

Dynamic Changes in Starch Metabolism and Endogenous Hormones during Stolon Formation in *Tulipa edulis*

Yuanyuan Miao, Qiaosheng Guo, Zaibiao Zhu¹, Xiaohua Yang, Changlin Wang, Yuan Sun, and Li Liu

Institute of Chinese Medicinal Materials, Nanjing Agricultural University, Nanjing 210095, People's Republic of China

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ABSTRACT. The stolon is the main asexual reproductive organ of the medicinal plant *Tulipa edulis* and has special morphology. In the greenhouse experiment presented herein, the dynamic changes in carbohydrates and related enzymes, proteins, and endogenous hormones of stolons during *T. edulis* stolon formation were investigated. The results showed that the content of total soluble sugar, sucrose, reducing sugar, fructose, and starch were all significantly enhanced in the middle period when stolon emerged and maintained at relatively high levels until the later period of stolon formation, while protein content decreased during stolon formation. The activities of amylase (AMY), sucrose phosphate synthase (SPS), and sucrose synthase (SS) peaked in the initial period and were negatively correlated with soluble sugars. However, adenosine diphosphoglucose pyrophosphorylase (AGPase) activity increased as stolon formation progressed, and the changes in soluble starch synthase (SSS), granule-bound starch synthase (GBSS) activities presented a single peak, reaching their maximums in the middle period. AGPase, SSS, and GBSS activities were all positively related to starch content. Moreover, quantitative real-time polymerase chain reaction (qRT-PCR) verified the changes in SS and SSS activities via the expression levels of the *SS*, *SSSI*, and *SSSII* genes. The gibberellin (GA) and zeatin riboside (ZR) content attained their maximum in the initial period of stolon formation. Indole-3-acetic acid (IAA) and abscisic acid (ABA) remained at high levels during the initial and middle period and decreased significantly during the later period of stolon formation, inversely to the ratio of ABA:IAA. Analysis of the physiological changes in *T. edulis* stolon indicated that the accumulation of soluble sugars and starch via various enzymes, a high level of IAA and a low ABA to IAA ratio mainly contributed to stolon development of *T. edulis*. This paper explored carbohydrate levels and endogenous hormones profiles during stolon formation, which provided the theory basis for further regulating stolon growth of *T. edulis*.

Tulipa edulis is a perennial herb of the genus *Tulipa* within the Liliaceae and is commonly distributed in the eastern provinces and regions of China, particularly growing on sunny hills, under sparse forest, or along riverbanks. It is also distributed in Japan and Korea. The dried bulb of *T. edulis* without tomentose tunic, *Guangcigu*, is currently an important medicine for the treatment of a variety of cancers, such as throat cancer, lymphoma, and breast cancer (Chinese Herbalism Editorial Board, 1999). Because of its medicinal value and industrial importance, the demand for *Guangcigu* has increased steadily in recent years, and the wild sources of *T. edulis* are, therefore, being reduced to a greater extent due to overexploitation (Bing et al., 2008; Miao et al., 2015).

The stolon is one of the main asexual reproductive organs of *T. edulis* and has unique morphology. It is similar to a rhizome in appearance, but it has no visible node, internode, or adventitious roots (ARs). It bulges outward and extends farther, to more than 10 cm at the bottom of the stem. The stolon is pushed deep into the soil or culture medium and develops a bulblet at the tip (Fig. 1A and B). Finally, the stolon shrivels up, and the bulblet, surrounded by a thin brown tunic, is left (Custers et al., 1992; Miao et al., 2015). The stolon plus the bulblet is called the dropper (Custers et al., 1992). The stolon

has been described in micropropagation culture in some *Tulipa* sp. plants as a transitional stage of bulblet formation (Custers et al., 1992; Koster, 1993). Nevertheless, few literature reports referred to the similar organ under natural conditions. Stolon formation in tulip (*Tulipa gesneriana*) has been observed in seedlings and small bulbs as well as in large bulbs, which grow in wet soil (Le Nard and De Hertogh, 1993). The number of stolons markedly influences the reproductive index. In addition to its role as a reproductive organ, the stolon of *T. edulis* is assumed to play an important role in its adaptation to diverse conditions. Therefore, it is necessary to explore the mechanisms involved in the formation of the stolon in *T. edulis* so that production can be improved to meet the high market demands.

Stolon formation is a process of organogenesis, which is defined as growth with an irreversible increase in size involving changes in morphology or anatomy at the intracellular or tissue level (Sinnott, 1960). Organogenesis, such as the initiation of an AR, lateral root (LR), stolon, or rhizome, should be considered to be a complex multistep process characterized by both cell division and cell enlargement, which are affected by endogenous hormones and nutritional factors with carbohydrates playing a central role (Mcintyre, 2001; Xu et al., 1998a, 1998b).

Studies of the influence of nutrition on organogenesis have shown that the relative amounts of carbohydrates can determine the path of bud development into a shoot or rhizome (Mcintyre, 2001). It was observed that once carbohydrate reserves were partly replenished and abundant, the growth of new rhizomes

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¹Corresponding author. E-mail: zhuzaibiao@njau.edu.cn.

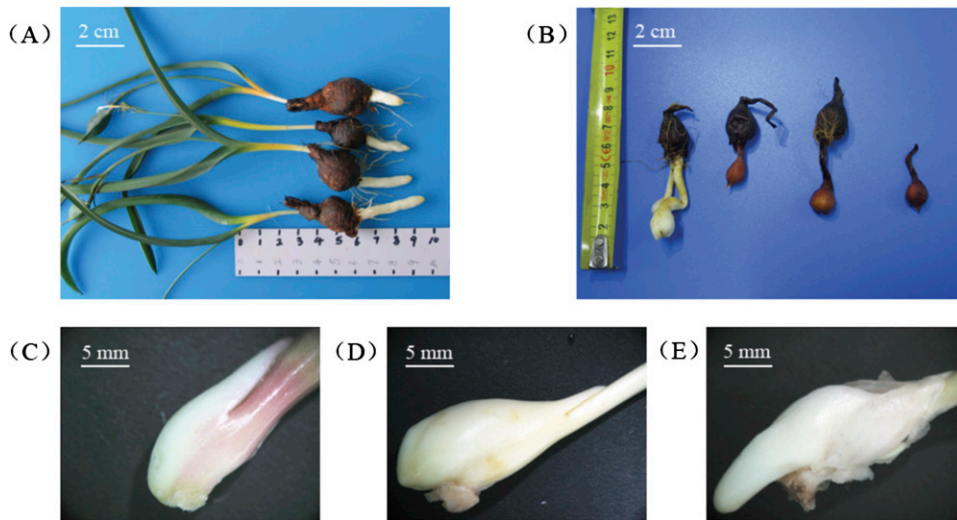


Fig. 1. (A) *Tulipa edulis* with elongated stolon; (B) *T. edulis* stolon developed into a new bulb; and (C, D, and E) *T. edulis* stolon formation at the initial, middle, and later stages.

was initiated (Gauci et al., 2009). The allocation and fluxes of carbohydrates in the cutting apparently play an important role in AR formation, as suggested by the fact that earlier stages of AR formation in plants are limited by carbohydrate availability, especially that of sucrose in the leaves (Klopotek et al., 2010; Rapaka et al., 2005). In addition, sugar is also likely to be an important determinant of LR formation and the tuberization process (Macgregor et al., 2008; Mitprasat et al., 2011). Fernie and Willmitzer (2001) have testified that soluble carbohydrates, especially sucrose, have been considered to be strong inducers of the formation of underground storage organs. Enhancing the level of sucrose in the stolon increases the number of initiated tubers (Jackson, 1999) and is helpful in the rhizome formation of *Nelumbo nucifera* (Cheng et al., 2013a) and the corm formation of *Sagittaria trifolia* (Cheng et al., 2013b). Similarly, Takahashi et al. (2003) previously showed that AR formation is stimulated by sucrose. Sucrose is translocated to the stem base to be cleaved into hexoses, which are used directly as a carbon source or converted into starch for storage (Akhkami et al., 2009). Moreover, sucrose can be taken up and metabolized, increasing LR formation emergence via a specific mechanism (Macgregor et al., 2008). In potato (*Solanum tuberosum*) tubers, a small fraction of starch is converted into the reducing sugars, glucose and fructose, to provide carbon skeletons and energy (Schreiber et al., 2014). Nevertheless, it is not too surprising that for a long time, starch formation was another parameter discussed as a requirement for tuber initiation and enlargement (Fernie and Willmitzer, 2001). The key enzyme involved in starch degradation is AMY, which decomposes starch into soluble sugars to maintain proper growth in plants (Mishra and Dubey, 2013). Among sucrose-metabolizing enzymes, SPS catalyzes the biosynthesis of sucrose and is regarded as a major control point in sucrose biosynthesis and its partitioning, utilization, mobilization, and cellular function in plants (Huber and Huber, 1996), whereas SS and invertase are involved in the cleavage of sucrose (Verma et al., 2012). Moreover, in starch-storing sinks, incoming sucrose is predominantly degraded via the SS pathway (Sung et al., 1989). Baroja-Fernández et al. (2009) showed that the enhancement of SS activity represents a useful strategy for increasing starch accumulation and yield in

potato tubers. The synthesis of starch requires the main participation of various enzymes including AGPase, SSS, and GBSS (Geigenberger, 2003; Mukerjea et al., 2010). Both adenosine diphosphate glucose (ADPG) synthesizing SS and AGPase activity patterns were found to correlate well with the amount of ADPG and starch content in plants (Baroja-Fernández et al., 2003). Cheng et al. (2013a, 2013b) discovered that a gene encoding GBSS enhanced messenger RNA (mRNA) levels during rhizome and corm development in starch synthesis.

The plant hormones auxin and cytokinin are central endogenous signaling molecules that positively and negatively regulate AR organogenesis, respectively (Agulló-Antón et al., 2014; Della Rovere et al., 2013), as observed in LR formation (Bielach et al., 2012; Ivanchenko et al., 2008). In addition, ABA is consistently thought to inhibit AR and LR induction (da Costa et al., 2013; Guo et al., 2012). In the rhizome and stolon, phytohormones such as IAA and GA are involved in the rhizome development signaling pathway, and auxin/IAA appears to act as a negative regulator of rhizome development, whereas GA acts as the activator of rhizome development (Hu et al., 2011). Xu et al. (1998b) observed that a high GA content promotes stolon elongation and inhibits storage organ (tuber) formation. In terms of bulbing ability in bulbous plants, Rice et al. (1983) enhanced tulip bulb production in vitro by soaking adventitious shoots developed into a bulb in GA₃. In contrast, treatments with GA₃ in explant shoots strongly inhibited the bulb formation of tulips in a study reported by Podwyszyńska and Ross (2003), and the application of inhibitors of GA biosynthesis, such as paclobutrazol, had a positive effect on bulb formation in initial explants of some bulbous plants including *Leucojum aestivum* (Ptak, 2014) and tulip (Podwyszyńska, 2006). ABA was found to stimulate the formation of bulb in *Hyacinthus orientalis* (Li et al., 2002). However, Podwyszyńska and Ross (2003) claimed that ABA has an adverse effect on tulip bulb formation, which was confirmed by demonstration that the formation of bulblets was hampered by ABA, particularly at higher concentrations, in the tarda tulip [*Tulipa tarda* (Maślanka and Bach, 2014)]. Numerous studies have reported that auxin exerts stimulatory effects on in vitro bulb formation. Podwyszyńska (2006) found that auxins promoted the efficiency of tulip bulb formation, and bulbing in lily (*Lilium*) depended on the presence of exogenous auxins, mainly naphthalene acetic acid [NAA (Van Aartrijk and Blom-Barnhoorn, 1981)]. There are conflicting indications regarding the roles of cytokinins in bulb formation in vitro. Exogenous benzyladenine stimulated bulb development in *Urginea maritima* (El Grari and Backhaus, 1987) but inhibited bulb formation in *Narcissus jonquilla* (Chow et al., 1992). The use of media with 6-benzyl-aminopurine promoted bulbing capacity together with NAA in bulb scale cultures of *Lilium ledebourii* (Bakhshaie et al., 2010) and *Muscari azureum* (Uranbey et al., 2010).

To the best of our knowledge, the development characteristics and formation process of the *T. edulis* stolon remain unknown. It would be highly significant to investigate the formation mechanism of the stolon for efficient cultivation. Thus, the aims of this study were 1) to investigate changes in carbohydrate content and related enzyme activities as well as protein and endogenous hormone content during stolon formation in *T. edulis* and 2) to clarify the relationships among nutrition, related enzymes, and endogenous hormones in response to stolon formation and to further elucidate the possible mechanism of stolon formation. For these purposes, nutrient (i.e., total soluble sugar, sucrose, reducing sugar, fructose, starch, and protein) content, the activities of related enzymes (i.e., SS, SPS, AMY, AGPase, SSS, and GBSS), and the content of endogenous hormones (viz. GA, IAA, ABA, and ZR) were measured at the location where the stolon of *T. edulis* formed.

Materials and Methods

PLANT MATERIAL AND GROWTH CONDITIONS. The experiment was performed under natural day/night conditions in the greenhouse of the Institute of Chinese Medicinal Materials at Nanjing Agricultural University, Nanjing, People's Republic of China. *Tulipa edulis* bulbs were obtained from Funan county, Anhui Province in Sept. 2013. Disease-free bulbs of uniform size (≈ 1.0 g) were selected for planting in polyethylene plastic pots (42 cm length, 42 cm width, and 5.5 cm depth) filled with taupe sandy loam. The gravimetric water content of taupe sandy loam at field capacity (FC) was 26.56%. The water content of the taupe sandy loam was adjusted gravimetrically every other day to 80% FC. The pots were arranged following a completely randomized block design. On the basis of the previous research, the process of stolon formation can be divided into the following three stages: 1) T1, the initial period of stolon formation, 41 d after planting (DAP; Fig. 1C); 2) T2, the middle period of stolon formation, 118 DAP (Fig. 1D); and 3) T3, the later period of stolon formation, 133 DAP (Fig. 1E). Fresh samples of stolons from these three stages were collected at the appropriate time and immediately frozen in liquid nitrogen after their fresh weight had been determined and then stored at -80 °C until analysis.

DETERMINATION OF CARBOHYDRATE CONTENT. Carbohydrate content was determined colorimetrically, essentially as described earlier (Mishra and Dubey, 2013; Xia et al., 2005). Briefly, ≈ 0.5 g frozen samples were homogenized with 4 mL 80% ethanol and extracted at 80 °C for 30 min, then centrifuged at 8000 g_n for 20 min. Samples were extracted thrice, and the supernatants were decolorized by activated carbon at 80 °C for 30 min and then diluted to 25 mL with water. After filtration, extracts were used for the measurement of soluble sugars including sucrose, reducing sugar, and fructose. For starch extraction, the precipitates left in the centrifuge tubes after sugar extraction were added 2 mL of water and then placed in boiling water bath for 15 min. After cooling, 2 mL of 9.2 M perchloric acid was added, and contents were stirred for 15 min and volumes were made up to 10 mL with water. Supernatants were collected after centrifuging the contents at 5000 g_n for 15 min. Residues were reextracted with 2 mL of 4.6 M perchloric acid. After centrifugation, all perchloric acid-containing supernatants were pooled and volumes were made up to 100 mL with water for starch content measurement. Total soluble sugar and starch concentrations were measured following the method of the anthrone reaction, and the

absorbance was recorded at 630 nm. The content of reducing sugar was determined using 3,5-dinitrosalicylic acid with the absorbance at 540 nm. The sucrose and fructose contents were determined using hydrolysis and resorcinol reactions, and the absorbance was measured at 480 nm.

ASSAY TO DETERMINE PROTEIN CONTENT AND AMY ACTIVITY. Samples of ≈ 0.25 g in weight were ground and extracted with 8 mL distilled water at 4 °C to separate a soluble fraction. The supernatant was taken after centrifugation at 4 °C and 8000 g_n for 15 min and used for the measurement of soluble protein content and AMY activity. The precipitates were extracted with 3 mL of 1 M NaOH at 90 °C for 20 min, and then centrifuged to obtain the supernatant for the determination of the insoluble protein content. The soluble and insoluble protein contents were measured according to Bradford (1976), using the method of coomassie brilliant blue G-250 with bovine serum albumin as a standard. AMY activity was measured using a test kit (C016; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The assay depends on unhydrolyzed starch, which turns blue in the presence of iodine, with absorbance at 660 nm when an excess of substrate starch is added to the reaction, based on the theory that starch can be hydrolyzed by AMY. AMY activity was measured based on the amount of hydrolyzed starch as determined by the shades of blue. One unit of enzyme activity was defined as the quantity of AMY required to hydrolyze 10 mg starch, and the enzyme activity was expressed as units per gram fresh weight. The reaction was conducted at 37 °C for 15 min.

SUCROSE METABOLISM-RELATED ENZYMES ASSAY. Test kits (A097, A098; Nanjing Jiancheng Bioengineering Institute) were employed for the assays of SS and SPS activities. 0.1 g of frozen sample was ground and extracted with 0.9 mL extracting solution [100 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 2 mM ethylenediaminetetraacetic acid (EDTA), 20 mM dithiothreitol (DTT), and 0.1% Triton X-100] in ice bath condition and centrifuged at 4000 g_n for 15 min at 4 °C. The supernatant was used for SS and SPS activity assays with the instruction of test kits. The reaction mixture containing supernatant (30 μ L), buffer (150 μ L 50 mM Tris-HCl, pH 7.0), promoter (2 μ L 0.75 M MgCl₂), and substrate [2 μ L 0.225 M uridine diphosphate glucose (UDPG), 3 μ L 0.5 M fructose for SS; 2 μ L 0.225 M UDPG, 3 μ L 0.5 M fructose-6-phosphate for SPS] was incubated in 37 °C for 30 min, and hydrolysate (2 mL 0.05 M NaOH) was added to reaction system for mixing to place in the boiling water bath for 8 min. The absorbance was recorded at 290 nm when the mixture cooled to room temperature. The activity of SS or SPS was expressed in terms of micromoles of sucrose per gram fresh weight per minute.

MEASUREMENT OF THE KEY ENZYMES OF STARCH SYNTHESIS. The extraction and determination procedures for AGPase, SSS, and GBSS were according to Nakamura et al. (1989) with a slight modification. Briefly, 0.1 g sample was homogenized with a pestle in a precooled mortar that contained 1 mL precooled extraction medium: 100 mM hydroxyethyl piperazine ethanesulfonic acid (HEPES)-NaOH (pH 7.6), 8 mM MgCl₂, 5 mM DTT, 2 mM EDTA, 12.5% (v/v) glycerol, 5% (w/v) polyvinylpyrrolidone 40, and 50 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 g_n for 10 min at 4 °C, and the supernatant was used for AGPase and SSS assays. The precipitate was suspended in the above extraction medium for GBSS activity measurement.

AGPase activity was assayed as follows: 20 μ L of supernatant was combined with 260 μ L of a reaction mixture containing

100 mM HEPES-NaOH (pH 7.5), 1.2 mM ADPG, 3 mM pyrophosphoric acid (PPi), 5 mM MgCl₂, and 4 mM DTT, and the mixture was incubated at 30 °C for 20 min, then placed in a boiling water bath for 1 min and cooled to room temperature. The mixture was centrifuged at 10,000 g_n for 10 min, and 240 μL of supernatant was mixed with 100 μL 10 mM nicotinamide adenine dinucleotide phosphate (NADP). Then, 10 μL 0.4 unit phosphoglucumutase and 10 μL 0.4 unit glucose-6-phosphate dehydrogenase were added to the reaction solution and mixed, and the increase in absorbance at 340 nm in 2 min was recorded. One unit of AGPase was defined as the quantity of enzyme needed to produce 1 μmol dihydronicotinamide adenine dinucleotide phosphate (NADPH), and the enzyme activity was expressed as micromoles per gram fresh weight per minute.

The supernatant and suspension described above were used for the SSS and GBSS activity assays, respectively. The assay of SSS activity was consistent with GBSS, using the method of Nakamura et al. (1989) with a slight modification. Briefly, 150 μL sample solution was mixed with 270 μL of 50 mM HEPES-NaOH buffer (pH 7.4) including 1.6 mM ADPG, 1.8 mg amylopectin and 15 mM DDT, and then the mixture was incubated at 30 °C for 20 min and afterward placed in boiling water for 1 min. The mixture was cooled rapidly in an ice bath, and then 150 μL of 50 mM HEPES-NaOH buffer (pH 7.4) containing 5 mM phosphoenolpyruvate, 200 mM KCl, 10 mM MgCl₂, and 1.5 unit pyruvate kinase were added, and the mixture was incubated at 30 °C for 30 min. The reaction mixture was then placed in a boiling water bath for 1 min and centrifuged at 10,000 g_n for 5 min after cooling. Next, 450 μL of supernatant was mixed with 300 μL 50 mM HEPES-NaOH buffer (pH 7.4) including 10 mM glucose, 20 mM MgCl₂, and 2 mmol·L⁻¹ NADP. Then, 10 μL 2.8 unit hexokinase and 10 μL 0.7 unit glucose-6-phosphate dehydrogenase were successively added to the above mixture and vigorously shaken immediately, and the change in absorbance at 340 nm in 2 min was measured. One unit of enzyme activity was expressed as micromoles per gram fresh weight per minute.

DETERMINATION OF ENDOGENOUS HORMONES. The extraction, purification, and determination of endogenous levels of IAA, GA, ABA, and ZR by an indirect enzyme-linked immunosorbent assay (ELISA) technique were performed as described by He (1993). The samples were extracted with ice-cold 80% methanol (v/v) containing 1 mM butylated hydroxytoluene to prevent oxidation using a mortar and pestle and then stored overnight at 4 °C. The extracts were collected after centrifugation at 12,000 g_n for 15 min at 4 °C. The residues were suspended in the same ice-cold extraction solution and stored at 4 °C for 1 h, then centrifuged again at 12,000 g_n for 15 min at 4 °C. The two resulting supernatants were combined and passed through a C₁₈ Sep-Pak cartridge (Waters, Milford, MA). The effluent was collected and dried in N₂. The residues were then dissolved in 0.01 M phosphate buffer solution (pH 7.4) to determine the levels of IAA, GAs, ABA, and ZR. In brief, microtitration plates (Nunc, Roskilde, Denmark) were coated with synthetic IAA, GAs, ABA, or ZR-ovalbumin conjugates in 50 mM NaHCO₃ buffer solution (pH 9.6) and left overnight at 37 °C. Ovalbumin solution (10 mg·mL⁻¹) was added to each well of the plates to block nonspecific binding. After incubation for 30 min at 37 °C, standard IAA, GAs, ZR, ABA, samples and antibodies were added and incubated for a further 45 min at 37 °C. Antibodies against IAA, GAs, ABA, and ZR were produced according to the method described by Weiler

et al. (1981). Horseradish peroxidase-labeled goat antirabbit immunoglobulin was then added to each well and incubated for 1 h at 37 °C. After incubation, the buffered enzyme substrate solution (orthophenylenediamine) was added to each well of the plates. The plates were then kept in the dark at 37 °C for the substrate reaction. After 15 min, 3 M H₂SO₄ was added to each well to end the substrate reaction. The absorbance was measured at 490 nm using the ELISA Recorder (model DG-3022 A; Huadong Electron Tube Factory, Shanghai, China). Calculations for the ELISA data were performed according to the method described by Weiler et al. (1981). In this study, the percentage recovery of each hormone was calculated by adding known quantities of standard hormone to a split extract. All the percentage recoveries were above 90%, and all the sample extract dilution curves paralleled the standard curves, indicating the absence of nonspecific inhibitors in the extracts. The results are the means of six replicates.

GENE EXPRESSION ANALYSIS BY qRT-PCR. To assay the expression levels of mRNA of putative key genes associated with stolon formation in *T. edulis*, qRT-PCR was performed using ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with fluorescence dye SYBR Green (TaKaRa, Dalian, China). On the basis of the transcriptome sequencing and annotation, the putative unigenes involved in sucrose and starch metabolism were identified, and three essential genes were selected for verification: *SS*, *SSSI*, and *SSSII* (Z.B. Zhu, unpublished data). The gene-specific primers were designed by Primer Premier 5.0 (Premier Corp., Canada) and the primer sequences are listed in Table 1. The PCR conditions were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 58 °C for 30 s. Each reaction was repeated three times, and *β-Actin* was used as an internal control. The relative expression levels of the selected genes were calculated using the 2^{-ΔΔCt} method: 2^{-ΔΔCt} = 2^{-[(Ct gene of interest - Ct internal control) Sample A - (Ct gene of interest - Ct internal control) Sample B]} (Zheng et al., 2014).

STATISTICAL ANALYSIS. The data presented in the figures were analyzed via one-way analysis of variance using SPSS Statistics 20.0 (IBM Corp., Armonk, NY), and significant differences in each index among the different development stages were determined using Duncan's multiple range test at a 0.05 *P* level. SPSS Statistics 20.0 was used to determine correlation coefficients.

Results

CONTENT OF TOTAL SOLUBLE SUGAR, SUCROSE, REDUCING SUGAR, AND FRUCTOSE. We observed significant differences in total soluble sugar, sucrose, reducing sugar, and fructose content during the stolon formation of *T. edulis* (Fig. 2A).

Table 1. Genes verified by quantitative real-time polymerase chain reaction (qRT-PCR) and primers used for qRT-PCR in stolons during *Tulipa edulis* stolon formation.

Gene	Primer name	5'-3'
<i>SS</i>	c96901-F	TTGTAACCTGGATGACCGA
	c96901-R	TTTGCCAGTTCTCTCAATCG
<i>SSSI</i>	c980201-F	TCTCGCAAGCTCCAGTACAA
	c980201-R	TGGAATTGAGCTCTTTCTCG
<i>SSSII</i>	c980202-F	CCTCCTTCGAGTATGTGGGT
	c980202-R	CGCTCGAGTTATCTTCACCA
<i>β-Actin</i>	c92841-F	TGTATGTTGCCATTCAGGCT
	c92841-R	ATCACCAGAATCCAGCACAA

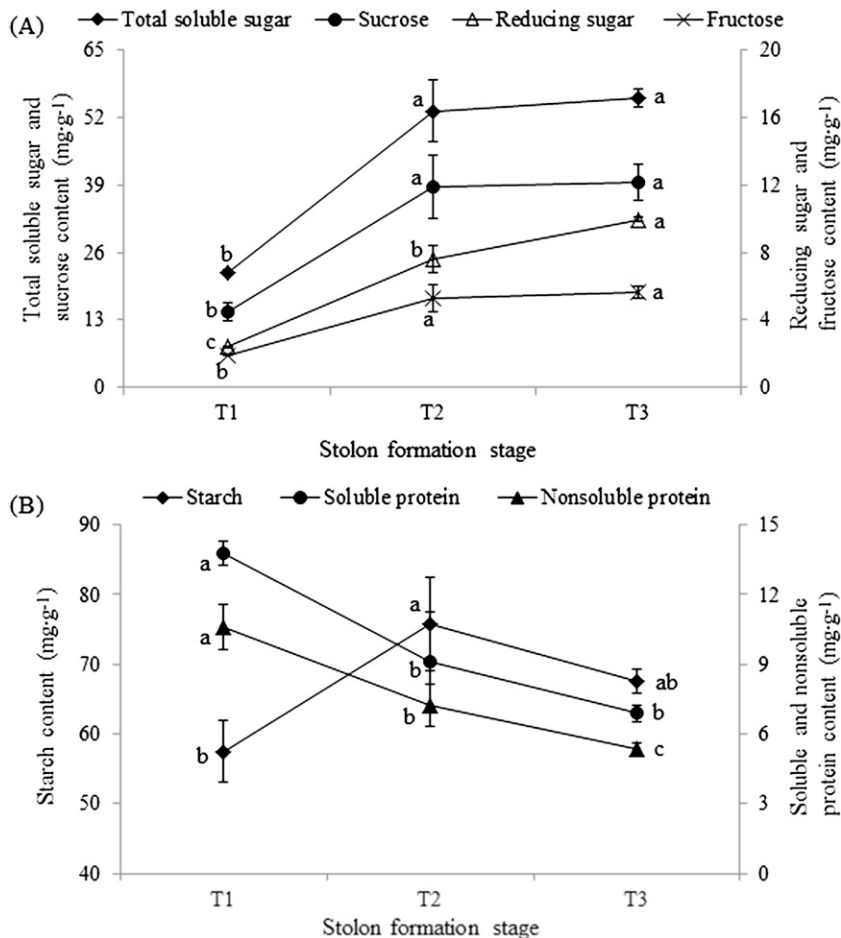


Fig. 2. Dynamic changes in (A) total soluble sugar, sucrose, reducing sugar, and fructose and (B) starch and protein content in stolons during *Tulipa edulis* stolon formation. T1, T2, and T3 indicate the initial, middle, and later periods of stolon formation, respectively. Different lowercase letters in each index indicate significant differences (Duncan's multiple range test at $P < 0.05$) among the three developmental stages. The error bars indicate SD of the three replicates.

The total soluble sugar, sucrose, and fructose content were all enhanced promptly in T2, showing increases of 139.14%, 165.20%, and 177.91% compared with T1, respectively, and then maintained a steady level until T3. The reducing sugar level increased continually throughout this period. The reducing sugar levels in T2 and T3 were 3.18- and 4.18-fold the value during T1, respectively.

CONTENT OF STARCH AND PROTEIN. The starch content was significantly higher in T2 than in T1 (Fig. 2B) and subsequently decreased in T3 by 10.84% in comparison with T2, which was not statistically significant. The soluble protein content decreased continuously during stolon formation. It was highest in T1 and lowest in T3, and it was as much as two times at its peak as at its nadir. There was a significant decline in the content of nonsoluble protein during stolon formation (Fig. 2B).

ACTIVITIES OF SS, SPS, AND AMY. The activities of SS and SPS decreased significantly from T1 to T2 and then increased slowly (Fig. 3A). They both reached the minimum values in T2, 8.89% and 31.45% lower than in T1, respectively. Moreover, SPS activity was always lower than SS. The activity of AMY showed a significant decrease during stolon formation, and the activity during T3 was 31.58% of the activity in T1.

ACTIVITIES OF AGPASE, SSS, AND GBSS.

The activity of AGPase increased gradually from T1 to T2, followed by a remarkable enhancement to a peak of $9.622 \mu\text{mol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ in T3 (Fig. 3B). SSS activity increased rapidly initially, peaking in T2, and the activity was 2.124 times the level in T1. Then, it significantly decreased, with a decline of 32.35% in T3 compared with T2. The trends of GBSS and SSS activity were similar. GBSS activity was significantly increased by 208.80% in T2 compared with T1, then decreased in T3 but remained significantly higher than in T1.

CORRELATION ANALYSIS OF CARBOHYDRATE CONTENT AND CARBOHYDRATE-RELATED ENZYME ACTIVITIES. There were significant positive correlations among total soluble sugar, sucrose, reducing sugar, and fructose content, and the positive correlations of starch, total soluble sugar, sucrose, and fructose content were also significant (Table 2). SPS activity was negatively correlated with total soluble sugar, sucrose, reducing sugar, and fructose content. A similar result was observed for the correlation between SS activity and soluble sugars, but the relationship was not significant. AMY activity had a significant negative correlation with total soluble sugar, sucrose, reducing sugar, and fructose content and was weakly negatively related to starch content and to AGPase, SSS, and GBSS activities. In addition, positive correlations were found between starch content and starch synthetic enzyme activities, and the correlation between starch content and GBSS activity was significant.

GA, ABA, IAA, AND ZR CONTENT AND THE RATIOS OF ABA TO IAA, GA, AND ZR.

Figure 4A shows that the content of GA descends throughout, occurring at a higher level in T1, decreasing significantly in T2, and then being somewhat reduced in T3 but remaining fairly flat compared with the T2 stage and at 67.52% of the T1 stage. A similar result was observed for ZR content. The IAA content increased and peaked in T2 but showed no significant difference between T1 and T2. There was a sharp decrease in T3, with only 47.79% of the T2 content. The ABA content in T2 decreased slightly but maintained about the same level as in T1, reaching the value of $91.720 \text{ ng}\cdot\text{g}^{-1}$, followed by a significant decrease during T3.

As shown in Figure 4B, the ABA:IAA ratio decreased steadily and achieved its lowest level during T2, and was then enhanced significantly in T3, showing an increase of 59.56% compared with the T2 period. No significant differences were found in the ratios of ABA:GA or ABA:ZR among the three stages during stolon formation.

EXPRESSION ANALYSIS OF SELECTED THREE GENES THROUGH qRT-PCR. From the qRT-PCR results (Fig. 5A), the gene expression level of SS was down-regulated during stolon formation, and the expression level of the SS gene was significantly higher in T1 than in T2 and T3. Conversely, the SSS gene showed a significantly higher transcriptional level in

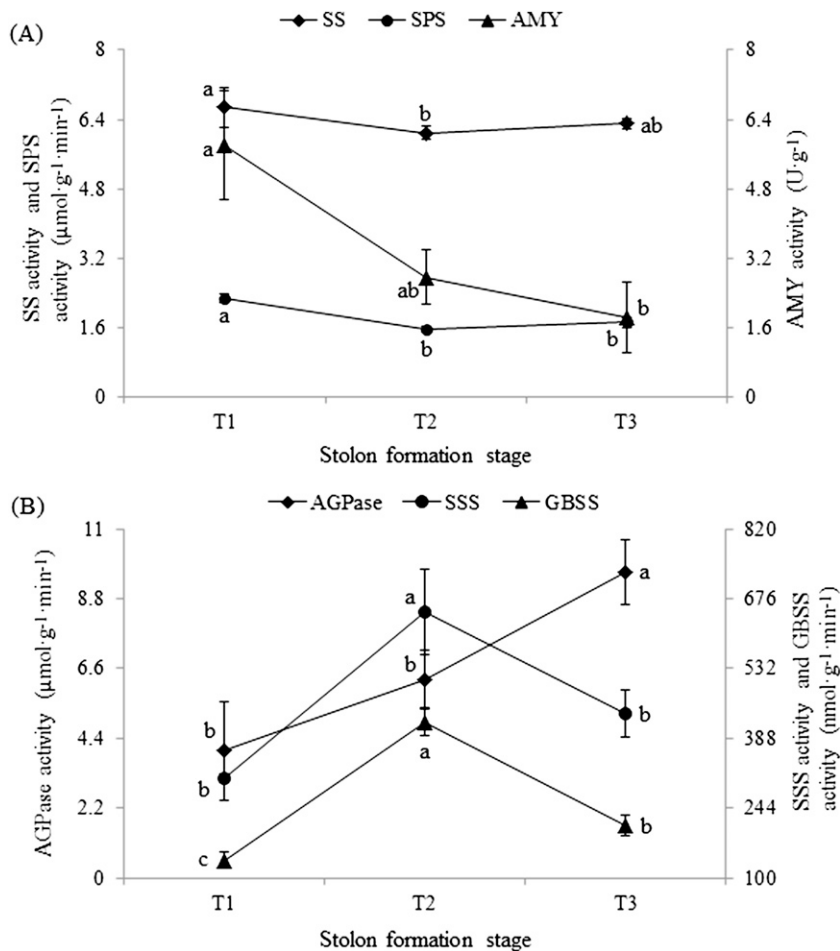


Fig. 3. Dynamic changes in (A) sucrose synthase (SS), sucrose phosphate synthase (SPS), and amylase (AMY) and (B) adenosine diphosphoglucose pyrophosphorylase (AGPase), soluble starch synthase (SSS), and granule-bound starch synthase (GBSS) activities in stolons during *Tulipa edulis* stolon formation. T1, T2, and T3 indicate the initial, middle, and later periods of stolon formation, respectively. Different lowercase letters in each index indicate significant differences (Duncan's multiple range test at $P < 0.05$) among the three developmental stages. The error bars indicate sd of the three replicates.

Table 2. Correlation analysis of carbohydrate content and carbohydrate-related enzyme activities in stolons during *Tulipa edulis* stolon formation.

	Total soluble sugar	Sucrose	Reducing sugar	Fructose	Starch	SS ^z	SPS	AMY	AGPase	SSS	GBSS
Total soluble sugar	1										
Sucrose	0.991**	1									
Reducing sugar	0.879**	0.890**	1								
Fructose	0.985**	0.984**	0.914**	1							
Starch	0.766*	0.740*	0.468	0.751*	1						
SS	-0.581	-0.572	-0.585	-0.619	-0.597	1					
SPS	-0.855**	-0.882**	-0.837**	-0.871**	-0.594	0.598	1				
AMY	-0.674*	-0.744*	-0.810**	-0.743*	-0.260	0.333	0.701*	1			
AGPase	0.761*	0.725*	0.849**	0.778*	0.295	-0.417	-0.585	-0.610	1		
SSS	0.525	0.580	0.528	0.575	0.566	-0.710*	-0.739*	-0.550	0.092	1	
GBSS	0.640	0.670*	0.423	0.630	0.745*	-0.660	-0.791*	-0.360	0.118	0.816*	1

^zSS = sucrose synthase; SPS = sucrose phosphate synthase; AMY = amylase; AGPase = adenosine diphosphoglucose pyrophosphorylase; SSS = soluble starch synthase; GBSS = granule-bound starch synthase.

*The level of significance (Pearson product-moment correlation) is $0.01 < P < 0.05$.

**The level of significance (Pearson product-moment correlation) is $P < 0.01$.

T3 than T1 and T2, and the difference in the expression level of *SSSI* between T1 and T2 was not significant (Fig. 5B). Compared with T1, the *SSSI* gene expression level increased significantly, by 710.25% and 583.39%, in T2 and T3, respectively. The analysis of the expression patterns of these genes was helpful to further understand the sucrose and starch metabolism during stolon formation.

Discussion

The formation of the stolon in *T. edulis* is similar to the formation of the stolon in potato in appearance. During stolon formation of *T. edulis*, meristematic cells in the specific tissue of the stem base generate a serial process of cell division and enlargement, and the tissue thereby bulges to form a stolon (Z.B. Zhu, unpublished data). The process of stolon formation is closely related to complex physiological changes such as the stolon in potato, involving many changes in nutrients, related enzyme activities, and endogenous hormone levels.

STOLON FORMATION AND CHANGES IN NUTRIENT CONTENT. Carbohydrates exist in the lily bulb as primary nutrients, not only as carbon frame providers of various types of chemicals but also as energy carriers required for substance synthesis, and play an important role in the growth, development, and physiological metabolism of the bulb (Li et al., 2014). Soluble sugars, mainly sucrose and reducing sugar, are the chief nutrients and carbon sources and can be directly used and transported in the bulb. Our work

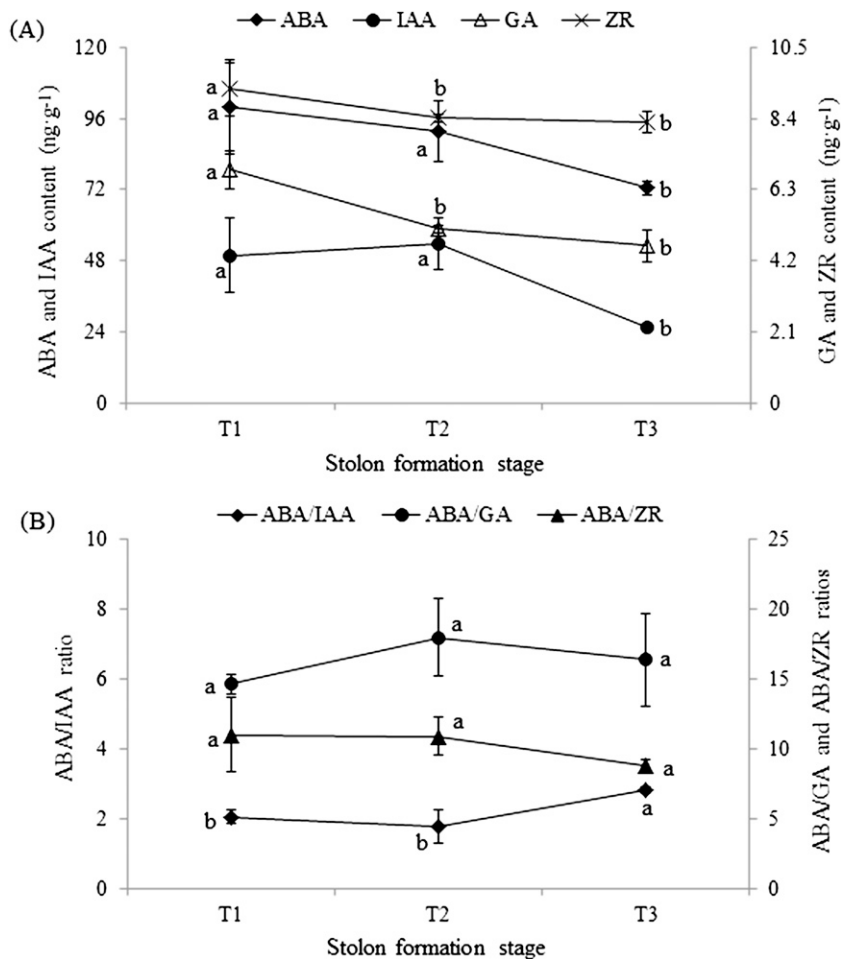


Fig. 4. Dynamic changes in (A) abscisic acid (ABA), indole-3-acetic acid (IAA), gibberellin (GA), and zeatin riboside (ZR) content and (B) the ratios of ABA:IAA, ABA:GA, and ABA:ZR in stolons during *Tulipa edulis* stolon formation. T1, T2, and T3 indicate the initial, middle, and later periods of stolon formation, respectively. Different lowercase letters in each index indicate significant differences (Duncan's multiple range test at $P < 0.05$) among the three developmental stages. The error bars indicate SD of the six replicates.

demonstrated that the total soluble sugar content was significantly increased in the middle period compared with the initial period of stolon formation (Fig. 2A). A likely explanation is that during the middle period in stolon formation, cells are highly active, accompanied by a great deal of cell division and cell enlargement, and that the accumulation of total soluble sugar could supply the nutrients and energy (Simard et al., 2013) for stolon organogenesis. The stolon still maintained a relatively high level of total soluble sugar during the later period, which was consumed less and amassed for further stolon development. As shown in Figure 2A, the ratio of sucrose to total soluble sugar can reach up to values ranging from 65.54% to 72.68%, and the changing trend of sucrose was consistent with the trend of total soluble sugar. A similar phenomenon was reported in lily bulbs by Shin et al. (2002) and Sun et al. (2005), supporting the idea that sucrose is the major form of total soluble sugar in long-distance transportation for direct participation in stolon formation in *T. edulis*. Reducing sugar content, including primarily fructose and glucose, was relatively lower than that of sucrose but showed a significant difference during stolon formation (Fig. 2A). It is inferred that

the change in reducing sugar embodies the supply and transformation of carbohydrates in stolon formation, as found by Sun et al. (2005) in lily, and the increase in reducing sugar when the stolon emerges coincides with the high metabolic activity of the dividing and rapidly growing cells (Simard et al., 2013). In addition, sucrose and reducing sugar typically act as a signaling molecule in plant development, so the specific mechanism of sucrose and reducing sugar in regulating stolon emergence by inducing the expression of related genes requires further investigation.

Starch is the main storage form of carbohydrates and also an important energy source in the bulb, and its metabolic characteristics are among the critical factors affecting organogenesis in plants (Qu et al., 1998). The results of the current study showed that starch content accumulated during the middle period when stolon emerged, in accord with the study in the haustorial development of *Santalum album* (Zhang et al., 2012), supporting the view that soluble sugar in the mother bulb was transported to the specific site of the stem for starch synthesis to satisfy the reserve substance and energy demands of active cells, including cell division and differentiation, during stolon morphogenesis. This result illustrated that starch accumulation was likely preparation for the initiation of new plant organs through substance and energy supplies or that it indicated an upcoming new developmental status in plants, such as organogenesis, in accordance with the viewpoint presented by Thorpe and Murashige (1968). In short, the starch and soluble sugars accumulation during the middle period, combined with their positive correlations (Table 2), suggested that the stolon developed in a

stage of high levels of carbohydrate. This result was consistent with the previous observations that starch and soluble sugar contents increased together in daughterbulb formation in lily and tuber initiation in potato (Fernie and Willmitzer, 2001; Xia et al., 2005). Protein is a vital nutrient and closely related to carbohydrate, and the two can be interconverted (Ribeiro et al., 2014). Therefore, the decrease in soluble protein content during stolon formation might be used to generate nutrient and energy sources to meet the requirements of the transportation and accumulation of carbohydrates as well as the increased cell number and cell volume in the stolon.

STOLON FORMATION AND SUCROSE AND STARCH METABOLISM. Sucrose and starch metabolism have provided a breakthrough for researching organogenesis via the close interaction between sucrose, starch and their related metabolic enzymes, which has been applied to other plants to study tuber and bulblet formation thoroughly and systemically (Li et al., 2014; Li and Zhang, 2003). In this work, we also discussed how sucrose and starch metabolism-related enzymes influence stolon formation with soluble sugars and starch. SPS and AMY activities peaked in the initial period of stolon formation (Fig. 3A). Once stolon

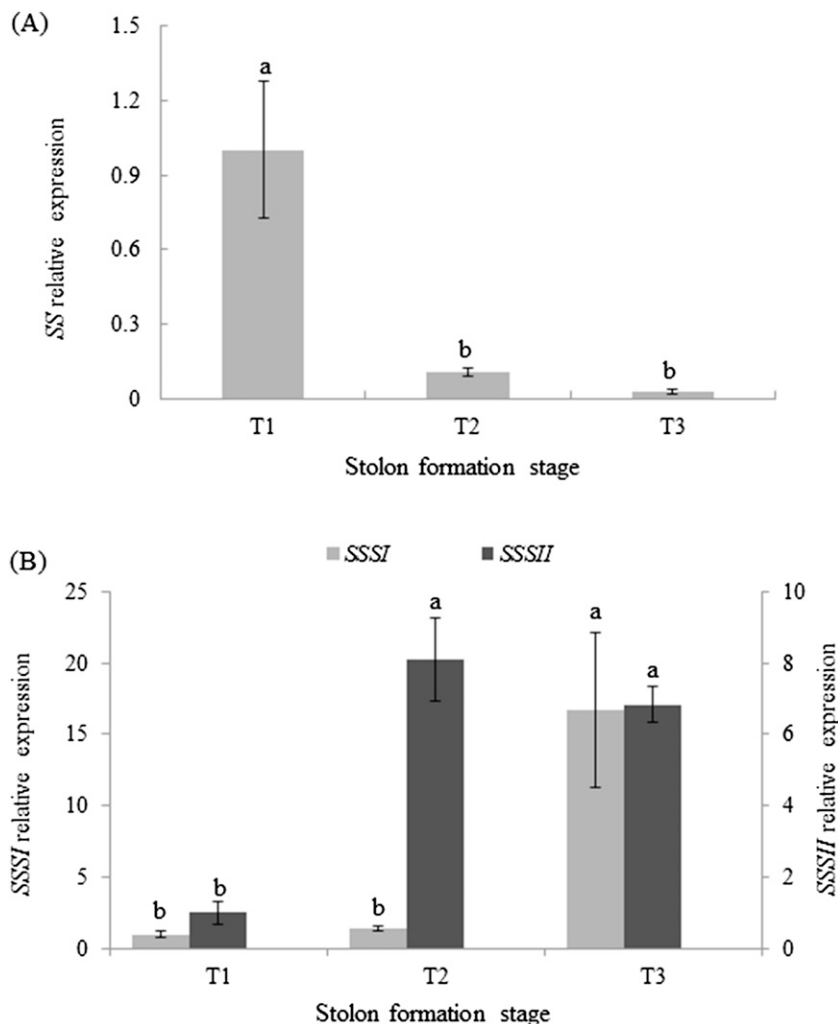


Fig. 5. Validation of selected three sucrose–starch metabolism-related genes (A) *SS* (sucrose synthase and (B) *SSS* (soluble starch synthase) from three developmental stages in stolons during stolon formation of *Tulipa edulis* with quantitative real-time PCR (qRT-PCR). T1, T2, and T3 indicate the initial, middle, and later periods of stolon formation, respectively. Different lowercase letters indicate significant differences (Duncan's multiple range test at $P < 0.05$) among the three developmental stages. The error bars indicate SD of the three replicates.

morphology was established, SPS and AMY participated immediately in material transformation and thereby offered energy for cell division and growth. Previous research demonstrated that SPS was an important enzyme in SS, and sucrose was positively related to SPS activity (Shin et al., 2002), in contrast to our results. The peak of SPS activity was achieved in the initial period and appeared to be backward to some degree; in the middle period, transforming reducing sugar transited from the mother bulb into sucrose (Huber and Huber, 1996), so the result assumed that sucrose content was significantly increased at the middle stage compared with the initial stage. However, sucrose might have an impact on the activities of sucrose-metabolizing enzymes through feedback adjustment, as sucrose was a metabolism regulatory factor (Chen et al., 2000; Koch, 1996), producing a balanced relationship between sucrose and SPS. Specifically, a high level of sucrose might inhibit the rise of SPS activity, while the reduction in SPS activity in turn might weaken the synthetic ability to turn reducing sugar into sucrose. However, the sucrose content did

not decline because sucrose was transited constantly from the mother bulb to the stolon for rapid replenishment. The above hypothesis would explain the significant increase in sucrose content in the middle period and its stabilization at a high level in the later period compared with the initial period of stolon formation. AMY attained its maximum activity in the initial period and promoted starch degradation to produce reducing sugar (Mishra and Dubey, 2013) for stolon emergence in the middle period, with the same performance lag as SPS. Combined with the reducing sugar transported continually from the mother bulb, reducing sugar content rose increasingly and hence showed a significantly negative correlation with AMY activity during stolon formation (Table 2).

Starch metabolism is a common dynamic process of starch synthesis and degradation. On the basis of the changes of starch content and AMY, AGPase, SSS, and GBSS activities and their correlations, starch synthesis played the leading role in starch metabolism, resulting in a condition of gradual starch accumulation. In the process of starch synthesis, sucrose served as the basic metabolic substance and was transported in the phloem over long distances and downloaded to the stolon location, becoming available for starch synthesis through metabolic process (Bánfalvi et al., 1996). In addition, SS is an exclusive necessary enzyme to prompt sucrose into the pathway of the sucrose–starch metabolism (Sung et al., 1989), and the inverse correlation relationship between sucrose content and SS activity suggests that the dominant role of the decomposition of SS in sucrose. In this study, the activity of SS peaked in the initial period of stolon formation, and it was confirmed by qRT-PCR approach that the *SS* gene was active at the initial stage but rapidly down-regulated to

a low level in the middle and later period of stolon formation (Fig. 5A). Therefore, the high level of SS activity in the initial period was beneficial for rapidly digesting sucrose into fructose and UDPG, not only providing a great driving force for cell growth by increasing cell osmotic pressure for stolon emergence in the middle period, but also generating a sucrose concentration gradient to facilitate the phloem loading and unloading of sucrose beneficial for continued stolon growth and for generating the preconditions (fructose, UDPG) of starch synthesis (Rouhier and Usuda, 2001; Wang et al., 1999). Fructose and UDPG were converted to glucose-1-phosphate or glucose-6-phosphate with a series of catalytic reactions (Fernie et al., 2002). In the present study, the gradual increase in AGPase activity as stolon formation progressed catalyzed the reaction between glucose-1-phosphate or glucose-6-phosphate and adenosine triphosphate to generate substantial amounts of precursor ADPG for starch synthesis. Following the production of ADPG as a substrate, starch synthesis proceeded via the catalytic reaction of SSS and GBSS (Mukerjea et al., 2010).

Therefore, the peak in starch content during the middle period resulted from the highest activities of SSS and GBSS compared with the other two periods and significant correlation between GBSS activity and starch content. The qRT-PCR results also confirmed this analysis by showing that the *SSSII* gene was up-regulated in the middle period compared with the initial and later period of stolon formation (Fig. 5B). However, *SSSI* gene expression was observed to show some differences in transcriptional level compared with the *SSSII* gene. It is possible that *SSSI* and *SSSII* are two key genes encoding SSS, playing different roles in different stages during stolon formation. The qRT-PCR results for *SSSI* and *SSSII* as a whole verified the change in SSS activity resulting from their high expression levels at the middle and later stage of stolon formation.

STOLON FORMATION AND CHANGES IN ENDOGENOUS HORMONES. Coinciding with changes in ZR content, GA content reached its highest value in the initial period and then declined during stolon formation. It may be because GA and ZR play dominant roles in stimulating cell division and elongation growth (Chope et al., 2012) during the initial period of stolon formation. As the peak GA and ZR levels in the initial period were accompanied by higher IAA content, it might be suggested that GA, ZR, and IAA were working more efficiently to prepare cell activities for stolon emergence. Previous researches reported that IAA was benefit for cell growth (Roumeliotis et al., 2012) and starch synthesizing metabolism (Borzenkova et al., 1998). Therefore, in the middle period, the IAA content peaked to prompt cell division, elongation and expansion, and simultaneously stimulates starch synthesis and assimilates translocation, triggering the initiation of the stolon in the middle period (Fig. 1D). This result was closely consistent with previous works in lily and tulip bulb formation (Podwyszyńska, 2006; Zheng et al., 2012). Nevertheless, there were drastic decreases in the GA, IAA, and ZR content in the later period, which demonstrated that cell growth became slow, and reserve materials were largely accumulated to enrich and fill the stolon after stolon emergence. A high concentration of ABA was previously considered to hamper tarda tulip bulblet formation (Maślanka and Bach, 2014), while the ABA level was relatively high when the stolon emerged in our study. Thus, there seems to be a balance of hormones, especially the ratio of ABA:IAA, which was significantly lower in the initial and middle period compared with the later period of stolon formation (Fig. 4B), suggesting that IAA played a stimulatory role in stolon formation via the suppression of ABA. It was concluded that the hormones synthesized in *T. edulis* stolon is apparently necessary for cell division and growth of the meristematic regions during stolon development.

Conclusions

Stolon emergence was indicated by the simultaneous accumulation of soluble sugars (sucrose, reducing sugar, and fructose) and starch, which was the cumulative effect of many enzymes. The endogenous level of IAA showed a positive regulation in stolon emergence. The highest content of IAA and lowest ratio of ABA:IAA were detected in the middle period of stolon formation, i.e., in stolon emergence. We report for the first time on the carbohydrate and endogenous hormones roles in stolon formation, which benefit for further regulation in *T. edulis* stolon growth.

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