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# Jointly silencing *BoDWARF*, *BoGA20ox* and *BoSP* (*SELF-PRUNING*) produces a novel miniature ornamental *Brassica oleracea* var. *acephala* f. tricolor variety

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**Abstract** The ornamental *Brassica oleracea* var. *acephala* f. tricolor is a good winter and spring foliage plant. Plant architecture is an important agronomic trait of plants, especially for ornamental plants with high ornamental and economic value. In this study, three miniature-related genes, *BoDWARF*, *BoGA20ox* and *BoSP* (*SELF-PRUNING*), were cloned and their tissue-specific expression patterns were analyzed. The results showed that the three genes were all highly expressed in young leaves and flowers, followed by the lateral roots, seeds and stems. To further achieve the purpose of miniaturization of plants, an RNAi expression vector, jointly targeting *BoDWARF*, *BoGA20ox* and *BoSP*, was constructed and transformed into kale plants. Smaller plant size and slower growth and development speed of flowers and roots were observed in jointly silenced kales. Brassinosteroids and gibberellin contents in leaves and flower buds of transgenic plants were significantly decreased. Furthermore, the expressions of brassinolide-, gibberellin- and flowering-related genes were down-regulated by varying degrees in silenced plants. These results suggest that *BoDWARF*, *BoGA20ox* and *BoSP* play important roles in plant architecture, and that brassinolide and gibberellin are important hormones controlling plant growth and architecture. This miniaturization strategy of kale provides an efficient approach for cultivation of new varieties of ornamental plants and crops.

**Keywords** *BoDWARF* · *BoGA20ox* · *BoSP* (*SELF-PRUNING*) · Miniaturization · Kale · Hormone

## Introduction

Plant architecture is an important agronomic trait in plants. For example, dwarfism in cereal crops has a positive influence on crop agronomy by increasing lodging resistance and decreasing the chances of damage due to wind and rain (Cai et al. 2012). For ornamental plants, with high ornamental and economic value, plant architecture is more important. For instance, Miniature Rosa chinensis is a new dwarf variety of the rose family. As its leaves and flowers are small and attractive, it does not occupy as much space,

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and its placement is easier to choose, it is favored by many people and has become one of the most popular potted flowers on the international flower market. The tomato cultivar Micro-Tom was produced for ornamental purposes by crossing Florida Basket and Ohio 4013-3 cultivars. Micro-Tom displays a very dwarf phenotype with small and red ripened fruits (Scott 1989), and it became a popular potted plant. Recently, this miniature tomato cultivar was also employed as a useful system to investigate fruit ripening and plant development. Thus, breeding miniature plant varieties meets the demand of intensive agriculture and the flower market. Although conventional breeding has made a great contribution to improving traits of plants, it is time-consuming. In recent years, genetic engineering has become an important approach for plant breeding.

Plant growth and development are regulated by both environmental and hormonal signals (Depuydt and Hardtke 2011). Eight principal classes of plant hormones have been characterized: auxin, abscisic acid, brassinosteroids, cytokinins, ethylene, gibberellins, jasmonates and strigolactones (Santner and Estelle 2009; Santner et al. 2009). Based on the phenotype of mutants with disrupted hormone biosynthesis or perception, only cytokinin, auxin, gibberellins and brassinosteroids are considered to be essential for growth (Depuydt and Hardtke 2011). Moreover, brassinosteroids (BRs) and gibberellins (GAs) are inferred to be two major growth-promoting hormones that have similar effects on promoting cell and stem elongation and division (Bai et al. 2012). Brassinosteroids (BRs) are growth-promoting steroid hormones in plants, regulating multiple aspects of physiological responses essential to both vegetative and reproductive development (Kim and Wang 2010; Clouse 2011). After their discovery in the 1970s, an array of experiments over the next decade suggested that these newly discovered plant compounds, with structural similarity to animal steroid hormones, regulate senescence, male fertility, pollen and root development and fruit ripening, and modulate the plant's response to numerous environmental signals (Mandava 1988). The biochemical definition of the BR biosynthetic pathway and the discovery of BR-deficient and BR-insensitive mutants in *Arabidopsis* and several crop plants provided convincing evidence that BRs were essential for normal plant development (Fujioka and Yokota 2003; Clouse and Sasse 1998; Bishop 2003, 2007; Altmann 1999). It has been proposed that BRs are synthesized via two parallel pathways, the early and late C-6 oxidation pathways, according to the C-6 oxidation status (Shimada et al. 2001). The tomato (*Solanum lycopersicum*) *DWARF* gene encodes a cytochrome P450 protein that has been shown to catalyze the C-6 oxidation of 6-deoxocastasterone to castasterone, which is a key step in the regulation of active BR production; the phenotype of the mutant is dwarf (Bishop et al. 1999). These previous studies have shown that regulating BR biosynthesis or signaling could control the development and height of plants. Gibberellins (GAs) are involved in many developmental processes, including seed germination, stem elongation, leaf expansion, flower, seed and fruit development (Peng et al. 1999; Sasaki et al. 2002). Modification of these processes by application of chemicals that alter the GA content has been common agronomic practice. For example, $GA_3$ was used to stimulate berry growth in seedless grape production (Christodoulou et al. 1968). Inhibitors of GA biosynthesis were used as growth retardants to control the stature of cereals and ornamental pot plants (Hedden and Hoad 1994). GA is one of the most important determinants of plant height (Sasaki et al. 2002; Qiao and Zhao 2011). Dwarfism is often caused by mutations in genes controlling the biosynthesis or signaling pathway of GA. For example, in the 1960s, a high-yielding semi-dwarf variety of rice, IR8, was achieved by introducing a major dwarfing gene (*sd-1*) that encoded a defective GA20-oxidase (*GA20ox*) gene (Spielmeyer et al. 2002), which led to the rice "green revolution" (Sui et al. 2012; Sasaki et al. 2002). At the same time, a dominant wheat semi-dwarf cultivar Norin 10 carrying Rht genes facilitated productivity and led to the wheat "green revolution" (Evans 1998). Norin 10 contained the Rht-B1b (formerly called Rht1) and Rht-D1b (formerly called Rht2) alleles that encode a mutant of DELLA protein, a GA signaling repressor (Hedden 2003). These studies demonstrated that controlling GA biosynthesis or signaling was crucial for the production of plants with suitable heights for modern production methods.

Interestingly, in addition to the hormones, the phenotype of Micro-Tom is attributed to the mutation of *SELF-PRUNING* (*SP*) gene, which regulates vegetative to reproductive switching of sympodial meristems and is the ortholog of *CEN* and *TFL1*,

which maintain the indeterminate status of inflorescence meristems in *Antirrhinum* and *Arabidopsis*, respectively (Pnueli et al. 1998; Bradley et al. 1996, 1997; Alvarez et al. 2005). A recessive allele of the *SP* gene confers accelerated termination of sympodial units in the inflorescence, resulting in limited growth of the shoot as well as a bushy, compact constitution and nearly homogeneous fruit setting (Yeager 1927; Went 1944; MacArthur 1932; Calvert 1965). In wild-type 'indeterminate' plants, inflorescences are separated by three vegetative nodes. In 'determinate' plants homozygous for the recessive allele of the *SP* gene, sympodial segments develop progressively fewer nodes until the shoot is terminated by two consecutive inflorescences (Pnueli et al. 1998). The phenotype of the recessive *sp* gene was one of the most important genetic traits in the development of modern agrotechniques for crop plant because the 'determinate' growth habit facilitates mechanical harvesting.

Kales (*Brassica oleracea* var. *acephala* f. tricolor) are widely cultivated as ornamental plants and belong to the Brassicaceae family (Zhang et al. 2012). Because numerous round leaves of kale are overlapping, resembling a rose with magenta center and gray–green outer foliage, and the color of leaves develops early and fades late, it is commonly known as "leaf peony". Its ornamental characteristics are well maintained even at −5 °C conditions (Liu et al. 2012), so it becomes an important flowerbed material in late autumn, winter and early spring of subtropical regions.

In this work, we wanted to obtain miniature kales which occupy a small area and have better ornamental value. From previous studies, we found that BR/GA biosynthesis or signaling genes could affect the height of plants, and the *SP/CEN/TFL1* family genes could control the development of inflorescence, which affected the height and proportions of plants. Silencing only one dwarf-related gene may not attain our desired degree of dwarfing, and jointly silencing the combination of genes that have a similar function may achieve better results. Thus, we constructed an RNAi expression vector jointly targeting *BoDWARF*, *BoGA20ox* and *BoSP*, which belong to the BR/GA biosynthesis and *SP/CEN/TFL1* family, respectively, and transformed it into kale plants. As we expected, miniaturized kales were obtained. This study provides a theoretical and technical foundation for future molecular breeding of ornamental plants.

## Materials and methods

### Plant materials and growth conditions

In this study, we used Beijing Red No. 1, a round-leaf kale variety, as the test material. For tissue culture, the kale seeds were washed with 70 % ethanol solution for 1 min and rinsed three times with sterile water, then washed in 1 % sodium hypochlorite solution for 12–15 min, rinsed eight times with sterile water, sowed on 1/2 MS solid agar medium (pH 5.8) under the conditions of illumination intensity of 1,000–2,000 lux 16 h/day, 25 ± 2 °C and 8 h/day of darkness, 18 ± 2 °C. On September 20, 2012, wild-type and transgenic plants with four leaves were transplanted into plastic pots, moved to the experimental field on 25 October, and managed routinely.

### Isolation of *BoDWARF*, *BoSP* and *BoGA20ox* gene fragments from kale and sequence analysis

Total RNA of kale was extracted using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Then 1 μg total RNA was used to synthesize first-strand cDNA through reverse transcription polymerase chain reaction (PCR; M-MLV reverse transcriptase, Takara, Dalian, China) with Oligo $d(T)_{18}$ primer (5′ TTT TTT TTT TTT TTT TTT 3′). 1–2 μL cDNA was used to clone the fragments of *BoDWARF*, *BoSP* and *BoGA20ox* genes with primer pairs (BoDWARF-F, BoDWARF-R), (BoSP-F, BoSP-R) and (BoGA20ox-F, BoGA20ox-R) (Table 1S) through high fidelity PCR (Prime STARTM HS DNA polymerase, Takara). The amplified products were tailed using DNA A-Tailing kit (Takara) and linked into pMD18-T vector (Takara). Positive clones were selected via *Escherichia coli* JM109 transformation and confirmed by sequencing (Invitrogen, Shanghai, China). Multiple sequence alignments were performed by DNAman version 5.2.2. The phylogenic tree was calculated by MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 (Kumar et al. 2004).

### Construction of *BoDWARF*, *BoSP* and *BoGA20ox* RNAi vector

In order to silence all three genes simultaneously, we first connected the above three specific gene fragments

together: the above plasmid SP::pMD18-T was digested by *Sal*I and *Eco*RI, then the *BoSP* gene fragment was purified and ligated into plasmid DWARF::pMD18-T double-digested by *Xho*I and *Eco*RI to obtain the recombinant plasmid SP/DF::pMD18-T. The above amplified fragment of *BoGA20ox* gene was linked into pGEM-T Easy vector (Promega), then digested by *Spe*I and *Eco*RI to obtain the *BoGA20ox* gene fragment, subsequently linked into plasmid SP/DF::pMD18-T double-digested by *Xba*I and *Eco*RI to obtain the recombinant plasmid SP/DF/GA20ox::pMD18-T, which includes the *BoSP*, *BoDWARF* and *BoGA20ox* gene fusion fragment. Subsequently, the three-gene fusion fragment was amplified from plasmid SP/DF/GA20ox::pMD18-T with DWARF (F + EX) and GA20ox (F + KB) primers (Table 1S), and double-digested with *Bam*HI/*Xba*I and *Kpn*I/*Eco*RI respectively, and linked into pHANNIBAL plasmid at *Bam*HI/*Xba*I restriction sites in the sense orientation and at *Kpn*I/*Eco*RI restriction sites in the antisense orientation. Finally, the double-stranded (ds) RNA expression unit, containing the cauliflower mosaic virus (CaMV) 35S promoter, *BoDWARF/SP/GA20ox* fragment in antisense orientation, *PDK* intron, *BoDWARF/SP/GA20ox* fragment in sense orientation and OCS terminator, was purified and linked into the plant binary vector pBIN19 with *Sac*I and *Xba*I restriction sites.

### Plant transformation of *BoDWARF/SP/GA20ox* RNAi vector

The resulted binary vector was transformed into *Agrobacterium* LBA4404 strain following the protocols described by Chen et al. (2004). *Agrobacterium*-mediated transformation was performed as follows: 7–10-day fully expanded (leaves not grown true) bands shank cotyledons were cut and cultured on the pre-culture medium (MS solid medium containing 1 mg/L 6-BA and 1 mg/L 2,4-D, pH 5.8) at 25 ± 2 °C under dark conditions for 48 h, then soaked in *Agrobacterium* bacteria solution for 15 min. Excess broth was sucked away by sterile absorbent paper, and culture was performed on co-culture medium (MS solid medium containing 1 mg/L 6-BA and 1 mg/L 2,4-D, pH 5.8), at 25 ± 2 °C under dark conditions for 48 h. After washing with sterile water, the explants were transferred to the bud-inducing medium (MS solid medium containing 4 mg/L 6-BA, 2 mg/L ZT, 5 mg/L $AgNO_3$, 500 mg/L Carb and 20 mg/L Kan, pH 5.8), and cultured in conditions of illumination intensity of 1,000–2,000 lux 16 h/day, 25 ± 2 °C and 8 h/day of darkness, 18 ± 2 °C, with the medium replaced every 3 weeks until green shoots grew to 2–3 cm. The shoots were then transferred to rooting medium (1/2 MS solid medium containing 0.2 mg/L NAA, 250 mg/L Carb and 5 mg/L Kan, pH 5.8) and cultured under the same condition of illumination and temperature. The transgenic plants were detected with primers NPTII-F (5′ GAC AAT CGG CTG CTC TGA 3′) and NPTII-R (5′ AAC TCC AGC ATG AGA TCC 3′).

### Quantitative real-time PCR analysis

RNA extraction and cDNA synthesis were performed as described above. The synthesized cDNAs were diluted twice with RNase/DNase-free water. Quantitative real-time PCR analysis was carried out using the CFX96™ Real-Time System (C1000™ Thermal Cycler, Bio-Rad). All reactions were performed using the SYBR® Premix Ex Taq II kit (Takara, China) in a 10-μL total sample volume (5.0 μL 2× SYBR Premix Ex Taq, 1.0 μL primers, 1.0 μL cDNA, 3.0 μL $ddH_2O$). To remove the effect of genomic DNA and the template from the environment, NTC (no template control) and NRT (no reverse transcription control) were performed. Three replications for each sample were done and standard curves were run simultaneously. Melting curve analysis of qPCR samples revealed that there was only one product for each gene primer reaction. The PCR products were sequenced to confirm the specific amplification. The expression of *ACTIN* (Zhang et al. 2012) and other two candidate housekeeping genes, *EF-1-α* (elongation-factor-1-α) and *Apr* (adenine phosphoribosyltransferase; Qi et al. 2010), were studied in different tissues of kale. Based on our results shown in Table 2S, *BoACTIN* gene was used as internal standard in tissues. The primers BoDWARF (RT)-F/BoDWARF (RT)-R, BoSP (RT)-F/BoSP (RT)-R and BoGA20ox (RT)-F/BoGA20ox (RT)-R (Table 1S) were used to determine the expression levels of *BoDWARF*, *BoSP* and *BoGA20ox* in wild-type and transgenic lines. Furthermore, the expression levels of GAs, BRs, auxin and inflorescence-related genes, such as *PRE1* (Paclobutrazol-resistant), *XTH5* (Xyloglucan endotransglucosylase/

hydrolase), *TCH4* (For touch, *XTH-22*), *PIF4* (PHYTOCHROME-INTERACTING FACTORs), *EXP1* (Expansion), *IAA3* (A member of the auxin-induced Aux/IAA family), *IAA19*, *SOC1* (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1) and *AP1* (APETALA1), were detected simultaneously. Primers are shown in Table 1S. The calculation method of relative gene expression was 2 (−Delta Delta C(T)).

### Measurement of parameters of plant architecture

In order to understand the differences between wild-type and transgenic lines, we measured the height, diameter, internode number and internode length of stem, width and length of mature leaves (4–5 circles from top), internode number, internode length and length of flower bolting, and length of root from wild-type and transgenic plants. Ten mature leaves of each plant were measured for width and length, ten biological repeats of each line were measured for length of root and six biological repeats of each line were measured for other parameters. Speed of bolting and stem growth of wild-type and transgenic lines were also counted. After the start of bolting, the bolting height was measured once every 2 days. For stem growth, stem height was measured 60 days after colonization, once every 10 days.

### Quantification of brassinosteroid and gibberellin contents

One-step double-antibody sandwich method enzyme-linked immunosorbent assay (ELISA) was used to detect the concentration of endogenous BR and GA in mature leaves and flower buds of wild-type and transgenic lines. For BR extraction, 0.5 g sample was ground in a mortar and homogenized in 5 mL extraction solution (isopropyl alcohol). Extracts were centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatant was analyzed by ELISA (Chai et al. 2013). For extraction of GA, 0.5 g sample was ground in an ice-cooled mortar in 5 mL 80 % (v/v) methanol extraction medium. The extract was incubated at 4 °C for 4 h and centrifuged at 4,000 rpm for 15 min at the same temperature. The supernatant was also analyzed by ELISA. The BR and GA ELISA experiments were carried out using the same procedure according to the manufacturer's instructions (http://www.wksubio.com/product/5794959.html). BR and GA contents were measured at 450 nm using a Bio-Rad680 microplate reader. The results are the means of three replicates. The correlation coefficients ($R^2$) of BR and GA standard curves were 0.997 and 0.991, respectively. The concentration of each sample was calculated from the linear regression equations.

### Measurement of chlorophyll contents

One gram of fresh mature leaves was ground to pieces in liquid nitrogen, extracted with 10 mL mixed solution of acetone and ethanol (2:1, v/v) for 48 h in the dark, and centrifuged at 5,000 rpm for 10 min at 4 °C. The absorbance of the supernatant was measured at 645 and 663 nm in a Hitachi UV–vis spectrophotometer using the above-mentioned mixed solution as a blank. Total chlorophyll content was calculated using the formula according to the method of Arnon (1949): $\text{Chl (mg/g)} = 20.29A_{645} + 8.02A_{663}$. The chlorophyll of each sample was extracted and measured in triplicate.

### Statistical analysis

The mean values of data and standard error of the means were calculated from the replicate measurements. Data were analyzed by Origin 8.6 (OriginLab) software, using the *t* test to assess significant differences between the means.

## Results

### *BoDWARF*, *BoSP* and *BoGA20ox* isolation and expression pattern analysis in kale

According to the sequences of *BoDWARF* (Bol032288), *BoGA20ox* (Bol042237) and *BoSP* (Bol026421) in Brassica Database (http://brassicadb.org), the three gene fragments were cloned with specific primer pairs (BoDWARF-F, BoDWARF-R), (BoSP-F, BoSP-R) and (BoGA20ox-F, BoGA20ox-R) (Table 1S), and sequenced. Phylogeny and amino acid analysis showed that BoDWARF belonged to a highly conserved cytochrome P450 family (Supplemental Fig. 1a) and had 88.53 % identity with BrDWARF and AtDWARF (Supplemental Fig. 1b); BoGA20ox belonged to the 2OG-Fe(II) oxygenase superfamily [which contains members of the 2-oxoglutarates

(2OG) and Fe(II)-dependent oxygenases] (Supplemental Fig. 2a) and shared high identity with BrGA20ox, RsGA20ox (*Raphanus sativus*) and AtGA20ox (*Arabidopsis*) (Supplemental Fig. 2b); BoSP belonged to the PEBP (phosphatidyl ethanolamine-binding protein) family (Supplemental Fig. 3a) and had 96.57 % identity with BrSP (Supplemental Fig. 3c).

Quantitative real-time PCR technology was performed to analyze the expression of *BoDWARF*, *BoSP* and *BoGA20ox*. The results showed that these three genes are all highly expressed in young leaf and flower. In case of *BoDWARF* and *BoGA20ox* a higher expression with respect to the other tissues is observed also in lateral roots and germinating seeds, tissues undergoing rapid growth. These results suggest that these three genes play important roles in the growth and development of the plant.

**Fig. 2** Phenotype of *BoSP*, *BoDWARF* and *BoGA20ox*-silenced lines and wild type. **a** *Top* view of phenotype of wild-type and transgenic plants 70 days after colonization. *Bar* = 50 mm. **b** *Top* view of phenotype of wild-type and transgenic plants 55 days after colonization. *Bar* = 50 mm. **c** Phenotype of leaves (4–5 *circles* from *top*) from wild-type and transgenic plants 60 days after colonization. *Bar* = 25 mm. **d** *Side* view of wild-type and transgenic plants 55 days after colonization. *Bar* = 36 mm. **e** Phenotype of wild-type and transgenic plants 140 days after colonization. *Bar* = 40 mm. **f** Root phenotype of wild-type and transgenic plants 140 days after colonization. *Bar* = 60 mm. **g** Phenotype of wild-type and transgenic plants 132 days after colonization. *Bar* = 60 mm. **h** Flower bud phenotype of wild-type and transgenic plants 135 days after colonization. *Bar* = 10 mm. *WT* wild-type. RNAi-2, RNAi-5, RNAi-6 were different transgenic lines

## Generation of transgenic kale plants

An RNAi construct targeting the specific fragment of *BoSP*, *BoDWARF* and *BoGA20ox* was created and

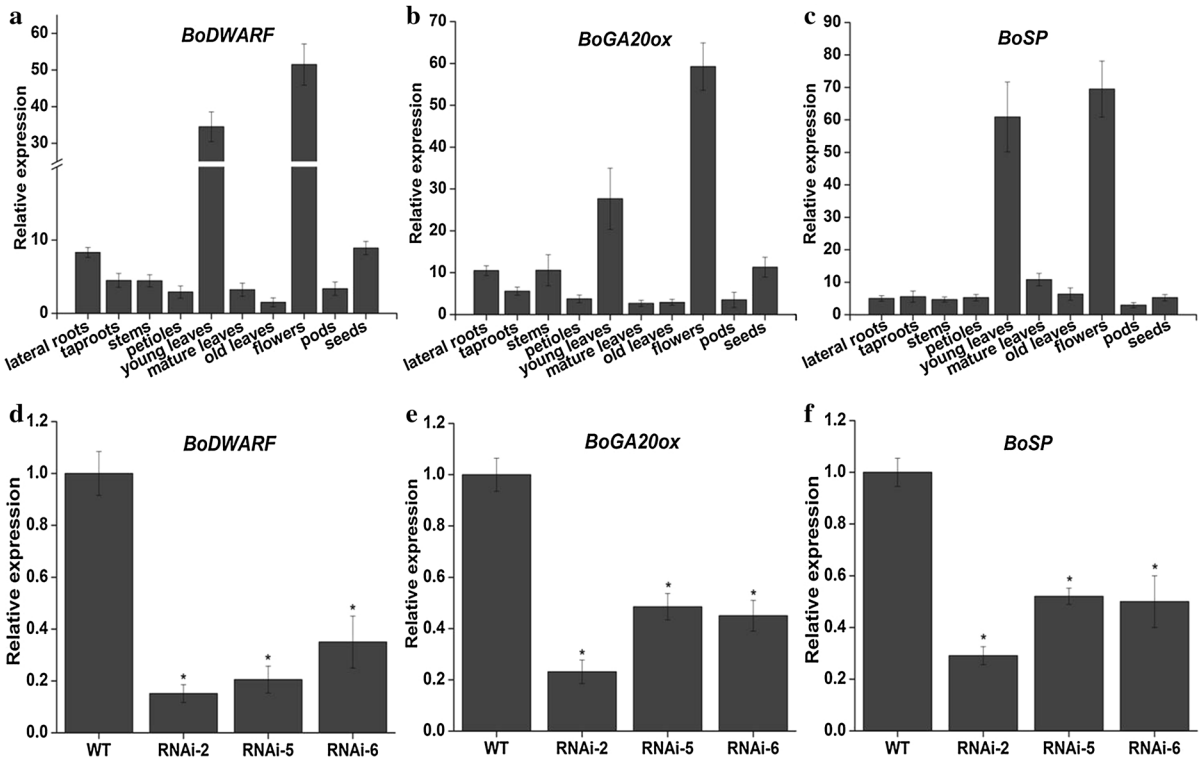
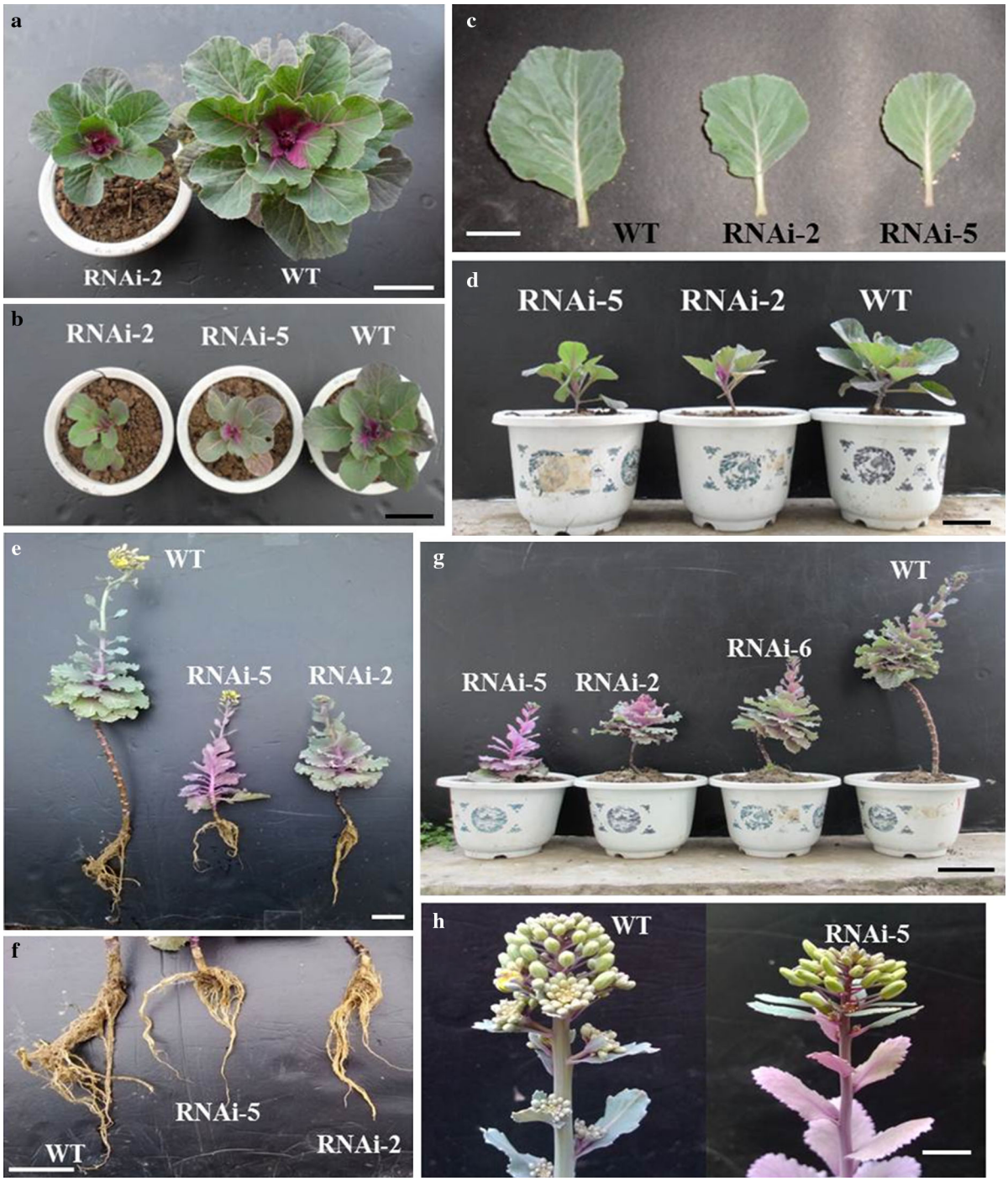



**Fig. 1** Expression pattern and silencing effects of *BoDWARF*, *BoGA20ox* and *BoSP* genes in wild-type kale and transgenic lines. Mean relative expression of *BoDWARF* (**a**), *BoGA20ox* (**b**), *BoSP* (**c**) in different kale tissues. Silencing effects of *BoDWARF* (**d**), *BoGA20ox* (**e**), *BoSP* (**f**) in different transgenic lines. *WT* wild type. RNAi-2, RNAi-5 and RNAi-6 were different transgenic lines. qRT-PCR analysis for *BoDWARF*, *BoSP* and *BoGA20ox* genes was performed on 4-month-old kale plants. Relative expression was normalized using a housekeeping gene *BoACTIN* (AF044573). The data represent mean from three replicates with two biological repeats. *Asterisks* indicate $P < 0.05$ between the wild type and others by *t* test. *Error bars* indicate SE. The expression levels of genes in the wild type were set to 1




transformed into wild-type plants Beijing Red NO.1 via *Agrobacterium*-mediated T-DNA transformation. Four independent transgenic lines were confirmed to be positive transgenic lines by PCR analysis using specific primers for the reporter gene *NPTII*. Quantitative real-time PCR results showed that transcripts of *BoSP*, *BoDWARF* and *BoGA20ox* were reduced by 70–82 % in transgenic lines compared with wild type, and the inhibitory effect of *BoDWARF* was the highest (Fig. 1d–f).

### Transgenic plants show smaller plant architecture

During the process of plant development, transgenic plants displayed a slower growth rate than the wild type, and their size was smaller than the wild type at the same time (Fig. 2a–e, g). To quantify the differences between wild-type and transgenic lines, we measured the height, diameter, internode number and internode length of stems, width and length of mature leaves (4–5 circles from top), and height, internode number and internode length of the flower stalks, respectively. Figure 3 showed that transgenic plants had shorter and thinner stems, smaller leaves and shorter flower stalks.

### Transgenic plants exhibit delayed bolting and flowering time and inhibited growth of roots

Under the same conditions, wild-type plants started bolting 120 days after field planting, 7 days later than the transgenic lines (Table 1). The mean time of the first flower of wild type was 134.5 days after field planting, and transgenic lines flowered after about 140–142 days under the same conditions (Table 1). Speed of bolting and stem growth of transgenic lines were also slower than wild type (Fig. 3k, l). To determine whether the development of roots in transgenic lines was affected, we observed the morphology of roots of mature plants. We found that wild-type plants had stronger and more taproots and lateral roots than transgenic lines (Fig. 2e, f).

### Gibberellin and brassinosteroid contents in transgenic plants were significantly decreased

In order to explore the underlying reason for the smaller shape of transgenic plants, endogenous gibberellin and brassinosteroid contents were determined in mature leaves and flower buds using an enzyme-linked immunosorbent assay. The results showed that the two plant hormone contents were significantly decreased in transgenic plants; in particular the brassinosteroid content declined by half in mature leaves (Fig. 3m, n).

### Chlorophyll content in transgenic plants was increased

In our study, transgenic kales had dark-green leaves. Total chlorophyll was extracted from leaf disks. Figure 3o shows that the transgenic lines contained approximately 10–15 % more chlorophyll than the wild type.

### Expressions of gibberellin-, brassinosteroid- and flowering-related genes were significantly affected in transgenic plants

To further characterize the molecular mechanism of growth rate and flowering of transgenic plants, a set of GA-, BR- and flowering-related genes were examined. Paclobutrazol-resistant family helix–loop–helix (HLH) factor, *PRE1*, is induced by GAs and BRs, and promotes cell elongation (Oh et al. 2012; Bai et al. 2012; Lee et al. 2006). The clock-controlled *PIF4* encoding a basic helix–loop–helix (bHLH) transcription factor plays crucial roles in regulating plant architecture in *Arabidopsis thaliana* (Oh et al. 2012; Nomoto et al. 2012a, b). Xyloglucan is one of the primary structural components of the plant cell wall. Therefore, the function of *XTH*s may affect cell shape and plant morphogenesis. *TCH4* and *XTH5* encoding xyloglucan endotransglucosylase/hydrolases, regulated by GAs and BRs, affect the recruitment of cell wall-modifying activity in response to environmental stress and growth (Xu et al. 1995; Campbell and Braam 1998; Iliev et al. 2002). Another plant cell wall expansion protein *EXP1*, regulated by GAs and BRs, unlocks the network of polysaccharides, permitting turgor-driven cell enlargement (Cosgrove 1998, 2000). In our study, except for *XTH5*, whose transcript showed a slight decrease in the stem of transgenic lines, the transcripts of *PRE1*, *PIF4*, *TCH4* and *EXP1* were all reduced by approximately half in leaves and stems of transgenic lines (Fig. 4a–e). These results indicated that the decreased expression of cell wall expansion and cell elongation genes in jointly silenced lines might affect plant cell elongation and development. Furthermore, SOC1, a MADS-box protein, plays an essential role in integrating multiple flowering signals to regulate the transition from vegetative to reproductive development (Moon et al. 2003). *AP1* is a floral meristem identity gene which is required for the transition of an inflorescence meristem into a floral meristem and is regulated by flowering signal (Mandel et al. 1992; Wilson et al. 1992). The two flowering-related genes were markedly down-regulated in jointly-silenced lines, and their transcripts showed an approximately 50 % decrease in leaves and 40 %

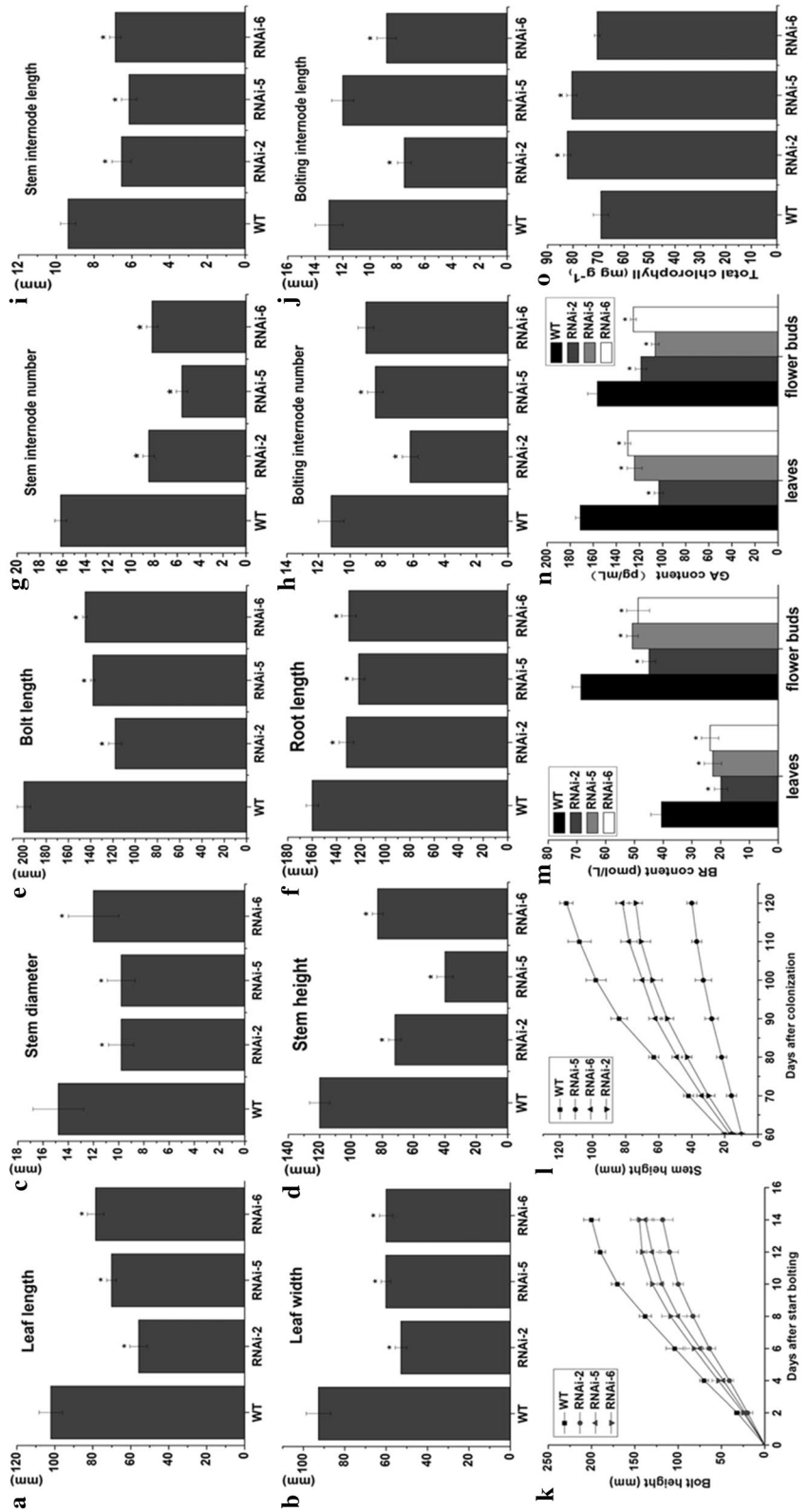



**Fig. 3** Growth and physiological index detection of wild-type and transgenic plants. Length (**a**) and width (**b**) of mature leaves (4–5 *circles* from *top*) (mm), $n = 10$. Diameter (**c**) and height (**d**) of stems (mm), $n = 6$. **e** Length of flower stalks (mm), $n = 6$. **f** Length of roots (mm), $n = 10$. Internode number of stems (**g**) and flower stalks (**h**) and internode length of stem (**i**) and flower stalks (**j**), $n = 6$. Bolting speed (**k**) and stem growth speed (**l**), $n = 6$. BR content (**m**) and GA content (**n**) in leaves and flower buds. **o** Total chlorophyll content in mature leaves. *WT*, wild type; RNAi-2, RNAi-5 and RNAi-6 are different transgenic lines. The data represent means from three replicates with two biological repeats. *Asterisks* indicate $P < 0.05$ between the wild type and others by *t* test. *Error bars* indicate SE

**Table 1** Time from field planting to start of bolting and first flowering for wild-type and transgenic lines ($n = 6$)

| Kale lines | Start bolting (days) | First flowering (days) |
|---|---|---|
| Wild type | 120.2 ± 0.50 | 134.5 ± 0.45 |
| RNAi-2 | 127.2 ± 0.45 | 142.8 ± 0.85 |
| RNAi-5 | 126.5 ± 0.48 | 140.3 ± 0.50 |
| RNAi-6 | 127.6 ± 0.38 | 142.5 ± 0.60 |

decrease in flower buds (Fig. 4f, g). In addition, BR- and auxin-regulated genes *IAA3* and *IAA19* were also dramatically down-regulated in transgenic lines: *IAA3* showed a 40 % decline and *IAA19* showed a 60 % decline in transcript level in leaves, and the transcripts of the two genes declined by 25 % in roots (Fig. 4h, i). These results suggested that down-regulated flowering-related and development-related genes in the

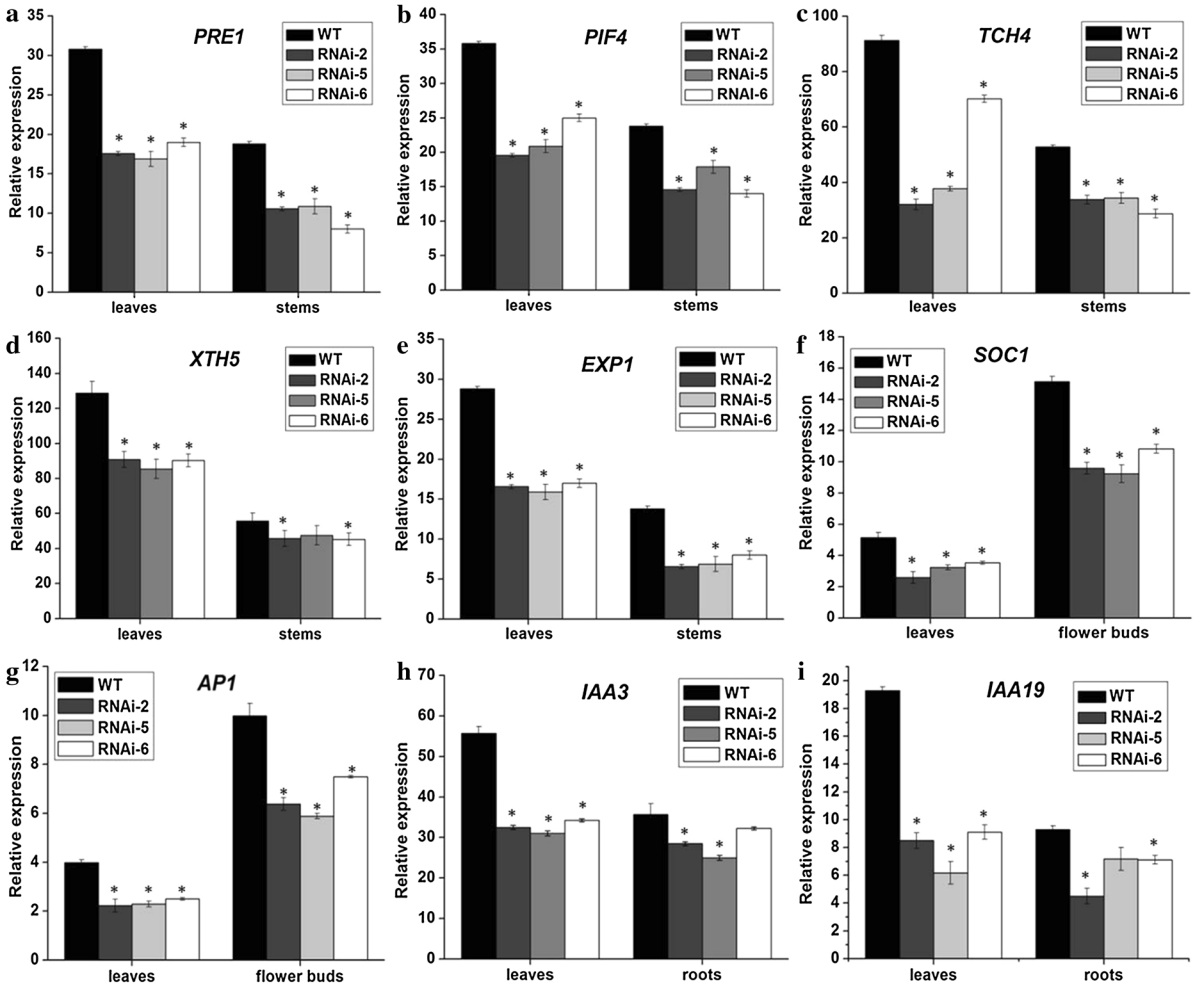



**Fig. 4** The detection results of brassinolide-, gibberellin- and flowering-related genes in wild-type and transgenic lines. Mean relative expression of *PRE1* (**a**), *PIF4* (**b**), *TCH4* (**c**), *XTH5* (**d**) and *EXP1* (**e**) in leaves and stems. Mean relative expression of *SOC1* (**f**) and *AP1* (**g**) in leaves and flower buds. Mean relative expression of *IAA3* (**h**) and *IAA19* (**i**) in leaves and roots. *WT*, wild type; RNAi-2, RNAi-5 and RNAi-6 are different transgenic lines. The data represent the means from three replicates with two biological repeats. *Asterisks* indicate $P < 0.05$ between the wild type and others by *t* test. *Error bars* indicate SE

transgenic lines might affect the growth, root development and flowering of plants.

## Discussion

In our study, we focused on miniaturization of ornamental plants. Through transgenic technology, we obtained a smaller plant architecture in ornamental kale. This study indicates that jointly silencing similar dwarfing genes is a feasible method of achieving miniaturization of plants, and lays the theoretical and technical foundation for molecular breeding of miniaturized flowers or ornamental plants in the future.

### An optimized transformation system of kale is established

Ornamental kale (*Brassica oleracea* var. *acephala* f. tricolor) is an edible, freezing-tolerant and excellent landscape plant for its varied and beautiful leaves. To improve its ornamental traits, transgenic technology has become more effective than traditional breeding methods. To date, many studies on tissue culture of kale have been done (XiuShu et al. 2009; Hosoki et al. 1989; Meng et al. 2005; Zhao et al. 2004), and genetic transformation of kale has also been studied (Cao et al. 2005; Hosoki et al. 1989; Poulsen 1996; De Block et al. 1989; David and Tempé 1988). In previous studies, many cultivars of kale were used, and Walcheria and Andersen have been used as materials. These results indicated that the genotype of kale was an important factor affecting the transformation efficiency. In this study, a new Chinese variety Beijing Red No. 1 was used as the material, and factors affecting the adventitious shoot regeneration in vitro were studied in detail. Different explants of kale were cultured on the media, and we found that, whatever the culture medium, cotyledon with petiole was the best explant. Phytohormone combinations and addition of $AgNO_3$ to the shoot regeneration medium were examined. Hormone combination with 6-BA 4 mg/L + ZT 2 mg/L was the best. The addition of 2 mg/L $AgNO_3$ to the medium could greatly increase the frequency of shoot regeneration. The optimum rooting medium was MS + NAA 0.1 mg/L + Carb 200 mg/L + Kan 5–15 mg/L. The transformation rate was 3.6 %.

### Reduction of GA and BR content slowed the growth rate and affected the morphological development of *BoSP–BoDWARF–BoGA20ox* silenced plants

To date, the functions of GA and BR associated with plant growth and development have been investigated intensively. Gibberellin is mainly concentrated on promoting the extension of stem, germination, seed dormancy, flowering, gender performance, root development, and aging suppression of leaf and fruit (Tyler et al. 2004; Kende and Lang 1964; Olszewski et al. 2002; Fu and Harberd 2003; Groot et al. 1987). In addition, BR plays important regulatory roles in seed dormancy and germination, organ differentiation, vascular tissue development, flowering and senescence, morphogenesis and other various important growth and development processes (Topping et al. 1997; Souter et al. 2002; Schlagnhaufer and Arteca 1985; Diener et al. 2000). The two plant hormones are involved in many aspects of plant growth and development, which necessarily require precise regulation of GA and BR levels in the plant body.

In this study, *BoSP–BoDWARF–BoGA20ox*-silenced plants showed slower growth speed and smaller size than wild type. To reveal the molecular mechanism of this phenotype, a set of GA- and BR-related genes that mainly focused on promoting cell elongation, such as *PRE1*, *PIF4*, *TCH4*, *XTH5* and *EXP1*, were detected in wild-type and transgenic lines. These genes were all significantly down-regulated in transgenic lines (Fig. 4a–e). In addition, the BR and GA contents of transgenic and wild-type kale plants were determined by ELISA. The two plant hormone contents were markedly decreased in transgenic plants, especially BR content in mature leaves. These results indicate that jointly silencing *BoSP*, *BoDWARF* and *BoGA20ox* in kale reduced the GA and BR levels in the plant, and down-regulated the expression of cell wall expansion and cell elongation genes, subsequently affecting plant cell elongation to produce smaller plants with slower growth speed.

### *BoSP–BoDWARF–BoGA20ox* silencing affected the flowering and root growth and chlorophyll content of the plant

In this study, transgenic plants showed a late flowering phenotype (Fig. 2e, h). In view of previous reports that

in *Arabidopsis* the flowering time of GA-biosynthetic and GA-signaling mutants were highly correlated with the expression level of *SOC1*, the *soc1* null mutant showed reduced sensitivity to GA for flowering, while overexpression of *SOC1* restored the non-flowering phenotype of *ga1-3*, which confirmed that the GA pathway provided a positive factor for *SOC1* activation (Moon et al. 2003). Moreover, *Arabidopsis* mutant *dd1* (*dwarf and delayed-flowering 1*) showed dwarfism and late flowering and was deficient in gibberellin biosynthesis, and the phenotype was restored by exogenous $GA_3$ (Magome et al. 2004). We detected the expression level of this flowering-related gene in the flower buds. The results showed that *SOC1* was markedly down-regulated in the jointly-silenced lines, unlike the other flowering-related gene *AP1* (Fig. 4f, g). Judging from these results, silencing of the GA-biosynthetic gene *GA20ox* results in reduced GA content in the flower buds, thereby down-regulating the expression level of *SOC1* and *AP1*, and perhaps eventually leading to delayed flowering of transgenic plants. We also note that many *Arabidopsis* BR synthesis and signaling pathway mutants showed extension of nutritional growth and delayed flowering time. For instance, under the same growth conditions, BR synthesis mutants *det2* (Fujioka et al. 1997), *dwf4* (Azpiroz et al. 1998), *cpd* (Li and Chory 1997) and BR-insensitive mutant *bri1* (Domagalska et al. 2007) all showed late-flowering phenotype. Studies have found that the endogenous BR content of the *det2* mutant was 10 % that of the wild type, and that *cpd*, *dwf4* and *bri1* mutants accumulated different BR precursors in vivo (Szekeres et al. 1996; Azpiroz et al. 1998; Noguchi et al. 1999). These data show that endogenous BR content and change of BR signal transduction were involved in flowering time regulation. Therefore, in our study, delayed flowering of transgenic lines may be due to reduced brassinolide content in the flower buds. In addition, the *SELF PRUNING* (*SP*) gene controls the regularity of the vegetative-reproductive switch along the compound shoot of tomato, which is homologous to *CENTRORA DIALIS* (*CEN*) from *Antirrhinum* and *TERMINAL FLOWER 1* (*TFL1*) from *Arabidopsis* (Carmel-Goren et al. 2003). *SP* has no effect on the architecture of inflorescence or on the timing of the first flower. However, by conferring early termination of the vegetative meristems in the sympodial shoot, its overall effect on plant architecture is much more substantial. The mutation of *SP* leads to the formation of the top flower of plant and miniature tomato phenotype (Martí et al. 2006). Overexpression of *SP* results in an increasing number of leaves between inflorescences and an increased leafiness of the inflorescence shoot itself (Pnueli et al. 1998). In this study, *BoSP* is the orthologue of *AtTFL*, which maintains the growth of stem apical meristem and inhibits the formation of floral meristem (Bradley et al. 1997).

Ectopic expression of *TFL1* in *Arabidopsis* caused both the vegetative and reproductive phases to be greatly extended, and the activity of the floral meristem identity gene *APETALA 1*, which was not directly inhibited by *TFL1*, was up-regulated compared to wild-type controls. However, these phenotypic and molecular effects complement those observed in the *tfl1* mutant, where all phases are shortened (Ratcliffe et al. 1998). Moreover, studies have shown that *TFL1* can indirectly inhibit *AP1* gene expression, thereby inhibiting the formation of floral meristem (Ahn et al. 2006; Danilevskaya et al. 2008; Liljegren et al. 1999). According to the above findings, we infer that silencing of the *BoSP* gene may generate early flowering plants, and thus stop the vegetative growth, finally lead to dwarf plant architecture, but this is opposite to the results we have observed where transgenic plants exhibited a late-flowering phenotype. A possible reason might be that the effect of *SP* silencing was not very strong, and the two hormones, BR and GA, could delay flowering, thus obscuring the effect of *SP* gene silencing. The reduced expression of the downstream gene *AP1* may be a similar reason. In addition, we observed that transgenic roots were shorter than the wild type (Fig. 2e, f). Previous experiments proved that BR regulates hypocotyl and root growth and development of dicots. The regulation was closely related to polar transport of auxin in the roots (Bao et al. 2004; Xu et al. 2006). BR- and auxin-regulated genes, *IAA3* and *IAA19*, which promote auxin-regulated root development and cell elongation (Nakamura et al. 2003; Vandenbussche et al. 2013), were detected in the transgenic roots. The results showed that the expression levels of the two genes were noticeably lower in joint RNAi lines than in wild type (Fig. 4h, i). These results indicated that reduced BR content in transgenic plants might affect the transport of auxin in roots, thus causing shorter roots. Moreover, jointly

silenced kales contained approximately 10–15 % more chlorophyll than wild-type plants (Fig. 3o). This phenotype was observed in GA-insensitive or GA-deficient mutants of *Arabidopsis*, which had high levels of chlorophyll (Koorneef et al. 1985).

## Simultaneous silencing of *BoSP*, *BoDWARF* and *BoGA20ox* genes is an effective way to achieve miniaturization of kale

Currently, dwarfing genes are concentrated in the GA, BR, auxin biosynthesis and signal transduction, and light signaling pathways (Ross et al. 2001; Yin et al. 2007). Genes of cytokinins and ethylene synthesis and signaling pathways (De Grauwe et al. 2005) may also become important factors for research into ornamental plant miniaturization. Moreover, cell wall synthesis and metabolism, temperature and other environmental factors, DNA methylation (Finnegan et al. 1996), microRNA (Achard et al. 2004) and transcription factors are important regulatory elements for plant growth and development. In this study, BR/GA biosynthesis genes, *BoDWARF* and *BoGA20ox*, and the flowering-related gene *BoSP*, were selected to construct an RNAi vector. Through transgenic technology, simultaneously silenced kale plants were produced and exhibited a dwarf phenotype. This result indicates that jointly silencing *BoSP*, *BoDWARF* and *BoGA20ox* genes is a feasible method to miniaturize kale plants.

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