RESEARCH ARTICLE

Ectopic expression of *liSHP2* from *Isatis indigotica* Fortune, a PLE-lineage MADS-box gene, influences leaf, floral organ and silique morphology in *Arabidopsis thaliana*

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Abstract In order to ascertain the regulatory mechanism of fruit development in *Isatis indigotica* Fortune, the complementary DNA (cDNA) sequence of the *SHATTERPROOF 2* (*SHP2*) orthologous gene was identified by Rapid Amplification of cDNA Ends technology and the corresponding gene was named *liSHP2*. The expression pattern of *liSHP2* was determined by quantitative reverse transcription-polymerase chain reaction and wild-type Col-0 *Arabidopsis* plants were transformed with the *liSHP2* gene using *Agrobacterium tumefaciens* and the floral-dip method. Expression analyses indicated that *liSHP2* was highly expressed in flowers, siliques and seeds. Compared to wild-type plants, *liSHP2* transgenic lines bolted earlier. Detailed phenotypic observations showed that the size of the rosette and cauline leaves in transgenic lines was reduced and the cauline leaves of the transgenic lines were incurved and displayed a funnel-like shape. During the reproductive growth stage, *liSHP2* transgenic plants produced shortened sepals and the flower buds were not encapsulated completely. Moreover, the petals of the transgenic lines were converted into staminateous tissues, accompanied by exposed stamens, short malformed

siliques and wrinkled valves, indicating a severe decline in fertility. These experimental conclusions are valuable as a reference for the breeding of medicinal plants.

Keywords *Isatis indigotica* Fortune · *liSHP2* · MADS-box gene · Floral differentiation · Silique development

Introduction

As one of the evidence types in taxonomy, the flower is the most complicated structure arising in the process of plant growth (APG 2003). The floral transition is composed of three stages, including formation of inflorescence meristems, production of floral meristems and genesis of floral organs, and is highly regulated by the genetic background of plants and environmental factors (Fornara et al. 2010). In research about auto-regulation signals of plants, it was found that the transcriptional factors of MADS (MCM1, AGAMOUS, DEFICIENS and SRF) family played important roles in the floral transition process. The transcriptional factors of MADS family were classified into two categories: Type I and Type II. Up to now, most reports about the plant proteins in this family are related to the Type II MADS transcriptional factors containing M, I, K and C functional domains.

SHATTERPROOF (*SHP*) genes are members of the MADS-box family. In the model plant *Arabidopsis*, two *SHP* genes were identified. They were named as *SHATTERPROOF 1* (*SHP1*) and *SHATTERPROOF 2* (*SHP2*), and were formerly known as *AGL1* (*AGAMOUS-LIKE 1*) and *AGL5* (*AGAMOUS-LIKE 5*). The ABCDE model of flowers has confirmed that *AG* (*AGAMOUS*), *SHP1* and *SHP2* are C-class floral homeotic genes, while *STK* (*AGAMOUS-LIKE 11*, *SEEDSTICK*) is a D-class floral

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homeotic gene (Kater et al. 2006). Both the euAG-lineage and the PLE-lineage of floral homeotic genes are derived from C-class precursor genes. *SHP1* and *SHP2* belong to PLE-lineage (Kramer et al. 2004).

In the stage of flowering, *SHP1* and *SHP2* are mainly involved in regulation of the development of pistil, carpels, ovaries and ovules. They are also the important transcriptional factors in regulation of silique dehiscence in the stage of ripeness (Liljegren et al. 2000). Moreover, *SHP1* and *SHP2* act redundantly to specify the valve margin and are known as the markers of this region. The siliques of *shp1 shp2* double mutant lack the dehiscence zone and are indehiscent. By constitutive expression of *SHP1* and *SHP2*, it was confirmed that these two genes were responsible for the differentiation of dehiscence zone and the lignification of the adjacent cells (Liljegren et al. 2000). Previous research indicated that *STK*, *SHP1* and *SHP2* were responsible for the development of ovules (Pinyopich et al. 2003; Battaglia et al. 2006). In the *stk shp1 shp2* triple mutant, integuments are converted into carpelloid tissues and ovules are transformed into leaf-like or carpel-like structures, illustrating that *SHP2* is also associated with controlling ovule identity (Brambilla et al. 2007). Overexpression of any one of these three genes would result in production of ovules on sepals (Ferrándiz et al. 2000; Battaglia et al. 2006). At the same time, *SHP1* and *SHP2* were found to be involved in the regulation of mucilage production in seeds. *shp1 shp2* seeds generated a thinner mucilage layer with an uneven distribution of pectins (Ehlers et al. 2016).

The biological functions of *SHP* homologous genes are conserved. *BnSHP1*, *BnSHP2a* and *BnSHP2b* are the *SHP* homologous genes identified in oilseed rape. When the expression level of *BnSHP1* was downregulated by RNA interference technology, the pods of oilseed rape showed an indehiscent phenotype (Kord et al. 2015). Overexpression of some D-class genes also produced phenotypes similar to overexpression of *SHP1* and *SHP2* in transgenic *Arabidopsis thaliana*, including *PeMADS7* cloned from *Phalaenopsis equestris*, *BdMADS2* and *BdMADS4* cloned from *Brachypodium distachyon* (Chen et al. 2012; Wei et al. 2013). These results demonstrate partial functional redundancy of C-class and D-class genes. However, the *SHP* homologous genes of different plant species also exhibit obvious variations in function, which might be seen as the result of the influences of geographical conditions and environmental factors on evolution.

Woad (*Isatis indigotica* Fortune) is a biennial herb and is distributed in many places of China. It possesses important medicinal value and its efficacies include heat-clearing, detoxifying, blood-cooling and sore-throat relieving. As a cruciferous plant species, woad produces terminal racemes and its flowers contain four intact whorls

of floral organs, including four sepals, four petals, six stamens (tetradynamous stamens) and one pistil. After pollination, the pistils develop into fruits. The fruits of woad are oval-shaped, laterally compressed, winged, and indehiscent after maturation, and are referred to as silicles in taxonomy. Owing to the higher medicinal value, protection and innovation of the germplasm resources by cross breeding or genetic improvement to woad are of great significance. Compared to model plant *Arabidopsis*, woad has a longer growth cycle and its requirements for growth conditions are more complex. Woad is usually planted in the spring of the first year. After vernalization by the low temperature in the winter, woad plants would flower and bear fruits in the next year.

At present, research about identification of gene function in woad is concentrated on the genes related to the synthesis of medicinal components, and similar research on MADS-box genes in woad are very few. Our laboratory has isolated the coding sequence, analyzed the expression patterns and determined the functions of *FRUITFULL* of *I. indigotica* (*IiFUL*) (Ma et al. 2017). In order to further investigate the function of woad genes in MADS-box family, the coding sequence of *SHATTERPROOF 2* of *I. indigotica* (*IiSHP2*) was isolated with the inflorescences as materials in the present work. Subsequently, analyses of bioinformatic features, expression patterns and biological functions were carried out. The results of this work can provide references for research about the regulatory network of woad genes associated with the development of flowers and fruits.

Materials and methods

Plant materials

The flowers of biennial woad plants grown in greenhouse were used as materials and were stored in -80°C freezer after quick freezing with liquid nitrogen.

Identification of the cDNA sequence of *IiSHP2* and bioinformatics analysis

In previous works of our laboratory, degenerate primers were designed according to the coding sequences of the MADS domains in the MADS transcriptional factors of other plant species and a number of the conserved fragments of the MADS-box family genes in woad were amplified (Ma et al. 2017). Sequencing results showed that one fragment was closely analogous to *SHP2* in *Arabidopsis* and the corresponding gene was named as *IiSHP2*. In the present work, specific primers were designed in accordance with the sequence of the conserved fragment

Table 1 The sequences of the primers used in RACE, quantitative RT-PCR and construction of expression vector

Primer name	Primer sequence (5'→3')
IiSHP2-5'RACE	GCGTCACACAAGACAGAGAGCTCATAAGC
IiSHP2-3'RACE	TGGGTTGATATACATGGAGGGTGGTGCG
qRTIiSHP2(F)	CATCAGTCGTGTCCGCTCCAA
qRTIiSHP2(R)	GCTGCTGTAGTCCCGCTCTTT
qRTIiActin(F)	ACGAGCTACCTGATGGACAAGT
qRTIiActin(R)	TTGAACCACCACTGAGGACGAT
IiSHP2-GFP(F)	CGCCCATGGAGGGTGGTGCGAGTAATGAAG
IiSHP2-GFP(R)	CGCACTAGTAACAAGTTGGAGAGGTGGTTG

(Table 1) and the cDNA (complementary DNA) sequence of *IiSHP2* was ascertained by Rapid Amplification of cDNA Ends (RACE) method. Total RNA was extracted from woad flowers with Trizol reagent (Invitrogen). cDNA used in 5'-RACE or 3'-RACE was prepared with the reverse transcriptase according to the protocol provided by SMARTer RACE cDNA Amplification Kit (Takara). Touchdown PCR (Polymerase Chain Reaction) was carried out with adapter primers and gene specific primers. After separation on a 1.2% agarose gel, the amplification products were ligated with pMD-18T vector (Takara) and competent cells of *Escherichia coli* strain DH5 α were transformed. Positive clones were sequenced after verification by colony PCR. The cDNA sequence of *IiSHP2* was assembled with the sequences obtained in 5'-RACE and 3'-RACE.

Homology searches and multiple alignments of the amino acid sequence encoded by *IiSHP2* were conducted with Protein Basic Local Alignment Search Tool (BLAST) in National Center of Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>). The amino acid sequences of *IiSHP2* and its corresponding orthologous proteins were also aligned with DNAMAN in DNASTar. The phylogenetic tree of MADS-box genes in different species was constructed using the MEGA7 software (Kumar et al. 2016). Molecular weight, isoelectric point and physico-chemical property of *IiSHP2* were analyzed with ProtParam online tools (<https://web.expasy.org/protparam/>).

Analyses of the expression patterns of *IiSHP2*

Total RNA was extracted from root, stem, leaf, inflorescence, flower, sepal, petal, stamen, pistil, silicle, fruit wing, valve and seed with Trizol reagent. The process of silicle development was subdivided into four stages. In the first stage, the fertilized pistil began to grow and no significant shrinkage could be observed in the other three whorls of floral organs. In the second stage, the growing silicles obviously protruded out of other floral organs which were partially withered. In the third stage, silicles elongated evidently and fruit wing grew quickly. In the fourth stage,

the length of the silicles was stable and the colour of the silicles was changed from green to tawny gradually. In analyses of the mRNA abundance of *IiSHP2* in fruit wing, valve and seed, the silicles at the third stage were used as materials. At this stage, other floral organs have fallen off completely from the green silicles.

Fluorogenic quantitative RT-PCR primers, qRTIiSHP2(F) and qRTIiSHP2(R), were designed according to the coding sequence of *IiSHP2* (Table 1). *Actin* (AY870652.1) of woad was used as a reference gene and the primers were qRTIiActin(F) and qRTIiActin(R) (Table 1). Two-step quantitative RT-PCR was carried out with FastStart Essential DNA Green Master Kit (Roche). In the first step, template was denatured at 95 °C for 10 min. In the second step, 40 cycles of 95 °C for 10 s and 60 °C for 30 s were conducted. The data from three biological repetitions were processed with $2^{-\Delta\Delta C_t}$ method. Root was used as the normalization sample. $\Delta\Delta C_t = (C_{tIiSHP2} - C_{tIiActin})_{Sample} - (C_{tIiSHP2} - C_{tIiActin})_{Root}$.

Cloning of the coding sequence of *IiSHP2* and construction of expression vector

pCambia1302 carrying a hygromycin resistance gene was used in construction of plant binary expression vector and DH5 α strain of *E. coli* was used as host in cloning of the coding sequence of *IiSHP2*. In addition, GV3101 strain of *Agrobacterium tumefaciens* was used in genetic transformation of *Arabidopsis*. Fresh woad flowers were ground into powder in liquid nitrogen and total RNA was extracted with Trizol reagent. cDNA was synthesized with reverse transcriptase using Primescript 1st Strand cDNA Synthesis Kit (Takara). Primers were designed according to 5'-terminus and 3'-terminus of the ORF of *IiSHP2*. Restriction site and protective bases were added to the 5'-end of the primers. In detail, the cutting site of *Nco* I was added to the 5'-end of the forward primer IiSHP2-GFP(F) and the cutting site of *Spe* I was added to the 5'-end of the reverse primer IiSHP2-GFP(R). The coding sequence of *IiSHP2* was amplified with high-fidelity PrimeSTAR GXL DNA

Polymerase (Takara) and was ligated into pCambia1302 after restriction digestion. The recombinant plasmid validated by sequencing was introduced into the competent cells of GV3101 strain by freeze-thaw method.

Screening and phenotypic observation of *liSHP2* transgenic *Arabidopsis* lines

The 35S::*liSHP2*-GFP fusion gene was transformed into the genome of wild-type Col-0 *Arabidopsis* by floral dip method (Clough and Bent 1998). The seeds of wild-type Col-0 *Arabidopsis* were scattered on the surface of the well-watered mixed matrix of nutrient soil, perlite and vermiculite (2:1:1), and were grown under 12 h light/12 h dark, 22 °C in the day/16 °C at night and 50% humidity. GV3101 strain of *A. tumefaciens* carrying the expression vector of *liSHP2* was propagated at 28 °C in YEB medium (beef extract 5 g/L, yeast extract 1 g/L, peptone 5 g/L, sucrose 5 g/L, MgSO₄·7H₂O 0.5 g/L, pH 7.0). Bacterial cells were precipitated from suspension cultures with an OD₆₀₀ of 0.8–1.0 and were resuspended in 50 mL infiltration solution (half strength hormone-free MS medium supplemented with 50 g/L sucrose) (Murashige and Skoog 1962) containing 10 µL silwet L-77 (Lehle Seeds). Flowers were removed from wild-type Col-0 *Arabidopsis* plants. The aerial parts of the Col-0 wild-type plants were immersed into the bacterial suspension for 5 min under vacuum condition ($P = 0.05$ MPa). The treated plants were grown overnight in the dark and the environment humidity was maintained in 30% RH. On the following day, the treated plants were transferred to normal illumination conditions and were grown to set seeds. Mature siliques were collected and seeds were screened on selective medium containing hygromycin. The phenotypic changes

of *liSHP2* transgenic plants were recorded and the functions of *liSHP2* were investigated.

Results

Determination of the cDNA sequence of *liSHP2*

A cDNA fragment between 250 to 500 bp and a cDNA fragment of about 1000 bp were amplified in 5'-RACE and 3'-RACE, respectively. Sequencing results showed that the length of the 5'-end cDNA fragment was 298 bp and the length of the 3'-end cDNA fragment was 920 bp (data not shown). By sequence assembly, the full-length *liSHP2* cDNA of 1020 bp in size was determined. In the cDNA sequence, a complete ORF of 741 bp encoding a 246-amino-acid protein could be found (Supplemental Fig. 1). Homology search in NCBI showed that the cDNA of *liSHP2* was closely related to *SHP2* (*AGL5*), a MADS-box gene in *Arabidopsis*, and the identity between *liSHP2* cDNA and *SHP2* cDNA reached up to 89%. The identity of the entire amino acid sequences between *liSHP2* and *SHP2* was 93.17%, indicating SHP homologous proteins were highly conserved in the process of evolution (Fig. 1). Protein BLAST search of the amino acid sequence in NCBI illustrated that *liSHP2* was constituted by M (1–73 aa), I (74–100 aa), K (101–185 aa) and C (186–246 aa) domains from N-terminus to C-terminus (Fig. 1). Predictive analysis of conserved domains also displayed that *liSHP2* contained MADS domain and K-box domain which were specific to the Type-II transcriptional factors in MADS family. Analysis of the physicochemical property showed that *liSHP2* was a basic protein and the isoelectric point was 9.23.

Fig. 1 Alignment of the amino acid sequences of SHP2 homologous proteins. Green indicates identical amino acids, pink and orange indicate different amino acids, black line shows MADS domain, purple line shows K domain. The AG motif I and AG motif II in the

C-terminal region are framed with black boxes. SHP2, *Arabidopsis thaliana* (NP_850377); EsAGL5, *Eutrema salsugineum* (XP_024016862.1); RsAGL5, *Raphanus sativus* (XP_018483390.1); LcSHP2, *Lepidium campestre* (CBY05405.1) (color figure online)

Multiple alignment of the amino acid sequences and construction of phylogenetic tree

The amino acid sequences of *LiSHP2* and SHP homologous proteins in different plant species were used for constructing the diagrams of multiple alignment and phylogenetic tree. Multiple alignment of amino acid sequence of *LiSHP2* with SHP-like proteins from *Arabidopsis*, *Eutrema salsugineum*, *Raphanus sativus* and *Lepidium campestre* indicated that these proteins share high similarity throughout the entire M, I, K and C regions. Furthermore, AG motif I and AG motif II were found in the C-terminus of *LiSHP2* containing hydrophobic and polar residues (Fig. 1). The phylogenetic relationship shows that *LiSHP2*

Fig. 2 Phylogenetic tree of MADS proteins from different plant species. The tree was constructed with neighbor-joining algorithm in MEGA 7. *LiSHP2* is framed in red box. SHP1, NP_001190130.1; SHP2, NP_850377.1; CsAGL5, XP_010506124.1; EsAGL5, XP_024016862.1; LcSHP1, CBY05404.1; LcSHP2, CBY05405.1; RsAGL5, XP_018483390.1; BnSHP1, NP_001302702.1; PrtSHP, AEI01160.1; TrSHP, ABB59995.1; PLENA, AAB25101.1; FAR, BAI68392.1; AG, NP_567569.3; TrAG, ABB59994.1; BnAG, XP_013719485.1; EsAG, XP_024005545.1; CbAG, ACD76828.1; CrAG, XP_023633847.1; STK, NP_001078364.1; PeAGL11, XP_011031538.1; RsAGL11, XP_018473770.1; BnAGL11, XP_013732865.1; EsAGL11, XP_006397127.1; FUL, NP_568929.1; TrFUL, ABB59991.1; AP1, NP_177074.1; TrAP1, ABB59990.1

Fig. 3 Expression pattern of *LiSHP2* in woad in different tissues (a), different floral organs (b), the silicles at different development stages and different parts of the silicles in stage 3 (c). Error bars represent the standard deviation

Fig. 4 Expression analysis of *LiSHP2* in transgenic *Arabidopsis* by qRT-PCR. Error bars represent the standard deviation. Asterisks indicate a significant difference ($P < 0.05$) between transgenic lines and wild-type Col-0 plants

could be grouped into the PLE-lineage of MADS-box genes (Fig. 2). These results verified that *LiSHP2* was a C-class floral homeotic gene and that it was the orthologous gene of *SHP2* from *Arabidopsis*.

Expression patterns of *LiSHP2* in woad

The expression levels of *LiSHP2* in different tissues and organs of woad were analyzed by real-time quantitative PCR. The results showed that the accumulation of *LiSHP2* mRNA in root, stem, leaf and inflorescence of the woad plants at the reproductive stage was very low (Fig. 3a). On

the contrary, the transcript abundance of *LiSHP2* in flowers was very high (Fig. 3a). Besides, the transcriptional abundance of *LiSHP2* increased gradually along with the development of flowers and silicles. In four types of floral organs, the difference of *LiSHP2* expression was insignificant in sepals and petals (Fig. 3b). The abundance of *LiSHP2* mRNA in pistils was the highest, and in stamens the expression level was also significantly higher than that in sepals and petals (Fig. 3b). In silicles, the expression of *LiSHP2* at stage 1 was very weak (Fig. 3c). In contrast, the expression levels of *LiSHP2* at the third and the fourth stages were increased significantly and were maintained persistently (Fig. 3c). Analysis of different parts of silicles at stage 3 confirmed that the expression level of *LiSHP2* in seeds was higher than that in fruit wings and valves, and the fruit wings exhibited the lowest abundance of *LiSHP2* transcript (Fig. 3c). These results suggest that *LiSHP2* gene in woad is not only involved in the development of pistil, carpel and silicle, but also might be directly or indirectly involved in the specialization of stamens.

Phenotypic variations of *LiSHP2* transgenic *Arabidopsis* plants

In order to investigate the biological functions of *LiSHP2*, an *LiSHP2-GFP* fusion gene controlled by the Cauliflower Mosaic Virus (CaMV) 35S promoter was introduced into the genome of wild-type *Arabidopsis*. By *Agrobacterium*-mediated genetic transformation and selection with hygromycin, twenty-one transgenic lines were obtained and all the lines showed severe phenotypic changes.

Fig. 5 Comparison of phenotypic characteristics and the number of rosette leaves between wild-type Col-0 and *LiSHP2* transgenic plants. **a** The 35S::*LiSHP2* plants (shown at right) flowered after producing 5–6 rosette leaves, while the wild-type plant (shown at left) did not flower until the eighth leaf was produced. **b** The rosette leaves of a wild-type Col-0. **c** The rosette leaves of an 35S::*LiSHP2* plant. Bar = 1 cm

LiSHP2 in transgenic lines 2, 4 and 8 was identified by qRT-PCR and it showed high expression levels (Fig. 4). The overexpressors produce 5–6 rosette leaves, while wild-type plants typically produce 10 rosette leaves (Fig. 5a–c). Moreover, the surface area of rosette leaves was significantly reduced in transgenic plants (Fig. 5b, c). Statistical data analysis also showed significant differences in the number of rosette leaves (Fig. 6).

The morphological development of flowers was also affected by *LiSHP2*. In wild-type Col-0 *Arabidopsis* plants, the flowers possess four normal whorls of floral organs (Fig. 7a). At the early development stage of flower buds, the petals are wrapped by sepals. Along with the growth of the flowers, the sepals will open and the petals will be exposed, accompanied by the development of stamens and pistil (Fig. 7a, b). However, the structure of the floral organs was changed notably in *LiSHP2* overexpressing plants (Fig. 7c–g). Compared to the flower buds of the wild-type plants at the same stage (stage 13 of the flower development in *Arabidopsis*), the sepals in *LiSHP2* overexpressing plants opened ahead of time, the petals were exposed prematurely (Fig. 7c). There was also a change in the orientation of the sepals in transgenic plants. In normal plants, the sepals clinged closely to the petals, incurved slightly and did not splay outwards. On the contrary, the sepals of the overexpressing plants stretched outwardly (Fig. 7d). The white petals of the wild-type *Arabidopsis* plants were wide and were arranged in a staggered pattern with the sepals. Unlike this, the petals of the transgenic plants were abnormal in development, their size became smaller, the colour of the top was yellowish, the shape

looked like a palm-leaf fan and the filamentous base was similar to the staminal column (Fig. 7c, e, f). Moreover, apetalous flowers were also found in certain transgenic plants (Fig. 7d, g).

Simultaneously, it was found that the development of the apical inflorescence of *Arabidopsis* could be influenced by overexpression of *LiSHP2* (Fig. 7h–m). Under the stereomicroscope, incomplete development of pistil could be observed in a number of flowers. These flowers only possessed one open carpel with exposed ovules and lacked the other carpel (Fig. 7i, k). At the same time, normal pollination could not occur in numerous flowers owing to the abnormal growth of stamens (Fig. 7j). In situation like this, the ovules were infertile and normal seeds could not be produced (Fig. 7k). It should be explained that the pistils without loss of carpel could not receive the pollen from ectopic stamens, and were unable to accomplish normal development as well (Fig. 7m).

The development of siliques and seeds were also obviously affected by *LiSHP2*. The flower buds of wild-type *Arabidopsis* plants produce only one pistil. After pollination, the pistil develops into a smooth-skinned silique containing well-developed seeds (Fig. 8a). In *LiSHP2* transgenic plants, the length of the siliques became shorter and the diameter of the siliques became smaller (Figs. 8a, 9). Seed abortion was found in siliques due to hypoplasia of pericarp and ovule. Compared to wild-type *Arabidopsis*, the regions of the siliques encapsulating the well-developed seeds bulged out and wrinkled valves were produced (Fig. 8b, c, e–g). Furthermore, at stage 17 of silique growth, the perianth of wild-type *Arabidopsis* plants falls off, but the valves are still dark green in colour. However, the perianth of the transgenic *Arabidopsis* plants did not fall off, the siliques entered a senescent state prematurely and the valve turned yellow gradually (Fig. 8a–g). In addition, siliques curved in varying degrees could be found in many transgenic plants (Fig. 8e–g).

LiSHP2 can also affect the development of cauline leaves. In wild-type *Arabidopsis* plants, the cauline leaves are found under the lateral branches, the blades are generally larger and form a flattened elliptic shape. Under normal circumstances, the wild-type cauline leaves are green and are in an expanded state with slight wrinkles (Fig. 10a). In *LiSHP2* transgenic *Arabidopsis* plants, the blade of the cauline leaves curled inwards along the stem in a longitudinal way (Fig. 10b–g). The blade of the cauline leaves under the fully-developed lateral branches curled inwards and were funnel-shaped (Fig. 10b–d, f), whereas the blade of cauline leaves at the positions producing the sprouts of lateral branches curled into a cylindrical shape (Fig. 10e, g). Compared to the wild-type *Arabidopsis* plants, the length-width ratio of the cauline leaves in transgenic lines decreased substantially (Fig. 10h).

Fig. 6 Statistical analysis of rosette leaf number between 35S::*LiSHP2* transgenic plants and wild-type Col-0 plants ($n = 10$). Error bars represent the standard deviation. Asterisks indicate a significant difference ($P < 0.05$) between transgenic lines and wild-type Col-0 plants

Fig. 7 Influence of *liSHP2* overexpression on morphological development of flowers. **a, b** Flower buds and flowers with four normal whorls of floral organs in wild-type Col-0 plants. **c–g** Flowers with four whorls of floral organs produced by *liSHP2* transgenic *Arabidopsis* plants. **h–m** Abnormal inflorescences and floral organs produced by *liSHP2* transgenic *Arabidopsis* plants. Bar = 1 mm

Discussion

As upstream transcriptional factors, MADS proteins participate in all stages of plant growth and development. In the ABCDE model of floral growth and development, the role of MADS-box genes is irreplaceable. C-class floral homeotic genes, such as *AG*, play an important role in maintenance of the identities of stamens and pistil, whereas the D-class floral homeotic genes, such as *STK*, control ovule development (Theissen 2001; Rijpkema et al. 2010).

In the present work, the coding sequence of a *SHP2* orthologous gene is isolated from *I. indigotica*. The amino acid sequence of *liSHP2* showed high similarity (93.17%) with *SHP2*. This means that *liSHP2* is likely to possess the same functions as *SHP2*. The result of multiple alignment illustrates that *liSHP2* is highly conserved with homologous proteins of other species, such as *SHP2*, *LcSHP2*,

EsAGL5 and so on (Figs. 1, 2). In phylogenetic analysis, *liSHP2* is grouped into PLE-lineage of C-class floral homeotic genes (Kramer et al. 2004). Moreover, two conserved motifs, AG motif I and AG motif II, are clearly found in the C-terminus of *liSHP2* (Fig. 1), further confirming the identity of *liSHP2* as a C-class MADS-box gene (Kramer et al. 2004).

Expression analysis showed that the transcript abundance of *liSHP2* was very low in vegetative tissues and was high in pistils (Fig. 3a). During fruit growth, the transcript abundance of *liSHP2* increased continuously and was maintained at a high level during later stages of silicle growth (Fig. 3b). These data indicated that *liSHP2* has similar functions with *SHP2* in pistil, ovule and fruit development (Fig. 3). In *Arabidopsis*, expression analysis suggests that the transcripts of *SHP1* and *SHP2* can be detected obviously in valve margin before and after

Fig. 8 Influence of *liSHP2* overexpression on silique and seed development of *Arabidopsis*. **a** Siliques of a wild-type Col-0 plant (lower) and an *liSHP2* transgenic plant (upper). **b–g** Siliques of *liSHP2* transgenic plants, with non-shedding perianth, wrinkled valves or curled shape. Bar = 1 mm

Fig. 9 Statistical analysis of the silique length of wild-type Col-0 and 35S::*liSHP2* transgenic *Arabidopsis*. Error bars represent the standard deviation. Asterisks indicate a significant difference ($P < 0.05$) between transgenic lines and wild-type Col-0 plants

fertilization, and also in gynoecium primordium, ovule, septum, nectary and style (Flanagan et al. 1996; Favaro et al. 2003; Colombo et al. 2010). Unlike *SHP2*, the

Fig. 10 Influence of *liSHP2* overexpression on cauline leaf development of *Arabidopsis*. **a** Fully-developed cauline leaf of wild-type *Arabidopsis* plant. **b–d, f** Fully-developed cauline leaves in *liSHP2* transgenic *Arabidopsis* plants. **e, g** The cauline leaves at an earlier stage of production of lateral branches. **h** Cauline leaves from wild-type (left) and transgenic (right) plants. In **a–g**, bar = 1 mm. In **h**, bar = 1 cm

expression level of *liSHP2* in stamens is about 25 times higher than in sepals and petals, indicating it is possibly involved in controlling stamen identity, because the expression patterns of genes often reflect their function (Lü et al. 2007).

Among the other plant species, including *Arabidopsis*, C-class and D-class genes retain not only their previous functions, but also may evolve new functions. For example, *MAwuAG*, an euAG-lineage gene, was restricted to reproductive organs, including stamens and carpels (Wu et al. 2012). *TrSHP*, a PLE-lineage gene, was mainly expressed in stamens, carpels and ovules, and *TrAG*, an euAG-lineage gene, was mainly expressed in floral meristems, stamens, carpels and ovules (Lü et al. 2007). Clearly, the expression pattern of *liSHP2* is more analogous to that of *TrSHP*. *STK* from *Arabidopsis* is a typical D-class floral homeotic gene. It is mainly expressed in ovules and seed coats, but not in stamens and pistils. Unlike *STK*, *DichSTK* is expressed only in pistils (Mizzotti et al. 2012). Although the functions of *STK*-like genes of different species overlap with those of C-class genes in regulating seed development, these genes do not regulate the development of stamens and pistils

under normal circumstances. According to the results of phylogenetic analysis, expression determination and genetic transformation, the function of *liSHP2* is consistent with the C-class genes in PLE-lineage.

Constitutive expression of *liSHP2* in *Arabidopsis* is the best way to further research its functions. *35S::liSHP2* transgenic plants produced plentiful phenotypic changes in the first, the second and the fourth whorls of floral organs, and in leaves (Figs. 5, 7, 8, 10). Early flowering is thought to be common in all kinds of transgenic *Arabidopsis* plants overexpressing MADS-box genes (Kotoda et al. 2002; Thiruvengadam et al. 2012; Hong et al. 2013). Our experimental results showed that the petals were converted into stameneous tissues in transgenic *Arabidopsis* plants overexpressing *liSHP2* and ovules appeared in the wrong area of carpels. Overexpression of *AG*, a C-class gene in euAG-lineage, or *SHP1* and *SHP2*, two C-class genes in PLE-lineage, produced similar results (Mizukami and Ma 1992; Favaro et al. 2003). The activity of *AG* could be substituted partially by ectopic expression of *SHP* genes, and introduction of *35S::SHP2* into the *ag* single mutant was sufficient to rescue the deficiency in stamens and carpels (Pinyopich et al. 2003). Apparently, the functions of euAG-lineage and PLE-lineage are partially redundant (Kramer et al. 2004). In addition, misexpression of *AG* in the second whorl of floral organs mimics the phenotypic changes of *apetala2* mutant (*ap2*) of *Arabidopsis* (Jack et al. 1997). In *liSHP2* transgenic plants, we found that the surface area of petals was reduced, the shape and the colour of petals was similar to stamens. At the same time, some plants could not produce petals. These results strongly suggest that *liSHP2*, as a C-class gene, was able to regulate and control the development of stamens and carpels in woad. In addition, the changes of petals could also be caused by antagonizing A-class genes, such as *liAP1* and *liAP2*.

The phenotypic changes of siliques and ovules were also given great attention in our research. In *Arabidopsis*, the specific expression of *SHP2* in valve margins endowed the dehiscence phenotype to siliques (Mühlhausen et al. 2013). *SHP1* and *SHP2* can regulate the lignification of valve margin cells redundantly and their single mutants are normal in phenotype (Flanagan et al. 1996; Smyth 2000). The siliques of *shp1 shp2* double mutant are indehiscent owing to disappearance of the dehiscence zone on the surface of siliques. All the internal cells of the valve were lignified in *SHP1* and *SHP2* overexpressing plants driven by CaMV 35S promoter and the perianth did not shed when the siliques were ripe (Liljegren et al. 2000). In comparison with the wild-type plants, the diameter of the siliques in *SHP1* and *SHP2* overexpressing *Arabidopsis* plants was smaller and precracked valves were found on the siliques (Liljegren et al. 2000; Mühlhausen et al. 2013).

Significantly, siliques of smaller diameter were also observed in *35S::liSHP2* overexpressing transgenic plants and the perianth also did not shed (Fig. 6). Short-sized siliques were found in *35S::liSHP2* plants, whereas this phenomenon was not found in *35S::SHP1* and *35S::SHP2* transgenic *Arabidopsis*. In addition, the early cracking siliques were also not found in *liSHP2* transgenic plants. *STK*, *SHP1* and *SHP2* are necessary for the development of ovules (Liljegren et al. 2000; Pinyopich et al. 2003). Previous experimental results confirmed that *STK* is a D-class gene and *AG* can promote *SHP* and *STK* expression in ovules (Pinyopich et al. 2003; Rodríguez-Cazorla et al. 2018). In *liSHP2* overexpressing plants, some ovules have been aborted and normal seeds did not develop, which caused a decline in seed yield. These results indicate that *liSHP2* is similar to *SHP1/SHP2* in regulation of the growth of siliques, ovules and seeds. However, some phenotypic differences may be due to the fact that the genes have retained their original functions and undergone slight changes due to differences in the evolutionary process between different plants.

CURLY LEAF (CLF) is involved in control of leaf morphogenesis and is necessary for stable repression of C-class floral homeotic gene *AG* (Goodrich et al. 1997). The *clf* mutant showed curled rosette and cauline leaves (Krizek et al. 2006). The phenotype of curled leaves in *35S::liSHP2* transgenic *Arabidopsis* indicated that ectopic expression of *liSHP2* in rosette and cauline leaves probably could inhibit the activity of *CLF*.

Conclusions

liSHP2 is specifically expressed in stamens, carpels and ovules. Multiple alignment and phylogenetic analysis indicated that *liSHP2* is a putative C-class PLE-lineage gene. Genetic transformation of *Arabidopsis* showed that *liSHP2* could change the morphology of floral organs in the first, second and fourth whorls. These results confirmed that *liSHP2* was involved in regulation of the development of stamens, pistils and seeds in *I. indigotica* Fortune. *liSHP2* is highly similar to *Arabidopsis SHP*, but shows slight changes in function.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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