Successive Induction of Invertase Isoforms During Flower Development in *Eustoma*

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In this study, we examined sucrose metabolism and expression of invertase, a sucrolytic enzyme, during vegetative and floral development in *Eustoma grandiflorum*, a widely cultivated ornamental plant. During vegetative growth, sucrose content was relatively lower in roots and unexpanded leaves than in expanded leaves. The activities of cell-wall invertase (CWIN) and vacuolar invertase (VIN) were higher in roots and unexpanded leaves, respectively, whereas the activity of cytoplasmic invertase (CIN) was higher in both organs. During flower development, although the contents of reducing sugars and sucrose were relatively unchanged, starch content was higher in elongated flower buds (stage 2), and we also detected a significant increase in CWIN activity. VIN and CIN showed contrasting changes in enzymatic activity, with the former being higher, and the latter lower in opened flowers (stage 3). Furthermore, we cloned two putative CWIN genes (*EgCWIN1* and *EgCWIN2*), one putative VIN gene (*EgVIN1*), and one putative CIN gene (*EgCIN1*), and examined the transcript levels of these four genes. Although we detected no clear correlations between invertase activities and the transcript levels of invertase genes in vegetative organs, we observed changes in the transcript levels of *EgCWIN1*, *EgVIN1*, and *EgCIN1* corresponding to changes in activities of the respective invertase during flower development. These results indicate that carbon partitioning during vegetative and floral development in *E. grandiflorum* is controlled by three invertase isoforms, and that differential gene expression underlies the successive induction of these invertase isoforms during flower opening.

Key Words: carbon partitioning, gene expression, starch, sucrose metabolism.

Introduction

Sucrose plays vital roles in both the vegetative and reproductive growth of plants. This disaccharide functions as the main photosynthetic carbon source that is translocated or stored to fuel cellular energy metabolism or to control cell expansion as an osmolyte; it also serves as a signaling molecule for gene expression (Ruan et al., 2010; Wan et al., 2018). The importance of sucrose has been demonstrated in numerous ornamental flowers, including roses and carnations, particularly with respect to petal growth and senescence (Hawker et al., 1976; Woodson and Wang, 1987; van Doorn and van Meeteren, 2003; Yamada et al., 2007; Horibe and Yamada, 2017).

Regulation of growth and development via carbon partitioning and other related factors has been studied in *Eustoma grandiflorum*, one of the most commercially important ornamental species. Flowering of *Eustoma* is controlled by temperature and day-length through floral promoter genes such as the *FLOWERING LOCUS T* (*FT*)-like gene (Nakano et al., 2011). During floral development in *Eustoma*, excision of the inflorescence branch and application of phytohormones such as cytokinin and gibberellin can be used to inhibit flower-bud blasting, whereas the provision of excess nitrogen can promote such blasting, thereby affecting assimilate partitioning (Ushio and Fukuta, 2010; Kawakatsu et al., 2012). Flower opening in *Eustoma* is accompanied by both cell division and cell expansion, and accumulation of glucose and sucrose in vacuoles has been suggested to be involved in the latter (Norikoshi et al., 2016a, b). Cell wall-related proteins such as expansins and xyloglucan endotransglycosylase/hydrolase (XTH) have also been implicated in cell wall loosening during flower opening (Ochiai et al., 2013a, b). Furthermore,
Microarray analyses have identified numerous genes that are differentially expressed during floral development in *Eustoma*, including those involved in carbohydrate metabolism (Kawabata et al., 2009, 2012). Sucrose treatment markedly extends the vase life of cut flowers and also increases anthocyanin concentrations in *Eustoma* (Shimizu and Ichimura, 2005). However, the involvement of sucrose-metabolizing enzymes or genes in vegetative or floral development in *Eustoma* has not yet been reported.

Invertase (INV: EC 3.2.1.26), one of the two known sucrolytic enzymes in plants, irreversibly catalyzes the hydrolysis of sucrose to glucose and fructose (Ruan et al., 2010). INV occurs as two types, acidic INV and neutral/alkaline INV, which differ with respect to their optimum requisite pH. Moreover, three isoforms, namely, cell-wall INV (CWIN), vacuolar INV (VIN), and cytoplasmic INV (CIN), are recognized depending on their cellular localization, each comprising a multi-gene family, the members of which are distinguished by their primary structures (Ji et al., 2005; Wan et al., 2018). Several studies on the physiological functions of INV using transgenic plants have revealed that CWIN and VIN are mainly responsible for the growth of sink organs, and that CWIN and CIN are involved in organ development, including flowering (Tang et al., 1999; Heyer et al., 2004; Zanor et al., 2009; Ruan et al., 2010). Sucrose synthase (SUS: EC 2.4.1.13) is another plant sucrolytic enzyme that could reversibly catalyze conversion of sucrose to UDP-glucose and fructose, and is also involved in sink development (Ruan, 2014).

The present study was conducted to obtain an overview of sucrose metabolism during vegetative and floral development in *Eustoma*. Investigation of carbohydrate content and activities of the three INV isoforms indicated the occurrence of differential regulation of sucrose catabolism among organs, and the successive induction of these INV isoforms during flower opening. Moreover, we identified four putative INV genes, three of which were found to be associated with changes in INV activities in different organs and during flower opening.

**Materials and Methods**

**Plant materials and growth conditions**

Seeds of *Eustoma grandiflorum* (Raf.) Shinn. ‘Piccorosa Snow’ (Sakata Seed, Yokohama, Japan) were sown in a 200-hole cell tray filled with culture soil comprising Nippi-engei baido (Nihon Hiryo, Tokyo, Japan) and vermiculite (7:3). The seeds thus sown were maintained in the dark at 10°C for four weeks, and subsequently grown on a culture shelf at 23°C under a 16-h photoperiod, with daytime illumination at a photosynthesis photon flux density (PPFD) of 70 μmol·m⁻²·s⁻¹ being provided by fluorescent lamps. After two months of growth, seedlings with three leaf pairs were transplanted to culture soil in 9-cm plastic pots, and grown in a growth chamber (LH-120RD; Nihonikakikai, Osaka, Japan) at 25°C under a 16 h photoperiod, and irradiated at 70 μmol·m⁻²·s⁻¹ PPFD with fluorescent lamps during the day. The seedlings were provided with solubilized fertilizer (OK-F-1; OAT agrio, Tokyo, Japan) once a week. Roots, expanded leaves (the fourth leaf pair), and unexpanded leaves (the sixth leaf pair) were collected one month after planting, and small flower buds with 10 mm-long petals (stage 1), elongated flower buds with 30 mm-long petals (stage 2), and opened flowers (stage 3) were collected at three months after planting (Fig. 1). Collected plant samples were dried in a convection oven (DY300; Yamato Scientific, Tokyo, Japan) at 80°C for three days, or were frozen with liquid nitrogen, and stored at −80°C until used for RNA or protein extraction.

**Extraction and measurement of carbohydrates**

Soluble sugars and starch were extracted from 200 mg of dried samples, and the contents of reducing sugars, sucrose, and starch were determined using the methods described by Harada and Ishizawa (2003).

**Protein extraction and enzyme assay**

Frozen samples were ground with a mortar and pestle, and the resulting powder (200 mg) was suspended in 1 mL of extraction buffer, containing 100 mM HEPES/KOH (pH 7.2), 5 mM MgCl₂, 1 mM EDTA, and 5 mM dithiothreitol. After centrifugation of the suspension at 15,000 × g for 10 min at 4°C, the supernatant was collected and stored. The pellet was washed with 0.2 mL of the extraction buffer and the supernatant obtained after further centrifugation was combined with the initially collected supernatant to obtain the soluble fraction. The final pellet was resuspended in 0.5 mL of extraction buffer to give the pellet fraction. The soluble fraction was desalted using a Sephadex PD MidiTrap G-25 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK), according to the manufacturer’s instructions.

INV activity was measured based on the methods described by Harada and Ishizawa (2003) and Tomlinson et al. (2004) with slight modifications. In brief, 100 μL
of the pellet fraction (for CWIN) or desalted soluble fraction (for VIN and CIN) were incubated in 600 μL of a reaction mixture containing 50 mM acetate buffer at pH 4.5 (for CWIN and VIN) or 50 mM HEPES/KOH at pH 7.2 (for CIN), and 100 mM sucrose at 37°C for 1 h. Following the addition of 100 μL of 1 M Tris/HCl (pH 8.0), the assay mixture was heated at 95°C for 5 min to stop the reaction. The increase in reducing sugars was determined spectrophotometrically in comparison with the respective zero-time references.

Database search and sequence analysis of INV genes

A homology search was performed on the NCBI website using the BLASTX program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Three INV genes from Arabidopsis thaliana [AtCWIN1 (At3g13790), AtVIN2 (At1g12240), and AtCIN7 (At1g35580)] were selected as query sequences, and the search was performed against nucleotides, expressed sequence tags (ESTs), and transcriptome shotgun assembly (TSAs) from Eustoma. Multiple alignments were performed with ClustalW using BioEdit software. Phylogenetic trees were constructed based on the neighbor-joining method using MEGA7.0 software.

Cloning and expression analysis of INV genes

Frozen plant samples were ground with a mortar and pestle, and total RNA was extracted from 100 μg of the resulting powder using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), including DNase treatment, according to the manufacturer’s instructions. cDNA was then synthesized from 2 μg of the isolated RNA using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan), with an oligo dT-adaptor primer. RT-PCR was performed to amplify cDNA fragments of the predicted INV genes using LA Taq HS (Takara Bio, Shiga, Japan) or KOD plus DNA polymerase (Toyobo), with gene-specific primers designed from known nucleotide sequences and an adaptor sequence for 3’ rapid amplification of cDNA ends (Table S1; Fig. S1). The amplified cDNA fragments were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and sequenced using a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences obtained have been deposited in the DDBJ/EMBL/Genbank database under accession nos. LC585262–LC585265.

For real-time RT-PCR, cDNA fragments of target genes were amplified from each template cDNA sample using the Fast SYBR Green reagent (Applied Biosystems), with gene-specific primers designed from the nucleotide sequence, including the 3’-untranslated region, of each gene (Table S1; Fig. S1), in a StepOne Plus Real-Time PCR System (Applied Biosystems). The PCR conditions were 95°C for 20 s, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. Plasmids obtained in advance were used as templates for the quantification standard. EgACT was used as the internal standard gene, as described by Nakano et al. (2011).

Results and Discussion

Carbohydrate content and INV activities during vegetative development

Eustoma seedlings transplanted at the three-leaf pair stage had produced a further three leaf pairs at one month after transplanting, of which the fourth and fifth leaf pairs were fully expanded, whereas the sixth leaf pair had yet to expand (Fig. 1A). Considering the predicted source or sink functionalities of each organ, carbohydrate content and INV activity were compared among roots, expanded leaves (the fourth leaf pair), and unexpanded leaves (the sixth leaf pair). The reducing sugar content in unexpanded leaves was found to be three times higher than that in roots, whereas there was no significant difference in contents between the expanded and unexpanded leaves (Table 1). The sucrose content in expanded leaves was twice as high as that in unexpanded leaves, while starch content did not differ significantly among the three assessed organs (Table 1). With regard to INV activity, we found that CWIN activity in roots was significantly higher than that in expanded leaves (Fig. 2A), whereas VIN activity was significantly higher in unexpanded leaves (Fig. 2B). CIN activity was significantly higher in both roots and unexpanded leaves than that in expanded leaves (Fig. 2C).

The aforementioned results are broadly consistent with the predicted source/sink functionalities of the respective organs (Ruan, 2014), with expanded leaves being characterized by sucrose accumulation and relatively lower INV activities, implying the source function, and roots and unexpanded leaves being characterized by relatively lower sucrose levels and higher INV activities, indicating a sink function. Furthermore, we could identify the different types and

| Organ or stage | Reducing sugar | Sucrose | Starch |
|---------------|---------------|---------|--------|
| Vegetative organ<sup>a</sup> |              |         |        |
| Root          | 35.0±7.5 a    | 40.2±9.9 ab | 52.4±19.3 a |
| Expanded leaf | 79.0±9.8 ab   | 81.0±3.4 b  | 30.6±3.3 a |
| Unexpanded leaf | 118.7±20.7 b | 33.9±12.8 a | 37.1±6.2 a |
| Flower bud    |               |         |        |
| Stage 1       | 113.3±22.3 a  | 63.5±16.6 a | 30.1±3.5 a |
| Stage 2       | 121.6±7.7 a   | 130.0±11.9 a | 61.0±4.9 b |
| Stage 3       | 129.3±37.5 a  | 87.1±24.9 a | 40.9±3.4 a |

<sup>a</sup> Sampled at one month after transplanting.
<sup>b</sup> Data are expressed as the mean ± SE of three (vegetative organs) and three or four (flower buds) separate samples. Significant differences (P < 0.05) detected among vegetative organs or flower bud stages using Tukey’s multiple comparison test are indicated by different letters.

| Carbohydrate content in Eustoma vegetative organs and flower buds. | Carbohydrate content (mg·g−1 DW) |
|-------------------------------------------------------------|---------------------------------|
| Vegetative organ<sup>a</sup> |                               |
| Root              | 35.0±7.5 a                   | 40.2±9.9 ab   | 52.4±19.3 a |
| Expanded leaf     | 79.0±9.8 ab                  | 81.0±3.4 b    | 30.6±3.3 a |
| Unexpanded leaf   | 118.7±20.7 b                 | 33.9±12.8 a   | 37.1±6.2 a |
| Flower bud        |                                |               |
| Stage 1           | 113.3±22.3 a                 | 63.5±16.6 a   | 30.1±3.5 a |
| Stage 2           | 121.6±7.7 a                  | 130.0±11.9 a  | 61.0±4.9 b |
| Stage 3           | 129.3±37.5 a                 | 87.1±24.9 a   | 40.9±3.4 a |

<sup>a</sup> Sampled at one month after transplanting.
<sup>b</sup> Data are expressed as the mean ± SE of three (vegetative organs) and three or four (flower buds) separate samples. Significant differences (P < 0.05) detected among vegetative organs or flower bud stages using Tukey’s multiple comparison test are indicated by different letters.
roles of accumulated carbohydrate in roots and unexpanded leaves; that is, whereas roots tended to accumulate starch following the unloading of photosynthates translocated through the phloem by CWIN, unexpanded leaves accumulated reducing sugars via the mediation of VIN, and supported the cell expansion associated with leaf development. In the later stages of plant development, CWIN may also be involved in the transition from vegetative to reproductive growth, as has been demonstrated in Arabidopsis (Heyer et al., 2004). CIN could contribute to providing more substrates for respiration in developing sink organs. Considering that a substantial amount of bornesitol is detected in Eustoma leaves as a soluble sugar (Norikoshi et al., 2016a), there may be additional regulatory mechanisms for carbohydrate storage or compartmentalization. On the basis of these observations, we speculate that all three INV isoforms play key roles in determining the sink capacity of vegetative organs in Eustoma, although the integrated control of carbohydrate metabolism by both developmental and environmental factors remains to be elucidated.

Carbohydrate content and INV activities during floral development

The Eustoma plants developed flower buds at three months after transplanting. The four floral organs, sepals, petals, stamens, and gynoecium, developed normally, and flowers opened following the elongation, expansion, and pigmentation of petals (Fig. 1B). We identified three distinct flower developmental stages: small flower buds with 10-mm-long petals (stage 1), elongated flower buds with 30-mm-long petals (stage 2), and opened flowers (stage 3), and we accordingly compared carbohydrate contents and INV activities among these stages. We found that the contents of neither reducing sugars nor sucrose changed significantly during flower development (Table 1). In contrast, the increase in starch content at stage 2 was twice that detected at stage 1, but thereafter decreased (Table 1). Furthermore, at stage 2, CWIN activity was about eight times higher than that at stage 1 (Fig. 3A), whereas VIN activity was found to have undergone a marked increase at stage 3 compared with the two earlier stages (Fig. 3B). Conversely, CIN activity decreased gradually during flower development and reached a minimum level at stage 3 (Fig. 3C).

The results obtained indicate alterations in the sink functionality of flowers during their development. The accumulation of starch accompanied by little or no change in the contents of reducing sugars and sucrose, along with induced CWIN activity in elongated flower buds, are indicative of substantial import of carbohydrates via accelerated phloem unloading (Ruan, 2014).

![Fig. 2. Invertase (INV) activities in Eustoma vegetative organs. Activities of cell-wall INV (CWIN, A), vacuolar INV (VIN, B), and cytoplasmic INV (CIN, C) were compared among roots (R), expanded leaves (EL), and unexpanded leaves (UL) one month after transplanting. Data are expressed as the mean ± SE of three separate samples. Significant differences (P < 0.05) detected using Tukey’s multiple comparison test are indicated by different letters above the bars.](image)

![Fig. 3. Invertase (INV) activities in Eustoma flower buds. Activities of cell-wall INV (CWIN, A), vacuolar INV (VIN, B), and cytoplasmic INV (CIN, C) were compared among flower buds at stages 1 (S1), 2 (S2), and 3 (S3). Data are expressed as the mean ± SE of three separate samples. Significant differences (P < 0.05) detected using Tukey’s multiple comparison test are indicated by different letters above the bars.](image)
The subsequent consumption of starch concomitant with a reduction in CWIN activity reflects the predominant usage of carbohydrate that promotes floral organ development, including the pronounced enlargement of petals associated with flower opening (Norikoshi et al., 2016b). This is consistent with observations made for several other ornamental plants, thereby highlighting the importance of starch synthesis and degradation in flower opening (van Doorn and van Meeteren, 2003; Horibe and Yamada, 2017), and an increase in CWIN activity during the intermediate stage of rose petal opening (Yamada et al., 2007). In the present study, however, we used whole flower buds for carbohydrate analyses, which may have contributed to masking the increase in soluble carbohydrates observed in petals during flower opening in some ornamentals, including Eustoma (Paulin and Jamain, 1982; Ichimura et al., 1998; Yamada et al., 2009; van Doorn and Kamdee, 2014; Norikoshi et al., 2016a). The observed induction of VIN activity and a decline in CIN activity in open flowers are consistent with an increase in VIN activity in petals at the later stages of flower opening observed in carnation and rose (Woodson and Wang, 1987; Yamada et al., 2007), or glucose accumulation in vacuoles (Yamada et al., 2009; Norikoshi et al., 2016a). Taken together, the opening of Eustoma flower buds reflects the modulation of carbohydrate metabolism with altered starch levels along with successive induction of INV isoforms. Further studies comparing carbohydrate contents and localization with the activities of the three INV isoforms among floral organs may reveal the significance of sucrose catabolism mediated by the successive induction of INV isoforms during floral development. Meanwhile, SUS could also play an important role in Eustoma flower development as it is known to be responsible for starch synthesis in other plants (Ruan, 2014).

Identification of INV genes in Eustoma and their expression during vegetative and floral development

Although to date there have been no reports regarding the nucleotide sequences encoding INV in Eustoma, we found that among tens of thousands of transcriptome shotgun assembly (TSA) accessions, largely attributed to Kawabata et al. (2012), there are several sequences showing homology to known INV genes from other plants. On the basis of our homology search using Arabidopsis genes encoding CWIN, VIN, and CIN, we selected five sequences with the highest score or longest nucleotide length for cDNA cloning (Fig. S1). Two similar coding sequences (CDSs) putatively encoding CWIN, comprising 1,731 and 1,740 bp, were derived from JT560374 and JT559138, respectively, with additional sequences determined by 3’ rapid amplification of cDNA ends (RACE). One CDS with a length of 1,752 bp, putatively encoding VIN, was derived from two different accessions, JT570125 and JT565816. Multiple alignment and phylogenetic analysis of CWIN and VIN proteins from Arabidopsis and rice revealed that each deduced amino acid sequence could be structurally classified as a predicted INV isoform, and the three identified genes were named EgCWIN1, EgCWIN2, and EgVIN1, respectively (Figs. 4 and S2). Furthermore, one partial CDS with a length of 990 bp, putatively encoding CIN, was derived from JT586730, with additional sequences determined by 3’ RACE, thus the observed increase in soluble carbohydrates observed in petals at the later stages of flower opening observed in carnation and rose (Yamada et al., 2009). In the present study, however, we used whole flower buds for carbohydrate analyses, which may have contributed to masking the increase in sucrose catabolism mediated by the successive induction of INV isoforms during floral development. Meanwhile, SUS could also play an important role in Eustoma flower development as it is known to be responsible for starch synthesis in other plants (Ruan, 2014).
forms encoded by different genes (Wan et al., 2018), we suspect that expression of other INV genes that were not identified in the present study may have contributed to the observed INV activities in these organs. It is also possible that other regulatory mechanisms are operating, including inhibition of INV activities by INV inhibitor proteins (Ruan, 2014). INV inhibitor proteins have been reported to inactivate CWIN and VIN, leading to alteration of cellular and developmental status, and multiple members with different organ-specificity of gene expression have also been reported for these inhibitors (Ruan, 2014; Wan et al., 2018). In Eustoma, selectively-induced INV inhibitor proteins may inactivate CWIN and VIN in leaves and roots, respectively. Further studies are necessary to identify key INV genes that are functional during vegetative development in Eustoma, which could then serve as potential target for the control of carbon partitioning among vegetative organs.

We also analyzed INV gene expression in flower buds at different stages, and found that transcript levels of EgCWIN1 were markedly increased at stage 2, as was CWIN activity, and this higher level of expression was maintained thereafter (Fig. 6A). In contrast, we detected no significant change in EgCWIN2 transcript levels, although these did tend to increase during flower development (Fig. 6B). Transcript levels of EgVIN1 gradually increased during flower development concomitant with a change in VIN activity (Fig. 6C), whereas there was a gradual decline in the level of EgCIN1 transcripts (Fig. 6D). Collectively, these results indicate that INV genes were differentially expressed, and that EgCWIN1, EgVIN1, and EgCIN1 play prominent roles in the changes in INV activities observed during flower development (Fig. 3). The transcriptional regulation of INV genes by environmental factors such as drought and light has been reported in other plants (Boyer and McLaughlin, 2007; Ruan et al., 2010; Rabot et al., 2012), and it has also been established that endogenous factors, such as soluble carbohydrates, function as signals that modulate INV gene expression (Rabot et al., 2012; Ruan, 2014). Identification of the key regulatory factors associated with the successive induction of sets of INV genes during Eustoma flower development will contribute to elucidating the general mechanisms underlying flower opening.

Concluding remarks

The findings of the present study provide basic information on carbohydrate catabolism associated with invertase enzyme activities during vegetative and floral development in Eustoma. Functional analysis of invertase genes will enable us to gain further insights into the role of carbon partitioning and utilization during plant development. In this regard, we confirmed that the Agrobacterium-mediated transformation technique reported by Nakano (2017) is applicable for the cultivar used in the present study (data not shown). In addition,
investigating the contribution of sucrose synthase, another sucrolytic enzyme in plants, for which two genes were identified in this study, would enable us to obtain a more complete picture of sucrose catabolism in *Eustoma grandiflorum*.

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