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CONSTITUTIVE, INSTITUTIVE AND UP-REGULATION OF CAROTENOGENESIS REGULATORY MECHANISM VIA *IN VITRO* CULTURE MODEL SYSTEM AND ELICITORS

(Mekanisme Regulatori dalam Pembentukan Karatenoid Secara Konsitutif, Institutif dan Regulasi Menaik Melalui Model Sistem *in vitro* dan Pengelisit)

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Abstract

Phytohormone abscisic acid (ABA) plays a regulatory role in many physiological processes in plants and is regulated and controlled by specific key factors or genes. Different environmental stress conditions such as water, drought, cold, light, and temperature result in increased amounts of ABA. The action of ABA involves modification of gene expression and analysis of in vitro callus model system cultures revealed several potential of constitutive, institutive and up-regulation acting regulatory mechanisms. Therefore, this study was aimed at establishing *in vitro* cultures as potential research tools to study the regulatory mechanisms of the carotenoid biosynthesis in selected plant species through a controlled environment. The presence and absence of zeaxanthin and neoxanthin in callus cultures and intact plants could be explained by changes in gene expression in response to stress. Abiotic stress can alter gene expression and trigger cellular metabolism in plants. This study suggested that the key factors which involved in regulatory mechanisms of individual carotenoid biosynthesis in a particular biology system of plants can be either be silenced or activated. Therefore, based on the results in this study environmental stress is made possible for enhancement or enrichment of certain carotenoid of interest in food crops without altering the genes.

Keywords: carotenogenesis, elicitors, regulatory mechanisms, constitutive, institutive, up-regulation

Abstrak

Di dalam kebanyakan proses regulasi fisiologi tumbuhan, fitohormon asid absisik (ABA) memainkan peranan penting dan mekanisme ini dikawal oleh gen-gen yang tertentu. Keadaan persekitaran yang ekstrem dan tegar seperti kemarau, banjir, kesejukan melampau, cahaya dan suhu juga akan mempengaruhi penghasilan ABA. Peningkatan ABA akan mengakibatkan perubahan dalam ekspresi genetik dan ini terbukti apabila analisis terhadap model sistem kalus secara *in vitro* menghasilkan 3 jenis mekanisme regulasi tumbuhan yang dikenali sebagai konstitutif, institutif dan regulasi menaik. Justeru itu kajian ini bertujuan merekabentuk satu model sistem *in vitro* menggunakan kultur kalus sebagai satu alat atau medium untuk mengkaji proses regulasi tumbuhan terpilih terhadap biosintesis karatenoid di dalam persekitaran terkawal. Hasil kajian mendapati kehadiran zeaxanthin dan neoxanthin di dalam kultur kalus dan tumbuhan asal merupakan petunjuk terhadap perubahan ekspresi genetik terhadap keadaan persekitaran yang ekstrem. Menariknya, mekanisme kehadiran sebatian ini boleh diaktifkan atau dihilangkan. Kepentingan hasil kajian ini adalah faktor persekitaran boleh dimanipulasi untuk meningkatkan produktiviti atau kualiti sesuatu tanaman tanpa melalui proses pengubahsuaian genetik.

Kata kunci: kerotenogenesis, pengelisit, mekanisme regulatori, konsitutif, institutif, regulasi menaik

Introduction

Carotenoids are yellow, orange and red pigments which secondly abundant in nature after the green chlorophylls. They exist with remarkable special properties as compared to other compounds, performing various functions and actions in enormous living organisms [1, 2]. Based on their highly double-bonded structures, carotenoids are not only important as potent antioxidants but some of them also serve as essential precursors to vitamin A formation such as β-carotene, α-carotene and β-cryptoxanthin. To date, there are more than 700 members of carotenoids' family which have been characterised in nature [1]. The carotenoids biosynthesis often commences with the formation of phytoene from condensation of two blocks of geranyl geranyl diphosphate (GGPP) molecules by phytoene synthase [3]. Through four desaturation forms of phytofluene, ζ-carotene, neurosporene and finally red coloured lycopene are converted from colourless phytoene by phytoene desaturase [4, 5]. The cyclization of lycopene with lycopene cyclases (β-cyclase and ε-cyclase) is a significant branch-point in carotenoid biosynthesis which resulted in the formation of α-carotene and β-carotene. Hydroxylation of α- and β-carotene will produce the well-known xanthophyll pigments zeaxanthin and lutein respectively. Violaxanthin is formed from zeaxanthin through epoxidation. This reaction sequence is reversible and de-epoxydation can convert violaxanthin back to zeaxanthin. Neoxanthin is synthesised and derived from violaxanthin. Cleavage products of this last plant carotenoid serve as precursors in the production of an important plant hormone, abscisic acid [6 - 8].

Over the past decade, several approaches of genetic manipulation or modification have been performed to increase the carotenoid content and composition in different plant species and tissues. As a result, many genes of the carotenoid biosynthetic pathway have been cloned such as dimethylallyl diphosphate synthase isomerase, geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase, ζ-carotene desaturase and β-carotene hydroxylase [6, 9-12]. When the first effort to engineer the rice endosperm was made, a gene encoding phytoene synthase was introduced. Unfortunately, no compounds downstream of fiction such as Lutein and β-carotene accumulated [13]. Subsequently, when the genes encoding phytoene synthase, lycopene β-cyclase and a bacterial phytoene desaturase (CrtI) were introduced, β-carotene accumulated, resulting in golden rice [14]. In recent work [15], expression of three *Erwinia* genes encoding phytoene synthase (CrtB), phytoene desaturase (CrtI) and of geranyl geranyl diphosphate (GGPP) into β-carotene which resulted golden potato with a deep yellow (golden) tubers. Similarly [16], revealed that through transformation of the *Or* gene into wild type cauliflower converts the white colour of curd tissue into distinct orange colour with increased levels of β-carotene. This result suggested that different plant species will react differently towards the stability of individual carotenoids accumulated in transgenic plants. Furthermore, the response in monocotyledonous and dicotyledonous species is also likely to differ. The overexpression of genes and modification of sink capacity in the carotenoid biosynthetic pathway have resulted in increased levels of carotenoids and proven as a new strategy to enhance carotenoids in food crops quantitatively and qualitatively but yet these techniques still need to become more precise to avoid the inadvertent introduction or expression of undesirable genes causing allergenicity, weediness or endanger natural ecosystems [17]. Hence, more detailed knowledge of the diversity of carotenoid pigments in plants and environmental factors influencing their accumulation is urgently required for better understanding of plant carotenogenesis regulation. The question remains, is which mechanism will stimulate carotenogenesis more effective. In order for this question to be answered, it is important to identify and to understand the key control factors for carotenoid accumulation in plants.

Ulam or traditional vegetables may become major sources of carotenoids in the Malaysian diet, but the extent of environmental and genetic influences on their carotenoid biosynthesis are poorly understood. Carotenoid biosynthesis are regulated by several factors and is susceptible to geometric isomerisation in the presence of oxygen, light and heat which causes colour loss and oxidation. The main problems associated with carotenoid accumulation arise from the inherent instability of pigments [18, 19]. In this study carotenoid biogenesis is investigated in selected ulam callus and shoots cultures as a potential model system for rapid initiation, extraction and analysis of carotenoids by providing stringent control of genetic, developmental and environmental factors. The value of this experimental system for investigating variables controlling carotenoid accumulation is then tested by assessing the effects of environmental variables, such as drought stress, light intensity and hormone strength on carotenoid accumulation.

Materials and Methods

Sample preparation

Intact leaves and callus cultures of *Morinda citrifolia* (mengkudu), *Ocimum basilicum* (selasih) and *Ruta angustifolia* (garuda) were freeze-dried for 72 hours, ground into fine powder and kept at -20°C until further analysis.

Extraction of carotenoid and saponification

0.1 grams of all samples were rehydrated with adequate amount of distilled water and extracted, partitioned to hexane and dried under a gentle stream of nitrogen free oxygen gas as detailed in Fatimah et al. [20]. Each of the samples was prepared in triplicate. The dried samples were saponified with a mixture of acetonitrile and water (9:1) and methanolic potassium hydroxide solution (10% w/v) in accordance to procedures described by Othman [17]. The extracts were then re-suspended in ethyl acetate (HPLC grade) prior to subsequent HPLC analysis.

HPLC analysis

The HPLC analysis of saponified carotenoids was performed on an Agilent model 1200 series comprised of a quaternary pump with auto-sampler injector, micro-degassers, column compartment equipped with thermostat and photodiode array detector. The column used was a ZORBAX Eclipse XDB-C_{18} end capped 5 μm, 4.6x150 mm reverse phase column (Agilent Technologies, USA). The eluents used were (A) acetonitrile:water (9:1 v/v) and (B) ethyl acetate. The column separation was allowed via a series of gradient such as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 mL min^{-1}. The temperature of the column was maintained at 20°C. The injection volume is 10 μL each. Detection of individual carotenoids was made at the wavelengths of maximum absorption of the carotenoids in the mobile phase: neoxanthin (438 nm), violaxanthin (441 nm), lutein (447 nm), zeaxanthin (452 nm), β-carotene (454 nm), β-cryptoxanthin (450 nm) and α-carotene (456 nm). Compounds were identified by co-chromatography with standards and by elucidation of their spectral characteristics using a photo-diode array detector. Detection for carotenoid peaks was in the range of 350 to 550 nm. Individual carotenoid concentrations were calculated by comparing their relative proportions, as reflected by integrated HPLC peak areas, to total carotenoid content determined by spectrophotometry. The total and individual carotenoid concentration would be expressed in terms of milligram per 1.0 g dry weight of freeze-dried matter (mg/g DW).

Tissue culture, callus and shoot initiation

Virus-free *in vitro* callus and shoots of *M. citrifolia, O. basilicum* and *R. angustifolia* were induced from leaf explants cultured on MS medium [21] supplemented with 1.0, 1.5 and 2.0 mg/l BAP and 1.0 mg/l 2,4-D of plant growth regulators (PGR) and incubated in a growth room at 24ºC day and night temperature, with a 16-h photoperiod at 80-85 μmol m^{-2} s^{-1} under cool white fluorescent light. Every 4 weeks the *in vitro* callus and shoots were subcultured on MS medium supplemented with 30g/L sucrose, 10 g/L agar and plant growth regulators. Media were adjusted to pH 5.7 and sterilized by autoclaving (15 min, 121ºC) and 50 ml aliquots poured into pre-sterilised 250 ml polycarbonate culture vessels (7 cm diameter x 8 cm high).

Effect of environmental factors on carotenoid biosynthesis

In three independent experiments the influence of light, water-stress and PGRs availability on carotenoid biosynthesis were tested in *Morinda citrifolia, Ocimum basilicum* and *Ruta angustifolia*. Callus harvested after 4 weeks from two culture vessels were pooled for each of three replicates established under the following conditions:

1. Light incubation under cool-white, fluorescent lamps (80-85 μmol m^{-2} s^{-1}; 16 h photoperiod)
2. Light incubation under cool-white, fluorescent lamps (80-85 μmol m^{-2} s^{-1}; 16 h photoperiod) with and without 50mg and 100 mg methyl salicylate acid to impose water-stress.
3. Light incubation under cool-white, fluorescent lamps (80-85 μmol m^{-2} s^{-1}; 16 h photoperiod) at three concentrations of PGR strength (1.0, 1.5 and 2.0).

Results and Discussion

In this study, three plant species were selected for evaluation of carotenoid biosynthesis in their respective *in vitro* cultures; *M. citrifolia* (mengkudu), *R. angustifolia* (garuda) and *O. basilicum* (selasih). These three species were

selected because of the following reasons: a) they are classified under three different families, namely Rutaceae, Rubiaceae and Lamiaceae, respectively; b) a preliminary study on *in vitro* cultures of *R. angustifolia* contained high amount of lutein, callus cultures of *M. citrifolia* had high levels of zeaxanthin whereas callus cultures of *O. basilicum* acummulated considerably high content of neoxanthin [Fatimah, unpublished data]. The preliminary studies have shown that different species accumulated different types of individual carotenoids, which was significantly affected by the enzymatic branching of lycopene in the biosynthetic pathway in combination with types of stress experienced by the plants [22].

Carotenoid accumulation in intact leaves and callus cultures of different colour strains of *M. citrifolia* in response to light.

When developing in the natural environment, the intact leaves of *M. citrifolia* accumulated predominantly zeaxanthin followed by lutein and β-carotene. The callus cultures of *M. citrifolia* possessed lesser carotenoids as compared to the leaves as shown in Table 1. The intact leaves were found to accumulate highest amount of total carotenoid content of about 58.59 ± 0.26 mg/g DW, followed by yellow callus (13.00 ± 0.08 mg/g DW) and green callus (2.17 ± 0.01 mg/g DW). This might be due to larger carotenoid biosynthetic capacity of the leaves in response to various external and internal stimuli in the natural environment. Referring to Table 1, highest accumulation of three individual carotenoids; lutein, zeaxanthin and β-carotene was observed in the intact leaves and the yellow calli seem to carry the characteristic of the intact leaves with additional accumulation of β-cryptoxanthin albeit in quite low concentration. Carotenoid accumulation in green calli was found to differ from the yellow ones. From the results, high level of zeaxanthin in the leaves might indicate that they have been exposed to strong light intensity because zeaxanthin is one component of the reversible reaction in the xanthophyll cycle where the synthesis of violaxanthin and zeaxanthin were made alternately in accordance to light conditions [23].

Table 1. Carotenoid accumulation in intact leaves and callus cultures of *M. citrifolia*

	Carotenoid content (mg/g DW)				
	Total carotenoid	**Lutein**	**Zeaxanthin**	**β-cryptoxanthin**	**β-carotene**
Intact leaves	58.59 ± 0.26	5.20 ± 0.00	52.08 ± 0.25	nd	1.31 ± 0.00
Green callus	2.17 ± 0.01	2.00 ± 0.00	nd	nd	0.16 ± 0.00
Yellow callus	13.00 ± 0.08	1.29 ± 0.00	11.53 ± 0.09	0.05 ± 0.00	0.14 ± 0.00

nd - non-detectable, not significantly different at $p < 0.0001$

Carotenoid accumulation in intact leaves and callus cultures of *O. basilicum* of different sources of explants and PGRs supplementation in response to light

Intact leaves of *O. basilicum* accumulated five individual carotenoids particularly neoxanthin, violaxanthin, lutein, α-carotene and β-carotene when grown in nature as shown in Table 2. The total carotenoid content of all samples was showing that the leaves accumulated more than 90-fold of total carotenoid content in comparison to all six batches of callus cultures. Different source of explants and PGR supplementation in this experiment resulted in more or less the same level carotenoids. Carotenoids are vital for protecting the photosynthetic system from oxidative destruction by light and other factors. In the natural environment, there are multiple of random stresses experienced by the plants, thus leading to high concentration of carotenoids residing in the intact leaves as shown in Table 2. Contrarily, many of the stresses are kept under control in the laboratory. This could possibly cause the callus cultures are behaving in the same manner and accumulated low content of lutein and β-carotene. Besides, the biosynthetic flux in the β, β branching of lycopene is inferred to stop at the formation of β-carotene since the light intensity was maintained. This is due to the fact that the formation of downstream carotenoid was meant for dissipating excess solar energy as heat [24, 25].

Table 2. Carotenoid accumulation in intact leaves and callus cultures of *O. basilicum*.

Source of explants	Samples	Carotenoid content (mg/g DW)					
		Total carotenoid	**Neoxanthin**	**Violaxanthin**	**Lutein**	**α-carotene**	**β-carotene**
-	Intact leaves	95.28 ±3.25	65.16 ±3.22	17.97 ± 0.50	9.66 ± 0.00	0.53 ± 0.11	1.95 ± 0.00
Shoot tips	S1	1.81 ± 0.01	nd	nd	1.62 ± 0.01	nd	0.19 ± 0.00
	S2	1.65 ± 0.03	nd	nd	1.52 ± 0.02	nd	0.14 ± 0.00
	S3	1.66 ± 0.01	nd	nd	1.51 ± 0.01	nd	0.15 ± 0.00
Leaves	L1	1.71 ± 0.00	nd	nd	1.57 ± 0.00	nd	0.14 ± 0.00
	L2	1.70 ± 0.01	nd	nd	1.55 ± 0.00	nd	0.15 ± 0.01
	L3	1.68 ± 0.00	nd	nd	1.54 ± 0.03	nd	0.16 ± 0.00

nd - non-detectable, significantly different at p <0.0001

Carotenoid accumulation in intact leaves and *in vitro* cultures of *R. angustifolia* in response to light

The leaves of *R. angustifolia* accumulated three major carotenoids where lutein was found in the highest level. The shoot cultures derived from stems contained the highest level of total carotenoids while callus cultures have the least carotenoid accumulation as tabulated in Table 3. The carotenoid accumulation of callus cultures was seen to be quite similar to that of intact leaves, although in varying concentrations. HPLC analysis has shown that lutein and β-carotene are two common carotenoids synthesized in all of the samples in comparable amounts. The retention of lutein and β-carotene as common carotenoids in each of the samples might be strongly correlated to their main functions in photosynthesis where the former is vital for its light-harvesting properties [26] while the latter is the first protective measures against free-radicals by-produced in the electron transport chain reaction [27]. There are other possibilities that could be significantly resulted in the carotenoid accumulation in the *in vitro* cultures; a) developmental factors might significantly affect the activity of lycopene β-cyclase, lycopene ε-cyclase and carotenoid hydroxylases to limit the supply of lutein [28], b) competing cyclization steps of lycopene by both β-cyclase and ε-cyclase enzymes which determined channelling of lycopene into respective branching in the pathway [22].

Table 3. Carotenoid accumulation in intact leaves and *in vitro* cultures of *R. angustifolia*

	Carotenoid content (mg/g DW)				
	Total carotenoid	**Neoxanthin**	**Lutein**	**β-cryptoxanthin**	**β-carotene**
Intact leaves	6.45 ± 0.56	nd	6.00 ± 0.11	0.04 ± 0.00	1.10 ± 0.01
Shoot cultures	14.70 ± 0.84	10.27 ± 0.62	2.75 ± 0.12	nd	1.68 ± 0.11
Callus cultures	1.76 ± 0.01	nd	1.54 ± 0.01	nd	0.22 ± 0.00

nd - non-detectable, significantly different at p <0.0001

Carotenoid accumulation in shoot cultures of *R. angustifolia* in response to light and elicitor

The results for a preliminary experiment to study the effect of salicylic acid (SA) on carotenogenesis in the shoot cultures was tabulated in Table 4. The salicylic acid was used as an elicitor to impose water stress onto the cultures [29]. The growth rate of the shoots has been found to be inversely proportionate with the SA concentrations. There were two interesting observations here: a) increasing neoxanthin production in treatment with 50 mg/L SA, and b) dropping of the other three carotenoids as SA concentration increased. Endogenously, SA may serve as a cellular

growth and death controller [30]. Apart from that, employing the SA exogenously could trigger stresses to the plants which would significantly change the regulatory mechanisms of photosynthesis [31]. An increased amount of phytohormone abscisic acid (ABA) can be seen in the plants while responding to different stress conditions like drought, water, temperature and light and activates the signal transduction pathways to transmit the information among cellular components. Eventually, changes in genes expression were induced which influence the carotenoid biosynthesis in plants. Therefore, from Table 4, it can be observed that cellular metabolism in the shoot cultures may have been altered to form more neoxanthin in Treatment 1 whereas the enzyme (zeaxanthin epoxidase) activity that catalyzes the irreversible formation of violaxanthin to neoxanthin significantly reduced due to high concentration of SA and restricting the supply of the ABA precursors.

Table 4. Carotenoid accumulation in shoot cultures of *R. angustifolia* in response to light and elicitor (salicylic acid)

	Carotenoid content (mg/g DW)				
	Total carotenoid	**Neoxanthin**	**Violaxanthin**	**Lutein**	**β-carotene**
Control (0 mg SA/L media)	12.49 ± 0.40	7.47 ± 0.35	1.30 ± 0.04	2.94 ± 0.08	0.79 ± 0.02
Treatment 1 (50 mg SA/L media)	16.51 ± 4.24	15.88 ± 2.16	0.75 ± 0.00	1.98 ± 0.01	0.18 ± 0.00
Treatment 2 (100 mg SA/L media)	1.50 ± 0.01	nd	nd	1.42 ± 0.00	0.07 ± 0.00

nd - non-detectable, significantly different at $p < 0.0001$

The individual carotenoid biosynthesis in a particular species may greatly differ due to many factors such as climate change, nutritional conditions, acidity or alkalinity of the soils, manipulating the biosynthetic pathway through the employment of *in vitro* culturing systems seems to offer benefits of keeping random environmental stresses at a minimal level. In the future, this system could help researchers in promoting better understanding to a complete regulatory mechanism of carotenoid biosynthesis.

Conclusion

The results suggest that a regulatory step in the carotenoid biosynthetic pathway versus environmental stress is mediated by ABA and involves the epoxidation of zeaxanthin to violaxanthin by zeaxanthin epoxidase during the first committed step in ABA biosynthesis. Zeaxanthin appears to be a key factor and indicator for the presence of environmental stress. Due to the presence and time of occurrence of environmental stress, some genotypes accumulated merely violaxanthin and neoxanthin in order to generate xanthoxin or precursors of ABA biosynthesis pathway. Not surprisingly, the response to such environments appeared to be highly genotype dependent and time duration exposed to stress. Another factor is the activity of functional enzymes and candidate enzymes that regulate carotenoid biosynthesis which will determine the type and quantity of individual carotenoids. By understanding the environmental factors that affected carotenoid biosynthesis, it should be possible to enhance the amount and type of carotenoid that accumulates in food crops. In conclusion, resistance or sensitivity of plants to stress depends on the species, type of stress and development age.

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