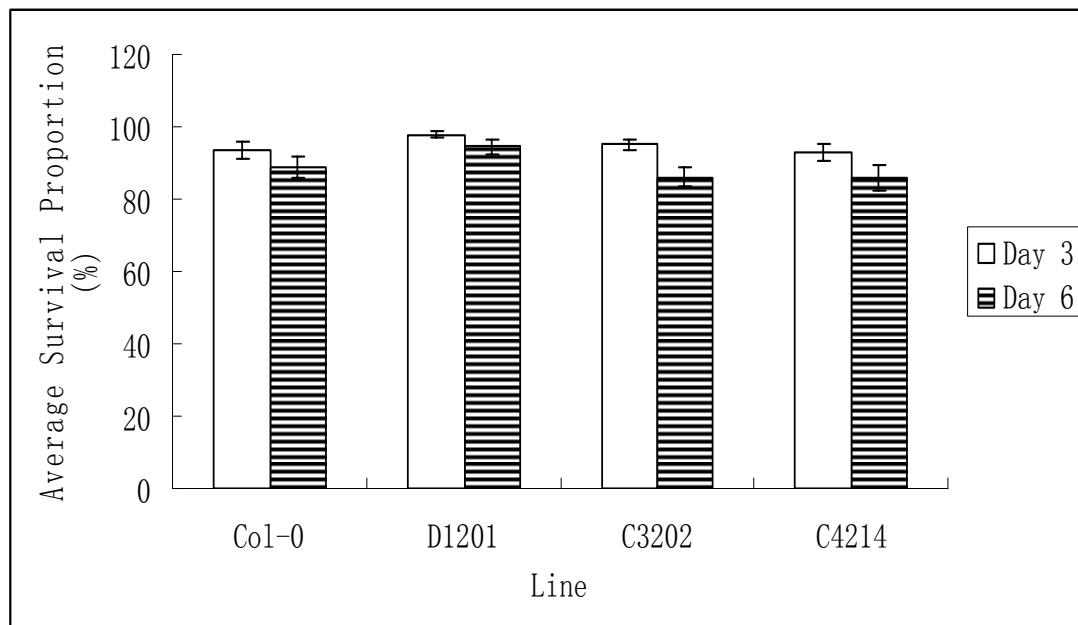
Figure 4.12 The percentage of surviving nymphs on different lines (Group M). The X axis indicates the lines tested and the Y axis indicates average survival proportion of the neonates. Error bars = SE.
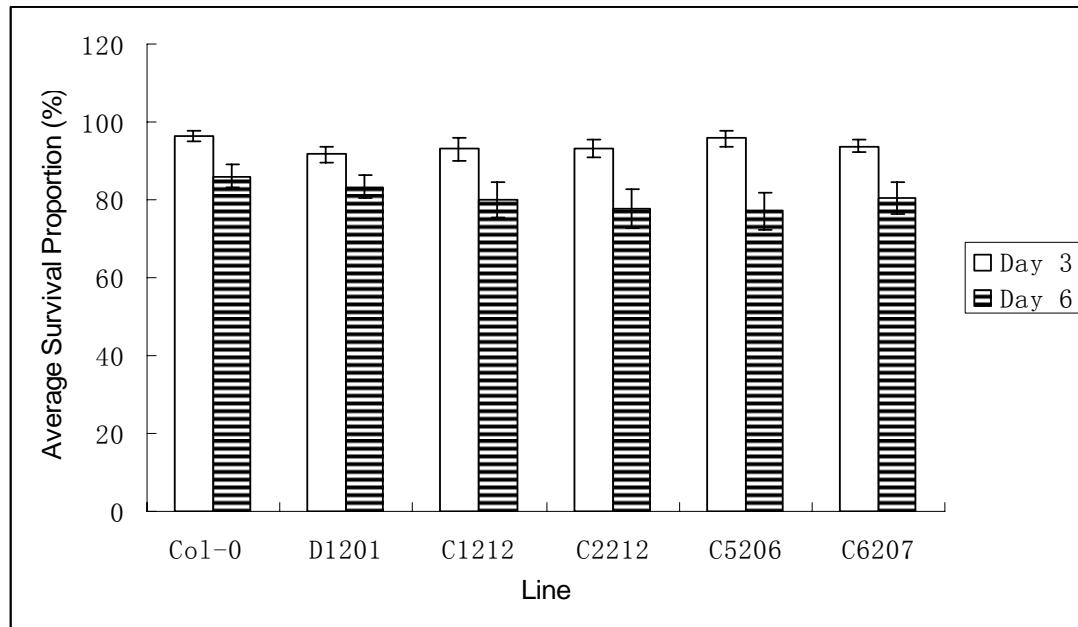
Figure 4.13 The percentage of surviving nymphs on different lines (Group S). The X axis indicates the lines tested and the Y axis indicates average survival proportion of the neonates. Error bars = SE.

4.3.5 Fecundity assay

The fecundity assay was performed as described in 4.2.5. To analyse the reproductive patterns of the adults on different Arabidopsis lines, the numbers of neonates produced by the surviving adults at each time point were compared. The K-S test showed that all the data points were subject to normal distribution. Therefore, the repeated measures ANOVA was used to reveal the reproduction patterns (Figure 4.14 (Group M) and 4.15 (Group S)). The lines were set as a fixed factor and the replicates were set as a random factor.

In Group M (Figure 4.14), except for Day 4, there was no significant difference between the patterns of reproduction of aphids feeding on different lines at different measuring time points (Table 4.14). However, a General Linear Model (GLM) test revealed a significant difference between the replicates throughout the experiment (Table 4.15).

In Group S (Figure 4.16), there was a significant difference between the numbers of neonates observed at Day 2 on different lines. However, no significant difference was spotted at other time points (Table 4.16). No significant difference was observed between replicates at most of the time points, except on Day 4 (Table 4.17). The LSD revealed that there was a significant difference between D1201 and the *ATA*-transformed lines. On Day 2, the numbers of neonates produced on wild type Col-0 and D1201 were higher than those on the *ATA* transformed lines.

The second analysis was comparing the r_m values of aphids feeding on different lines. Various transformation methods were applied. However, neither the original data nor the transformed data followed normal distribution or any other commonly used distribution. Therefore, the non-parametric K-W test was applied to compare the pooled r_m values for all the replicates of aphids feeding on each line (Figure 4.16 (Group M) and 4.17 (Group S). The interaction plots (Figure 4.18 (Group M) and 4.19 (Group S) and LSD test was also performed to enhance the sensitivity of the K-W test.

In Group M (Figure 4.16), the K-W test showed if the r_m values were pooled, there was no significant difference in the r_m values between lines (H = 6.20, d.f. = 3, p = 0.102); However, the LSD test showed that the r_m values of aphids on wild type Col-0 were the same as those on D1201, but the r_m values on C3202 and C4214 were only lower than those on D1201.

In Group S (Figure 4.17), the K-W test showed if the r_m values were pooled, there was no significant difference in r_m values between lines (H = 9.73, d.f. = 5, p = 0.083); However, the LSD test showed the r_m values of aphids on wild type Col-0 were

higher than those on line D2212 and C6207 and it was the same as those on other lines, including D1201.

In Group M (Figure 4.18), the interaction plot showed that the numbers of total neonates in both second and third replicates were lower than the first replicate. The performance of aphids on Line C3202 was different from those on other lines.

In Group S (Figure 4.19), the interaction plot showed the numbers of the neonates produced in the second replicate were lower than those in first and third replicate. In the second replicate, the performance of aphids on the *ATA* transformed line was much lower than those on the controls.

The mortality of the adults on the *ATA*-transformed lines was much higher in both groups in the fecundity test (Figure 4.20 (Group M) and 4.21 (Group S)). The 2-sample t-test was applied to compare the percentage of the surviving adults (P_{AS}) in pairs, which means the P_{AS} values on the *ATA*-transformed lines were compared with those on both wild type Col-0 and D1201 respectively.

In Group M (Figure 4.20), there was no significant difference between the P_{AS} values of the aphids on wild type Col-0 and D1201. The P_{AS} values of adults on both C3202 and C4214 were significantly lower than those on wild type Col-0 and D1201 (Aphids on C3202 and C4214 have same P_{AS} values) (Table 4.18).

In Group S (Figure 4.21), there was no significant difference in P_{AS} values between the two control lines, wild type Col-0 and D1201. When compared with wild type Col-0, the P_{AS} values of adults were significantly lower on C1212 and C6207 (Aphids on C1212 and C6207 had same P_{AS} values). The P_{AS} values of aphids on the remaining *ATA*-transformed lines appeared to be same as those on wild type Col-0. The P_{AS} values of adults on D1201 were same as those on the *ATA*-transformed lines (Table 4.19).

Table 4.14 Repeated measures ANOVA of neonates observed at each time point (Group M). The significant difference was highlighted with **bold**

Time	d.f.	F	p
Day 2	3,81	0.59	0.622
Day 4	**3,71**	**0.534**	**0.036**
Day 6	3,66	0.39	0.759
Day 8	3,63	0.48	0.700
Day 10	3,61	0.12	0.950

Table 4.15 GLM test of neonates produced in different replicates (Group M). The significant differences were highlighted with **bold**

Time	d.f.	F	p
Day 2	**2,81**	**41.97**	**<0.001**
Day 4	**2,71**	**7.39**	**0.001**
Day 6	**2,66**	**7.62**	**0.001**
Day 8	2,63	2.77	0.071
Day 10	**2,61**	**4.92**	**0.011**

Table 4.16 Repeated measures ANOVA of neonates observed at each time point (Group S). The significant differences was highlighted with **bold**

Time	d.f.	F	p
Day 2	**5,131**	**4.98**	**0.001**
Day 4	5,126	1.32	0.260
Day 6	5,115	1.79	0.121
Day 8	5,110	1.10	0.366
Day 10	5,104	0.21	0.957

Table 4.17 GLM test of neonates produced in different replicates (Group M). The significant differences were highlighted with **bold**

Time	d.f.	F	p
Day 2	2,131	1.24	0.292
Day 4	**2,126**	**5.21**	**0.007**
Day 6	2,115	2.04	0.134
Day 8	2,110	1.36	0.261
Day 10	2,104	1.42	0.247

Table 4.18 2 sample t-test of the percentage of surviving adults in the fecundity assay (Group M). The significant differences are highlighted in **bold**.

Lines compared	Z values	p values
Col-0 Vs D1201	-0.71	0.48
Col-0 Vs C3202 and C4214	**2.16**	**0.030**
D1201 Vs C3202 and C4214	**2.95**	**0.003**

Table 4.19 2 sample t-test of the percentage of surviving adults in the fecundity assay (Group S). The significant differences are highlighted in **bold**.

Lines compared	Z values	p values
Col-0 Vs D1201	1.25	0.234
Col-0 Vs C1212 and C6207	**2.24**	**0.025**
Col-0 Vs C2212	1.92	0.055
Col-0 Vs C5206	1.59	0.112
D1201 Vs C1212 and C6207	0.98	0.325
Col-0 Vs C2212	0.67	0.503
Col-0 Vs C5206	0.34	0.731
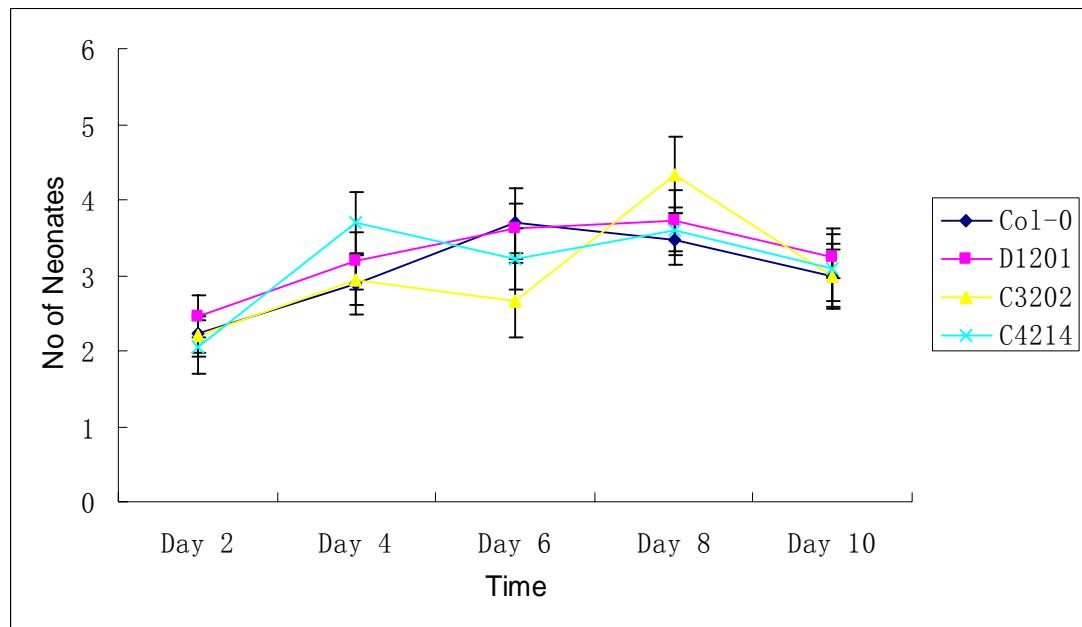
Figure 4.14 Numbers of neonates observed at each time point (Group M). The X axis indicates the different measuring day point and the Y axis indicates the number of neonates observed. Error bars = SE.
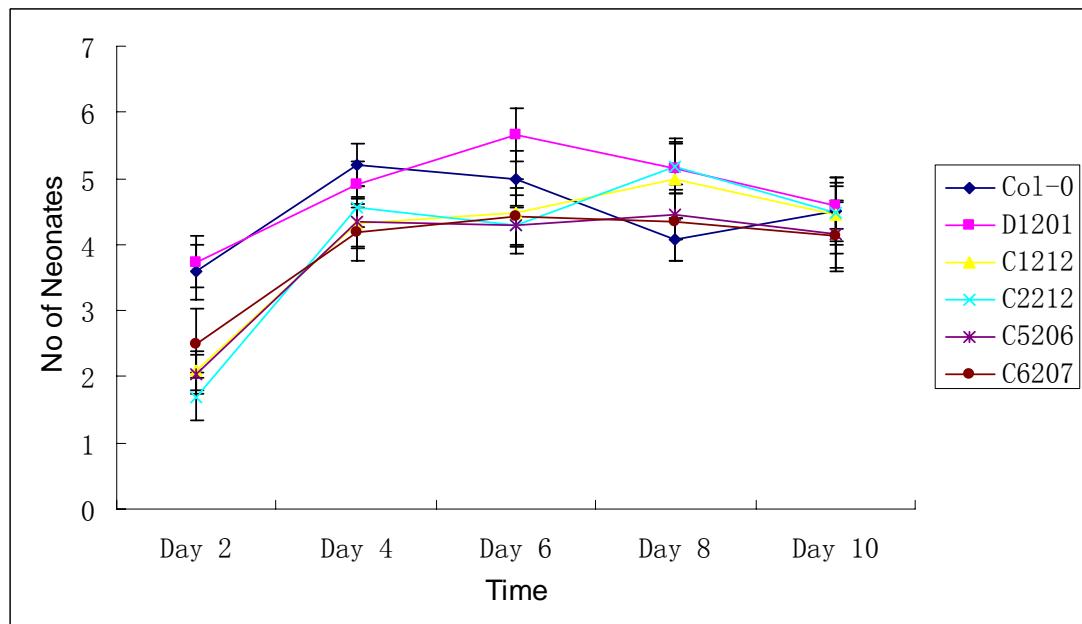
Figure 4.15 Numbers of neonates observed at each time point (Group S). The X axis indicates the different measuring day point and the Y axis indicates the number of neonates observed. Error bars = SE.
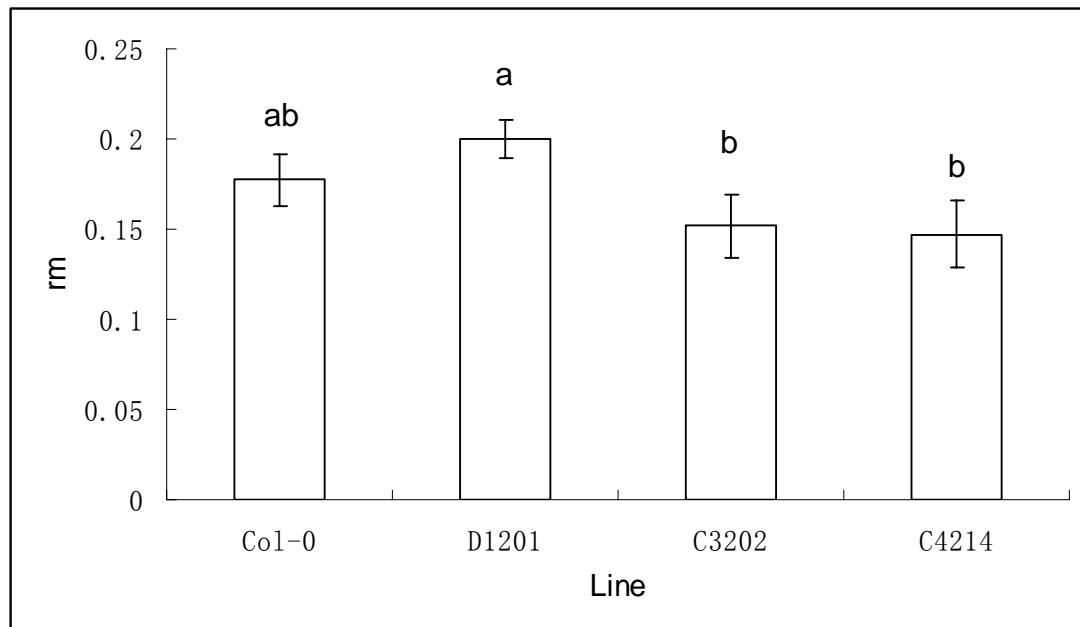
Figure 4.16 The r_m values of aphids on different lines (Group M). The X axis indicates the different lines tested and the Y axis indicates the r_m of aphids. Error bars = SE. The letters above each data bar indicates the result of the LSD Test.
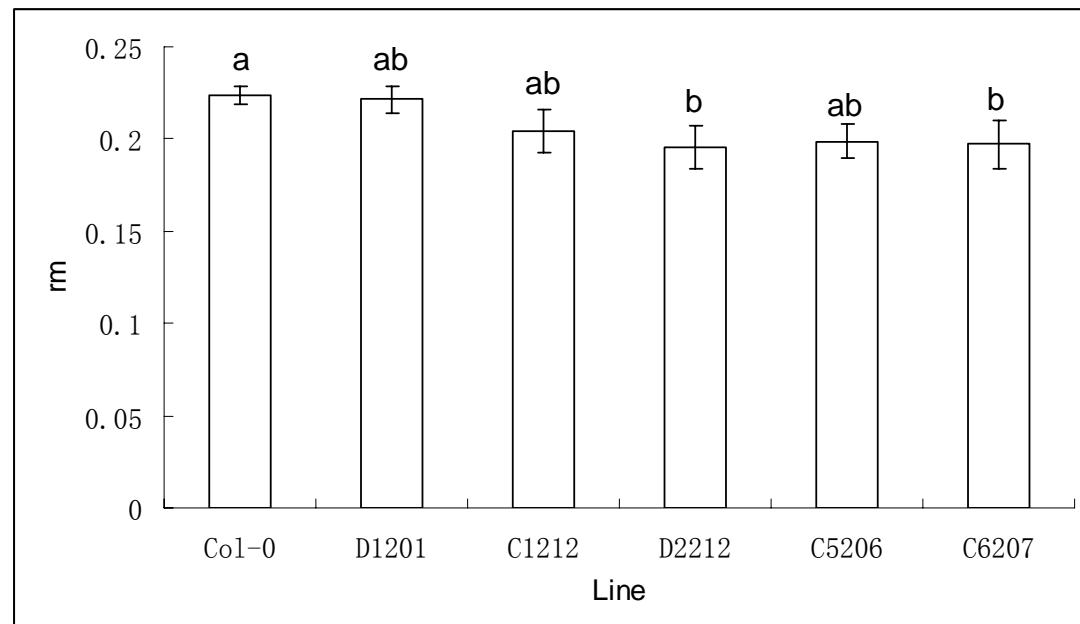
Figure 4.17 The r_m values of aphids on different lines (Group S). The X axis indicates the different lines tested and the Y axis indicates the r_m of aphids. Error bars = SE. The letters above each data bar indicates the result of the LSD Test.
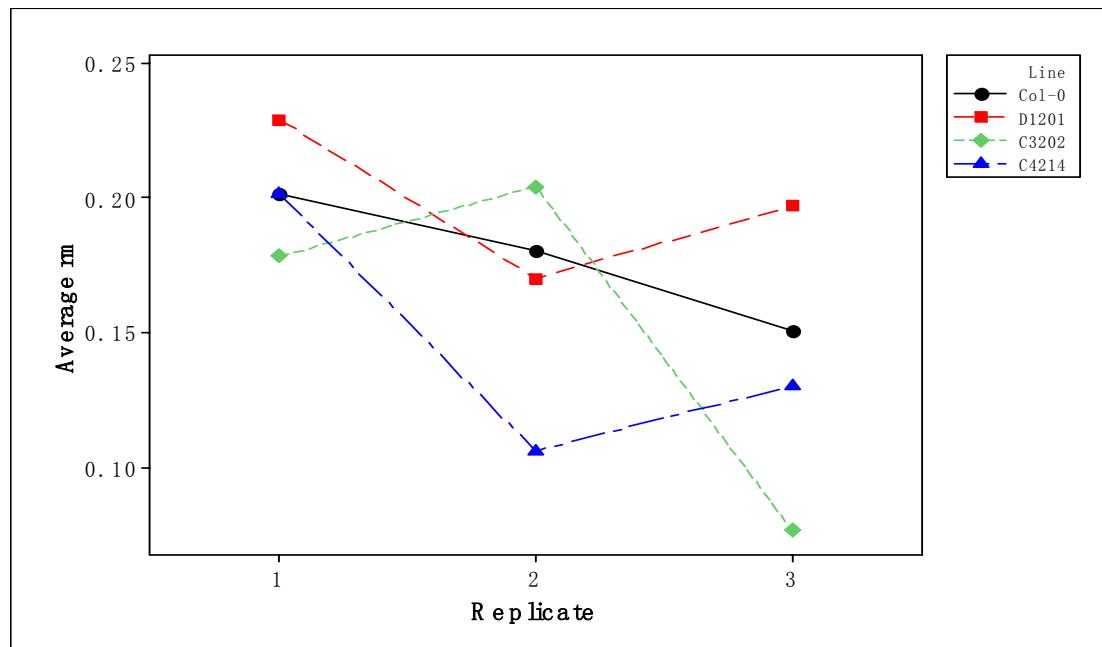
Figure 4.18 Interaction plot of the factors in fecundity test: lines and replicates (Group M). The X axis indicates three replicates tested and the Y axis indicates the mean r_m of each line.
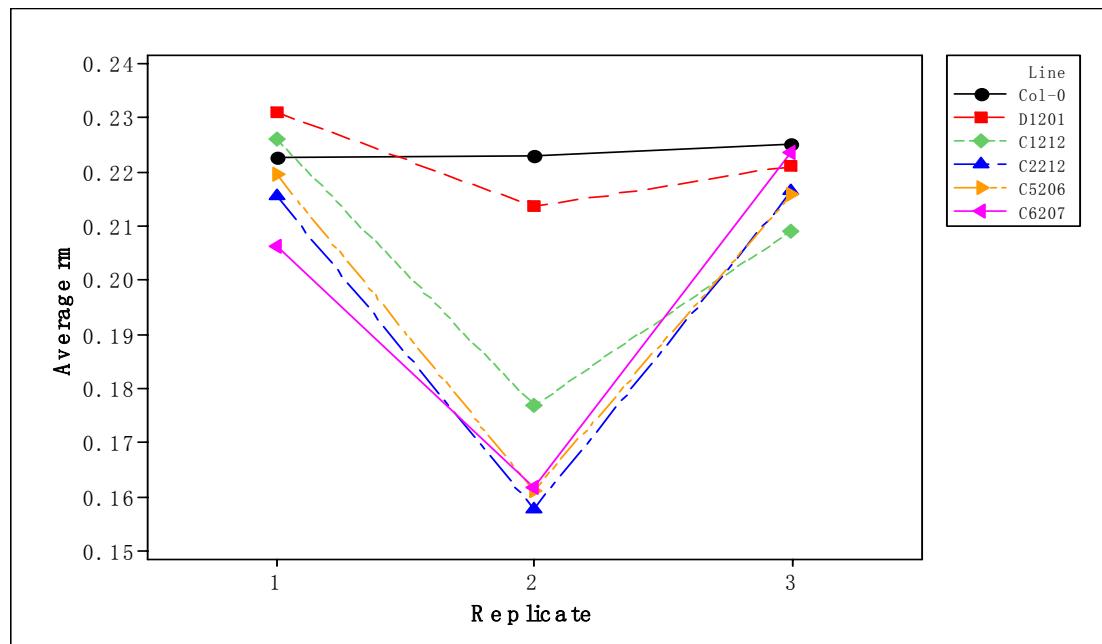
Figure 4.19 Interaction plot of the factors in fecundity test: lines and replicates (Group S). The X axis indicates three replicates tested and the Y axis indicates the mean r_m of each line.
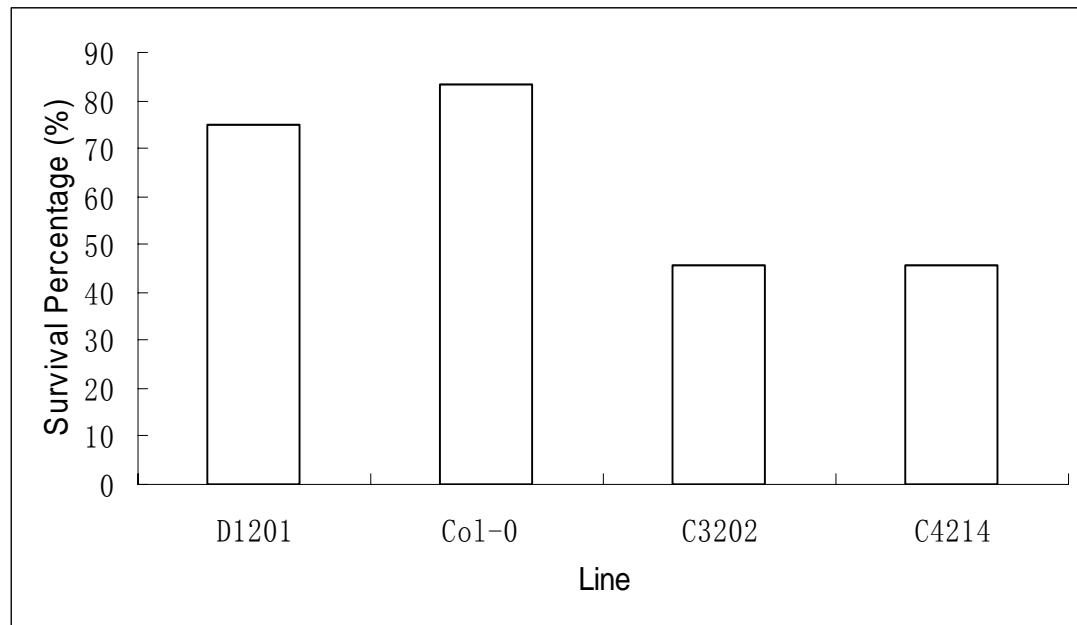
Figure 4.20 The percentage of surviving adults in the fecundity test (Group M). The X axis indicates the lines tested and the Y axis indicates the percentage of surviving aphids.
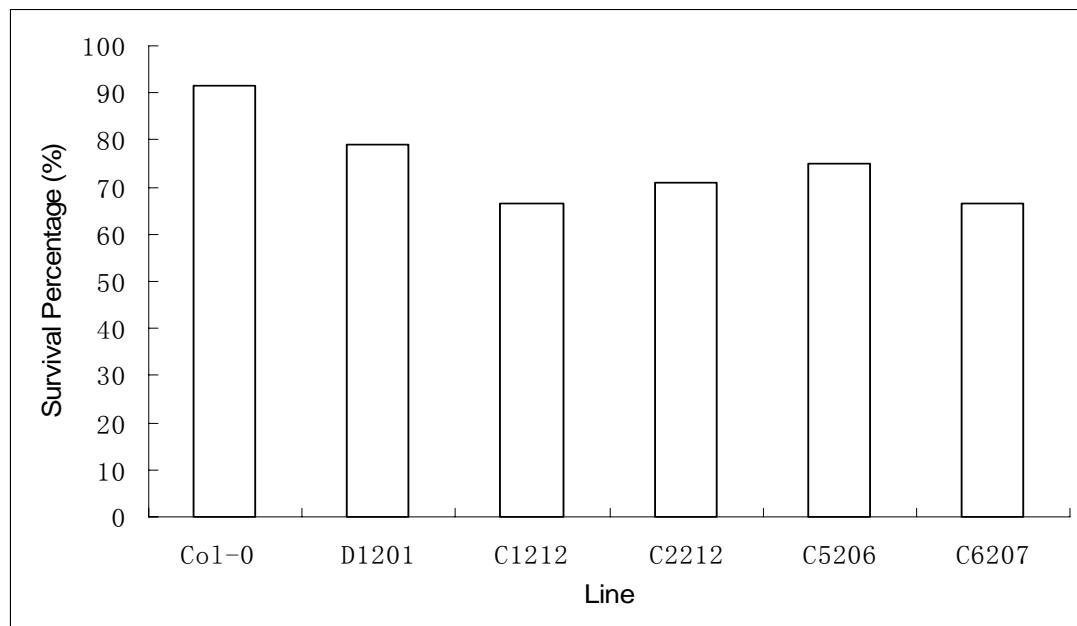
Figure 4.21 The percentage of surviving adults in the fecundity test (Group S). The X axis indicates the lines tested and the Y axis indicates the percentage of surviving aphids.

4.3.6 The relationship between Allium triquetrum agglutinin concentration, plant age, and resistance against aphids

Protein was extracted from Arabidopsis leaves of 4 week and 8 week. Seven dilutions (Table 4.1) of 2mg/ml BSA was used to produce a standard curve of protein concentration (Figure 4.22). The standard transformation equation for calculating the protein concentration was calculated from the standard curve.

$$Y = 3.748X - 0.0839 \qquad \text{(Equation 4.3)},$$ where X represents the absorbance at 750 nm and Y represents concentration of the protein sample.

The high R^2 indicated that the standard curve fit the data well. The concentration of the protein extracts of both the young and old plants were calculated with Equation 4.3. About 54 mg of each protein sample was used for western blotting (Figure 4.23 and 4.24).

The western blots (Figure 4.23 and 4.24) showed that three bands were detected in the protein extracts of all the *ATA*-transformed lines; these were 12 kDa, 75 kDa, and 100 kDa, regardless of the plant age. Two bands only were detected in the control D1201 protein extracts (75 kda and 100 kDa). Only one band was detected in wild type Col-0 (75kDa). The 12 kDa band corresponds to ATA (Chapter 3). The 75 kDa band must be wild type Arabidopsis protein. The 100 kDa band seems to be caused by the *Agrobacterium*-mediated transformation (Chapter 3). The results showed that stronger lectin signals were detected in all the protein samples extracted from 8 week old *ATA*-transformed plants than those from 4 week old counterparts, which supports the assumption that there is more lectin in older plants. A well-controlled fecundity test was done to test if the ATA concentration was correlated with aphid resistance. The fecundity test was selected because large variations were observed between replicates (Figure 4.12 and 4.13), which is possibly related to plant age. Two *ATA*-transformed lines (C1212 and C6207) were selected for the following three reasons: (1) they are single copy transformed plants; (2) their phenotype is similar to the wild-type plants (Chapter 3) (3) they have relatively high ATA concentrations. The K-S test showed the data were normally distributed, and therefore, a two-way repeated measures ANOVA was performed to compare the neonates produced at different time points feeding on different lines (Figure 4.24) and a one-way ANOVA was used to compare the r_m values (Figure 4.25).

The repeated measures ANOVA test (Figure 4.25) showed that there was a significant difference in the numbers of neonates reproduced on different days ($F = 15.343$, d.f. = 4, $p < 0.001$), but the difference did not come from different lines ($F = 1.070$, d.f. =

12, p = 0.387). The major source of the difference was the different ages (F = 3.362, d.f. = 4, p = 0.011). The test also revealed that if the data collected at different time points were pooled, there was a significant difference between both lines (F = 4.815, d.f. = 3, p = 0.005) and ages (F = 31.243, d.f. = 1, p < 0.001). The LSD test suggested that if the data collected on different measuring day points were pooled, the aphids produced fewer neonates on C6207 than those on the other lines.

The one-way ANOVA (Figure 4.26) showed that there was a significant difference between different treatment (each combination of line and age) (F = 7.926, d.f. = 7, p < 0.001). The LSD test showed that the r_m on all the 4 week old plants were identical while the r_m on 8 week old C6207 were significantly lower than the other lines of the same age. Except D1201, the r_m on the 8 week old plants were all significantly lower than their 4 week old counterparts.
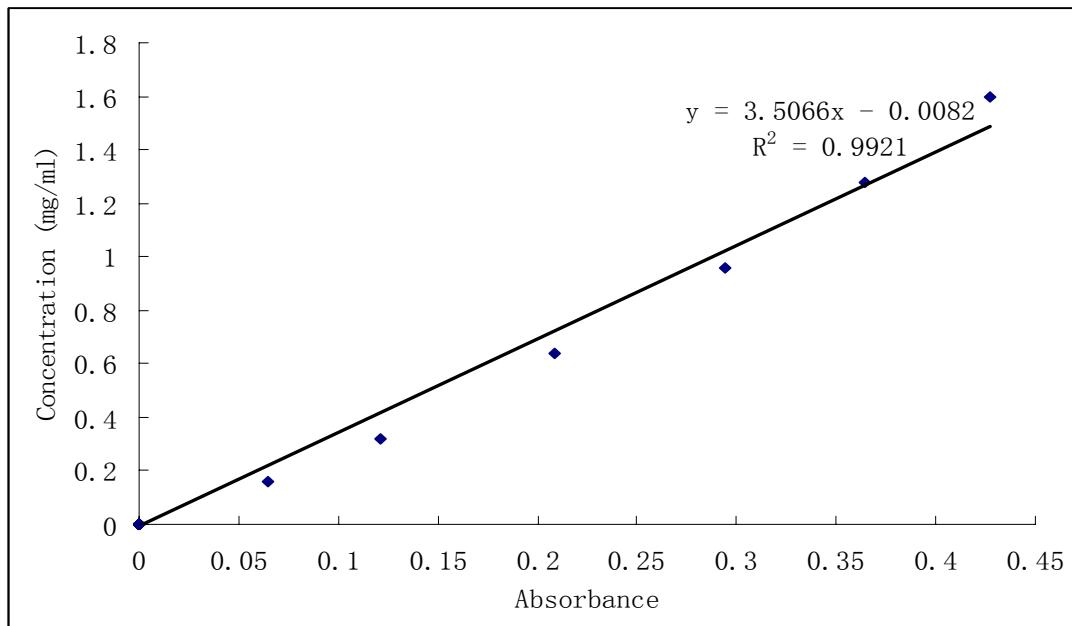
Figure 4.22 Standard curve of Bio-Rad DC protein assay. The X axis indicates the absorbance of the protein samples at 750 nm and the Y axis indicates the concentrations of the protein samples.
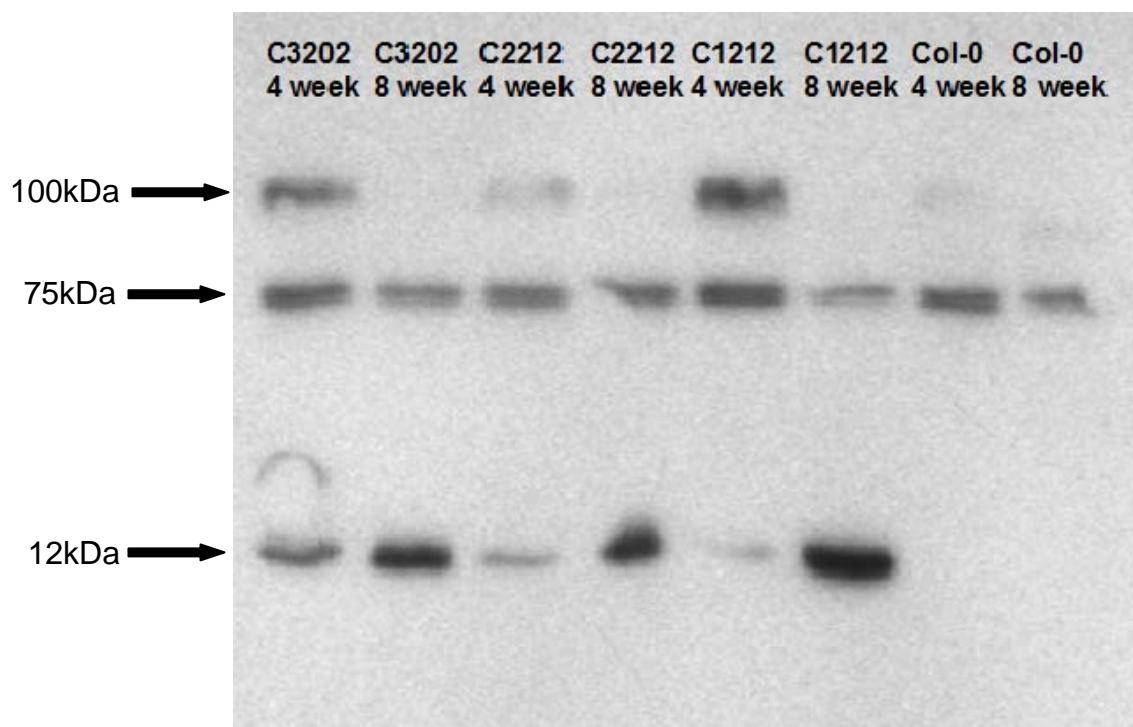
Figure 4.23 Comparison of ATA concentrations in plants (Col-0, C1201, C2212, and C3202) of different age by western blotting with anti-ASAL polyclonal antibody.
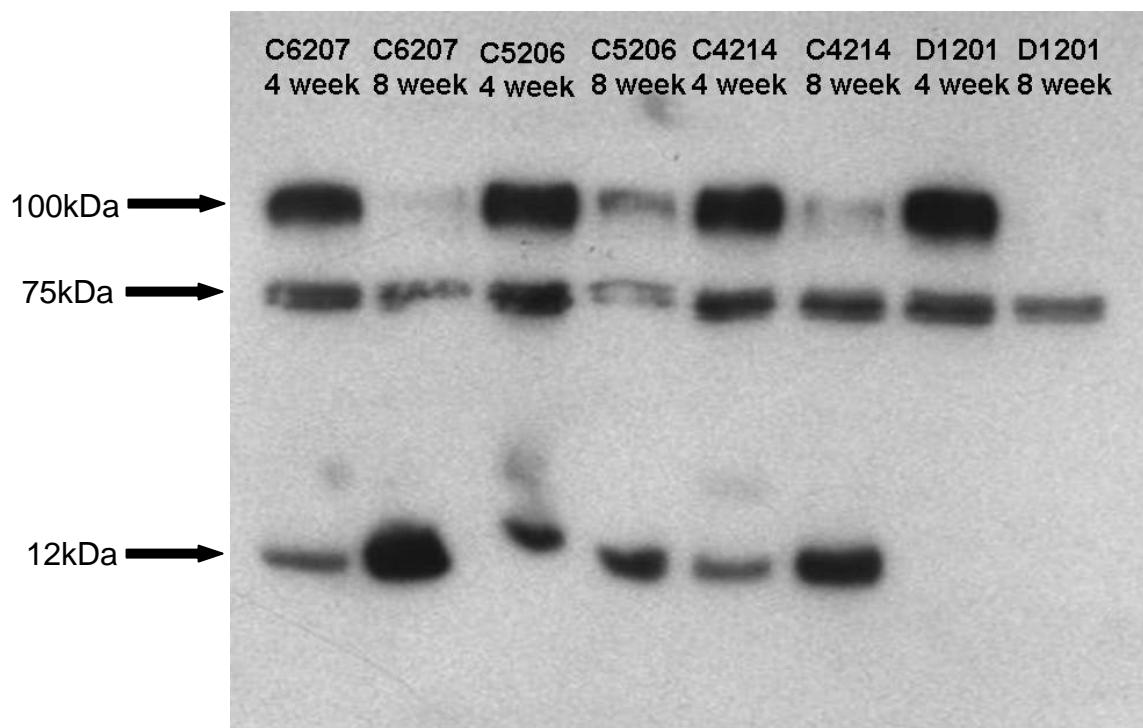
Figure 4.24 Comparison of ATA concentrations in plants (D1201, C4207, C5206, and C6207) of different age by western blotting with anti-ASAL polyclonal antibody.
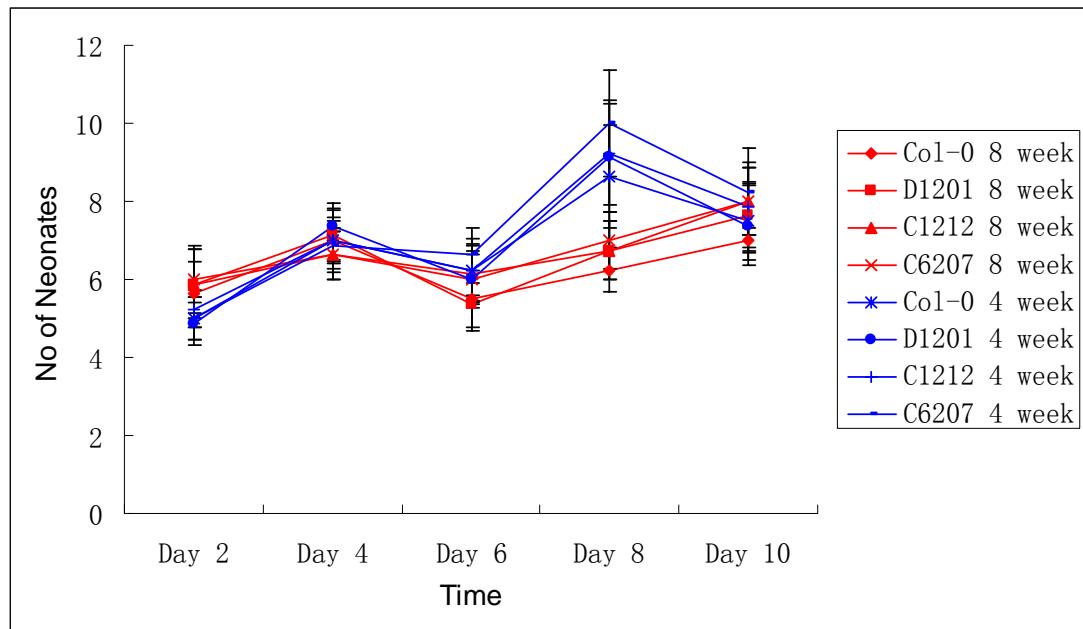
Figure 4.25 Numbers of neonates observed on young and old plants at each time point.
The X axis indicates measuring time points and the Y axis indicates the number of
neonates observed. The lines in red represent the results on 8 week old plants and the lines
in blue represent the results on 4 week old plants. Error bars = SE.
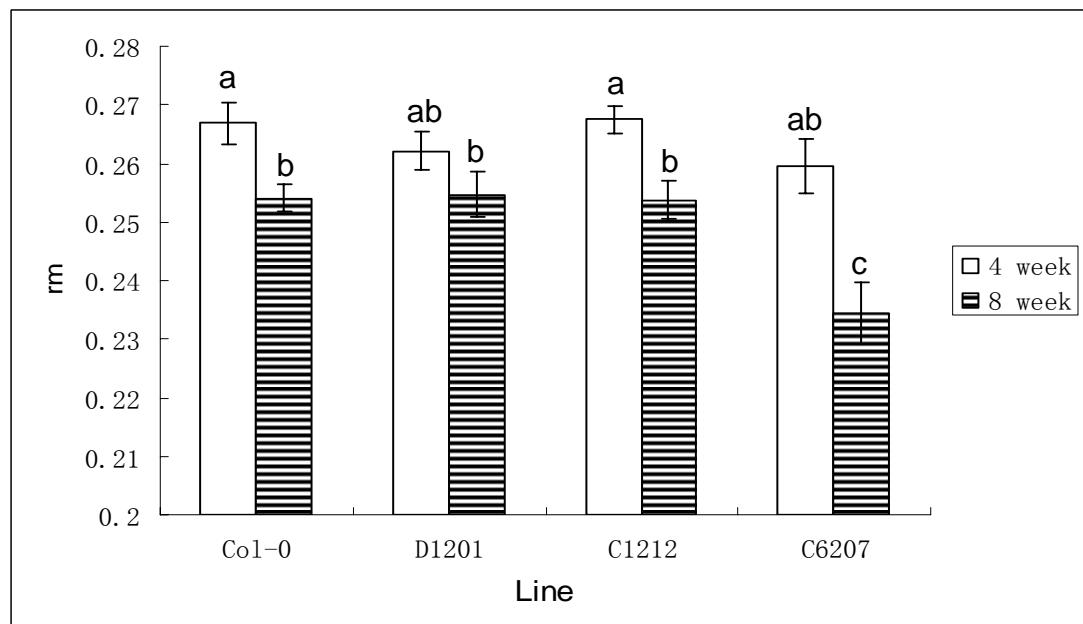
Figure 4.26 The r_m values of aphids on plants of different age. The X axis indicates the
lines tested and the Y axis indicates the r_m of aphids. Error bars = SE.

4.3.7 Resistance against insects other than aphids

When preparing the plants for the plant age controlled fecundity test in 4.3.6, an unexpected thrips invasion took place in the growth room and all the plants were infested. However, it was interesting to note that C6207 plants appeared to be least damaged (Figure 4.27).

Figure 4.27 showed the plants were obviously no longer suitable for aphid bioassays. However, because they were grown in a homogeneous environment, the dry weight of the above ground part can somewhat represent the damage caused by thrips. Therefore, the above ground part of the plants were harvested and dried at 60°C for 3 days to ensure the weight did not change. The weight of the dried plants was measured with a five place balance and the data were compared with one-way ANOVA (Figure 4.28).

The results of the one-way ANOVA showed there was a significant difference in the dry mass of different lines (F = 18.855, d.f. = 3, p < 0.001). The LSD test showed that C6207 had the highest dry mass, wild type Col-0 and C1212 had identical dry mass, and D1201 had the lowest dry mass.

Figure 4.27 Thrips infested Arabidopsis plants.
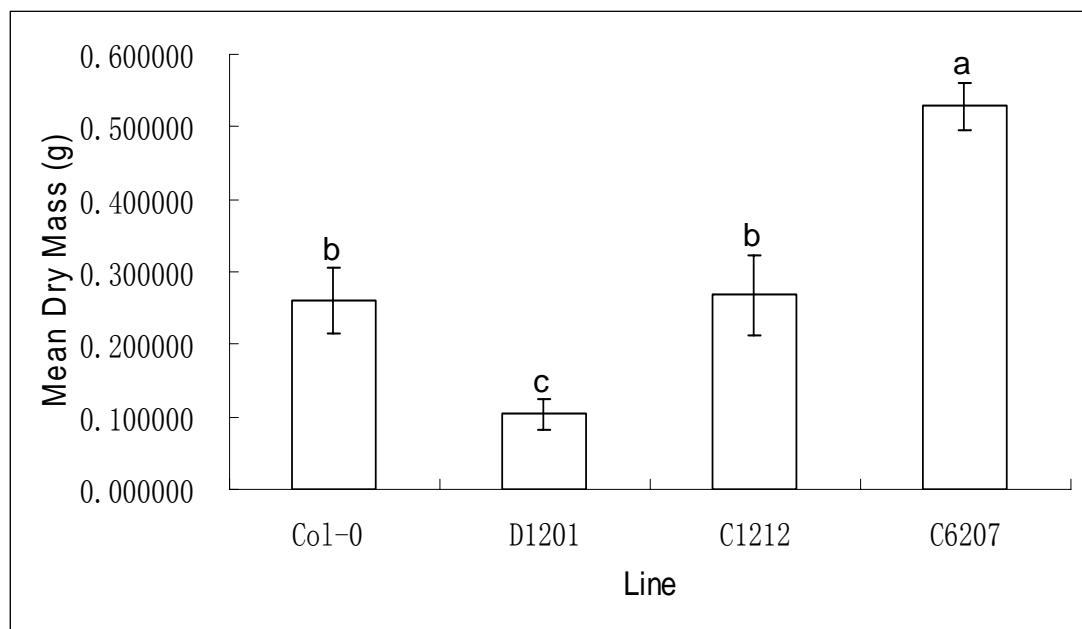
Figure 4.28 Dry mass of thrips infested Arabidopsis. The X axis indicates the lines tested and the Y axis indicates the mean dry mass of the plants. Error bars = SE.

4.4 Discussion

Since plant lectins were first associated with insecticidal properties against homopteran pests (Rahbe and Febvay, 1993), several lectins have been found to be toxic to pests in this order. Among these candidates, *Galanthus nivalis* agglutinin (GNA) and *Allium sativum* leaf agglutinin (ASAL) were the two most promising and well-studied (Hilder *et al*., 1995; Sauvion *et al*., 1996; Gatehouse *et al*., 1996; Nagadhara *et al*., 2003 Bandyhyay *et al*., 2001; Dutta *et al*., 2005). Recently, a leaf-specific lectin gene, *Allium triquetrum agglutinin (ATA)*, has been isolated from *Allium triquetrum*, (wild garlic leaf) (Chapter 2). Western blotting showed that the subunit of this lectin was 12 kDa and the deduced amino acid sequence showed high sequence similarity to GNA and ASAL. The typical mannose-binding domains were also detected in this new protein. All the evidence supports that ATA is a novel mannose-binding lectin. Considering that GNA and ASAL have showed different levels of resistance to homopteran pests when studied using both lectin-containing artificial diets and transgenic plants, it is reasonable to assume that ATA also confers resistance to homopteran pests.

Root pests, stem and fruit borers and sap-feeding pests are not easy to control by chemical sprays. Developing transgenic plants expressing toxins throughout their tissues provides a possible solution. In the present study, a genetic engineering approach was applied to express the novel lectin (ATA) in the model plant Arabidopsis. Whether this novel lectin has insecticidal properties against the homoptera pest *Myzus persicae* and the way in which the expressed ATA affects the behaviour, development, survival and fecundity of *M. persicae* were investigated. The investigation was undertaken with the wild type Col-0, one control line transformed with empty vector (D1201), and six independent *ATA*-transformed homozygous T$_3$ lines (C1212, C2212, C3202, C4214, C5206, C6207).

4.4.1 Choice and non-choice behaviour tests

In the choice behaviour test (4.3.1), when a choice of D1201 and another line (*ATA*-transformants or wild type Col-0) was given to the aphids, the percentages of aphids that made a choice were identical in different choice scenarios at each time point. Therefore, the presence of *ATA*-transformed leaves did not affect the ability of aphids to locate a host. However, the percentages of aphids that made a choice were different at different time points. After 30 min, 74% - 78 % of the aphids made a choice and this proportion increased to 85% - 88% after 2 hr, but it decreased after 24 hr to 79% - 84%.

The changes of the percentages were not unexpected because *M. persicae* is a generalist phloem feeder. It usually takes more time for the generalists to choose a host than the specialists since they depend not only on secondary metabolites but on other nutritional and chemical cues to make a decision (Tosh *et al*., 2003). The more cues they utilize, the harder to make a decision. The decreasing percentage of aphids making a choice after 24 hr is probably due to decreasing quality of the excised leaves. The present design of the choice behaviour test could not reveal whether the odour of the *ATA*-transformed plants was altered, but *M. persicae* usually does not depend much on volatile to distinguish host and non-host plants (Troncoso *et al*., 2005). However, the present experiment allowed aphids to compare the tastes and quality of both the control plants and *ATA*-transformed plants. That the same percentages of aphids chose D1201 in different choice scenarios indicates that *M. persicae* was not able to detect ATA in the plant material. The results of the choice behaviour test suggest that the presence of ATA in Arabidopsis did not reduce the quality of the plant as a host either, because if the quality of the plants is low, aphids are more likely to leave and return in later hours.

It was thought that aphids may behave differently when they are in a non-choice scenario because the non *ATA*-transformed leaves in the choice behaviour test may serve as a kind of refugia and dilute the insecticidal effects of ATA in the choice scenario. Therefore, the non-choice behaviour test was performed (4.3.2). In the group using multi-copy transformed *ATA*-transformed plants (Group M), it is interesting to see that significantly fewer aphids stayed on C3202 and C4214 than those on the control leaves 15 min after they were introduced onto the leaves, but the numbers of aphids that stayed on the leaves of all lines were identical after 30 min and the difference seen at 15 min turned up again after 24 hr. In the group using single-copy *ATA*-transformed plants (Group S), the numbers of aphids that stayed on the different lines were not different in the first 90 min and more aphids stayed on the control leaves after 24 hr. The fact that more aphids left C3202 and C4214 in the first 15 min suggests that ATA, as a "foreign" protein, may have made the host plant taste different. It has been previously reported that such behavioural difference was observed in a study with *Acyrthosiphon pisum* and an artificial diet containing Concanavalin A (Con A) (Sauvion *et al*., 2004). However, this difference may also result from the pleiotropic effects of the transgene to the metabolism of the host plants. The multiple copies of transgenes are believed to cause more changes to the host plants (Wenck *et al*., 1997). Because the difference of feeding behaviour of aphids on C3202 and C4214 at 15 min was not observed in the choice behaviour test, such a difference is more likely to be a mixture of the effects of ATA and pleiotropic effects. The effects of ATA or

the pleiotropic effects alone do not alter the feeding behaviour. After 24 hr, although the average numbers of aphids staying on leaves always decreased, there were still more aphids on the control leaves, which suggests that a decline in quality made the *ATA*-transformed leaves less suitable as a host than the controls. There are two possible explanations for this phenomenon. First, it may take a certain amount of time for the aphids to detect the existence of ATA, or ATA does not have any rapid deleterious effect on aphids. Second, it is well documented that lectin can bind to the receptors in the brush border membrane vesicle in the midgut of homopteran pests (Bandyhyay *et al*., 2001; Dutta *et al*., 2005) and may decrease the permeability of the membrane (Bandyhyay *et al*., 2001). The reduction of the permeability of the membrane can affect the absorbance of nutrients, together with the deceasing leaf quality, making the aphids fail to absorb enough nutrient and turn away.

Although some reports argued that aphids can only select their host after they have landed and inserted their stylets (Kennedy *et al*.,1959; 1959a), it is still widely believed that various factors are related to the aphid behaviour before stylet insertion, including gustatory cues , epicuticular waxes, trichome, substrate texture, topology and colour (Reviewed by Powell *et al*., 2006). Nevertheless, it is almost certain that the stylet penetration is attempted as a reflex following tarsal contact with any solid surface (Powell *et al*., 1999), even in the presence of repellent cues (Griffiths *et al*., 1982; Phelan and Miller *et al*., 1982). Based on the results of both choice and non-choice behaviour tests, it appears that the transformation itself and expressing *ATA* in Arabidopsis should not change all the cues involved in attraction of *M. persicae*. Although a potential behavioural difference was observed in the non-choice behaviour test at 15 min in Group M, aphids soon resumed feeding. If an observation were performed 15 min after the introduction of aphids in the choice behaviour test, this behavioural difference might also have been noticed. Further experiments with both naïve and ATA-conditioned aphids in the non-choice behaviour test could be undertaken to verify this behavioural difference. The long term effects of ATA (after 24 hr) observed in the non-choice behaviour test was not obvious in the choice behaviour test, which was possibly due to the diluting effect of the presence of the control leaves. There are plant genes conferring resistance to aphids acting through phloem-based deterrent factors, as have been shown for resistance of lettuce to *Nasonovia ribisnigri* (*Nr* gene) (Van Helden and Tjallingii, 1993; Van Helden, 1995) or for resistance of melon to *Aphis gossypii* (*Vat* gene) (Chen *et al*., 1996; 1997). However, it can be concluded that the alteration of host selection and ingestion behaviour of *M. persicae* was due to the toxicity of ATA instead of a deterrent effect or a sensory-mediated

process, which is in accordance with the result of the study by Sauvion *et al*., (2004) with *A. pisum* feeding on Con A in an artificial diet.

4.4.2 Nymphal mean relative growth rate (MRGR) and survival test

In Group M, the MRGR values of nymphs on C3202 and C4214 were identical to that on the wild type plant and the MRGR values on C4214 was lower than that on empty-vector control line D1201. Similar observations were obtained in Group S, only the MRGR values of nymphs on C2212 were lower than those on the control plants. MRGR value is a parameter reflecting nutritional quality better than other fitness components (Wellings *et al*., 1980). Therefore, these results indicate that the presence of ATA did not significantly affect the nutrient ingestion of nymphs. The results of the nymphal survival test were similar to the MRGR test. In Group M, the survival rates of nymphs on C3202 and C4214 were lower than on D1201 but the same as those on the wild type plants at both time points (Day 3 and Day 6). In Group S, the survival rates were usually the same at all times. The difference between the survival rates on C5206 and wild type Col-0 on Day 6 was significant but moderate (9%). One possible reason for this small difference is that the expression level of ATA is not high enough. The deleterious effects of lectins on aphids and other homopteran insects have been absolute in moderately high doses (100 – 500 μg/ml) (Bandyopadhyay *et al*., 2001, Roy *et al*., 2002; Yao *et al*., 2003) but lectins at low concentration may even increase the weight gain of *M. persicae* (Sauvion *et al*., 1996). Because ATA has not been purified, the absolute expression level in the plants can not be determined.

The results of both the MRGR and survival tests also suggest that ATA may not effectively reduce the fitness of *M. persicae* nymphs. Similar results have been reported previously. In other studies, GNA has been reported only to reduce the fecundity of *M. persicae* (Down *et al*., 1996; Gatehouse *et al*., 1996). In a study with mustard aphids and mustard expressing ASAL, ACA (*Allium ceva* agglutinin), and ASLA::ACA fusion protein, respectively, it was found that although the presence of ASAL::ACA lead to the lowest LC50, the majority of the dead insects were adults not nymphs (Hossain *et al*., 2006). Sadeghi *et al* (2007) also reported that no difference in neonate growth and survival occurred on *ASAL*-transformed tobacco. In the present study, similar results were observed. In the fecundity test, significantly more adults feeding on the *ATA*-transformed Arabidopsis died than on the control plants (4.3.5), which suggests that *M. persicae* nymphs are less sensitive to ATA than adults. The insecticidal properties of lectins must

relate to their binding property and lectin receptors in the digestive tract have been isolated in various insects (Bandyopadhyay *et al*., 2001; Sauvion *et al*., 2004a; Dutta *et al*., 2005). The fact that nymphs are less sensitive than adults to lectins may indicate that the quantity and formation of these lectin receptors are different in adults and nymphs. This can probably be validated by ligand blot analysis of protein extracts of digestive tract tissue of both adults and nymphs. Another possible explanation is that when adults are giving birth to neonates, they require more nutrients than when in their nymphal stage and are therefore more vulnerable to nutrient shortage. The effects of ATA on nutrient ingestion would be amplified in this situation and lead the adults to death. Although not statistically significant, it seems nymphs performed better on D1201 than on the wild type plants. Since the expression of *nptII* report gene does not alter the host plants (Bergelson *et al*., 1996; Ouakfaoui and Miki, 2005), this better performance should be related to the unexpected effects of the transformation.

4.4.3 Fecundity assay

        In the fecundity test, the numbers of neonates produced by all the surviving adults at each interval on different plant lines were identical (Figure 4.8 and 4.9). The only exception is that more neonates were observed on the control line D1201 than on the *ATA*-transformed lines on Day 2 in Group S. Another major difference was that more adults died on C3202 and C4214 in Group M than those on C1212, C2212, C5206, and C6207 in Group S (Figure 4.14 and 4.15). It has been reported previously that the presence of lectin can alter the dynamics of fecundity by shifting the reproductive peak by more than one day at a relative low dose (Sauvion *et al*., 1996). Similar stimulatory effects caused by lectins at low concentrations have been observed with the cowpea weevil, *Callosobruchus maculatus* (A. Gatehouse, unpublished data). Therefore, this difference in fecundity was likely to be the sublethal effect of lectins, which also happened in the present study. Although the two groups (Group M and Group S) of fecundity test were performed at different times, the mortality of the adults on the control plants in both groups were very similar. Therefore, the differences of mortalities on the *ATA*-transformed lines in the two groups could represent toxicity differences between the lines. However, considering the change of the growth parameters of D3202 and D4214 (Chapter 3), it is also possible that the nutrient levels in these two lines were low, and therefore aphids obtained even less nutrients in the presence of lectin.

When comparing the intrinsic rate of increase (r_m) of the aphids that successfully reproduced, no significant difference was observed between the *ATA*-transformed lines and the controls. This could be partially due to the less powerful non-parametric Kruskal-Wallis test because the least significant difference (LSD) test revealed that the aphids on C3202 and C4214 had lower r_m than those on the control line D1201 and the aphids on C2212 and C6207 had lower r_m than those on both of the control lines. However, the LSD test was not protected by ANOVA, so it was not very reliable. It has also been noticed that there were quite big variations of r_m between different replicates, especially in Group S (Figure 4.19). In Group S, the r_m of aphids on the *ATA*-transformed plants in the second replicate were much lower than their counterparts in the first and third replicates whereas the r_m of aphids on the control plants stayed constant. The most likely reason for this variation is the difference in plant age. In the second replicate, older plants were used because of the infection of an unknown pathogen on the plant prepared for the experiment. Lectins are usually very stable, so it is reasonable to assume that driven by the constitutive expressing promoter CaMV 35S, ATA is likely to accumulate in the plant. Therefore, measuring expression of ATA in the plant is actually measuring the accumulation of ATA. A similar age-dependent pattern of increasing lectin concentration in older plants was observed in *GNA*-transformed rice. (Hilder *et al*., 1995).

4.4.4 The relationship between *Allium triquetrum agglutinin (ATA)* concentration, plant age, and resistance against aphids

Based on the results of the previous aphid bioassays, it was assumed that there is a relationship between ATA concentration and plant age. Western blotting of both 4 week old and 8 week old plants supported the assumption that there was more lectin in older plants (Figure 4.23 and 4.24). The 12 kDa protein band (due to ATA) was detected in all the *ATA*-transformed lines, but more ATA was produced in all the 8 week old plants than in their 4 week old counterparts. An approximate 100 kDa band was detected in all the transgenic plants but not in the wild type, which matched the result in Chapter 3 (3.3.6). Interestingly, this band also showed a correlation between plant age and concentration. But by contrast with ATA, its concentration decreased with plant age. Because this band was also detected in D1201, it cannot be related to ATA, but it is still of interest to identify it in the future because of this age-concentration correlation. Another 75 kDa band, which was not detected in the western blotting in Chapter 3 (3.3.6), appeared in all the tested plants. Lectin is abundant in plants (Carlini and Grossi-de-sa, 2002), so this 75 kDa band may

represent an Arabidopsis origin lectin or lectin-like protein. The amount of this 75 kDa protein is relatively weak and constant, so it should not affect the aphid bioassays too much. The repeated fecundity test proved that there was a correlation between the lectin concentration and its anti-reproduction ability (Figure 4.25). It is not surprising to see the numbers of neonates produced on the 8 week old plants are all lower than on their 4 week old counterparts because theoretically, levels of constitutive defence compounds increase, and nutrient and water content decreases in older plants (Karban and Myers, 1989). According to the preference-performance hypothesis (Jaenike; 1978), *M. persicae* should therefore prefer younger leaves, which is in contrast with the work with yellow sugarcane aphid, *Sipha flava* (Forbes) and Johnsongrass, *Sorghum halepense* (Gonzales *et al*., 2003). In their work, they found that the yellow sugarcane aphid preferred older leaves. The results proposed that when designing the experiments, the experimental designs of other insect-plant system should not be generalised. The aphids on 8 week old D6207 plants gave birth to significantly fewer neonates than on other 8 week old lines (22% fewer). This significant difference was not observed in the 4 week old plants. Together with the results in the previous fecundity test, the way in which ATA reduces aphid reproduction is dependent on the concentration of the lectin, and ATA needs to reach a certain threshold concentration to be effective.

Another interesting finding is the obviously different patterns of aphid reproduction on plants of different age (Figure 4.24). The aphids on the older plants exhibited a good start after two days but failed to reach their reproduction peak after eight days. The fact that the reproduction patterns were identical in the plants of the same age indicates that the presence of ATA simply reduced the number of neonates reproduced instead of shifting the timing of reproductive peak. That such a clear reproduction pattern was not observed in the previous fecundity test is due to the variation in plant age. Therefore, it is important to strictly control the plant age in fecundity test. Various research groups have reported the ability of lectin to reduce the fecundity of aphids (Down *et al*., 1996; Gatehouse *et al*., 1996; Dutta *et al*., 2005; Hossain *et al*., 2006). Because of the stability of lectin, the lectin can survive its passage through the insect alimentary tract and be absorbed unchanged into the circulation. Then it may pass to any distant site within the body via the circulating hemolymph. After oral uptake from an artificial diet, the mannose-binding lectin GNA was recovered throughout the hemolymph of the rice brown plant hopper ovariole (Powell *et al*., 1998). Similarly, ASAL was detected in the ovary by western blotting, indicating that ASAL was taken up by the insects and reached the organ, which is a sink for circulatory nutrients (Dutta *et al*., 2005). After the lectin reaches the

ovariole or ovary, it may bind to certain receptor(s) and affect fecundity. Potential receptors of other lectins in midgut of a few homopteran insects have been isolated by ligand blot analysis, and therefore, the same technique can be used to recover the receptor(s) of ATA in the ovariole and ovary of *M. persicae*.

4.4.5 Thrips infected Arabidopsis

Unlike aphids, thrips have only one mandibular stylet, or if a second is present it is greatly reduced and non functional. The one fully formed mandibular stylet is used to pierce an entry hole in plant cells or pollen grains, wherein the maxillary stylets can easily enter the cell and suck out the contents (Hunter *et al*., 1994). Many thrips are pests of commercial crops due to the damage caused by feeding on developing flowers or vegetables which causes discoloration, deformities, and reduced marketability of the crop. In the present study, after infection by thrips, C6207 plants followed normal development while deformities were very obvious on other lines (Figure 4.26). The result of the comparison of the dry mass of all the plants matches the observations in the previous aphid bioassays surprisingly well. The mean dry mass of C1212 was same as that of wild type Col-0, indicating if the lectin level is not high enough, the resistance is low. And the lowest dry mass of D1201 corresponds to the best performance of aphids on this line. This preliminary observation was not performed under well-controlled conditions, such as same infestation time and same starting population, but considering that the positions of the plants were random when grown in the growth room, the result should not be completely meaningless. By contrast with the study of Bt Cry toxin (Dutton *et al*., 2004), most of the studies of lectins seem to focus on the life parameters and behavioural changes of insects. Therefore, more effort should be devoted to testing whether the lectin-transformed plant can survive the infection of pests (Gatehouse *et al*., 1997).

4.5 Conclusions

Myzus persicae is a notorious pest, especially because as a vector, it transmits many virus diseases (Kennedy *et al*., 1962). Since the presence of ATA does not change the host selection behaviour of *M. persicae*, the transmission of virus diseases could not be controlled by ATA. Although a recent study suggests that ASAL has the potential to impair the transmission of symbionin-mediated luteovirus (Banerjee *et al*., 2004), the transmission of non-circulative viruses remains a problem. ATA has only minor effects on

the development and survival of nymphs. But the results suggest that ATA can reduce the fecundity of *M. persicae* and cause relatively high mortality to the adults. The deleterious effect is likely to be related to the concentration of ATA. It is possible that there is a high lethal threshold that kills both adults and nymphs and a low lethal threshold which only kill adults. Under the low threshold, the lectin does not kill but decreases the fecundity and the higher ATA concentration leads to the greater decrease. This proposal is also supported by the work of Sauvion *et al*. (2004a). In their research on growth of *Acyrthosiphon pisum* on artificial diets incorporating Concanavalin A (Con A), they found that at a Con A concentration up to 400 µg/ml, lectin binding only occurred in the stomach region; however, at higher concentration (800 µg/ml), the whole digestive tract bound lectin. It is worth mentioning that aphids seem to perform better on the empty vector-transformed plants, D1201 than on the wild type and *ATA*-transformed plants. This change is probably due to the unexpected effects of transformation, which should be carefully studied and ensured to have no implication prior to any application of transgenic crops. This also highlights the importance of using the empty vector transformed plants as negative controls. Generally speaking, it emerged that *M. persicae* is less sensitive to mannose-binding lectins than are specialists (Sauvion *et al*., 1996). *M. persicae* is one of the most generalist aphid species. Its ability to use a large range of food sources suggests an effective adaptation system to cope with various defence mechanisms in host plants. Therefore, ATA may confer resistance better to other specialist sap-feeders than generalists. However, greenhouse experiments with *Aphis gossypii* indicate that a strain with a low rate of population increase and low carrying capacity can nevertheless outcompete a strain with superior population parameters if the former infests the plants first (Rochat, 1997). Aphids invest most of their energy in production of offspring rather than on fat reserves, so the decrease in reproduction of *M. persicae* is meaningful. Moreover, a recent study shows that GNA can also impair the development of *Sopdoptera littoralis* (Sadeghi *et al*., 2008). The present observation suggests that ATA can reduce plant damage caused by thrips, so ATA may also have a broad-spectrum pest resistance.

Chapter Five

5 Summary and General Discussion

5.1 Summary of findings

5.1.1 The existence of lectin genes in Allium triquetrum

By using the rapid amplification of cDNA ends (RACE) technique, several lectin or lectin-like genes were detected in the transcriptome of *A. triquetrum* (2.3.2). From these, a leaf-specific lectin gene, *Allium triquetrum* agglutinin (*ATA*) was selected and cloned. The open reading frame of *ATA* is 522bp encoding a 173 amino acid polypeptide. *ATA,* and its deduced amino acid sequence, have high sequence similarities to previously reported monocot mannose-binding lectin genes (Figure 2.8). Three putative mannose-binding subdomains were detected in the mature protein.

5.1.2 The molecular evolution evidence

The molecular evolution analysis supports the conclusion that *ATA* is a leaf-specific lectin gene and also suggests that ATA is likely to be a homodimer. Focusing on the relationship between the predicted subdomains in ATA suggests that the formation of present ATA is possibly the result of subdomain duplications (see 2.4).

5.1.3 The transformation of Allium triquetrum agglutinin into Arabidopsis and its expression

Once the status of ATA as a monocot mannose-binding lectin was established by sequence alignment and molecular evolution analysis (see 2.3.3), it was introduced into Arabidopsis, by the *Agrobacterium*-mediated floral dip method, for studying the unexpected transgenic effects (Chapter 3) and insecticidal effects (Chapter 4) of ATA. Six homozygous *ATA*-transformed lines were selected. The transformation and expression of *ATA* in Arabidopsis was verified by PCR (Figure 3.2 and 3.3), R/T PCR (Figure 3.4, 3.5, and 3.6), and western blotting (Figure 3.7 and 3.8). The western blots revealed that the size of mature ATA is approximately 12 Kda which resembles most of the reported mannose-binding lectins. The subsequent molecular modeling of ATA indicates that it has a similar

three-dimensional structure as *Galanthus nivalis* agglutinin (GNA). The molecular modeling work also reinforces the assumption that mature ATA is a homodimer.

5.1.4 The effects of expression of Allium triquetrum agglutinin on Arabidopsis

The *ATA*-transformed lines showed a variety of phenotypic characteristics, such as early flowering, fewer leaves, lower dry mass, and reduced seed production (Chapter 3). Multi-copy transgenes (as in C3202 and C4214) usually have more pleiotropic effects (Wenck *et al*., 1997) and this was seen in their phenotypes. It was thought that there might be a trade-off between expression of ATA and seed production in Arabidopsis because all the *ATA*-transformed lines showed lower seed yield than the wild type plants. However, the reduced seed production could not be correlated with the expression levels of ATA in Arabidopsis (Figure 3.30, Figure 4.23, and Figure 4.24). In Figure 4.24, the expression level of ATA in C6207 was the highest among all the *ATA*-transformed lines, but the same line had the most similar seed yield to the wild type. In fact, C6207 showed fewer morphological changes than all the other lines in various growth parameter characterization tests, which suggests that the effects of expressing *ATA* ectopically maybe minor. The results of choice (4.3.1) and non-choice (4.3.2) tests with *M. persicae* actually proved that (except for production of volatile chemicals, which could not be tested in the present experimental design), a range of plant features that affect *M. persicae* host selection were not significantly altered in *ATA*-transformed lines.

5.1.5 The effects of Allium triquetrum agglutinin on the feeding behaviour of Myzus persicae

The presence of ATA in Arabidopsis has minor effects on the feeding behaviour of *M. persicae* in several ways. In the choice behaviour test, the experimental design allowed comparison of the behaviour of aphids towards the empty vector-transformed plants, the wild type and the *ATA*-transformed plants. That the same numbers of aphids made a choice in different choice scenarios indicated that ATA did not affect the ability of aphids to locate the host. Thus, ATA does not block the chemosensory sensilla of aphids that would prevent them from locating food, which is in accordance with the previous work with other lectins (Hilder *et al*., 1995; Sauvion *et al*., 2003). The same numbers of aphids chose all three types of plants in different choice scenarios suggesting that the

features of the tested plants that affect how *M. persicae* chooses a host have not been altered either by the unexpected transgenic effects or by the expression of ATA itself. However, some repellent effects of ATA to aphids were observed at the start of the non-choice behaviour test, being possibly correlated with ATA concentration. But the results suggest that this does not have a major effect on the feeding behaviour of the aphids (4.3.2).

5.1.6 The insecticidal effects of Allium triquetrum agglutinin on the development of Myzus persicae

5.1.6.i Development of aphids

Although the mean relative growth rates (MRGR) of nymphs on two *ATA*-transformed lines are lower than those on the controls, the result could not be correlated with the ATA expression level. Thus, this reduced MRGR could not be completely ascribed to the presence of ATA. Previous work demonstrated that low doses of lectin can induce a significant increase in weight gain (Sauvion *et al.*, 1996). Therefore, ATA possibly has minor effects on the nutrient ingestion of *M. persicae* nymphs (4.3.3).

5.1.6.ii Survival of aphids

More adult aphids died on the *ATA*-transformed plants in the fecundity test (4.3.5) whereas the same numbers of nymphs survived on the *ATA*-transformed plants and control plants (4.3.4). This suggests that adult *M. persicae* are more sensitive to ATA than the nymphs. This survival difference could be due to a difference in the numbers and types of lectin receptors in adults and nymphs, or due to a greater vulnerability to nutrient shortage.

5.1.6.iii Fecundity of aphids

The aphids on four lines showed lower fecundity than those on the control plants. The fact that ATA affects the fecundity of aphids can be linked to the high mortality of adults in the fecundity test, indicating that ATA confers greater insecticidal effects towards adults.

5.1.7 The relationship between Allium triquetrum agglutinin concentration and plant age

The western blotting of young and old plant material reveals that the ATA content was higher in older plants. The fecundity test showed that the insecticidal effects of ATA depend on the concentration of ATA in both young and old plants. Together with the results of other aphid bioassays (Chapter 4), it can be concluded that there are possibly two thresholds for the lethal effect, a higher lethal dose for nymphs and a lower lethal dose for adults. Below the lower lethal dose, the sublethal effects appear to be reduced fecundity.

5.1.8 The insecticidal effects of Allium triquetrum agglutinin on thrips

An unexpected observation suggests that *ATA*-transformed Arabidopsis may have good resistance to thrips (4.3.7). Line C6207 having the highest expression level of ATA showed outstanding fitness (Figure 4.26) and the best resistance to aphids, which suggests that its resistance against thrips is not accidental.

5.2 General discussion

"Two conditions must be satisfied in order to produce transgenic plants with enhanced resistance to sap-sucking insects. First, gene products must be indentified which are effective against sap-suckers and whose encoding genes can be obtained and transferred. Secondly, an effective means of delivery of the chosen gene products to the insects, which feed exclusively on phloem sap, must be developed for use in transgenic plants." (Hilder *et al*., 1995)
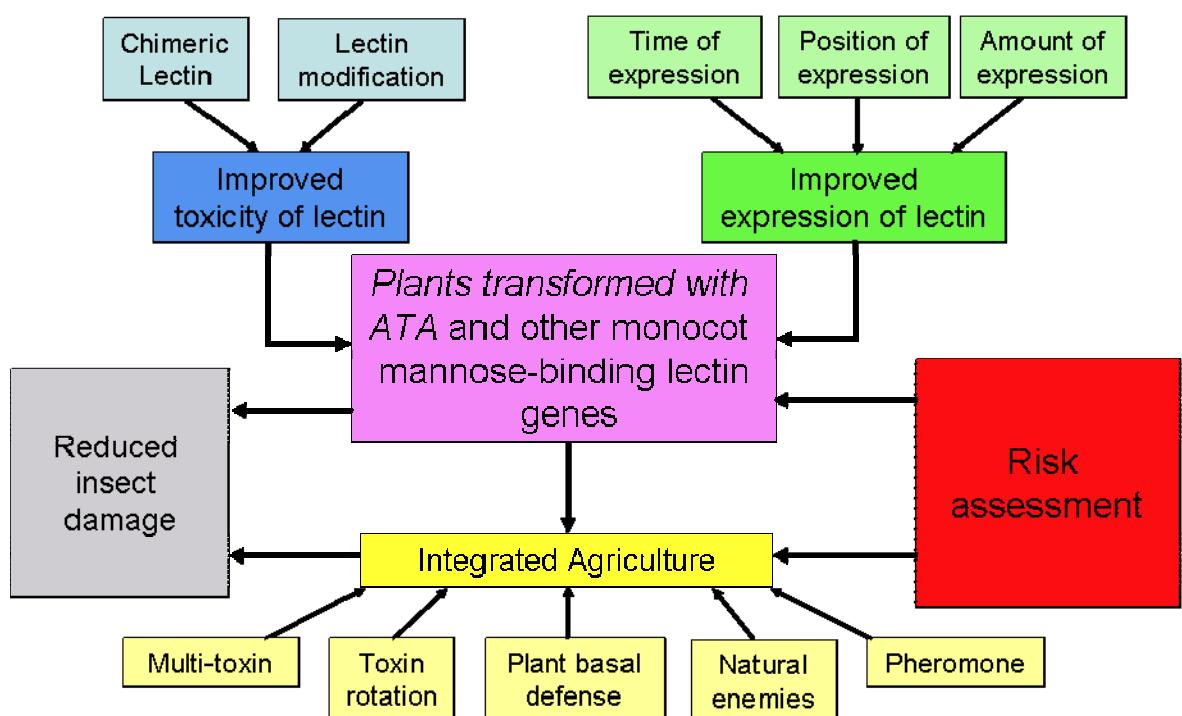
Figure 5.1 The future of ATA and other monocot mannose-binding lectins in agriculture.

5.2.1 How to improve the insecticidal effects of Allium triquetrum agglutinin?

Inspired by the success of *Bt*-transformed crops, the study of monocot mannose-binding lectin has never stopped since the isolation of GNA (Van Damme *et al*., 1987). However, these lectins usually only confer partial resistance to sap-feeding pests (Down *et al*., 1996; Gatehouse *et al*., 1996; Sauvion *et al*., 1996; Yao *et al*., 2003; Sadeghi *et al*., 2007) and strong detrimental effects of the lectins on sap-feeding pests have required moderately high doses of lectins (Sauvion *et al*., 1996; Bandyopodhyay *et al*., 2001; Roy *et al*., 2002; Yao *et al*., 2003). In the present study, the novel lectin, ATA, also confers only partial resistance against *M. persicae* and the dose dependant toxicity of this lectin is also

very likely (4.3.3-4.3.6). Nevertheless, such partial resistance against sap-feeding insects is still considered significant because no other insecticidal agents for transgenic use against sap-feeding pests have proven to be adequately effective (Ferry *et al.*, 2004). However, according to the definition by the Environmental Protection Agency (EPA, USA), the adequate protection should be >95% mortality of target insects. Therefore, if the insecticidal effects of ATA can be improved, it could have a promising future. With respect to the improvement of effectiveness, two aspects should be considered, the toxicity of ATA itself and the expression of ATA in crops. Figure 5.1 summarizes the process of improvement of plants transformed with *ATA* and other monocot lectin genes for use in agriculture.

5.2.1.i The toxicity of lectins

One way to improve the toxicity of lectins is producing chimeric proteins or fusion proteins. The type-2 ribosome inactivation protein (RIP) is a naturally occurring example of chimeric proteins. A typical type-2 RIP consists of two chains, a B-chain for binding of carbohydrates and delivery (Frigerio and Roberts, 1998) of an A-chain for cleavage of ribosomes. Since binding of the B-chain resembles the way that the lectins work, therefore, type-2 RIP is actually regarded as a lectin (Van Damme *et al.*, 1998). The type-2 RIP provides an ideal model for a chimeric protein. Recently, a chimeric protein produced from Bt Cry1Ac toxin and the ricin B-chain has proved to be more toxic towards the Egyptian cotton leafworm, *Spodoptera littoralis,* than Cry1Ac alone and conferred resistance towards a homopteran insect the leafhopper, *Cicadulina mbila*, which has not been observed previously. If monocot mannose-binding lectins, such as ATA, are fused with other toxins, for example, Bt Cry toxins or protease inhibitors, the chimeric proteins may have better resistance to sap-feeding pests. In fact, chimeric proteins with two or more lectins can show better resistance to sap sucking pests. For example, a chimeric protein of *Allium sativum* leaf agglutinin (ASAL) and *Allium ceva* agglutinin (ACA) has lower LC50 than either ASAL or ACA alone. When mustard was transformed with the fused gene, *ASAL::ACA*, a higher mortality to aphids was seen than in mustards expressing ASAL or ACA alone (Hossain *et al.*, 2006). As all of these lectins are also from Alliacae, it is possible that if ATA is fused with ASAL or ACA, better resistance may also be achieved.

Another way to improve the toxicity of ATA is by protein modification. The toxicity of Bt Cry1Ab toxin towards larvae of the gypsy moth, *Lymantria dispar*, increased up to 40 fold by modifying the amino acid residues in loop regions of domain II

(Rajamohan *et al*., 1996). This technique has not been used on lectins to increase the toxicity yet, but it has been reported that the change of a few amino acids which are involved in sugar binding and variations in the length of loops did not affect the overall three-dimensional structure of the protomer of lectins (Young and Oomen, 1992; Sharma and Surolia, 1997), which suggests that it is possible to modify ATA without changing its overall structure. Some lectins have been proved to be more toxic to certain insects than others, so the crucial residues involved in the toxicity can be identified by comparing amino acid sequences, and thus the toxicity of some lectins can be increased by modifying these residues.

5.2.1.ii The expression of lectins

When discussing "expression", three factors need to be considered: time, position, and amount. In the present study, the expression of *ATA* was driven by a CaMV 35S promoter, so it is not surprising that the constitutive expression of ATA will bring some costs to the plants. Indeed, it has been demonstrated that the expression of ATA can decrease seed production (3.3.12). It is reasonable to assume if the expression of ATA is not activated until the plants are attacked by the insects, which can be achieved by using inducible promoters, the costs can be reduced. As their name says, the activity of these promoters is induced by the presence or absence of biotic or abiotic factors. The currently available inducible promoters can be divided into physically-regulated promoters and chemically-regulated promoters. Physically-regulated promoters are usually not appropriate for resistance to aphids in agriculture because these promoters are regulated by light, temperature, salt stress, and wounding. Except wounding, all the other factors are not easy to control in open fields. The feeding of *M. persicae* can induce a variety of defence responses in Arabidopsis (Girling *et al*., 2008). If the promoters that control the expression of the genes involved in the defence responses are cloned, they can be used to drive the expression of *ATA* induced by infestation of aphids. The chemically-inducible promoters can be modulated by chemical compounds, such as ethanol, antibiotics, and hormones (reviewed by Gatz, 1997), which either turn off or turn on gene transcription. If a chemically-inducible promoter is attached to *ATA*, the expression of *ATA* can be activated by simply spraying chemicals when the infestation of aphids is observed.

Sap-feeding pests exclusively feed on the phloem sap, in which the protein concentration is generally very low, so the specific expression of lectins in phloem can increase the concentration of lectins in the phloem sap (Dutta *et al*., 2005). However, there

is no substantial evidence to suggest that usage of a phloem-specific promoter attached to the lectin gene can lead to better protection for the plants (Dutta *et al*., 2005; Saha *et al*., 2007). Nevertheless, using tissue-specific promoters may reduce the transgenic effects as well because the total amount of expressed ATA in the plant is reduced. Inducible promoters and tissue-specific promoters may also help to minimize the potential negative effects to non-target organisms because there is less chance for them to be exposed to the toxins.

It is possible higher copy numbers of the transgene in plants can lead to higher expression, but the present study suggests that multi-copy transgenes cause larger phenotypic differences (Chapter 3). Therefore, increasing the copy number of the transgene in the host genome is obviously not a sensible choice. There are two other ways to increase the total amount of expressed ATA in plants. Some DNA sequences, called enhancers, can increase the transcription levels by cis-acting (Khoury and Gruss, 1983). If these enhancers can be applied in the production of *ATA*-transformed plants, the expression level of ATA may also be increased. Same as the two types of promoters described above, there are two special types of enhancers, tissue-specific enhancers and inducible enhancers, so the enhancers can be applied together with the same type of promoters to get synergetic effects. Within the plant genome, there are areas, such as subtelomeric regions, which exert positive position effects due to their high transcriptional activity (Topping *et al*., 1991). The *ATA* integrated in this kind of region may be strongly expressed. The site-specific transformation technique (Nanto *et al*., 2005) makes it possible to integrate *ATA* into those positive effect positions. However, posttranscriptional gene silencing can be triggered by threshold concentrations of the transgene transcript (Schubert *et al*., 2004). Therefore, the strategies described above to increase the expression of *ATA* may not always work.

5.2.2 The problems which may occur with Allium triquetrum agglutinin in Agriculture

The success of *Bt*-transformed crops inspired the research with lectins whereas the problems that have been experienced when using Bt Cry toxins are lessons for the future application of ATA and other lectins. The problems include evolution of resistant insects (Tabasnik 1994; Perez and Shelton, 1997; Ferre and Van Rie; 2002) and the concerns about the potential risk of using Bt Cry toxin (Losey *et al*., 1999; Poppy, 2000; Meissle *et al*., 2005; Wei *et al*., 2008). Certainly, the same problems will occur with ATA

and other lectins if they are also commercialized and it is of benefit to address these problems before they turn up.

5.2.2.i Resistance of insects to lectins

Following the introduction of *Bt*-transformed plants, insects have gradually evolved resistance to the Bt Cry toxins (Tabasnik 1994; Perez and Shelton, 1997; Ferre and Van Rie; 2002). Till now, there is no report of insect resistance to lectins. However, because the most likely way in which lectins work is binding to receptors in the insect digestive tracts, insects may be able to overcome the toxicity of lectins by changing the amount of the receptors as how they cope with Bt Cry toxins and protease inhibitors (Ferre and Van Rie; 2002 and 1.7.3.ii). There are two ways to deal with the insect resistance: pyramiding of toxins and rotation of toxins.

The present study suggests that ATA is a homodimer (2.3.3.ii and 3.3.7), but the other published mannose-binding lectins have various quaternary structures, such as homotetramer, heterotetramer, and heterodimer (Van Damme *et al*., 1998), which suggests that different mannose-binding lectins probably have preferable binding receptors in insects. If the specific binding does exist, insects must require multiple simultaneous mutations or the recombination of different mutations from several sources to overcome the pyramiding of the lectins, which is not likely to take place in nature (Gatehouse and Gatehouse, 1998; Maqbool *et al*., 2001; Zhao *et al*., 2003; Gould, 2003).

The adaptation cost is another principal that can be used to deal with insect resistance. In general, adaption to one environment can impose fitness costs in other environments (Ghalambor *et al*., 2004). In the case of Indian Meal Moth grown in the presence of different Bt Cry toxins, a receptor shift has been observed (Van Rie *et al*., 1990). The insects resistant to Cry1A became more sensitive to the Cry1C toxin, providing an ideal demonstration of the potential of the toxin rotation strategy. As discussed above, the mannose-binding lectins may bind to different receptors in insects, so the toxin rotation strategy is also suitable for lectins. This also highlights the importance of isolating more mannose-binding lectins from potential sources, which is one of the major aims of this project.

It is also possible to pyramid or rotate mannose-binding lectins with other toxins, such as Bt Cry toxins, protease inhibitors, and lectins in other families. The combination of different toxins can not only broaden the target spectrum but also reduce the risk of

outbreaks of secondary pests that might otherwise occur when the main pest is no longer present as a competitor.

5.2.2.ii The concerns about the risk of using lectins

The concerns can be divided into two parts: the concern about the transgene and the concern about the techniques. It is true that a few lectins are toxic towards higher mammals, such as Concanavalin A and type 2 ribosome inactivation proteins whereas many lectins are safe, such as ATA and other mannose-binding lectins. However, some papers claimed to be unbiased, but they only focused on the toxic lectins and ignored those which are safe. As Poppy (2000) pointed out the public concerns about genetically modified (GM) crops are increasing by the "continuing twists and turns as new research is published, the conclusions of which are often misrepresented and even misinterpreted by the public". It is every scientist's duty to make sure that the public treat new technology with rationality instead of outrage. Indeed, there were lots of failed cases, even crises because of new technologies, but obviously stopping-eating is not the right way to prevent choking. The correct attitude towards new technologies should be used with caution. Selecting the right gene is one major issue of caution when using lectin-transformed plants. The major public concern about transformation is the unexpected effects of the transgene and the ethical concerns. ATA originates from plants, so it raises less ethical problems. The present study showed the unexpected effects of ATA on the host plants were very minor. The public may also worry about the presence of selection markers in GM plants. The marker-free techniques (Dale and Ow, 1991) can be applied in lectin-transformed plants to increase the acceptance by the public. The concern about the transgene focused on the toxicity of the "foreign" protein towards humans and non-target organisms. In a 90-day safety study in Wistar rats fed rice expressing GNA, no obvious adverse effects were observed (Poulsen *et al.*, 2007). *A. triquetrum* is consumed raw or cooked by people who are fond of wild food, so ATA should be even safer than GNA. The potential hazard of ATA on non-target insects cannot be predicted based on the present study. So far, the direct deleterious effects of other mannose-binding lectins on non-target insects are still controversial (Romeis, 2003; Wakefield *et al.*, 2006; Hogervorst *et al.*, 2006). Certainly, more work is required to assess the toxicity of ATA against non-target organisms before it is applied in the practical use. To summarize, based on the present study, ATA-transformed plants are not likely to cause any environmental crisis or big public concern.

However, this heavily depends on that all the information is precisely transferred to the public.

5.3 Future work – What next?

This thesis describes the characterisation of a novel lectin gene, *Allium triquetrum* agglutinin (ATA), its transformation into Arabidopsis and a study of the effects of the presence of ATA on *Myzus persicae*. It is appropriate to conclude with a few suggested further studies.

In the present study, a positive correlation between plant age and ATA concentration in the transgenic plants was observed (4.36) and the toxicity of ATA depends on its concentration. It was also assumed that there are two possible lethal concentration thresholds of ATA for *M. persicae*, a lower one for adults and a higher one for neonates (4.4.2 and 4.4.3). The easiest and most robust way to verify the assumption is using artificial diets incorporated with ATA, in which the concentration of ATA can be precisely controlled. Mannose-binding lectins can be isolated from the original plants by affinity chromatography. The same technique can also be used to purify the ectopically expressed ATA and compare its biological activity with naturally occurring ATA.

If the hypothesis of the two lethal thresholds is proved by using artificial diets, it will be very interesting to find out the reason. It is possible that because adults need to give birth to offspring, they are more vulnerable to nutrient shortages. But it is also possible that the amounts of ATA receptors in nymphs and in adults are different. Ligand blot analyses on the protein extract of digestive tract tissue have been used to study the potential receptors in insects of mannose-binding lectins (Majumder *et al*., 2004; Dutta *et al*., 2005). By comparing the signal strength of the ligand blots of both adults and nymphs, the mechanism of how ATA works can be understood better.

As discussed in 5.2.2.ii, the present study did not specify the potential harm of ATA on non-target organisms. Therefore, the ecological risk assessment (ERA) and the tiered-risk assessment must be performed (Poppy, 2000; 2003). The ERA is used to decide the concerned environmental value (assessment endpionts) and parameters to describe the assessment endpoints (measurement endpoints).Once both type of endpoints were decided, the tiered-risk assessment needs to be performed to assess risk in three different levels, namely, laboratory studies under worst case scenario conditions, semi-field extended laboratory studies, and field studies. Only if the results of all the three tiers are acceptable, could ATA be practically used in agriculture.

Some unexpected observations showed that ATA may also confer resistance to thrips (4.3.7). However, because the conditions were not well controlled, the conclusion was not reliable. Nevertheless, ATA possibly confers resistance to other insect species. The present study suggests *M. persicae* may not be target organism that ATA can control most effectively (Chapter 4), and therefore, bioassays with other insect species, especially other sap-feeders, will reveal better target organisms.

The partial resistance against *M. persicae* that ATA showed in the present study may not be impressive, but several authors suggest that biocontrol is more efficient when combined with partially-resistant plants rather than with completely resistant plants (Van Emden, 1991; Wellings and Ward, 1994), which means ATA can act synergistically with other biocontrol techniques. In addition to the studies on mannose-binding lectins, scientists are also working hard on other possible methods to control insects, such as induced plant basal defence, natural enemies, and pheromones. In the future, ATA and other mannose-binding lectins can be an important component of the integrated agriculture. Last but not the least, because the mechanisms of different combinations of insect-controlling measures are very likely to be different, the risk of different combinations of insect-controlling measures are also different. Therefore, in whatever way that ATA is applied, alone or integrated, intact or modified, proper tiered risk assessment in the framework of ERA must be performed case by case before any measures are used.

Appendix 1 Buffers and Media

Buffers

1× Blocking buffer = 3% (w/v) skimmed milk powder in TBS

Buffer E = 125mM Tris-HCl pH8.8, 1% (w/v) SDS, 10% (v/v) glycerol, 50mM $Na_2S_2O_5$

Buffer Z = 125mM Tris-HCl pH6.8, 12% (w/v) SDS, 10% (v/v) glycerol, 22% (v/v) $HOCH_2CH_2SH$, 0.01(w/v) bromophenol blue

Coomassie brilliant blue stain = 40% (v/v) CH_3OH, 6% (v/v) Glacial CH_3COOH, 0.125% Coomassie brilliant blue.

10×Orange G DNA loading buffer = 0.25% (w/v) Orange G, 15% (w/v) ficoll in water

Protein Gel Destain buffer = 40% (v/v) CH_3OH, 7 % (v/v) Glacial CH_3COOH

1×Protein gel running buffer = 25mM Tris-HCl pH 8.3, 192 mM Glycine, 0.1% (w/v) SDS

10×TAE = 0.4 M Tris-base, 11.4% (v/v) glacial CH_3COOH, 10 mM EDTA (disodium), adjust pH to 7.6 with glacial CH_3COOH.

1×TE = 10 mM Tris-HCl pH8.0, 1mM EDTA

Tris buffered saline (TBS) = 20mM Tris-HCl pH 7.5, 150mM NaCl.

TBST = 0.1 % (v/v) Tween 20 in TBS

Western blot buffer = 25mM Tris-HCl pH8.3, 192 mM NH_2CH_2COOH, 20% (v/v) CH_3OH. 0.1% (w/v) SDS.

Media

L-broth (LB) = 1.6% (w/v) Bactotryptone, 1% (w/v) Yeast extraction, 0.5% (w/v) NaCl.

L- agar = As for L-broth with the addition of 1.5% (w/v) Agarose.

SOC media = 2% (w/v) Bactotryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose

0.5×MS = 0.22% (w/v) Murashige and Skoog (MS) basal salt mixture, 15% (w/v) Sucrose, Adjust pH to 5.8 with 1 M NaOH.

0.5×MS agar = As for 0.5× MS with the addition of 0.8% (w/v) Agarose

Orange G, Coomassie brilliant blue, Tween 20, and bromophenol blue were purchased from Sigma-Aldrich (Dorset, UK). **Yeast extraction** is from Oxoid (Hampshire, UK). **Agarose for DNA electrophoresis** is from Melford (Ipswich, UK). **Agarose for media**

and **MS basal salt mixture** are from Duchefa (Haarlem, The Netherlands). **All the other chemicals** were from Fisher Scientific (Leicestershire, UK).

Appendix 2 Primers and Antibody

Primers

Primer Name	Sequence
CY AP	5'-GGCCACGCGTCGACTATCA(T)$_{16}$-3'
CY SPEC AG1	5'-ATGCAGGATGCTGCAACCT-3'
CY DEG AG1	5'-ATGCAG(A)GAT(C)GAC(T)TGC(T)AAC(T)CT-3'
CY AUAP	5'-GGCCACGCGTCGACTATCA-3'
Allium tri R2	5'-GCGCGGCTGTTTGAGTCATAAACCA-3'
35S Sal F	5'-CCCGACGTACCCCTACTCCAAAAAT-3'
35S Stu R	5'-GGAGGCCTCTGGATTTTAGTACTGGATT -3'
Allium ORF F	5'-CATCTAGATGGCCTATTCAGTAACTTGTAAA-3'
Allium ORF R	5'-CCGAGCTCAAGCAATAGCAACATCTCCCA-3'
35S F	5'-CACTATCCTTCGCAAGACCCT-3'
35S R	5'-CCAAAGCGAGTACACAACTCG-3'
Uni F	5'-GTAAAACGACGGCCAGT-3'
Uni R	5'-GGAAACAGCTATGACCATG-3'

Antibody

The anti-ATA antibody is the sera of rabbits raised against the polypeptide QSLEEGPYRLAMQDDC (EUROGENTEC, Southampton, UK). The rabbits were bleeded four times (Once before the injection of the polypeptide and three times after the injection).

Appendix 3 BlastP result of *Allium triquetrum* agglutinin deduced amino acid sequence
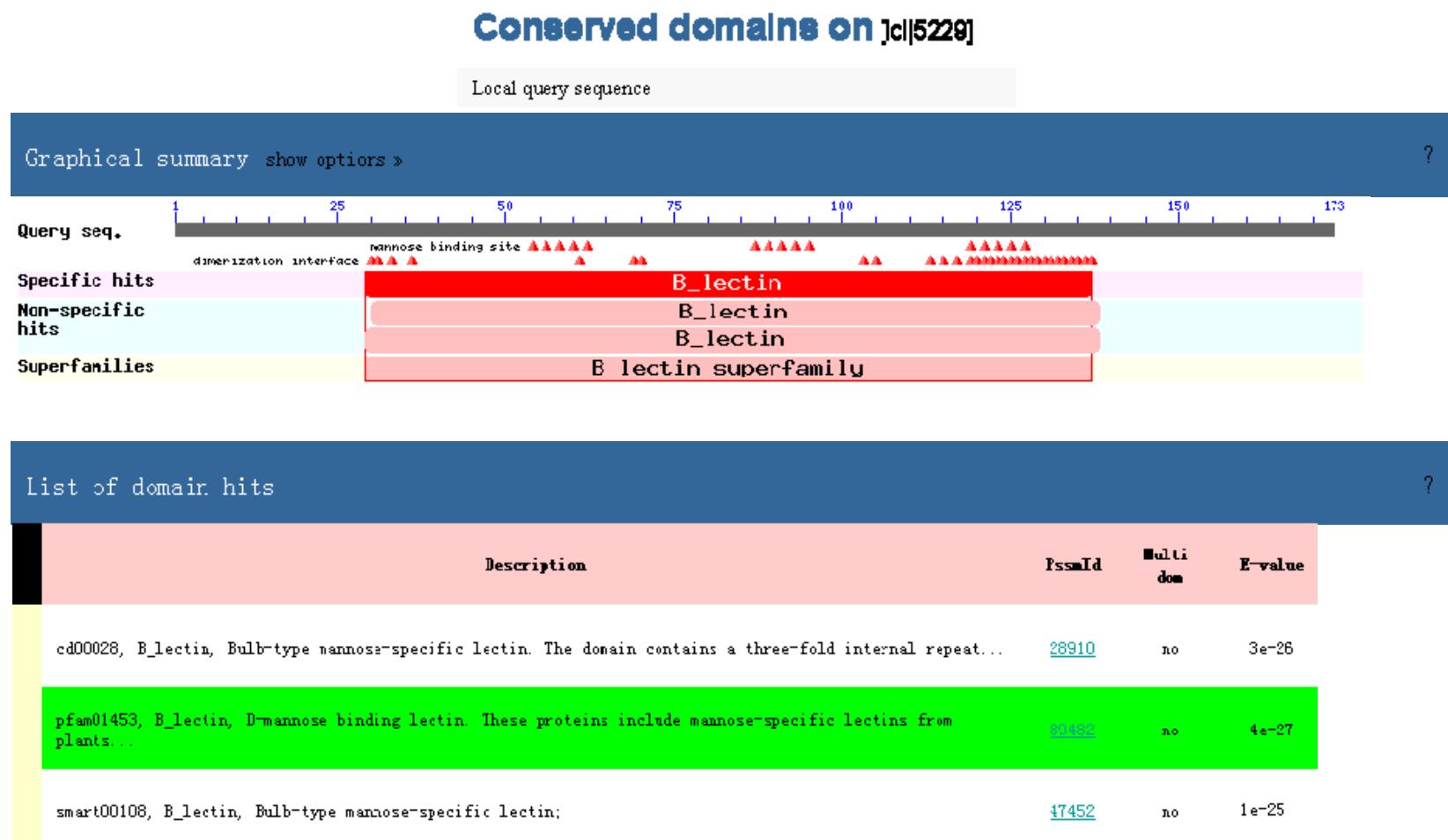
Appendix 4 Predicting the signal peptide of
Allium triquetrum agglutinin


```
>Sequence                length = 70
# Measure    Position    Value    Cutoff    signal peptide?
  max. C      29         0.646    0.32      YES
  max. Y      29         0.671    0.33      YES
  max. S      12         0.992    0.87      YES
  mean S     1-28        0.837    0.48      YES
       D     1-28        0.754    0.43      YES
# Most likely cleavage site between pos. 28 and 29: CMG-RN
```
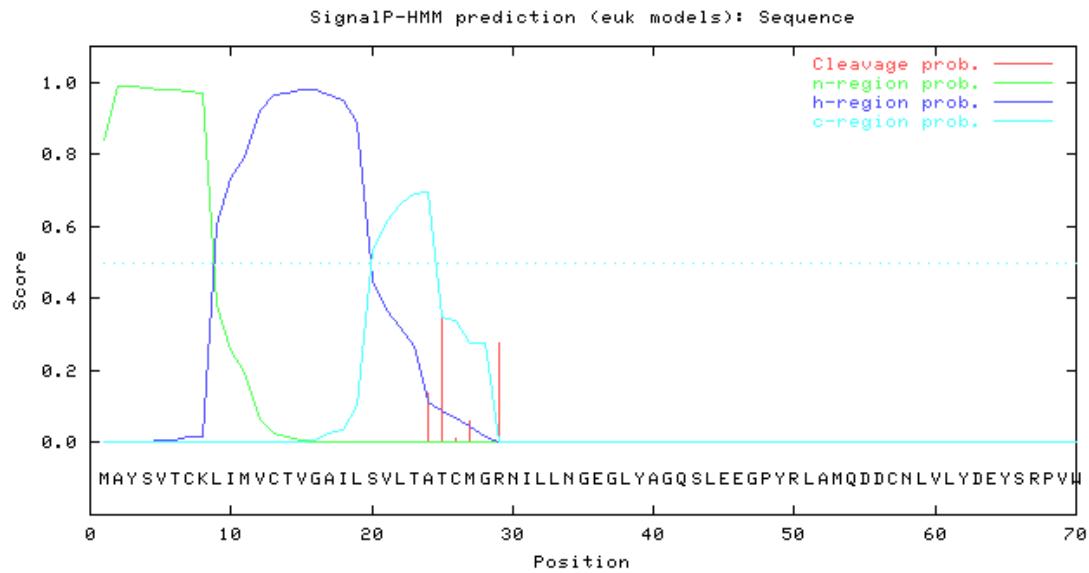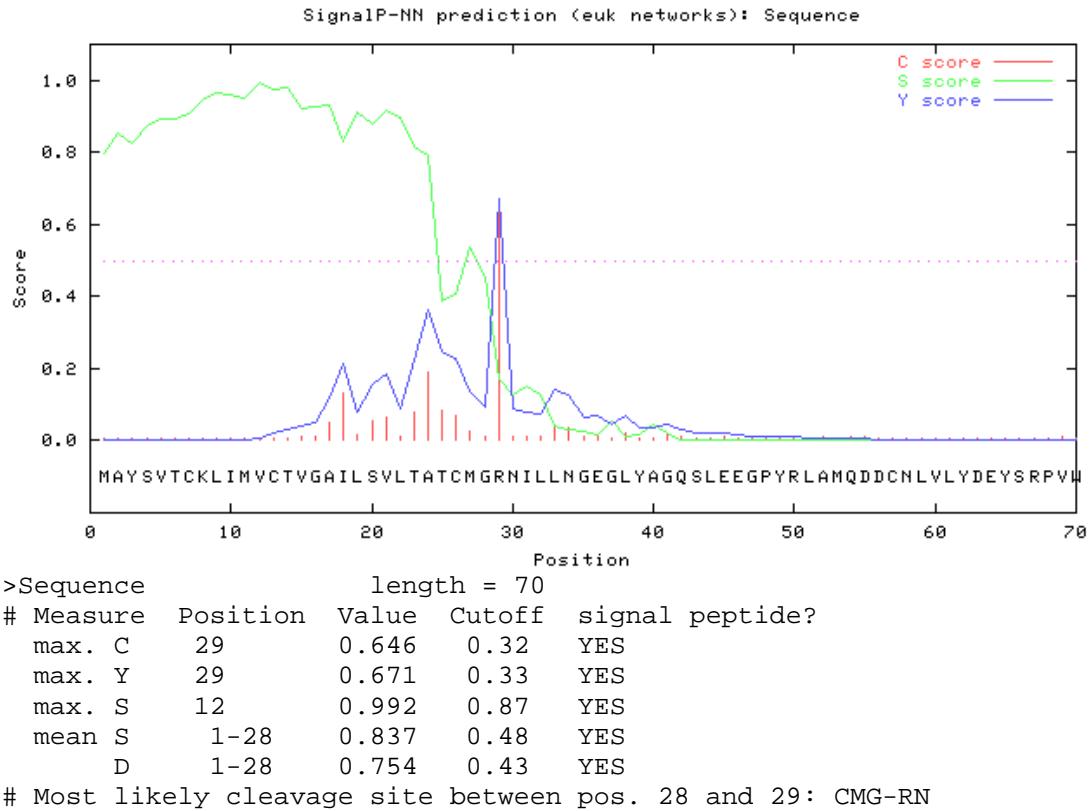

>Sequence

```
Prediction: Signal peptide
Signal peptide probability: 0.842
Signal anchor probability: 0.147
Max cleavage site probability: 0.349 between pos. 24 and 25
```

Appendix 5 DNA sequence of T-DNA of pGreen-0029

0001 AGATCTTGGCAGGATATATTGTGGTGTAACGTTATCAGCTTGCATGCCGG 0050

0051 TCGATCTAGTAACATAGATGACACCGCGCGCGATAATTTATCCTAGTTTG 0100

0101 CGCGCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATTGCGGGACT 0150

0151 CTAATCAAAAAACCCATCTCATAAATAACGTCATGCATTACATGTTAATT 0200

0201 ATTACATGCTTAACGTAATTCAACAGAAATTATATGATAATCATCGCAAG 0250

0251 ACCGGCAACAGGATTCAATCTTAAGAAACTTTATTGCCAAATGTTTGAAC 0300

0301 GATCTGCTTGACTCTAGCTAGAGTCCGAACCCCAGAGTCCCGCTCAGAAG 0350

0351 AACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGC 0400

0401 GATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTT 0450

0451 CAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACA 0500

0501 CCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCAT 0550

0551 GATATTCGGCAAGCAGGCATCGCCCTGGGTCACGACGAGATCCTCGCCGT 0600

0601 CGGGCATCCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCC 0650

0651 TGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCG 0700

0701 AGTACGTCCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGG 0750

0751 TAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGAT 0800

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0901 CAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCC 0950

0951 TCGTCTTGGAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAG 1000

1000 AACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGC 1050

1051 CGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCG 1100

1101 GCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCCTCGATCGAG 1150

1151 TTGAGAGTGAATATGAGACTCTAATTGGATACCGAGGGGAATTTATGGAA 1200

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1351 CTGTCAGTTCCAAACGTAAAACGGCTTGTCCCGCGTCATCGGCGGGGGTC 1400

1401 ATAACGTGACTCCCTTAATTCTCATGTATCGATAACATTAACGTTTACAA 1450

1451 TTTCGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCG 1500

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1551 AAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTA 1600

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4851 GCAACCTCTCGGGCTTCTGGATTTCCGATCCCCGGAATTAG          4891
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* The CaMV 35S promoter is highlighted in red.

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