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IDENTIFICATION OF A NOVEL GA-RELATED BUSH MUTANT IN PUMPKIN (*CUCURBITA MOSCHATA* DUCHESNE)

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Abstract

Pumpkin (*Cucurbita moschata* Duchesne) bush mutant plants were characterized by short stems. The sensitivity of pumpkin bush mutant plants to exogenous hormones was identified in this study. Results revealed that internode elongation of bush mutant plants could respond to gibberellins (GA_{4+7} and GA_3), but not to indole acetic acid (IAA) and brassinosteroids (BR); by contrast, the mutant phenotype of bush mutant plants could not be fully rescued by GA_{4+7} and GA_3. The internode of bush mutant plants yielded a lower *KS* expression level than that of vine plants. Therefore, pumpkin bush mutant plants were designated as GA-related mutant plants eliciting a partial response to GAs; the action of IAA and BR might not be involved in the internode growth of pumpkin bush mutant plants, specifically *Cucurbita moschata* Duch.

Key words: Auxin, Brassinosteroids, Bush, Gibberellins, Pumpkin.

Introduction

In higher plants, a complex hormonal system likely plays a crucial role in controlling growth and development (Ali *et al.*, 2014; Naz & Khatoon, 2014). Hormones control growth and development throughout the life cycle of plants and function as intermediates in response to environmental signals, such as light and temperature. Such signals alter the rate of hormone biosynthesis and catabolism, as well as responsiveness of plants to hormone. For instance, dwarf mutants related to hormone biosynthesis and signaling have been identified (Hentrich *et al.,* 1985; Noguchi *et al.,* 1999; Symons *et al.,* 2002). Dwarf mutants have been used as valuable tools in hormonal studies in a wide range of plant species (Nadhzimov *et al.,* 1988; Sponsel *et al.,* 1997; Fukuta *et al.,* 2004). Hormonal studies have also been conducted on gibberellins (GAs) (Nadhzimov *et al.,* 1988), auxin [particularly indole acetic acid, (IAA)] (Symons *et al.,* 2002), and brassinosteroids (BR) (Noguchi *et al.,* 1999). GAs, IAA, and BR are plant growth regulators of cell and plant size; these hormones also control mutations that impair biosynthesis or sensitivity causing dwarfism. Hormonal responses observed in dwarf mutants categorized as hormone-sensitive and hormone-insensitive (Hentrich *et al.,* 1985; Koornneef *et al.,* 1985; Azpiroz *et al.,* 1998). Hormone-sensitive dwarf mutants are deficient in endogenous hormones; as a result, these mutants are dwarf in stature, but can be induced to grow to normal height through exogenous hormone application (Hentrich *et al.,* 1985; Azpiroz *et al.,* 1998). By contrast, hormone-insensitive dwarf mutants cannot be normalized through exogenous hormone treatment (Koornneef *et al.,* 1985). These hormone-sensitive dwarf mutants indicate that such hormones are necessary to determine plant stature. Therefore, plant growth response to exogenous hormone application should be investigated when new dwarf mutants are characterized to identify potential hormone-biosynthetic mutants.

In this study, a bush-type plant was selected from the 'Cga' cultivar of the tropical pumpkin *Cucurbita moschata* Duchesne (Wu *et al.,* 2007). Bush plants were characterized by shorter internodes, earlier flowering, a higher ration of female to male flowers and smaller fruit than vining 'Cga' plants (Wu *et al.,* 2007). Genetic analysis indicated that the bush plants had a monogenic inheritance, in which the bush genotype was dominant (*Bu*) to the vine genotype (*bu*) (Wu *et al.,* 2007). The internal development of pumpkin was determined by investigating the differences in endogenous hormones between bush and vine plants. Endogenous hormones (GA_3, GA_4, and IAA) levels were characterized and compared in different growth periods of *C. moschat*a bush and vine plants (Wu *et al.,* 2008). Results revealed that the internodes of *C. moschat*a bush plants contained lower levels of GA_3, IAA, and GA_4 than those of *C. moschat*a vine plants. GA_3 was also deficient in leaves and roots of bush plants. The GA_3 contents measurement result of Wu *et al.* (2008) is in accordance with that of Cao *et al.* (2005), indicating that *C. moschat*a bush plant is a GA_3-responsive mutant. Wu *et al*. (2008) further determined GA_4 and IAA contents and found that *C. moschata* bush mutant plant may be sensitive to these hormones, as indicated by low hormone contents. In addition to elongated internodes, other developmental factors, such as leaf size and petiole length, maybe sensitive to exogenous hormones, such as GA_4, IAA, and BR. However, exogenous hormone sensitivities of *C. moschata* bush mutant plants remain poorly understood.

This study was conducted to characterize and compare the sensitivities of *C. moschata* bush mutant plant to exogenous hormones (GA_{4+7}, GA_3, IAA, and BR). This study also aimed to provide novel insights into hormone sensitivity of pumpkins with different growth habits.

Materials and Methods

Plant material: The bush-type plant was selected from a cultivar 'Cga' of tropical pumpkin (*Cucurbita moschata* Duchesne) (Fig. 1). Seedlings of bush and vine plants could be distinguished at third leaf stage. Bush plants had short internodes length (4.1±0.4 cm vs 10.2±0.6 cm), short vine length (14.5±4.9 cm vs 175.6±28.2 cm) and small number of internode (3.5±0.7 vs 17.2±1.6) compared with vine plants at 60 d after planting. Vine plants produced more male flowers (21.1±4.3) than bush plants (13.7±3.3). In contrast, bush plants flowered 8 d earlier (40.6±2.9 d vs 48.6±2.7 d) than vine plants. Although bush plants had a higher rate of fruit abortion than vine plants, they had a larger total number of fruit than vine plants in a plant basis (Wu *et al.,* 2007). Seeds of wild type and bush plants were germinated and grown in a rectangle pot containing a nutrient solution (Japan gardening) on the research farm of the Institute of Vegetable Science, Zhejiang University, Hangzhou, China. Each pot had 6 plants.

Hormone treatment of plants: Spraying method was used to test the sensitivity of plant stature to exogenous hormones. For GA_{4+7} treatment, Bush and wild type plants were sprayed with 0.15 mM GA_{4+7} (Shenghua Baike biotechnology co. ltd, Zhe Jiang, China) at three leaf stage. Exogenous GA_{4+7} were sprayed every 3 d for five times in total. Seven days after the treatments, the length of elongating internodes, petiole length, leaf length and width were measured. Bush and wild type plants were also sprayed with various concentrations of GA_3 (0.3, 0.6, 0.9, 1.2, and 1.5 mM, Sigma, G7645), IAA (50, 100, and 150 μM, Sigma, I-2886) and BR (50, 100, and 150 μM, Shenghua Baike biotechnology co. ltd, Zhe Jiang, China) solutions respectively at three leaf stage. Exogenous hormones were sprayed every 3 d for five times in total. Three days after the treatments, the length of elongating internodes, petiole length, leaf length and width were measured.

RNA extraction and cDNA synthesis: Total RNA was extracted from 500 mg of the frozen internodes of bush and vine pumpkin plants by using Trizol^{TM} extraction method (Invitrogen, Carlsbad, CA). The RNA quality, integrity and quantity were determined by running 2 μL of total RNA in a formaldehyde denaturing gel. For cDNA synthesis, total RNA from 15 representative individual plant of each phenotype was pooled. One μg of total pooled RNA was used initially for first strand synthesis, followed by double-stranded cDNA synthesis using SMART^{TM} cDNA Library Construction Kit (Clontech, USA) according to the manufacturer's instructions.

RT-qPCR analysis: For RT-qPCR, 20 μL of samples were run in triplicate on an ABI Prism 7000 Sequence Detection System and Applied Biosystems software using a tenfold dilution of 1 μL of cDNA and SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific primers (forward: 5'-TTTTCAAAGGGAAGAGAAGC -3'; reverse: 5'-GCAGCTGCTGTTGTGGCTGG -3'). Relative quantitation of gene expression was calculated and normalized to *β-actin* (forward: 5'-CCACCAATCTTGTACACATCC-3'; reverse: 5'-AGACCACCAAGTACTACTGCAC-3'). Three biological and three technical replicates were included in RT-qPCR.

Statistical analyses: The experiments were setup in a completely randomized design. Each replicate contained mixed sample randomly collected from nine seedlings of each type. Three replications per plant type were used with measurement date as a block. Data means and standard errors (SD) were then averaged from four repeated experiments. The data were analyzed by an analysis of variance (ANOVA) using DPS (version 7.05) (Ruifeng Info Technology Ltd, Hangzhou, China), and significant differences among treatment means were calculated by Duncan's multiple range test ($p<0.05$).

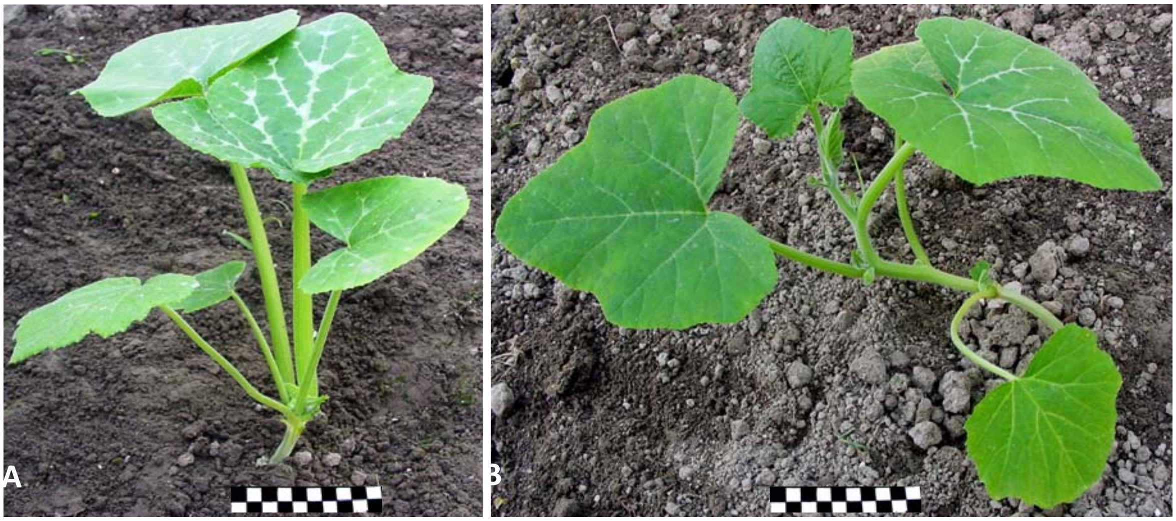

Fig. 1. Bush mutant (A) and wild type (B) 20 d old plants in *Cucurbita moschata* Duch. Bars = 10 cm.

Results

Effect of GAs on the biological of *C. moschata*: Wu *et al.* (2008) revealed that the internodes of *C. moschata* bush plants contain lower levels of GA_4 than those of *C. moschata* vine plants. In our study, the effects of exogenous GA_{4+7} on the seedlings of pumpkin bush mutant plants and wild-type plants were examined. GA_{4+7} could partially recover a 1.2-fold increase in the internode length of bush mutant plants (Fig. 2). GA_{4+7} could also elicit a response from wild-type plants; furthermore, GA_{4+7} could cause a 1.5-fold increase in internode length (Fig. 2). Petiole length, leaf length, and width of bush and wild-type plants were also measured after exogenous GA_{4+7}. Our results showed that GA_{4+7} affected these developmental factors of bush mutant plants and wild-type plants (Fig. 2).

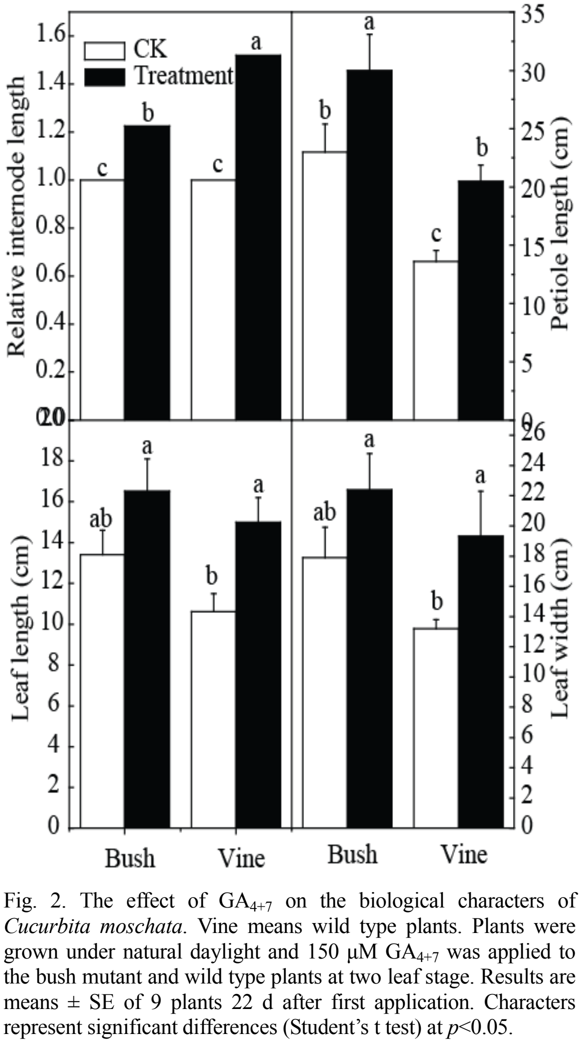

Fig. 2. The effect of GA_{4+7} on the biological characters of *Cucurbita moschata*. Vine means wild type plants. Plants were grown under natural daylight and 150 µM GA_{4+7} was applied to the bush mutant and wild type plants at two leaf stage. Results are means ± SE of 9 plants 22 d after first application. Characters represent significant differences (Student's t test) at $p<0.05$.

Vine length, petiole length, leaf length, and width of bush mutant plants and wild-type plants treated with exogenous GA_3 were also measured. The results showed that 0.9 mM GA_3 elicited the greatest effect on internode elongation of bush mutant plants and wild-type plants (Fig. 3). Conversely, GA_3 did not affect the petiole length of bush mutant plants. By contrast, 0.3 and 0.9 mM GA_3 suppressed the growth of the petiole length of wild-type plants (Fig. 3). Various GA_3 treatments did not also affect the leaf length of bush mutant plants. Conversely, various GA_3 treatments inhibited the increase in leaf length of wild-type plants (Fig. 3). Likewise, various GA_3 treatments prevented the increase in leaf width of bush mutant plants and wild-type plants (Fig. 3). GA_3 also caused the leaf color of bush mutant plants to change from normal light green to dark green (data not shown).

The internode length of bush mutant plants and wild-type plants treated with various GA_3 concentrations were measured to further identify the greatest effect of GA_3 concentration on internode elongation of bush mutant. The results showed that internode length of bush mutant plants was responsive to exogenous GA_3 application in a concentration-dependent manner (up to 0.9 mM); among various concentrations, 0.9 mM GA_3 elicited the highest effect on internode elongation of bush mutant plants (Fig. 4). Compared with the application of 0.9 mM GA_3, the application of other high GA_3 concentrations, such as 1.2 and 1.5 mM, failed to further recover the internode elongation of bush mutant plants (Fig. 4). GA_3 application induced similar responses between wild-type plants and bush mutant plants. In contrast to 0.9 mM GA_3, 1.2 mM GA_3 elicited the highest effect on internode elongation of wild-type plants (Fig. 4).

Suppression of internode elongation of pumpkin bush mutant plants through exogenous BR application: Bush mutant and wild-type plants were treated with various concentrations of BR. The results of internode length measurement revealed that BR slightly suppressed internode elongation of bush mutant plants and wild-type plants. Bush mutant plants elicited a more significant response to BR application than wild-type plants. The internode lengths of bush mutant plants were reduced by 50%, 58.1%, and 40% at 50, 100, and 150 µM BR treatments compared with those of control plants, respectively. By contrast, the internode lengths of wild-type plants were reduced by 12%, 16.1%, and 10% at 50, 100, and 150 µM BR treatments, respectively (Fig. 5). Moreover, various concentrations of BR did not affect the development of petiole length, leaf length and width of bush mutant plants and wild-type plants (Fig. 5).

Effect of IAA on the biological characteristics of *C. moschata*: Bush mutant plants and wild-type plants were treated with three concentrations of IAA. The results of internode length measurement revealed that IAA slightly suppressed the internodes elongation of bush mutant plants and wild-type plants. Bush mutant plants and wild-type plants elicited similar responses to IAA application. The internode lengths of bush mutant plants were reduced by 12.5%, 8.9%, and 5.1% at 50, 100, and 150 µM IAA treatments compared with those of control mutant plants, respectively. By contrast, the internode lengths of wild-type plants were reduced by 7%, 11%, and 10.9% at 50, 100, and 150 µM BR treatments compared with that of control wild-type plants, respectively (Fig. 6). Various IAA concentrations did not affect the development of petiole length, leaf length and width of bush mutant plants and wild-type plants (Fig. 6).

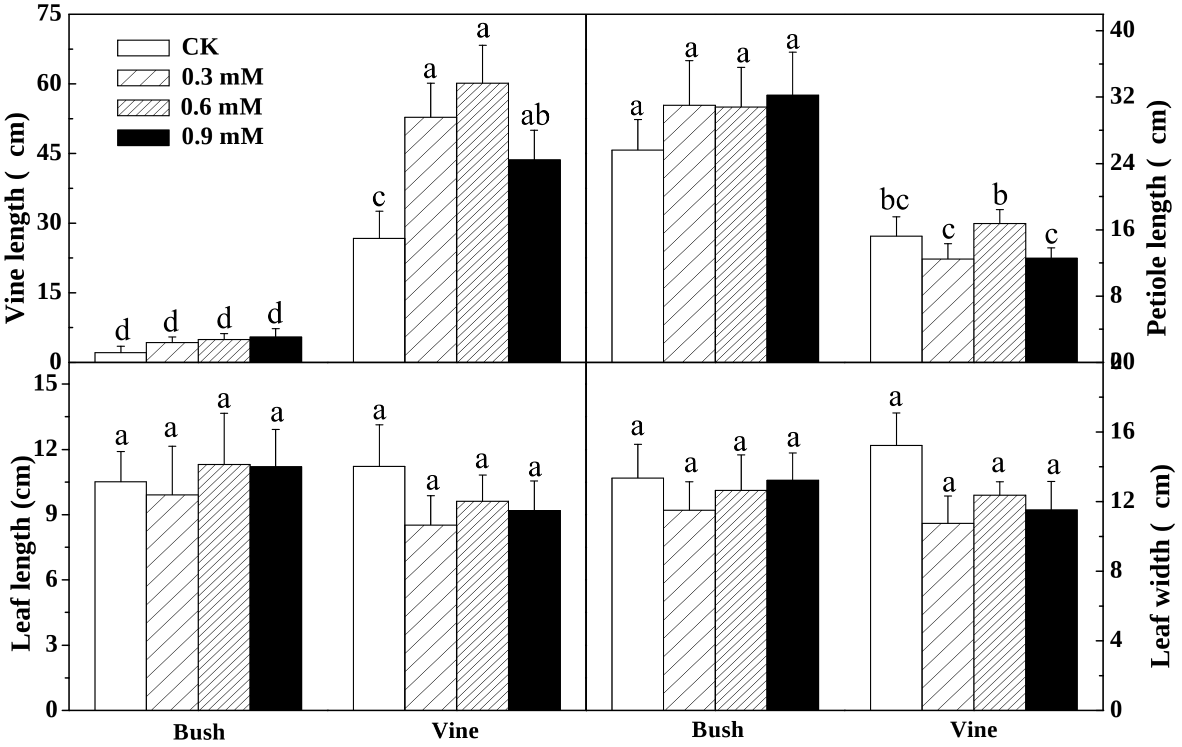

Fig. 3. The effect of various concentrations of GA_3 on the biological characters of *Cucurbita moschata.* Vine means wild type plants. Plants were grown under natural daylight and GA_3 at various concentrations was applied to the bush mutant and wild type plants at two leaf stage. Results are means ± SE of 9 plants 18 d after application. Characters represent significant differences (Student's t test) at $p<0.05$.

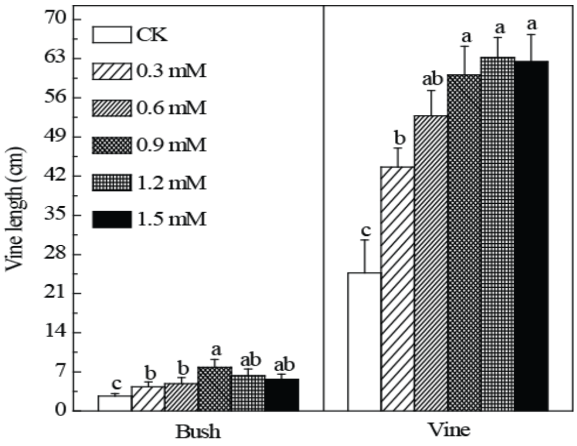

Fig. 4. Effect of various GA_3 concentrations on the internode length of bush mutant and wild type plants in *Cucurbita moschata* Duch. Vine means wild type plants. Plants were grown under natural daylight and GA_3 at various concentrations was applied to the bush mutant and wild type plants at two leaf stage. Results are means ± SE of 9 plants 18 d after first application. Characters represent significant differences (Student's t test) at $p<0.05$.

Lower *KS* expression level of the internode of bush mutant plants than that of vine plants: Plants can sense internal and external hormone status, and then adapt to changing hormone conditions by modifying gene expression. mRNA levels of several genes involved in GA biosynthesis were analyzed to verify whether GA biosynthesis of bush mutant seedling was changed. The result clearly indicated that the internodes of bush mutant plants yielded a lower *KS* expression level than those of wild-type plants (Fig. 7); the internodes of bush mutant plants and wild-type plants exhibited similar expression levels of *CPS*, *KO*, *KAO*, *GA20ox*, *GA3ox*, and *GA2ox* (data not shown).

Discussion

Sensitivities of pumpkin bush mutant plants and wild-type plants to exogenous GA_{4+7}, GA_3, BR, and IAA were investigated in this study. The results indicated that internode elongation in pumpkin bush mutant plants was partially recovered by GA_{4+7} and GA_3 but suppressed by BR (Figs. 2, 3, 4, and 5). In addition, IAA did not affect internode elongation of bush mutant plants (Fig. 6). Although pumpkin bush mutant plants exhibited reduced endogenous GA_3, GA_4, and IAA levels (Wu *et al.,* 2008), pumpkin bush mutant plants were not GA-sensitive mutants because these plants cannot be normalized by exogenous GA_{4+7} and GA_3 as indicated by our results. However, bush mutant plants cannot also be considered as GA-insensitive mutants because internode elongation could be induced by 188.9% and 52.9% when these plants were treated with GA_3 and GA_{4+7} (Figs. 2, 3, and 4). Therefore, pumpkin bush mutants can be considered as GA-related mutant eliciting a partial response to GAs.

This finding is similar to that obtained by Cao *et al.* (2005). The causal gene controlling bush phenotype is not primarily involved in either GA biosynthesis or GA action. Other functions might have caused the retarded growth of *C. moschata* bush mutant plants. The sensitivity of pumpkin bush mutant plants is different from that of *Arabidopsis ga1* and pea *lh* dwarf mutants, which are hormone-sensitive mutants (Zeevaart & Gage, 1993; Davidson *et al.,* 2004). Pumpkin bush mutant plants also differ from maize GA-insensitive dwarf mutant (Harberd & Freeling, 1989). The results obtained in this study are consistent with observations in tomato *dgt* and d^x dwarf mutants and pea *lk* and *lw* dwarf mutants (Ross & Reid, 1986; Jolly *et al.,* 1987; Nadhzimov *et al.,* 1988; Scott, 1988), which can be partially recovered by exogenous hormone application. However, wild-type phenotypes of tomato and pea dwarf mutants could not be fully recovered through exogenous hormone application. As such, a reasonable hypothesis cannot be easily presented to interpret conflicting results related to endogenous hormone levels and exogenous hormone application based on current knowledge. Nevertheless, other data may help elucidate this uncharacterized mechanism. The causal gene of pumpkin bush mutant plants might not have a major role in GA biosynthesis or signaling pathway. Bush mutant phenotype might have originated from other unknown functional pathways, whereas the partial sensitivity of bush mutant plants to GA_3 and GA_{4+7} was influenced by the inhibited unknown functional pathways. The action of IAA and BR might not be involved in the internode growth of *C. moschata* bush mutant plants. Furthermore, if BR and IAA participate in the regulation of internode elongation of bush mutant plants, physiological activity may be inhibited by the mutation of causal gene controlling bush phenotype. Wu & Cao, (2008) identified differentially expressed genes during internode development of the pumpkin bush mutant and reveled that these genes are involved in metabolism and energy, unknown functional protein, unknown gene, stress and defense, cell wall biosynthesis or modification, gene-encoding transcription factor and signal transduction. A novel gene encoding *NADH dehydrogenase* was then isolated from pumpkin plants. RT-PCR analysis showed that *CmBu1* was expressed at a high level in the internodes and hypocotyls and was expressed stronger in elongated than in fully expanded internodes, which further revealed that *CmBu1* gene might play important roles in vine elongation of tropical pumpkin (Wu & Cao, 2010). However, none of genes related to plant hormone were identified by Wu & Cao, (2008).

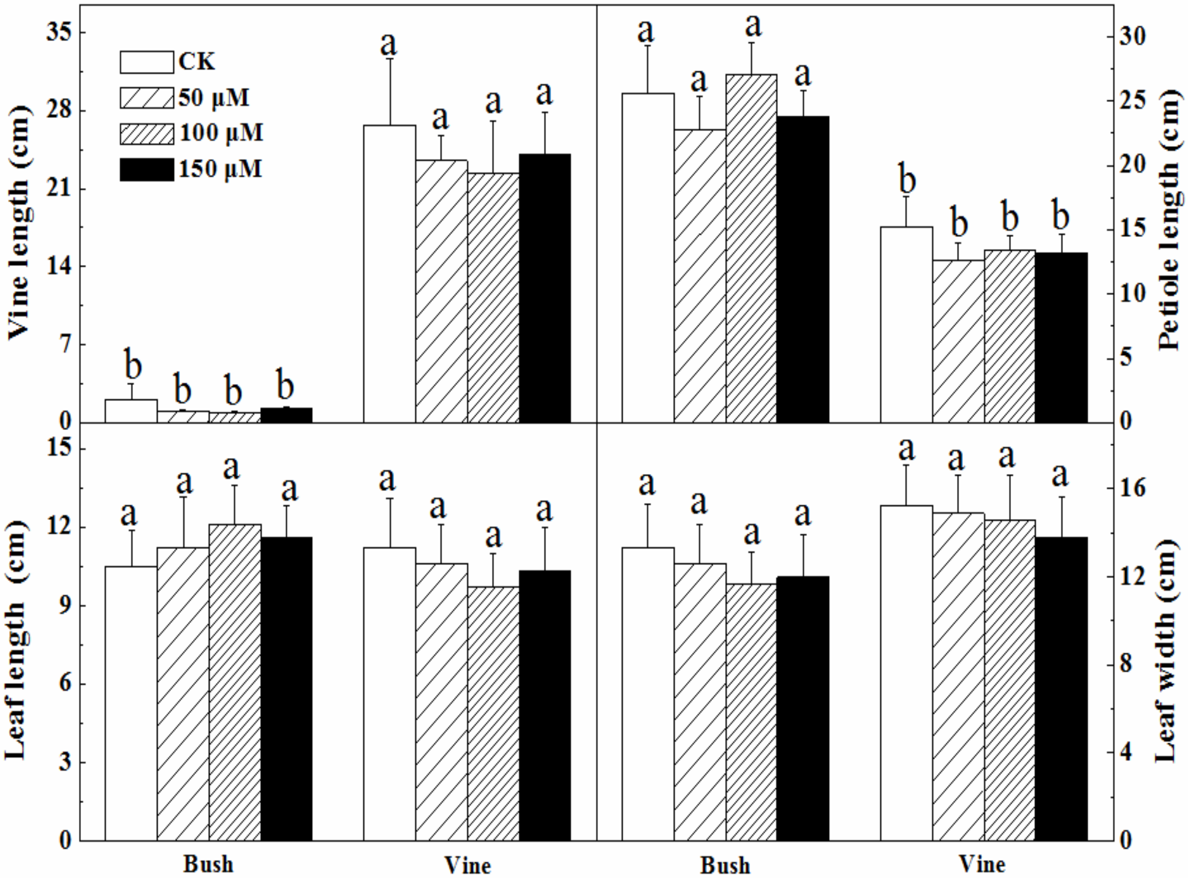

Fig. 5. The effect of BR on the biological characters of *Cucurbita moschata.* Vine means wild type plants. Plants were grown under natural daylight and BR at various concentrations was applied to the bush mutant and wild type plants at two leaf stage. Results are means ± SE of 9 plants 18 d after first application. Characters represent significant differences (Student's t test) at $p<0.05$.

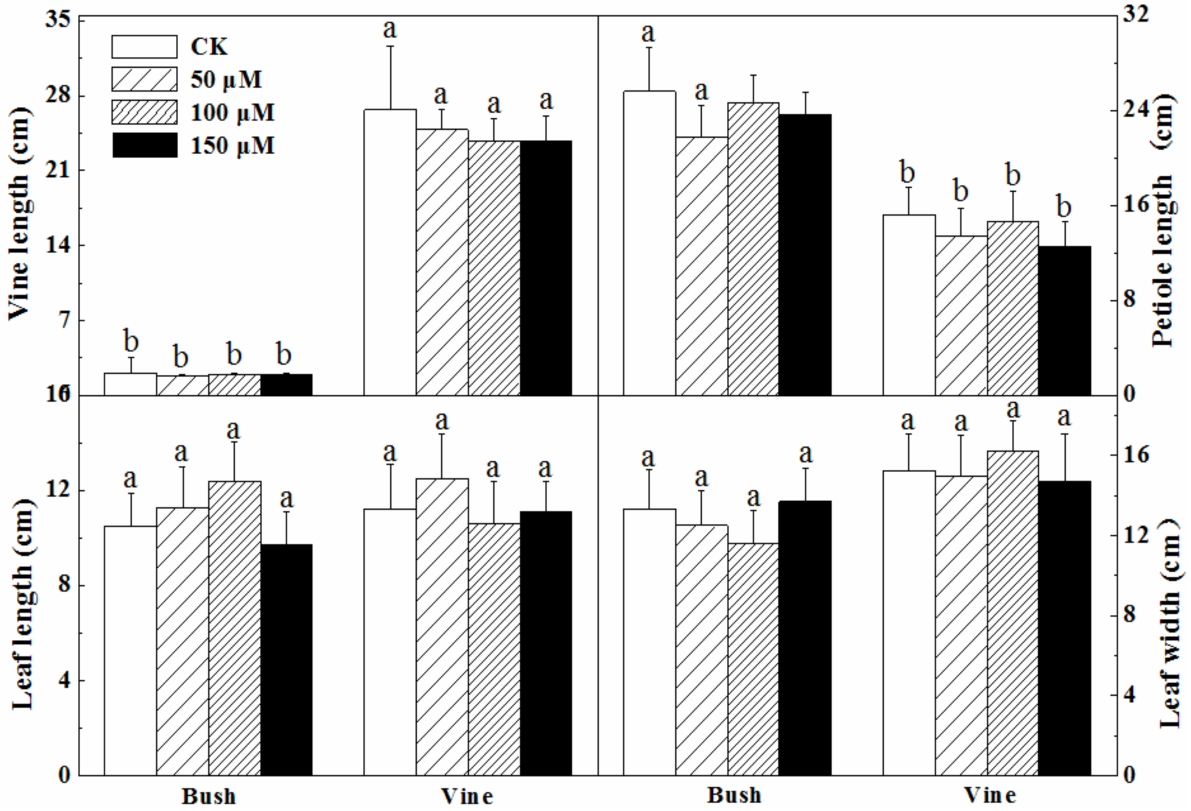

Fig. 6. The effect of IAA on the biological characters of *Cucurbita moschata.* Vine means wild type plants. Plants were grown under natural daylight and IAA at various concentrations was applied to the bush mutant and wild type plants at two leaf stage. Results are means ± SE of 9 plants 18 d after application. Characters represent significant differences (Student's t test) at $p<0.05$.

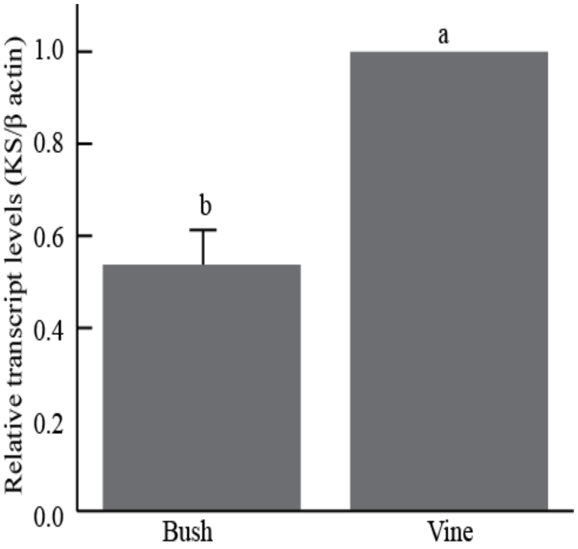

Fig. 7. The expression level of *KS* in the internode of bush mutant and Vine plants. At the third leaf stage, the 1st internodes of bush and vine phenotypes were collected and frozen immediately in liquid nitrogen for RNA extraction and cDNA synthesis. A total of 15 representative plants were sampled. Levels of *KS* mRNA were normalized to the *β-actin* mRNA levels in the same samples. The data are expressed as means ± SE (n = 3) relative to the value of Vine plants (defined as 1). Characters represent significant differences (Student's t test) relative to Vine plants at $p<0.05$.

Besides the result of this study, there are still some other reports on the hormone sensitivity of *Cucurbita pepo* L. bush mutants. Bukovac & Wittwer, (1956) and Pisani, (1958) obtained a similar result to this study, which revealed that the internode elongation of *Cucurbita pepo* L. could be induced by exogenous GA_3 application under both field and greenhouse conditions. However, Denna & Munger, (1963) obtained a different result from that of this study, which revealed that the bush mutant of *Cucurbita pepo* L. was a GA-sensitive mutant. The internode elongation of *Cucurbita pepo* L. bush mutant plants could be fully rescued by exogenous GA_3 application, while the wild-type plants had no sensitivity to GA_3. In addition, the internode elongation of *Cucurbita pepo* L. bush mutant plants could also be partially rescued by various concentrations of IAA (Denna & Munger, 1963). The results of this study revealed that both BR and IAA suppressed the internode elongation of bush mutant (Figs. 5, 6). The differences in the results obtained from different studies could be explained by the difference in genetic background of *Cucurbita pepo* L. and *Cucurbita moschata* Duch. Also, *Cucurbita pepo* L. and *Cucurbita moschata* Duch. have different causal genes controlling the bush phenotype and these genes have different hormone sensitivities.

Moreover, the results reported by Denna & Munger,

(1963) and Cao *et al.* (2005) revealed that the petiole elongation could be induced by IAA and GA_3 application. However, the results of this study revealed that the petiole elongation was suppressed by IAA and BR application (Figs. 5, 6), while GA_{4+7} and GA_3 had no effect on petiole elongation of pumpkin (Figs. 2, 3). Phenocopies of the elongated phenotype of tomato, including a distinctive change in leaf shape, can be induced by GA treatment of normal tomato plants (Jones, 1987). Matsukura *et al.* (1998) also reported that GA_3 enhanced the growth of the secondary leaf sheath, but auxin did not. However, the results of this study showed that the leaf shape (length and width) of the pumpkin bush mutant plants had no response to exogenous applied GA_{4+7}, GA_3, IAA and BR (Figs. 2, 3, 5, and 6), suggesting that internode elongation of pumpkin bush mutant plants might be regulated by a different system with leaf shape and petiole growth.

Acknowledgements

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