Assessment of the Response of Beta Carotene Enhanced Transgenic Soybeans to *Soybean Mosaic Virus (SMV)*

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ABSTRACT Beta-carotene, a defense chemical, is synthesized by the carotenoid biosynthesis pathway. In the present study, a transgenic soybean line, with a single copy insertion of *phytoene synthase* and *carotene desaturase genes*, having high beta-carotene content was studied for its response to systemically inoculated *Soybean mosaic virus (SMV)*. Beta-carotene-enhanced transgenic soybean showed similar leaf and seed symptoms, viral RNA, and protein expression compared to the non-genetically modified (GM) 'Kwangan' control. Total antioxidant contents in the non-GM 'Kwangan' line were increased after SMV attack in both leaves and seeds; however, the antioxidant contents in the beta-carotene-enhanced soybean line have no significant changes. In addition, both GM and non-GM soybean were detected increased lipid hydroperoxide concentrations in leaves and seeds after SMV infection, even though they did not reach a statistical significant level. Absciscic acid (ABA) levels in beta-carotene-enhanced transgenic soybean seeds was determined 35-fold increase after SMV infections caused a lower seed germination rate and a higher SMV transmission rate to subsequent generations, compared to those of non-GM 'Kwangan'. Thus, we concluded that the additional production of beta-carotene did not confer resistance of beta-carotene-enhanced transgenic soybean to SMV infections, but caused mass accumulations of ABA in seeds.

Keywords Transgenic soybean, Beta-carotene, Antioxidant, Lipid hyperoxide, Absciscic acid, *Soybean mosaic virus*

INTRODUCTION

Biosynthesis of beta-carotene involves the conversion of geranylgeranyl diphosphate to phytoene by *phytoene synthase*, followed by the action of five enzymes of the carotenoid biosynthesis pathway, namely phytoene desaturase, zeta-carotene desaturase, zeta-carotene isomerase, carotenoid isomerase, and lycopene beta-cyclase (Zhu *et al.* 2013). Some studies have suggested that carotenoids, particularly beta-carotene and lycopene, might contribute to neutralization of reactive oxygen species and prevent oxidative damage to proteins, lipids, carbohydrates, and DNA, thereby enhancing the cancer preventive activity as well as resistance and survivability of plants under abiotic and biotic environmental stress (Burton and Ingold 1984; van Poppel 1993; Slaga 1995). Furthermore, beta-carotene

has been shown to have a powerful effect in boosting natural killer cell activity and enhancement of anti-viral activity in elderly men. Human trials suggested that beta-carotene supplementation may enhance the number and function of various immune cells, especially for human immunodeficiency virus and chronic hepatitis C virus infected patients (Delmas-Beauvieux *et al.* 1996; Santos *et al.* 1998).

In plants, several studies suggested that the antioxidant activity of plants under salt, high temperature, drought, and pathogen stress could be enhanced by increasing their beta-carotene, vitamin C, and lycopene content (Rosenfeld *et al.* 1998; Du *et al.* 2010; Kim *et al.* 2012b; Wang *et al.* 2014). Recent years, transgenic plants have been developed by metabolic engineering to enhance the production of carotenoids, such as beta-carotene (pro-vitamin A), vio-

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laxanthin, and zeaxanthin, thereby increasing the nutrient value and stress tolerance of plants. For plants such as canola (*Brassica napus*), *Arabidopsis*, maize (*Zea mays* L.), and soybean (*Glycine max* L.), which contain a native carotenogenic pathway, genetic engineering was performed to enhance the catalysis of an enzyme in the carotenoid biosynthesis pathway, for example, by overexpression of *phytoene synthase* or *beta-carotene hydroxylase*, resulting in the enhanced production of carotenoids (Shewmaker *et al.* 1999; Davison *et al.* 2002; Lindgren *et al.* 2003; Zhu *et al.* 2008; Schmidt *et al.* 2014).

Soybean (*Glycine max* L.) is one of the world's most important crops, being a source of oil and protein, as well as secondary metabolites. Several breeding and genetic engineering efforts have been made to improve nutrients and their functions in soybean. Kim *et al.* (2012a) reported the introduction of a recombinant *phytoene synthase-2A-carotene desaturase* (PAC) gene, under the control of a seed-specific beta-conglycinin promoter, in the Korean soybean variety, 'Kwangan', and approximately 45-fold higher beta-carotene production (110.7 µg/g) was detected in the transgenic soybean seeds compared to the non-genetically modified (GM) counterpart (2.4 µg/g). As well, Schmidt *et al.* (2014) reported transgenic soya bean (*Glycine max*) plants were developed by overexpressing a seed-specific bacterial *phytoene synthase* gene and accumulated 845 µg/g dry weight beta-carotene, with seed protein content increase by 4% (w/w) and abscisic acid (ABA) content decrease by 40% in cotyledons compared to those of non-transgenic counterpart. However, they revealed significant differences on ABA-responsive transcription factor gene expression in transgenic lines which indicated seed composition trait alternations were attributed to altered ABA hormone levels. Besides, many previous studies have suggested that ABA levels could be alternated by overexpression of *phytoene synthase* (Fray *et al.* 1995; Shewmaker *et al.* 1999; Busch *et al.* 2002; Lindgren *et al.* 2003). Thus it can be seen that beta-carotene additional production could bring more impacts on carotenoid and ABA biosynthetic pathway consequently result in alternation of ABA levels. ABA plays a key role in modulating plant responses to difference biotic and abiotic stresses. Alazem *et al.* (2014) studied the effects of ABA

pathway on the accumulations of *Bamboo mosaic virus* (BaMV) and *Cucumber mosaic virus* in different hosts of *Arabidopsis thaliana* and *Nicotiana benthaminana* and results indicated ABA biosynthesis and ABA signaling pathway could increase plant resistance to BaMV.

Here, we assessed the responses of beta-carotene-enhanced transgenic soybean to *Soybean mosaic virus* (SMV), a causative agent of the most prevalent soybean disease in Korea. By comparing the symptom development, viral protein accumulation, agronomic traits, antioxidant and lipid hydroperoxide concentration, and ABA levels in plants or seeds for beta-carotene-enhanced transgenic soybean and non-GM counterpart 'Kwangan', we attempt to answer three questions: (i) Beta-carotene additional production whether or not strengthens resistant ability to SMV. (ii) After virus infections, the differences of agronomic traits and cell damage degrees between beta-carotene-enhance GM soybean and its non-transgenic counterpart. (iii) Alternations of ABA levels in beta-carotene-enhanced transgenic soybeans before and after virus infections.

MATERIALS AND METHODS

Plant materials

Plant materials used in the present study included beta-carotene-enhanced transgenic soybean line '7-1-1' (GM) and its non-GM counterpart, soybean variety 'Kwangan'. The GM line '7-1-1' (T₅ generation) was characterized to contain a single copy of transgene, intergenically inserted between ~10,873,131 and 10,872,988 bp on chromosome 14, 9.469 kb downstream of the *Glyma14g12130.1* gene (Qin *et al.* 2015). This line showed a stable beta-carotene mRNA expression and had an orange-colored endosperm. Agronomic traits such as plant height, seed weight, seed appearance, and date of maturity of this line were similar to those of 'Kwangan', except for germination, which was late by two days on an average.

Systemic virus inoculation

Infectious full-length cDNA clones of SMV-G7H strain

was used as the virus source and a susceptible soybean variety ‘Seokryangput’ as the maintenance host (Seo *et al.* 2009). One hundred seeds of ‘Kwangan’ and beta-carotene-enhanced GM line were planted in a greenhouse with nighttime and daytime temperatures of 22°C and 27°C, respectively. A gap of two days between the seeding of non-GM and GM lines was implemented to maintain the seedlings at a similar growth stage. When the first pair of foliage leaves was fully expanded after two weeks, the seedlings were selected for systemic inoculations. Approximately 5 g SMV-infected young soybean leaves were ground in 50 mM potassium phosphate, pH 7.5, added to a total volume of 20 ml. The extract was inoculated onto two foliage leaves per plant, with 50 µl of inoculum applied per leaf, by direct rub-inoculation with carborundum, as described by Seo *et al.* (2009).

After nine days, two weeks, four weeks, and 40 days of inoculations, leaves of the infected plants were observed for symptoms and the fully expanded second, third, and fourth leaves were respectively sampled and stored at –80°C until used for real-time polymerase chain reaction (PCR), double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA), and antioxidant and lipid hydroperoxide analysis. For the observation of leaf symptoms, systemic inoculations for non-transgenic ‘Kwangan’ and beta-carotene-enhanced GM lines, as described above, were performed thrice. A total of fifty plants with successful systemic infections were respectively selected for both lines and transferred to pots, with an aim to harvest soybean seeds after maturity. To evaluate the effects of SMV on yield components, number of pot per plant, 100-grain weight, and total grain weight, were measured and calculated from each fifty plants of both the soybean lines. SMV seed transmission rate was evaluated as the proportion of SMV-infected seeds to total seeds per plant. Furthermore, 400 seeds respectively harvested from

SMV-infected GM and non-GM soybean plants were sown in the same greenhouse to investigate seed germination rate and SMV transmission rate to the next generations. Seed germination rate was calculated as the proportion of germinated seeds to the total seeds sown, whereas the transmission rate to next generations was calculated as the proportion of seedlings with SMV infection symptoms to the total number of germinated seeds.

Real time PCR analysis

Total RNA was extracted from the second, third, and fourth leaves of eight plants each of the infected beta-carotene-enhanced soybean line and non-GM ‘Kwangan’, and three mock-inoculated plants of both of the soybean lines using TRI reagent (MRC, Cincinnati, OH, USA) and chloroform. The extracted RNA was treated with RNase-free DNase I, which was subsequently inactivated with ethylenediaminetetraacetic acid by heating at 65°C for 5 minutes. cDNAs were synthesized using amfiRivert cDNA synthesis platinum master mix (Genedepot, Barker, TX, USA) as described in the manual. Briefly, 1 µg of total RNA was denatured at 70°C for 5 minutes along with 10 µM of oligonucleotide primer. The reverse transcription reactions were incubated at 42°C for 60 minutes with 1 µl of amfiRivert cDNA synthesis platinum enzyme Mix and 10 µl of amfiRivert cDNA synthesis platinum 2× buffer, followed by heat inactivation at 85°C for 5 minutes.

Real time PCR was performed for SMV in triplicates for each treatment of both the soybean lines, using *lectin* gene as an internal control. A total of 20 µl of reaction mixture consisted of 10 µM primers, 50 ng cDNAs, and 10 µl amfiSure qGreen Q-PCR Master Mix (2×), low ROX (Genedepot). The reactions were carried out in Stratagene Mx3005P system (Agilent Technologies, Santa Clara, CA, USA) using the following protocol: predenaturation temperature at 95°C for 3 minutes, followed by 40 cycles of

Table 1. Sequences of primers for real time polymerase chain reaction to assess the *Soybean mosaic virus* mRNA expression in non-genetically modified soybean variety ‘Kwangan’ and beta-carotene-enhanced transgenic soybean leaves.

Genes	Forward sequence (5’-3’)	Reverse sequence (5’-3’)	Product size
<i>Soybean mosaic virus</i> (target)	TGATGGTAGCATGCACCCAG	TGTTACCCACTGCCCAACAA	118 bp
<i>Lectin</i> (housekeeping)	GCCGCTTCCTTCAACTCAC	AACCTGCATGTGTTTGTGGC	103 bp

95°C for 10 seconds and 60°C for 60 seconds. Negative controls were prepared, including a no template control, where nuclease-free water was added to the reaction instead of template cDNAs, and samples from the mock-inoculation of SMV. Transcript levels were calculated using the $2^{-\Delta\Delta C_t}$ method and compared to the expression levels of *lectin*. Specific primer sequences used for the internal control i.e., soybean *lectin* gene and the SMV gene are provided in Table 1.

DAS-ELISA analysis

The accumulation of viral protein in the leaves at different positions, two weeks after the inoculation, or leaves after different periods of infection, as well as in different organs, for each of the eight treatments was determined for from both the SMV-inoculated soybean lines. To prepare protein samples, 500 µl of General extraction buffer (Agdia Inc., Elkhart, IN, USA) was added to 50 mg of grinded soybean leaves and seeds and vortexed followed by centrifugation for 30 minutes at 4°C at 10,000 ×g and the supernatant was collected. The protein was quantitated using Quant-iTTM protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) on a QubitTM fluorometer (Invitrogen, Waltham, MA, USA). DAS-ELISA was performed using DAS-ELISA Reagent Set (Agdia Inc.) of SMV following the manufacturer's instructions.

Analysis of total antioxidant content

For analyzing the total antioxidant content, 100 mg dried and frozen soybean leaves were collected from each of the three SMV infected and mock-inoculated GM and non-GM soybean plants. After harvest, seed samples were collected and 200 mg was grinded for antioxidant analysis. The samples were grinded in 1 ml 0.05 M phosphate buffer (0.05 M phosphate potassium, 0.9% [w/v] NaCl, 0.1% [w/v] glucose), mixed by vortexing and centrifuged at 4°C, 12,000 rpm for 10 minutes. The supernatant (20 µl) was used for analyzing the antioxidant content using a total antioxidant status assay kit (Calbiochem).

Lipid hydroperoxide analysis

To measure lipid hydroperoxide (LPO), 100 mg dried and frozen soybean leaves were collected from each of the

three SMV infected and mock-inoculated GM and non-GM soybean plants. Also, seed samples were collected after harvest from three plants for each treatment respectively and 200 mg was grinded for LPO analysis. 100 mg or 200 mg of ground leaves or seeds with 1 ml HPLC-grade water addition were mixed by vortexing and incubated at 85°C in a water bath for 2 hours. Extract R-saturated methanol (Calbiochem, Merck Millipore, Darmstadt, Germany), prepared by adding 100 mg Extract R to 15 ml methanol, was separately added to 500 µl of homogenate and vortexed followed by the addition of 1 ml of cold deoxygenated chloroform and vortexing. After centrifugation at 1,500 ×g for 5 minutes at 0°C, 500 µl of bottom chloroform layer was collected separately and stored on ice until use. The 50 µl extracted samples were measured as described in the manual of lipid hydroperoxide assay kit (Calbiochem).

Abscisic acid levels analysis

Seed samples were collected from each four SMV-infected or mock-inoculated GM and non-GM soybean plants, respectively. After grinded, 50 mg seed samples for each treatment were extracted with 80% (v/v) methanol at 4°C for 3 hours. The methanol extracts were centrifuged at 3,000 ×g for 10 minutes to remove debris and dried under vacuum. The powder was dissolved in 50 ml of 13 Tris-buffered saline buffer (25 mM Tris-HCl, 100 mM NaCl, 0.1 mM MgCl₂, and 0.3% [w/v] NaN₃), as Dong *et al.* (2014) description. The ABA content was determined by competitive ELISA using an anti-ABA antibody according to the protocol of the Phytodetek ABA Test Kit (Agdia Inc.).

Statistical analysis

Microsoft Excel (Microsoft, Redmond, WA, USA) was used to calculate the mean values and standard deviations for all the agronomic traits, viral mRNA and viral protein contents, as well as the antioxidant and LPO concentrations and ABA contents in both soybean lines. T-tests were performed for the values for viral mRNA and viral protein contents, antioxidant and LPO concentration and ABA contents in the beta-carotene-enhanced GM and non-GM 'Kwangan' leaves and/or seeds, using Microsoft Excel.

RESULTS

Leaf and seed symptoms upon systemic SMV inoculations

After nine days of systemic SMV inoculation, slight mosaic symptoms were observed on the second foliage leaves as shown in Fig. 1. Compared to the mock-inoculated plants, the leaves of SMV inoculated plants two weeks after the inoculations showed significant chlorotic

spots and mosaic symptoms (Fig. 1C, H). After four weeks and after 40 days of systemic inoculations, significant necrotic spots and mosaic symptoms were observed on both the soybean lines (Fig. 1D, E, I, J).

Seed transmission rates of SMV were investigated for virus infected non-GM 'Kwangan' and beta-carotene-enhanced transgenic lines. Non-GM 'Kwangan' line and beta-carotene-enhanced GM line showed 20.5% and 43.5% seed transmission rate (Table 2) for the G7H SMV

Fig. 1. Leaf symptoms of two soybean lines after *Soybean mosaic virus* systemic inoculation. (A, F) Non-infected control of 'Kwangan' and genetically modified (GM) soybean, respectively. (B-E) Infected symptoms after 9, 14, 30, and 40 days, respectively in 'Kwangan'. (G-J) Infected symptoms after 9, 14, 30, and 40 days, respectively in GM soybean line.

Table 2. Comparison of yield components and seed transmission rates of SMV between greenhouse-grown non-GM soybean variety 'Kwangan' and beta-carotene-enhanced GM line after systemic virus infection.

Soybean varieties	No. of pods per plant	100-grain weight (g)	Total grain weight (g)	Seed transmission rate of SMV (%)	Seed germination rate of mock-inoculation (%)	Seed germination rate after SMV infection (%)	SMV ²⁾ transmission rate to next generations (%)
Kwangan (non-GM)	20.3±4.55	10.6±1.20	4.5±0.90	20.5±20.1 (0.0-56.0)	47.8±1.6	45.5	8.3
β-carotene enhanced (GM)	21.4±3.60	10.3±1.12	4.5±0.74	43.5±21.3 (0.6-90.2)	37.1±12.5	20.1	14.4
T-test	0.27 ^{ns}	0.33 ^{ns}	0.05 ^{ns}	1.09 ^{ns}	1.07 ^{ns}	-	-

Values are presented as mean±standard deviation or mean±standard deviation (range).

^{ns}Represents no significance at $P < 0.05$ level.

²⁾SMV: *Soybean mosaic virus*, GM: genetically modified, ns: not significant.

Fig. 2. Seed symptoms of non-infected control and *Soybean mosaic virus* (SMV) infected soybean seeds of non-genetically modified (GM) soybean variety 'Kwangan' and beta-carotene-enhanced transgenic soybean line. (A, E, I) Whole seed, seed endosperm and seed coat of mock-inoculated 'Kwangan', respectively. (B, F, J) Whole seed, seed endosperm and seed coat of SMV inoculated 'Kwangan', respectively. (C, G, K) Whole seed, seed endosperm and seed coat of mock-inoculated GM soybean, respectively. (D, H, L) Whole seed, seed endosperm and seed coat of SMV inoculated GM soybean, respectively. (M, O) Mock-inoculated seedlings of non-GM 'Kwangan' and GM soybean line, respectively. (N, P) SMV infected seedlings by seed transmission of non-GM 'Kwangan' and GM soybean line, respectively.

strains, respectively. Significant differences in the number of pods per plant, 100-grain weight, and total grain weight were not observed between the non-GM 'Kwangan' and transgenic line after systemic SMV infections. Seed coat pigmentation and mottling was observed on SMV infected seeds of both the soybean lines as shown in Fig. 2B and D.

After separation of seed coat from the endosperm, pigmented seed coat was confirmed in both the soybean lines (Fig. 2J, L). Furthermore, the endosperm became dull colored after viral infection (Fig. 2F, H).

The seed germination rate was approximately 20.1% in the transgenic line but 45.5% in the non-GM 'Kwangan'

line. The low germination rate might be attributed to transgenic soybean itself, as a germination rate of 37.1% was determined for non-SMV infected beta-carotene-enhanced transgenic seeds. However, SMV transmission might have much more effect on the beta-carotene-enhanced transgenic seeds compared to the non-GM counterpart. Additionally, the SMV transmission rate to next generations in the beta-carotene-enhanced transgenic seeds was observed to be 14.4%, whereas that for non-GM 'Kwangan' was 8.3%. In SMV infected beta-carotene-enhanced transgenic soybean seedlings, many necrotic spots and mottling symptoms were observed; these were relatively few in the non-GM 'Kwangan' seedlings (Fig. 2N, P).

SMV mRNA expression and protein accumulation in soybean leaves and seeds

The expression of viral mRNA in the soybean leaves after two weeks of systemic inoculations was quantitatively detected using real time PCR analysis for both the non-GM 'Kwangan' and beta-carotene-enhanced transgenic soybean line. Although large individual variations occurred, there was no statistically significant difference in

the viral mRNA expression in the second, third, and fourth leaves between the non-GM and GM lines (Fig. 3).

Viral protein accumulation in leaves at different positions and after different periods post inoculation was

Fig. 3. Relative amount of *Soybean mosaic virus* (SMV) viral mRNA in non-genetically modified (GM) soybean variety 'Kwangan' and beta-carotene-enhanced transgenic soybean leaves by real time polymerase chain reaction after two weeks of systemic infections. 2nd, 3rd and 4th: the second, the third and the fourth leaf, respectively counted from the inoculated leaf. Error bars: the maximum and minimum values of viral mRNA expression. ns: no significance at $P < 0.05$ level by T-tests.

Fig. 4. *Soybean mosaic virus* (SMV) protein accumulations in leaves and seeds in non-genetically modified (GM) soybean variety 'Kwangan' and beta-carotene-enhanced GM line by double-antibody sandwich enzyme linked immunosorbent assay test. (A) SMV protein accumulations of the second and third leaf after two weeks of systemic infections. (B) SMV protein accumulations in leaves after two and four weeks of systemic infections. (C) SMV protein accumulations in leaves and seeds. Error bars: the maximum and minimum values of protein accumulation contents. ns: no significance at $P < 0.05$ level by T-tests.

detected by DAS-ELISA. The results suggested that there was accumulation of viral protein in the leaves to similar levels in the beta-carotene-enhanced GM and the non-GM 'Kwangan' lines (Fig. 4A, B). Compared to the high accumulation of viral proteins in the leaves, seeds showed only one twentieth of the viral protein content of the leaves. Furthermore, the seeds of beta-carotene-enhanced GM line had much higher viral protein content compared to those of the non-GM 'Kwangan' line; however, the result was not statistically significant (Fig. 4C). In conclusion, the viral protein profile of the seeds was consistent with the seed appearance and virus transmission rate, indicating that the high production of carotenoids did not enhance the resistance of plants to SMV.

Total antioxidant content in soybean leaves and seeds

Antioxidant content of the leaves of mock- and SMV-inoculated plants of both the soybean lines was analyzed. Compared to the non-GM 'Kwangan' plants, the total antioxidant content in the leaves and seeds of mock-inoculated beta-carotene-enhanced GM line was higher; the difference in the content of seeds being statistically significant ($P < 0.05$). After SMV inoculation, non-GM 'Kwangan' line showed a significant increase in the total antioxidant content of leaves but not much change was evident in the beta-carotene-enhanced GM line (Table 3). Compared to the mock-inoculated plants, the antioxidant content of the seeds showed a significant increase after

SMV infection in the non-GM 'Kwangan' line; however, little decrease in the content was observed in the beta-carotene-enhanced GM line after infection. This suggests that the additional production of carotenoids as a result of the introduction of *PAC* gene might cause a decline in the levels of other defense chemicals, such as phenolic compounds. Similar antioxidant activities in both the leaves and seeds of the non-GM 'Kwangan' and GM lines upon virus infection revealed that the introduction of *PAC* gene had no positive impact on the resistance of soybeans to virus attacks (Table 3).

Lipid hydroperoxide analysis in soybean leaves and seeds

Compared to the mock-inoculated controls, both the SMV inoculated soybean lines showed an increase in the concentration of LPO in the leaves as well as in the seeds (Table 3). The LPO levels in the leaves of beta-carotene-enhanced GM line after virus inoculation were slightly higher than that in the non-GM 'Kwangan' line, indicating that relatively more accumulation of oxygen radical occurred in the transgenic soybean leaf cells after the SMV infection. However, compared to the non-GM 'Kwangan' line, relatively lower LPO concentrations were observed in both the mock and virus inoculated GM soybean seeds. Nevertheless, the differences in the concentrations observed in both the leaves and seeds of non-GM 'Kwangan' and beta-carotene-enhanced GM line were not significant

Table 3. Antioxidant and lipid hydroperoxide concentrations in the leaves and seeds of both mock inoculated and nine days infected for non-GM soybean variety 'Kwangan' and beta-carotene-enhanced transgenic lines, respectively.

Cell damage parameters	Soybean lines	Positions	Mock inoculated (negative control)	T-test	SMV systemic inoculated	T-test
Antioxidant concentration (mM)	Kwangan (non-GM ²⁾)	Leaves	1.82±0.36	-	2.13±0.09	-
		Seeds	2.81±0.05	-	3.25±0.12	-
	β-carotene enhanced (GM)	Leaves	2.17±0.06	1.29 ^{ns}	2.23±0.30	0.46 ^{ns}
		Seeds	3.42±0.09	8.76*	3.35±0.01	1.06 ^{ns}
Lipid hydroperoxide (μM)	Kwangan (non-GM)	Leaves	45.26±13.80	-	47.00±5.43	-
		Seeds	65.34±3.16	-	85.30±10.98	-
	β-carotene enhanced (GM)	Leaves	47.22±12.80	0.15 ^{ns}	55.93±2.02	2.18 ^{ns}
		Seeds	59.28±5.19	1.41 ^{ns}	72.15±9.48	1.28 ^{ns}

Values are presented as mean±standard deviation.

^{ns}Represents no significance at $P < 0.05$ level. *Represents significance at $P < 0.05$ level.

²⁾GM: genetically modified, ns: not significant.

Table 4. Absciscic acid contents in beta-carotene-enhanced transgenic soybean seeds compared to those of non-transgenic counterpart 'Kwangan' before and after SMV infections.

Soybean varieties/abscisic acid	Mock inoculated seeds (ng/ml)	SMV ²⁾ inoculated seeds (ng/ml)
Kwangan (non-GM)	4.54±1.39	29.15±20.90
β-carotene enhanced (GM)	6.11±2.87	218.48±101.72
T-test	0.70 ^{ns}	2.58 [*]

Values are presented as mean±standard deviation.

^{ns}Represents no significance at $P < 0.05$ level. ^{*}Represents significance at $P < 0.05$ level.

²⁾SMV: *Soybean mosaic virus*, GM: genetically modified, ns: not significant.

before or after virus infection.

Absciscic acid levels in mock- and SMV-inoculated soybean seeds

The phytohormone ABA levels in seeds were quantitated for mock- and SMV-inoculated beta-carotene-enhanced GM soybean line and non-GM counterpart 'Kwangan', respectively. In mock-inoculated soybean seeds, beta-carotene-enhanced GM line was determined to contain 6.11±2.87 ng/ml ABA compared to non-GM level of 4.54±1.39 ng/ml. T-test analysis of ABA levels indicated no significant difference between GM and non-GM soybean line. Similarly, ABA levels of SMV-inoculated soybean seeds were determined to contain 29.15±20.90 ng/ml ABA in non-GM 'Kwangan' and 218.48±101.72 ng/ml ABA in beta-carotene-enhanced GM line. This result caused a significant difference on ABA levels between GM and non-GM soybean lines after SMV infections by T-test analysis (Table 4).

DISCUSSION

Over the last two decades, beta-carotene, vitamin A, and vitamin C have been verified to play important roles in the enhancement of the immune system of animals and in protection against numerous infections (Tjoelker *et al.* 1990). In addition, beta-carotene as one of defense chemicals has been reported could be distinctly increased under biotic and abiotic environmental stresses in plants (Rosenfeld *et al.* 1998; Du *et al.* 2010; Kim *et al.* 2012b; Wang *et al.* 2014). Therefore, it is worth noting that amounts of endogenous beta-carotene would be favorable

for strengthening the plant defense system under biotic stresses, such as virus attacks. The present study was conducted to assess the response of beta-carotene-enhanced transgenic soybean plants and their seeds to systemic SMV infections. Our results indicated that the mRNA expression and protein accumulation of SMV, mosaic symptoms, agronomic traits, total antioxidant and LPO concentrations in the seeds and leaves of non-GM 'Kwangan' and beta-carotene-enhanced GM line did not differ significantly. However, a 35-fold increase of ABA level in beta-carotene-enhanced GM soybean seeds was determined compared to that of non-GM 'Kwangan' after virus infections, which is considered as a major reason of the occurrences on a high transmission rate of virus to subsequent generations and a low germination rate in beta-carotene-enhanced transgenic soybean seeds.

In addition, Ha *et al.* (2010) developed pro-vitamin A producing rice by introduction of the same transgene cassettes to that of beta-carotene-enhanced transgenic soybean. However, both of transgenic rice and soybean are confronted with the same problem: viral origin 2A linker was used to achieve simultaneous expressions of two genes in carotenoid biosynthesis pathway, instead of two cassettes expressions of two genes. Actually, we did not find homologous sequence between 2A and most plant virus sequence in NCBI database, which suggested that probability of homologous virus recombination happening was nearly rare. Up to present, no evidence indicated 2A sequence has negative effects on transgenic plants, animals, GMO food and feed, and environments. In the present study, plant performances, virus accumulations and cell damage degrees between transgenic soybean line and non-transgenic 'Kwangan' have no significant differences,

implied that 2A sequence had a very remote chance of adverse effect on transgenic soybeans, virus and environments.

Previous studies demonstrated that beta-carotene-enhanced transgenic soybean lines overexpressing *Psy-2A-crtI* (*PAC*) under the control of seed-specific beta-conglycinin promoter had an average total carotenoid content of 1,008 µg/g dry weight in the leaves, compared to 882 µg/g dry weight present in the non-GM 'Kwangan' plants. Furthermore, in the beta-carotene-enhanced soybean seeds, the average carotenoid content was 110.7 µg/g dry weight, which was 45-fold higher than that in the non-GM 'Kwangan' seeds (Kim *et al.* 2012a). However, the 25% increase or 45-fold higher carotenoid content in the leaves and seeds, respectively, did not confer higher resistance or tolerance against virus attacks in the beta-carotene-enhanced transgenic soybean lines in this study. On the contrary, more than 50% lower germination rate was observed in the SMV infected beta-carotene-enhanced GM seeds compared to the mock-inoculated controls, whereas little difference in the germination rate was observed between the SMV-infected and the mock-inoculated non-GM 'Kwangan' seeds. Lindgren *et al.* (2003) studied the relationship between carotenoids or ABA and the germination rate for several transgenic *Arabidopsis* lines by introduction of *phytoene synthase* under the control of seed-specific promoter. This study revealed a positive correlation between carotenoids and ABA, but a negative correlation between the germination rate and carotenoids or ABA content. Moreover, there were positive correlations between the production of ABA and lutein or violaxanthin, but not of zeaxanthin. The beta-carotene-enhanced GM soybean lines used in the present study were detected to produce 77% beta-carotene and 20% alpha-carotene instead of 96.6% lutein in seeds of non-GM 'Kwangan', and two new components, namely beta-cryptoxanthin and violaxanthin, were also accumulated (Kim *et al.* 2012a). According to previous studies, high productions of beta-carotene and violaxanthin were assumed to produce additional ABA through the ABA biosynthetic pathway. In this study, ABA levels in seeds

were detected for both soybean lines and results suggested beta-carotene-enhanced GM soybean had a higher ABA content than that of non-GM 'Kwangan', but the differences did not reach statistical significant (Table 4). This finding is consistent with previous studies that have demonstrated the improvement of endogenous ABA levels could lead to delay germination or low germination rate (Lindgren *et al.* 2003).

Otherwise, ABA levels in seeds were also detected for both soybean lines after SMV infections and results indicated that ABA content of beta-carotene-enhanced GM soybean seeds was increased 35-fold to that of mock-inoculated seeds, but only 7-fold increase was observed on non-GM 'Kwangan' seeds in this study. Alazem *et al.* (2014) elucidated that ABA plays multifaceted roles in the defense of plants against viruses. For one thing, it increases the susceptibility of plants by repressing the induction of hypersensitive response, reactive oxygen species, and salicylic acid production, and for another, it restricts systemic movement of the virus. In the present study, whether ABA plays a crucial role or not could not give a definite answer, according to non-significant difference between GM soybean and non-GM 'Kwangan' on responses against virus attacks. However, the high SMV transmission rate to next generations in the beta-carotene-enhanced GM soybean line might be attributed to a sharp increase of ABA level (Table 2, 4). It can be seen from this research findings that additional production of beta-carotene made no difference on antiviral property of soybeans, but numbers of beta-carotene productions would cause alternations of other metabolite compositions including phytohormone ABA levels, that would be concerned about plant responses to virus attacks.

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