Effects of Abscisic Acid on Capsanthin Levels in Pepper Fruit

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ABSTRACT. Abscisic acid (ABA) is an important plant hormone that plays an important role in stress responses. Previous studies have suggested that ABA can also accelerate ripening in climacteric and nonclimacteric fruit. Capsanthin is a carotenoid that confers red coloration to mature pepper (*Capsicum annuum*). However, the effect of ABA on capsanthin accumulation in pepper fruit has not been thoroughly studied. Herein, we aimed to evaluate the effects of ABA treatment on capsanthin accumulation in pepper fruit and on the expression of key genes involved in the capsanthin biosynthetic pathway. For this purpose, we treated pepper fruit with ABA at green mature stage. Our results indicate that ABA treatment increased capsanthin content in pepper fruit, with the best result obtained with 150 mg L⁻¹ ABA solution. Application of exogenous ABA also increased the expression levels of the capsanthin synthesis genes phytoene synthase (*Psy*), lycopene β-cyclase (*Lcyb*), β-carotene hydroxylase (*Crtz*), and capsanthin/capsorubin synthase (*Ccs*), likely explaining the significant capsanthin content increase in pepper fruit.

In recent years, food insecurity has become an important concern for human health (Friel and Ford, 2015). Synthetic food pigments are a serious health threat, as some of them include carcinogenic substances (Caroy et al., 2012). At the same time, use of natural pigments in food production is increasing worldwide (Tian et al., 2014a). Capsanthin (3,3′,5′,7′-tetrahydroxy-β-k-caroten-6’-one) is one of the primary ingredients for red pigments. It accumulates in chromoplast thylakoids in the pericarp of ripe red pepper fruit and contributes up to 60% of the total carotenoids in these species (Perez-Galvez et al., 2003; Suzuki and Mori, 2003). With the increasing demand for natural pigments, capsanthin is extensively used in food and cosmetic industries (Tian et al., 2014a).

Capsanthin synthesis is regulated by capsanthin/capsorubin synthase (*Ccs*) present in membrane fractions of pepper fruit (Bouvier et al., 1994). Capsanthin is the end product in the pepper carotenoids biosynthetic pathway. Its biosynthesis starts with phytoene synthase (*Psy*) converting two molecules of geranylgeranyl diphosphate into phytoene. In turn, phytoene becomes lycopene through desaturation, and then lycopene β-cyclase (*Lcyb*) catalyzes a cyclization on both ends of lycopene to create α-carotene and β-carotene. β-carotene hydroxylase (*Crtz*) converts β-carotene to β-cryptoxanthin, zeaxanthin, and antheraxanthin. Ccs converts antheraxanthin into capsanthin (Guzman et al., 2010). These biosynthetic enzymes are directly involved in the red coloration of pepper fruit (Moehs et al., 2001; Ronen et al., 1999). Among all of these biosynthetic enzymes, Ccs is the rate-limiting enzyme; yellow coloration in pepper fruit depends on a *Ccs* gene deletion or mutation, which disrupts capsanthin synthesis (Thorup et al., 2000).

Previous research mainly focused on the molecular mechanisms of capsanthin biosynthesis, while little is known about the effects of phytohormones, such as ABA, on capsanthin accumulation. ABA has a number of functions in plant growth, mainly in the regulation of stress resistance (Dalal and Inupakutika, 2014; Etehadnia et al., 2008; Huang et al., 2015). Recent studies have also found that ABA plays an important role in the regulation and control of fruit ripening (Chai et al., 2011; Jia et al., 2011; Luo et al., 2014), especially in nonclimacteric fruit (Jia et al., 2011; Sun et al., 2012). For example, Jia et al. (2011) found that ABA stimulates strawberry
Table 1. Primers used for quantification of capsanthin/capsorubin synthase (Ccs), phytoene synthase (Psy), lycopene β-cyclase (Lcyb), and β-carotene hydroxylase (Crtz) genes' expression level in pepper fruit.

| Gene | Accession no. | Forward primers (5′ → 3′) | Reverse primers (5′ → 3′) |
|------|---------------|---------------------------|---------------------------|
| Ccs  | X76165.1      | GCTTCGGCTAGTGAAAGTTTC     | CTCAAGGCCCACCTATGATGAGAC  |
| Psy  | X68017.1      | CCGAATGCTACACATTACTCCCG   | GACTTCTCAAGTCATAGCCTCCC   |
| Lcyb | X86221.1      | GAAACCTCAGTGTCCTGTC       | ATCAAAGACCTCTTGTAGCC      |
| Crtz | Y90225.1      | AAAGCGATACTGAAATAG      | AACTGAAATAACCGCCATAAGAA  |
| UBI-3 | AY486137.1 | TGTCACATCGTCTCTGTTG      | CACCCCAAGCACAAATAAGAC    |
| GAPDH | AJ246013.1 | ATGATGATGTGAAAGCAGCG   | TTCCACTGGTGCTGCTAC        |
| β-TUB | EF495259.1 | GAGGGTGAGTGAGCAGT       | CTTTACGTCATCTGCTGC       |

*These accession of genes can be found from GenBank (National Center for Biotechnology Information, Bethesda, MD).
*Primers were designed using Primer Premier 5.0 (PREMIER Biosoft Intl., Palo Alto, CA).
*Ubiquitin conjugating protein (UBI-3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and beta tubulin (β-TUB) were used as housekeeping genes which were previously reported by Wan et al. (2011).

With the demand for capsanthin increasing with the growth of food industry, exogenous hormone application to improve capsanthin content in pepper fruit can represent a potential solution, along with breeding programs. Keeping in mind the importance of capsanthin, our present study examined the effects of fruit ripening process is also a process of pigment accumulation. Martinez et al. (1996) found that color change is an important parameter in evaluating the ripening process in strawberry fruit; color change from green to red is a very important indicator of tomato ripening (Su et al., 2015); and fruit ripening involves a complex series of events, which include changes in color (Kachhwaha and Gehlot, 2015). For pepper fruit, the formation of capsanthin is closely related to fruit ripening (Guzman et al., 2010).

Overall, ABA plays an important role in the regulation of fruit ripening and carotenoid biosynthesis. However, there is no relevant report on ABA regulating capsanthin accumulation. Red pepper fruit ripening involves a transition from green to red fruit coloration, depending on capsanthin accumulation. Capsanthin cannot be synthesized in young fruit, and capsanthin gradually is accumulated during the color-changed period of fruit ripening (Guzman et al., 2010). Therefore, we hypothesized that ABA affects capsanthin accumulation in pepper fruit as this hormone regulates pepper fruit ripening.
1 week, the seeds were sown on plates containing holes in a controlled environment with 60% humidity and 16/8-h (day/night) photoperiod at 25/18 °C (day/night) temperature. Plates (59 mm top width, 35 mm bottom width, 110 mL volume) had 4 × 8 holes (50 mm hole depth). The growth medium was a sterile mixture of peat:sand:perlite (1:1:1). The photosynthetic photon flux density in the controlled-environment chamber was 278 μmol·m⁻²·s⁻¹. Hoagland’s solution (Hoagland and Arnon, 1950) was added every 2 d. Plants were transplanted into 20 × 14-cm white pots after 8–10 true leaves appeared. The growth medium was a sterile mixture of peat:sand:perlite (1:1:1). Each pot contained one plant and was put in a plastic tunnel, and the pots (total 100) were arranged according to randomized complete block design. These experiments were replicated three times between 2012 and 2014.

**Optimization of ABA Concentration.**

ABA (Sigma-Aldrich, St. Louis, MO) was weighed in darkness and dissolved in 1 mL anhydrous ethanol. Then, ABA solution was diluted to different concentrations (900, 600, 300, 150, and 100 mg·L⁻¹) in distilled water (Li et al., 2014; Romero et al., 2013; Wang et al., 2007). ABA treatment was performed by immersing fruit at the green mature stage (25 d after flowering) in the diluted ABA solution (900, 600, 300, 150, or 100 mg·L⁻¹) for 10 s; untreated control fruit were immersed in water for 10 s. Each treatment contained five fruit. These fruit were tested for capsanthin content at red fruit period (43 d after flowering).

**Capsanthin Extraction and Analysis.**

Capsanthin was extracted from the samples as described previously (Tian et al., 2014b). A 2.0-g sample of pericarp tissue (multiple pieces taken from each fruit) was incubated in 15.0 mL acetone containing 0.1% butylated hydroxy-toluene. After shaking and incubation on ice in the dark for 10 min, the samples were centrifuged at 1917 g for 10 min at room temperature and the extract was transferred to a clean tube. Samples were re-extracted until the extracts were colorless (normally three times). All extracts were pooled in a separator. Pooled extracts were dried in a rotary evaporator at 35 °C (Morais et al., 2002). The residue was dissolved in 5.0 mL acetone, filtered through a 0.20-μm membrane filter before high-performance liquid chromatography (HPLC) injection.

Aliquots were concentrated under nitrogen gas, sealed and frozen at −20 °C until HPLC analysis (Wall et al., 2001). HPLC was performed as described previously (Tian et al., 2014b). For HPLC, samples (20 μL) were analyzed on an HPLC column [5 μm, 150 × 4.6 mm (Shim-pack VP-ODS C-18; Shimadzu, Kyoto, Japan)]. The eluent consisted of (A) acetonitrile:2-propanol:water (39:53:8) and (B) acetonitrile:2-propanol (60:40). The gradient profile was 0–30 min from 0% to 100% B. The flow rate was set at 0.3 mL·min⁻¹ and the column temperature at 40 °C. Standard solution of capsanthin (0.001–0.1 mg·L⁻¹) was used to make calibration curve at 454 nm. Capsanthin was identified by its absorption

![Fig. 2. The effect of pepper fruit maturity stage on capsanthin accumulation after abscisic acid (ABA) treatment. Treatment was performed by immersing fruit in ABA solution (150 mg·L⁻¹) for 10 s; untreated control fruit were immersed in distilled water for 10 s; YF = young fruit period (10 d after flowering); GM = green mature (25 d after flowering); CC = color-changed period (30 d after flowering); RF = red fruit period (43 d after flowering). Duncan’s multiple-range test was used, and least significant range analysis at 5% significance is shown in lowercase letters in the same day of control and treatment. All experiments were carried out in triplicate. Means followed by the same letter do not differ significantly.](image1)

![Fig. 3. Capsanthin content in pepper fruit after abscisic acid (ABA) treatment. ABA treatment was performed at the green mature stage (25 d after flowering) by immersing fruit in the diluted ABA solution (150 mg·L⁻¹) for 10 s; untreated control fruit were immersed in distilled water for 10 s. All values are means ± SE. Mean separation was performed by Duncan’s multiple-range test. Least significant range analysis at 5% significance is shown in lowercase letters. All experiments were carried out in triplicate. Means followed by the same letter do not differ significantly.](image2)
The highest capsanthin content was observed after ABA treatment occurred in fruit treated at the mature green stage (Fig. 2). Capsanthin content in fruit increased up to 150 mg L−1 ABA solution then decreased at higher concentrations. In contrast to low ABA concentrations, too high ABA concentrations resulted in fruit abscission, rotting, and wrinkling.

Optimum ABA concentration. The content of capsanthin increased then decreased with increasing ABA solution concentration (Fig. 1). Capsanthin content in fruit increased up to 150 mg L−1 ABA solution then decreased at higher concentrations. In contrast to low ABA concentrations, too high ABA concentrations resulted in fruit abscission, rotting, and wrinkling.

Optimum stage for ABA treatments. The highest capsanthin content after ABA treatment occurred in fruit treated at the mature green stage (Fig. 2).

Capsanthin content changes in ABA-treated fruit. There was a gradual decrease in antheraxanthin content in fruit from the mature green to ripening stage, whether treated or not (Fig. 4). As antheraxanthin is a capsanthin biosynthetic precursor (Guzman et al., 2010), we hypothesized that antheraxanthin was gradually converted into capsanthin. We observed significant differences in antheraxanthin content between control and treatment groups (Fig. 4), with the content in treated fruit lower than that of the control group at 6, 9, 12, 15, and 18 d after ABA treatment.

Effect of ABA on the expression of capsanthin biosynthetic genes at different maturity stages. The expression levels of Psy and Lcyb were higher in the control group than in fruit treated with 150 mg L−1 ABA 3 d after treatment, while from 6 to 18 d after treatment, the expression levels of these genes were higher in ABA-treated fruit (Fig. 5A and B). We observed the biggest difference in Lcyb expression between control and treated fruit at 9, 15, and 18 d after

Results

Fig. 4. Antheraxanthin content in pepper fruit after abscisic acid (ABA) treatment. ABA treatment was performed at the green mature stage (25 d after flowering) by immersing fruit in the diluted ABA solution (150 mg L−1) for 10 s; untreated control fruit were immersed in distilled water for 10 s. All values are means ± SE. Means separation was performed by Duncan’s multiple-range test. Least significant range analysis at 5% significance is shown in lowercase letters. Means followed by the same letter do not differ significantly.
We measured small differences in \( \text{Crtz} \) expression between control and ABA treatment 3 d after treatment, while from 6 to 18 d, \( \text{Crtz} \) expression was higher in ABA-treated fruit than controls (Fig. 5C), with the biggest differences in expression between the two groups observed 12, 15, and 18 d after treatments. ABA-treated fruit had higher \( \text{Ccs} \) expression compared with the controls at 6 and 18 d after treatment, with expression peaking 12, 15, and 18 d after ABA treatment (Fig. 5D).

**Effect of ABA on fruit weight and yield during fruit development and ripening.** The average fruit weight and the yield were measured between treatment and control groups, respectively. The results showed that there were no significant differences between the average fruit weight of treatment group and that of control group (Fig. 6A). Similarly, there were no significant differences between the yield of treatment group and that of control group (Fig. 6B). Compared with the control, whether the yield or the average fruit weight, it did not reduce after fruit was treated with ABA solution.

**Discussion**

To explore the effects of ABA in fruit development, exogenous ABA treatment by spraying the whole plant or dipping fruit are usually carried out. Comparing the two different treatment methods, low ABA concentration was used in the spraying method to avoid its harm to leaves (Wang et al., 2013); in contrast, without side effects on leaves, relatively high ABA concentration was used in the dipping method to obtain better phenotype changes (Li et al., 2014; Romero et al., 2013). In this experiment, fruit were treated with ABA solution by dipping fruit. Experimental data showed that some ABA concentrations were too high, not only causing no improvements in capsanthin content (Fig. 1), but also resulting in fruit abscission, rotting, and wrinkling, in contrast to low ABA concentrations. Combined with the previous research results, through trial and error, it was found that ABA (150 mg \( \text{L}^{-1} \)) had an obvious effect on capsanthin content in pepper fruit. The study results show that ABA treatment enhanced the expression...
of the capsanthin biosynthetic genes *Psy*, *Lcyb*, *Crtz*, and *Ccs*, stimulated antheraxanthin (capsanthin precursor) to convert into capsanthin, and led to an increase in capsanthin content.

To confirm the best fruit ripening stage for ABA treatments, pepper fruit were treated with ABA in young fruit stage, mature green stage, color turning period, and red fruit stage. The results showed that the highest capsanthin content was achieved when fruit were treated at the mature green stage (Fig. 2). Premature treatment had little effect on the capsanthin content because *Ccs* is not expressed in that stage (Lefebvre et al., 1998). On the other hand, late ABA treatment cannot affect capsanthin content after its biosynthesis has been completed (Guzman et al., 2010). *Ccs* begins to express in the mature green stage in ABA-treated fruit (Lefebvre et al., 1998); at this time, ABA enhanced *Ccs* expression, with a beneficial effect on the synthesis of capsanthin. These findings are consistent with previous studies. Barickman et al. (2014) found that ABA treatments had a significant effect on lycopene and β-carotene concentrations in ‘MicroTina’ tomato fruit tissue. They also found that ABA has a positive impact on tomato fruit carotenoids, and ABA’s most important function is in the pre-ripening stage of fruit tissues, when it triggers ethylene production causing an increase in carotenoid production (Barickman et al., 2014). They demonstrated that ABA’s most important function is in the pre-ripening stage of fruit tissues, when it triggers ethylene production causing an increase in carotenoid production (Barickman et al., 2014).

Antheraxanthin is an important carotenoid and is a direct precursor in the synthesis of capsanthin. Here, the levels of antheraxanthin decreased in pepper fruit after ABA treatment. At the same time, ABA treatment enhanced *Ccs* expression levels, a gene encoding for a key factor for the conversion of antheraxanthin to capsanthin. This suggests that the increase in *Ccs* expression likely accelerated the transformation of antheraxanthin, leading to a rapid decrease in the antheraxanthin content in ABA-treated pepper fruit.

Previous studies have found that ABA-induced *Ccs* expression depends on an ABA-sensitive cis-regulatory element in the *Ccs* promoter (Bouvier et al., 1998). In our experiments, ABA treatment resulted in higher *Ccs* and *Crtz* expression from day 6 until day 18 after treatment. With the increased expression of these genes (Fig. 5C and D), capsanthin gradually accumulated in pepper fruit (Fig. 3). This indicated that there was a close relationship between gene expression and capsanthin accumulation. Exogenous ABA application stimulated the expression of *Ccs* and *Crtz*, ultimately promoting capsanthin accumulation in pepper fruit.

Previous research has demonstrated the potential of ABA to enhance the anthocyanin contents of grape (*Vitis vinifera*) when applied directly to the berries, while having little or no influence on mean fruit cluster weights and fruit yield (Gua et al., 2011). One study showed that ABA (50 mg·L⁻¹) did not significantly influence kiwifruit (*Actinidia deliciosa*) fruit weight (Cruz-Castillo and Woolley, 2006). This is in agreement with our results that showed that pepper yield and fruit weight did not change after ABA treatment. The improvement of capsanthin content will naturally lead to the improvement of yield of capsanthin, because yield and average fruit weight of pepper fruit were the same as that of the control by ABA treatment. It may be possible to use ABA as a viable and novel approach to increase capsanthin yield in agricultural production.

**Fig. 6.** Comparison of pepper fruit weight (A) and yield (B) between treatment and control groups. Abscisic acid (ABA) treatment was performed at the green mature stage (25 d after flowering) by immersing fruit in the diluted ABA solution (150 mg·L⁻¹) for 10 s; untreated control fruit were immersed in distilled water for 10 s. All values are means ± st. Mean separation was performed by Duncan’s multiple-range test. Least significant range analysis at 5% significance is shown in lowercase letters; all samples were carried out in triplicate. Means of fruit weight followed by the same letter do not differ significantly between treatment and control group. Means of yield followed by the same letter do not differ significantly between treatment and control group.
Conclusion

ABA treatment significantly enhanced capsanthin content in pepper fruit at the green ripening stage. In particular, 150 mg L⁻¹ ABA solution could significantly increase the capsanthin content of pepper fruit. Exogenous ABA application also increased the expression levels of the capsanthin biosynthetic genes Psy, Lcyb, Crtz, and Ccs, resulting in a significant increase in the fruit capsanthin content. Therefore, we demonstrate that it is feasible to improve content and yield of capsanthin by ABA treatment.

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