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Proteomic and gene expression analyses during bolting-related leaf color change in *Brassica rapa*

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ABSTRACT. Bolting and flowering are key processes during the growth and development of Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*). Understanding the molecular mechanisms underlying bolting and flowering is of significance for improving production of the vegetable. A leaf-color change from bright green to gray-green has been observed following differentiation of the flowering stem and before bolting in the vegetable, and is considered to be a signal for bolting. Proteomics in meristem tissues of an inbred line (C30) were analyzed by two-dimensional electrophoresis during the transition period. We found that some proteins were specifically expressed while others were differentially expressed. Among these, 17 proteins were specifically expressed before the color change, 18 were specifically

expressed after the color change, 21 were downregulated during the color change, and 29 were upregulated. Mass spectrometric analysis (MALDI-TOF-TOF/MS) was used to analyze 17 protein spots, and four proteins (subunit E1 of vacuolar-type H^+ transporter ATPase, the large subunit of Rubicon, S-adenosylmethionine synthetase, and tubulin α-2) were identified. qPCR analysis was conducted to quantify the expression of genes encoding these proteins during the transitional period. The expression of *BrVHA-E1*, *BrSAMS*, *BrrbcL*, and *BrTUA6* was significantly different before and after the leaf-color change, suggesting that these genes might be involved in regulating flower differentiation and bolting.

Key words: Chinese cabbage; Bolting; Leaf color; Protein; Gene expression

INTRODUCTION

Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*) is one of the most important cruciferous vegetables. Bolting has evolved as an important developmental stage for brassica plants. Under certain light regimes, the cabbage changes from vegetative to reproductive growth after going through vernalization. The biology of flowering and bolting biology has been extensively studied at the molecular level in both Chinese cabbage and *Arabidopsis thaliana*. However, most of these studies have focused on the analysis of quantitative trait loci, regulatory pathways, and gene networks (Lin et al., 2005; Greenup et al., 2009; Turnbull, 2011; Wang et al., 2014; Blümel et al., 2015; Chung et al., 2015; Tao et al., 2015; Zhang et al., 2015, 2016), and the proteomics of bolting and flowering are poorly understood.

Two proteins in Chinese cabbage were found to be specifically expressed at the bolting stage (Ao and Li, 1997). Vernalization induced the production of a 58-kD protein and the loss of another protein, possibly due to the expression of vernalization-related genes or the metabolism of DNA or proteins (Li et al., 2007). Three significantly enhanced bands were observed on SDS-PAGE during the analysis of soluble proteins extracted from *Brassica oleracea* at the bolting stage, with molecular weights of 48.4, 18.8, and 14.5 kD, while an 80.4-kD protein was lost (Yang et al., 2009).

In a previous study, we found that leaf color changed from bright green to gray-green before bolting in vernalized Chinese cabbage, which was followed by elongation of the flowering stem. The color change was most marked in the early flowering cultivars. When observed under a microscope, the meristem can be seen to protrude to form the flower, followed quickly by color change if the plant is grown under normal conditions. We have demonstrated that the leaf-color change is associated with changes in the levels of hormones, pigments, and proteins. The ratio of chlorophyll a/b and levels of IAA (indole-3-acetic acid) and GA3 (gibberellin A3) peaked at the point of the color change (Han et al., 2011). In the present study, we aimed to investigate the proteomics in the meristem tissues between the color change stages in order to better understand the developmental biology and molecular regulatory mechanisms underlying flowering and bolting in the Chinese cabbage.

MATERIAL AND METHODS

Materials

An inbred line of Chinese cabbage (C30), developed at Northeast Agricultural University, was seeded in 8 x 8-cm pots containing nutrient-rich soil. The seedlings were subjected to vernalization in a light incubator after the cotyledons were fully laid open at 8°/3°C (day/night) with a 16/8-h light/dark period for 25 days. The vernalized plants were transplanted to the field for normal growth. The differentiation of flower buds was confirmed by microscopy (**Figure S1**). When the color of the new leaf (which was about 1 cm in size) changed from bright green to gray-green (Figure 1), leaves smaller than 1 cm and their surrounding meristem tissues were collected and flash-frozen in liquid nitrogen for subsequent use.

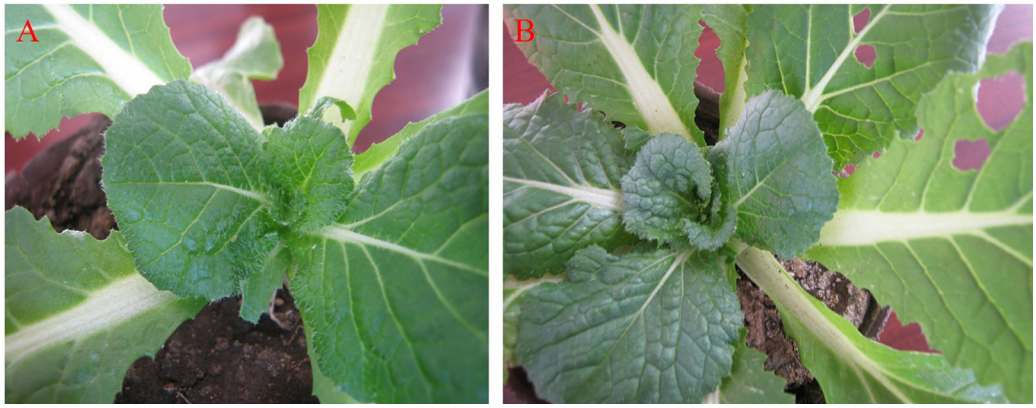

Figure 1. Leaf color change during bolting in Chinese cabbage. Leaf color before (**A**), and leaf color after (**B**) bolting.

Protein extraction

Proteins were extracted as previously described (Shao et al., 2009), with some modifications. Briefly, frozen tissue (1 g) was ground to a fine powder in liquid nitrogen, added to pre-chilled TCA (trichloroacetic acid) (7 mL containing 0.07% β-mercaptoethanol), vortexed, and incubated overnight at -20°C. The mixture was centrifuged at 15,000 *g* at 4°C for 30 min, and the pellet was resuspended in ice-cold acetone (7 mL containing 0.07% β-mercaptoethanol), vortexed periodically, and incubated at -20°C for 2 h. The procedure was repeated and the pellets were vacuum-dried. For protein extraction, 1 mg of the pellet was suspended in 10-20 µL lysis buffer [7.0 M urea, 2.0 M thiourea, 4% (w/v) CHAPS, 60 M DTT, and 0.5% (v/v) IPG buffer], and incubated in a water bath at 35°C for 2 h or freeze-thawed three times (with full vortex). The extraction was centrifuged as described above and the protein content in the supernatant was quantified using the Bradford method (Kruger, 1994).

Two-dimensional gel electrophoresis

The proteins were analyzed using two-dimensional gel electrophoresis as previously

described (Liu et al., 2009), with some modifications. Protein (200 μg) in loading buffer [7.0 M urea, 2.0 M thiourea, 4% (w/v) CHAPS, 60 M DTT, and 0.5% (v/v) IPG buffer] was loaded and analyzed on an Isoelectric Focusing System IPGPhor II™ using a 24-cm strip at pH 4-7, according to the manufacturer instructions. Electrophoresis was performed at 20°C after hydration at 50 V for 12 h, followed by 500 V for 1 h, 1000 V for 1 h, 1000-8000 V for 1 h, 8000 V for 10 h, and 500 V for 10 h. The second SDS-PAGE was performed on an Ettan DALTsix Electrophoresis System at 18°C with a 10 mA initial current and a 20 mM resolution current applied when the samples were moved out of the IPG strips. The proteins were visualized by silver staining for 30 min. Each assay was repeated three times.

Gel chromatogram analysis

The stained gels were scanned using a 24-color or gray scale with an Imagescan (Amersham) at a resolution of 600 dpi with default brightness and contrast settings. The chromatograms were analyzed using the ImageMaster™ 2D Platinum software (Version 5.0, Amersham) in order to determine the number of protein spots, optical density, area, and volume. The protein abundance was determined before and after the leaf-color change, and proteins were identified as being differentially expressed if the difference in their abundance was greater than 3-fold.

Mass spectrometry and homology search

Spots that were differentially expressed were isolated for mass spectrometry analysis as previously described (Chen et al., 2009) at the Proteome Research Center (Huada Inc., Beijing, China) using a 4700 Proteomics Analyzer (Applied Biosystems, USA). The peptide mass fingerprints were analyzed using Mascot (http://www.matrixscience.com). Protein homologs were searched on the NCBI, MSDB, and EST-Viridiplantae databases. To ensure reliability, each protein was sequenced for at least 15% of the total predicted protein using a minimum of five peptide fragments with less than 1 Dalton mass error and at least one unhydrated restriction site.

RNA extraction and qPCR

Expression and analysis of the proteins selected in this study were based on a Mascot database search with a limit score of 70, the tested spots whose molecular weights and iso-electric points were close to related species. Total RNA was extracted from leaf and meristem tissue samples using the TRIzol method. First-strand cDNA was synthesized using RevertAid First-Strand cDNA Synthesis Kit (TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer instructions in a 20-μL reaction volume containing 4 μL total RNA. Primers were designed based on the DNA sequences identified from the *Brassica* genome database (http://brassicadb.org) using DNAMAN 6.0. Genes and their specific primers used for qPCR included *BrVHA-E1*, forward 5'-GGCAAGATTATGAGAAGA-3', reverse 5'-ATGGCATTG ACAATATCA-3'; *BrrbcL*, forward 5'-GGAGTTCCTATCGTAATG-3', reverse 5'-CCATGAT TCTTCTGTCTAT-3'; *BrSAMS*, forward 5'-TATAGTCCAAGTGTCGTA-3', reverse 5'-CAAGGTTAATAGCCATCA-3'; and *BrTUA6*, forward 5'-TTGTATGATCTCCAACTC-3', reverse 5'-CAACCTCTTCATAATCCT-3'. The primers were synthetized at Beijing Genomics

Institution. The efficiency of the primers used in this study was at least 96%. qPCR was performed using a TransStart™ Top Green qPCR SuperMix kit (TransGen Biotech Co., Ltd.) with four replicates. Real-time PCR assays were performed on a Biorad iQ5 real-time PCR detector system (Bio-Rad). qPCR conditions were as follows: pre-denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s. Melting curve analysis was completed following amplification. The GAPDH gene was used as an endogenous quantification control.

RESULTS

Proteomic analyses

Electrophoresis experiments were performed in triplicate using the same experimental conditions and parameters, with soluble proteins obtained before and after the leaf-color change, from the inbreeding line C30. Soluble proteins extracted from plant tissues were well-separated on SDS-PAGE after two-dimensional electrophoresis. After silver staining, clear protein spots were visible with uniform distribution, excellent resolution, and repeatability. Representative images of the samples before and after the leaf-color change are shown in Figure 2. The samples contained similar species and amounts of proteins before and after the color change, indicating that most of the proteins were stable during that period. There were some stage-specific proteins with isoelectric points between 4.5 and 7.0, and molecular weights between 13 and 74 kD. The number of protein spots detected was 1126 and 1147 before and after the color change, respectively.

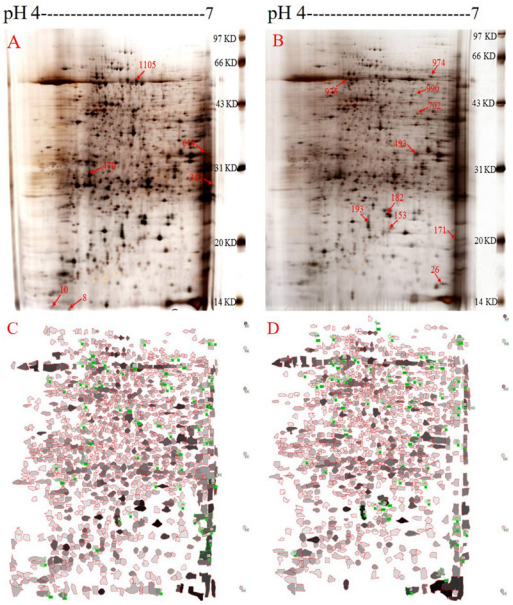

Figure 2. Two-dimensional SDS-PAGE showing protein dynamics in Chinese cabbage. Protein dynamics before the leaf-color change (**A**), protein dynamics after the leaf-color change (**B**). Analogue results before the leaf-color change (**C**), and analogue results after the leaf-color change (**D**).

Eighty-five proteins with changes in expression of 3-fold or more during the color change period were identified after analysis with the PDQuest software. Of these, 17 proteins were specifically expressed before the color change, 18 were specifically expressed after the change, 21 were downregulated during the change, and 29 were upregulated.

Mass spectrometric analysis and protein homology search

Five differentially expressed proteins with at least a 3-fold change in expression, and 12 specifically expressed proteins were selected for mass spectrometric analysis. The reproducibility and abundance of these proteins were confirmed. They were easily distinguished from other protein spots and were easily isolated. The protein spots were identified only when the Mascot Protein Scores were greater than 50 ($P < 0.05$). Among the 17 proteins, 16 were identified in a MASCOT database search, and most presented some differences between the predicted and actual molecular weights and isoelectric points. Five of those (spots number 470, 493, 975, 974, and 990) had similar predicted and actual molecular weights and isoelectric points. Spots 974 and 493 were the same protein (RuBisCO large subunit). Spot 702, one of the identified proteins, was an unknown protein. Spots 10, 26, 153, 171, and 342 were hypothetical proteins. The other proteins were possibly involved in transcriptional regulation, signal and energy transduction, growth, and metabolism, or were structural proteins (Table 1). Based on the Mascot scores (**Figure S2**), proteins on spots 470, 493, 975, and 990 were most homologous to *A. thaliana* subunit E of vacuolar-type H^+ transporter ATPase, the large subunit of *Brassica* RuBisCO, *A. thaliana* S-adenosylmethionine synthetase, and canola tubulin α-2, respectively.

Expression of the four identified genes

Amino acid sequences were used to search the *Brassica* genome database (http://brassicadb.org); *Bra*033155, *Bra*028087, *Bra*023084, and *Bra*018825 were highly homologous with the identified proteins. These clones were designated *BrVHA-E1*, *BrrbcL*, *BrTUA6*, and *BrSAMS*, respectively, and their expression was analyzed by qPCR using mRNA extracted from leafs before and after the color change. *BrTUA6*, *BrrbcL*, and *BrSAMS* were upregulated after the color change, while *BrWHA-E1* was downregulated (Figure 3). In the two-dimensional protein analysis, the proteins on spots 975 and 493, which corresponded to *BrTUA6* and *BrrbcL*, respectively, were expressed only after the color change, while the protein on spot 470, which corresponded to *BrVHA-E1*, was only expressed before the color change. The protein coded by *BrSAMS* was upregulated after the color change. These data indicated that the protein and gene expression results were consistent based on the protein analysis and qPCR results. However, the levels of *BrrbcL* and *BrVHA-E1* expression, but not of *BrSAMS* and *BrTUA6*, differed significantly ($P < 0.05$) before and after the leaf-color change. Further studies are required to analyze the expression profiles.

DISCUSSION

Bolting is a complex physiological and biochemical process in Chinese cabbage, which involves the participation of many genes. The dynamics of protein expression during

Table 1. Identification of differentially expressed protein homologs.

Spot No.	Homologous protein	Protein ID	Molecular function	Species	pI/MW	Score	No. of matching peptides	Coverage	E value	Time of expression
8	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|306482423	Magnesium ion binding, monooxygenase activity, ribulose-bisphosphate carboxylase activity	*Stilpnogyne* sp SH-2010	4.69/13	73	11	21%	0.04	Before color change
10	Hypothetical protein SORBIDRAFT_06g028450	gi|242074314	ATP binding, chromatin binding	*Sorghum bicolor*	4.48/13	76	23	17%	0.062	Before color change
342	Hypothetical protein	gi|3702319	Unknown molecular function	*Arabidopsis thaliana*	6.93/26	68	10	50%	0.082	Before color change
470	TUF; proton-transporting ATPase, rotational mechanism	gi|15237054	Proton-transporting ATPase activity, rotational mechanism	*Arabidopsis thaliana*	5.01/30	70	11	46%	0.031	Before color change
1105	mitochondrial F1 ATP synthase beta subunit	gi|17939849	ATP binding, kinase activity, magnesium ion binding, potassium ion binding, pyruvate kinase activity	*Arabidopsis thaliana*	5.75/55	197	25	54%	1.8e-14	Before color change
26	Hypothetical protein OsI_00723	gi|125524740	Unknown molecular function	*Oryza sativa* Indica Group	6.58/15	73	8	38%	0.043	After color change
153	Hypothetical protein ARALYDRAFT_343774	gi|297821421	Unknown molecular function	*Arabidopsis lyrata* subsp *lyrata*	5.69/21	55	9	47%	0.18	After color change
182	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|247893136	Magnesium ion binding, monooxygenase activity, ribulose-bisphosphate carboxylase activity	*Brassica rapa* subsp *chinensis*	5.64/23	100	11	40%	8.8e-05	After color change
493	RuBisCO large subunit	gi|1346967	Magnesium ion binding, monooxygenase activity, ribulose-bisphosphate carboxylase activity	Chloroplast *Brassica oleracea*	6.15/33	174	24	40%	3.5e-12	After color change
702	Unknown	gi|217075356	Unknown molecular function	*Medicago truncatula*	6.10/40	65	6	35%	0.27	After color change
974	RuBisCO large subunit	gi|1346967	Magnesium ion binding, monooxygenase activity, ribulose-bisphosphate carboxylase activity	Chloroplast *Brassica oleracea*	6.38/54	147	21	37%	1.8e-09	After color change
975	Putative tubulin alpha-2/alpha-4 chain	gi|34733239	GTP binding, GTPase activity, protein binding, structural constituent of cytoskeleton	*Brassica napus*	4.90/54	134	19	46%	3.5e-08	After color change
171	Hypothetical protein SORBIDRAFT_09g019590	gi|242087855	Unknown molecular function	*Sorghum bicolor*	6.79/20	88	18	26%	0.0013	Upregulated
193	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|290586314	Magnesium ion binding, monooxygenase activity, ribulose-bisphosphate carboxylase activity	*Brassica juncea*	5.33/22	130	12	41%	8.8e-18	Upregulated
614	Maturase K	gi|319976508	RNA-directed DNA polymerase activity	Unidentified plant	6.91/33	58	10	40%	0.13	Downregulated
990	Copper ion binding / methionine adenosyltransferase	gi|15228048	ATP binding, methionine adenosyltransferase activity/protein binding	*Arabidopsis thaliana*	6.11/46	103	10	21%	4.4e-05	Upregulated

pI: isoelectric point; MW: molecular weight.

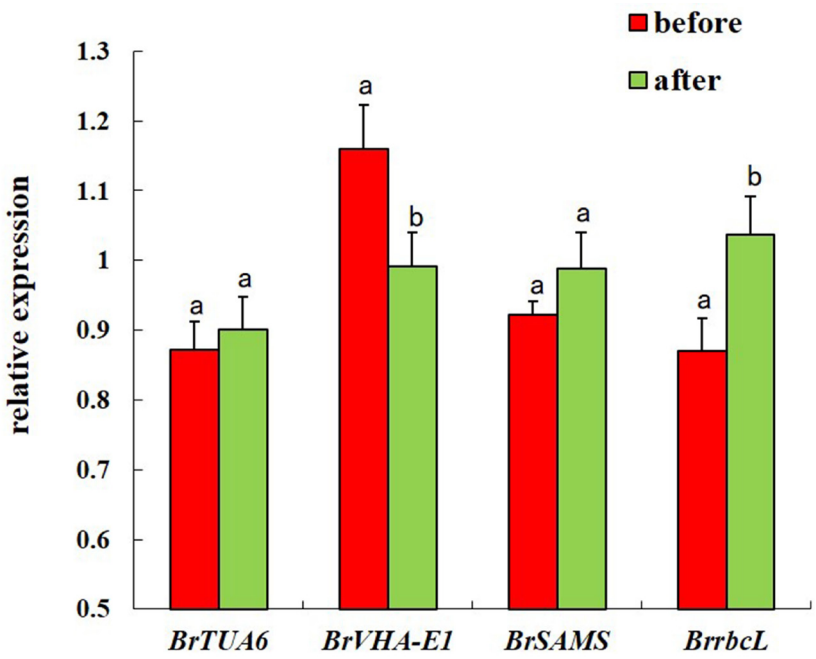

Figure 3. Expression of four genes encoding differentially expressed proteins before and after leaf color change. The internal control gene is GAPDH, a and b above the bars are significant differences, same letters mean nonsignificant differences.

the bolting stage can be visualized using two-dimensional SDS-PAGE. In this study, 1126 and 1147 protein spots were detected before and after the leaf-color change, respectively. Only 85 protein spots that quantitatively or qualitatively changed during the bolting stage were associated with the color change. The levels of specific and differential proteins were low, probably because the two processes were sampled over a small time period. These proteins might be involved in transcriptional regulation, signal and energy transduction, cell growth and metabolism, or they could be structural proteins. It is likely that they are involved in the bolting process.

S-adenosylmethionine synthetase (SAMS) is a key enzyme involved in plant metabolism, which catalyzes the synthesis of S-adenosylmethionine (SAM) from methionine and ATP. SAMS is the precursor of ethylene and polyamine, which are regulators of development and stress resistance in many plants. SAMS is more highly expressed in the stems than in the leaves of *A. thaliana* and is localized to vascular tissue (Peleman et al., 1989). In carnation flower tissues, the expression of SAM decreases as the flowers blossom (Woodson et al., 1992). In the present experiment, the SAMS protein was upregulated following the color change, suggesting that it may be involved in the regulation of bolting. However, the level of *BrSAMS* expression was not significantly different ($P < 0.05$) before and after the leaf-color change. We speculated that homologues of *BrSAMS* might exist in the Chinese cabbage genome. We found three *BrSAMS* genes in a *Brassica* genome database using bioinformatic analysis. Further study is needed to analyze the expression profiles of these genes.

Microtubules exist widely in the cytoskeleton, and are important for cellular morphology, cell division, transportation, energy transfer, and signal transduction, and α-tubulin is the basic unit of microtubules. Genes encoding different isomers of the protein are differentially expressed in different tissues and development stages. A number of studies have been conducted on plant tubulins (Carpenter et al., 1992; Uribe et al., 1998; Xu et al., 1999; Li et al., 2007). Transgenic expression of GFP-TUA6 revealed that transverse microtubules form during the elongation stage of the inner cell wall in *A. thaliana* (Chan et al., 2011). Microtubules on the outer cell wall are changeable, and tend to arrange transversally during the period of fastest growth. The arrangement of microtubules on the walls of the outer epidermal cells affects the rate at which the cells elongate. We found that *BrTUA6* proteins were expressed specifically after the leaf-color change, suggesting that changes in the arrangement of microtubules might occur in order to prepare for the elongation of flowering stems and bolting. However, levels of *BrTUA6* expression were not significantly different before and after the leaf-color change. Twelve α-tubulin genes have been identified in a *Brassica* genome database. Bioinformatic analysis revealed that *Bra*018825 (*BrTUA6*) shares 93.4% homology with *Bra*039648 (*BrTUA6*) at the nucleic acid level, and their deduced amino acid sequences are identical. Furthermore, their expression levels were not significantly different, possibly because *TUA* has multiple homologous genes, and the primers used for qPCR analysis are not nonspecific. Further study is needed to gain a better understanding of the genomic structure of *BrTUA6*.

Vacuoles act as reservoirs of ions and metabolites in higher plant cells. V-ATPase is a highly conserved proton pump found on vacuolar membranes, which regulates the movement, metabolism, and transportation of vacuolar proteins through the acidification of organelle lumens. This enzyme possesses multiple subunits and is involved in the regulation of a number of cell growth and metabolic processes (Matsuoka et al., 1997; Schumacher et al., 1999; Seidel et al., 2008). In the present study, we found that *BrVHA-E1* was only expressed before the leaf-color change using two-dimensional SDS-PAGE and was found to be significantly downregulated after the leaf-color change. It is possible that *BrVHA-E1* is only associated with the differentiation of flower buds. This result is consistent with a previous report stating that VHA-E1 is involved in regulating early embryogenesis in *A. thaliana* (Strompen et al., 2005).

Ribulose-1,5-bisphosphate carboxylase oxygenase, or RuBisCO, is the key enzyme involved in the Calvin cycle of carbon assimilation during photosynthesis. RuBisCO is made up of eight small subunits and eight large subunits, in which its active centers are located. Moreover, the Calvin cycle is the fundamental pathway of carbon assimilation to synthesize starch, sucrose, and other products. The activity of enzymes in this pathway directly determines the net photosynthetic yield. Kraus and Kraybill (1918) proposed that increases in the C/N ratio would cause plants to flower. Conversely, sucrose signal transduction regulates the expression of a large number of genes, and thereby influences a variety of important physiological and developmental processes. For example, sucrose has been found to directly regulate the expression of the flower regulator LFY (LEAFY) (Blazquez et al., 1998; Ohto et al., 2001) and is therefore important for regulating flowering in plants. In our study, we found that some large RuBisCO subunits were specifically expressed or upregulated after the leaf-color change. In addition, *BrrbcL* was more highly expressed after the color change than before the color change, suggesting that the assimilation efficiency might increase after the color change. The increase would boost the synthesis of photosynthetic products, leading to an increased C/N ratio, and the subsequent occurrence of bolting and flowering in the plant.

Microtubules are ubiquitous structures in the cytoskeleton, which contain α-tubulin as

the basic unit. Different isomers of α-tubulin protein encoding genes have different expression patterns during specific developmental stages or in the cells of specific tissues (Carpenter et al., 1992; Xu et al., 1999; Li et al., 2007). In the present study, we observed differential expression of α-tubulin during the process of leaf-color change, although *BrTUA6* was not differentially expressed. These results suggest that variation in microtubule arrangement could be associated with floral axis elongation and bolting following leaf color change. Therefore, we could predict and identify *cis*-acting elements in the promoter sequences of *BrTUAs*, and the expression of different genes. The bolting process could be controlled by regulating the expression of *BrTUAs*, on account of *BrTUAs* containing *cis*-elements that are responsive to light, temperature, hormonal signals, and tubulin-specific drugs. The same controls could be used for *BrSAMS*. Based on a Mascot search, only four spots were verified with molecular weights and isoelectric points that were close to those of related species. However, the other spots had different molecular weights and isoelectric points, and might contain additional information.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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Supplementary material

Figure S1. Differentiation of flower buds was confirmed by microscopy. Vegetative growth phase (**A**), flower bud differentiation (**B**), complete flower bud differentiation (**C**).

Figure S2. Spectra of differential protein generated by the MALDI-TOF-TOF/MS (matrix-assisted laser desorption/ionization time-of-flight/time-of-fligh mass spectrometer). No. 470 (**A**), No. 493 (**B**), No. 990 (**C**), and No. 975 (**D**).