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Research article

Ectopic expression of *IiFUL* isolated from *Isatis indigotica* could change the reproductive growth of *Arabidopsis thaliana*

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ABSTRACT

The coding sequence of *IiFUL* in *Isatis indigotica* was isolated and was used in transformation of Arabidopsis. *IiFUL* overexpressing Arabidopsis plants exhibited early flowering phenotype, accompanied with the reduction of flower number and the production of terminal flower on the top of the main stems. In development process, the flowers located on the top of the main stems generated a lot of variations in phenotype, including abnormal swelling of pistil, withering and numerical change of stamens and petals, appearance of stigmatoid tissues and naked ovules at the margin or inside of sepals. Besides, secondary flower could be formed within the flowers on the top of the main stems. These observations illustrated that *IiFUL* mainly affected the development of inflorescence meristems and pistils, but its ectopic expression could also disturb the normal growth of other floral organs. Moreover, the fertile siliques produced by the lateral inflorescences of *IiFUL* overexpressing Arabidopsis plants showed indehiscent phenotype, and the shape of the cauline leaves was changed significantly. The results of quantitative real-time PCR revealed that higher transcriptional levels of *IiFUL* could be detected in flowers and silicles of *I. indigotica*. In comprehensive consideration of the previous reports about the dehiscence phenotype of Arabidopsis siliques and the fact that the siliques of *IiFUL* overexpressing Arabidopsis plants were indehiscent in the present work, it can be speculated that high expression of *IiFUL* in pericarp is likely the reason why the silicles of *I. indigotica* possess an indehiscent phenotype.

1. Introduction

FUL (*FRUITFULL*) is a MADS-box gene of the model plant Arabidopsis and belongs to *AP1/SQUA-LIKE* subfamily (Litt and Irish, 2003). The genes in this subfamily are involved in regulation of multiple reproductive growth stages, including floral transition, meristem determinacy, floral organ differentiation and fruit ripening (Jia et al., 2015). In Arabidopsis, *FUL* can control the flowering time, the formation of inflorescence meristems, the phenotypes of leaves and the development of siliques (Ferrándiz et al., 2000a). In other words, FUL participates in promotion of floral transition in early stages of reproductive growth and also in control of the development of carpels and siliques at late stages of flowering (Gu et al., 1998).

In other plant species, the homologous genes of *FUL* possess additional and diversified functions. In tomato, *FUL1/2* can influence the process of fruit ripening together with *RIN* (*RIPENING INHIBITOR*) and *TAGL1* (*TOMATO AGAMOUS-LIKE1*). The complex formed by RIN and FUL1/2 can promote the sustained accumulation of aromatic and sweetish substances in flesh, and the wall softening of the flesh cells by an ethylene-independent pathway (Bemer et al., 2012). *FUL1* (*TM4*, *TDR4*) of tomato shows high homology with Arabidopsis *FUL*. The interaction between FUL1 and RIN (SEP-like protein) is probably related with the maturation of the fleshy fruit. In the meantime, FUL1 possibly functions in regulation of the flowering process of tomato by interaction with TM3 (SOC1-like protein) (Leseberg et al., 2008). As an orthologous gene of *FUL*, *DEFH28* (*DEFICIENS-Homolog 28*) of snapdragon can induce and promote the formation of floral meristems in late development stages of inflorescence meristems. At the same time, *DEFH28* can control the development of carpels, just like *FUL* of Arabidopsis (Müller et al., 2001). In wheat, the *FUL* paralogue *WFUL1/VRN1* plays important roles in response to low-temperature induction and in initiation of vernalization, and its expression can also promote the development of floral organs (Preston and Kellogg, 2007; Kinjo et al., 2012). In grapevine, *VFUL-L* is involved in floral transition and development of carpel, fruit and tendril (Calonje et al., 2004). It can be seen that the homologous genes of *FUL* possess multiple functions and play important roles in floral transition, carpel differentiation and fruit development. In other words, the transcriptional factors encoded by *FUL* homologous

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genes are indispensable to reproductive growth of plants.

In most plants, finish of fruit development and release of seeds are the signs that the reproductive growth has been completed. Although the shapes of the angiospermous fruits are highly diversified, the ovary wall in pistil of most plant species will develop into pericarp and the fertilized ovule will develop into seeds. The silique of Arabidopsis is derived from the pistil containing two carpels. In siliques, two valves are connected to the replum located at the periphery of septum (Mühlhausen et al., 2013). At the late stages of ripening, the siliques of Arabidopsis will form a dehiscence zone between valve and replum, and open at this position to release the seeds into the external environment. The dehiscence zone consists of two different cell layers, the lignified layer and the separation layer. The lignified layer is close to the valves and contains a stripe of lignified cells which are connected to the lignified endocarp. The separation layer is adjacent to replum and is constituted by small thin-walled cells (Rajani and Sundaresan, 2001). In the ripening process of siliques, the whole valves will dry and shrink. However, the lignified cells will stay in a rigid state, which will create an internal tension in the valves. Along with the degeneration of the middle lamellae between the cells of the separation layer, the valves will crack at this predetermined breaking zone.

The dehiscent process of Arabidopsis siliques is associated with a sophisticated regulatory network constituted by a number of transcriptional factors. *SHP1* (*SHATTERPROOF1*) and *SHP2* (*SHATTERPROOF2*) are specially expressed in dehiscence zone and the redundant MADS-box proteins encoded by them can activate *IND* (*INDEHISCENT*) and *ALC* (*ALCATRAZ*) (Liljegren et al., 2000). IND is related with the lignification of the valve margin cells and the differentiation of both the lignified layer and the separation layer (Liljegren et al., 2004; Wu et al., 2006; Sorefan et al., 2009; Lenser and Theißen, 2013). In contrast, ALC is only involved in formation of separation layer (Rajani and Sundaresan, 2001). The specific expression of *SHP1*, *SHP2*, *IND* and *ALC* in dehiscence zone of the siliques is critical to establishment of this region and is related with negative regulators. *FUL* is mainly expressed in valves and is associated with differentiation and expansion of valve cells, and the MADS-box protein encoded by *FUL* can inhibit the expression of *SHP1* and *SHP2* in valve (Mühlhausen et al., 2013). RPL (REPLUMLESS) expressed in the replum is also involved in regulation of dehiscence zone formation as a negative regulator of valve margin identity genes (Roeder et al., 2003; Marsch-Martínez et al., 2014). In addition, AP2 (APETALA2) is found to be able to inhibit the expression of *RPL* and *SHP* (Ripoll et al., 2011).

Mutants of *SHP1*, *SHP2, IND*, *ALC*, *FUL*, *RPL* and *AP2* all showed impaired dehiscence phenotype. In *ind* or *alc* single mutants and *shp1/2* double mutants, the dehiscence zone could not be formed (Liljegren et al., 2000, 2004; Rajani and Sundaresan, 2001; Wu et al., 2006). In *rpl* single mutants with a partial indehiscence phenotype, *SHP1*, *SHP2*, *IND* and *ALC* were ectopically expressed and the replum cells were converted into valve margin-like cells (Roeder et al., 2003; Sorefan et al., 2009). The siliques of *ful* single mutant and overexpressing line showed indehiscent phenotypes. In *ful* single mutant, *SHP1/2*, *IND* and *ALC* were ectopically expressed and the valve cells were converted into valve margin cells, and the siliques of *ful* mutant were shorter in comparison with the wild-type siliques due to the failure of the valves in elongation (Gu et al., 1998; Ferrándiz et al., 2000b). The siliques of *FUL* overexpressing lines showed a normal size, but lacked the dehiscence zone, indicating ectopic expression of *FUL* could inhibit the identity genes functioned in differentiation of dehiscence zone (Ferrándiz et al., 2000b; Østergaard et al., 2006). In *ap2* single mutant, the expansion of the replum and the lignified layer led to a delayed opening of fruits, owing to the upregulation of *RPL*, *SHP2* and *IND* (Ripoll et al., 2011).

Woad (*Isatis indigotica* Fort.) is a traditional Chinese medicine plant and belongs to Cruciferae. Up to now, the research works of molecular biology in woad were mainly associated with the biosynthesis of lignans, the active pharmaceutical ingredients (Hu et al., 2011; Ma et al., 2016; Zhang et al., 2016), and no reports about the genes related with floral transition in woad could be found. The typical fruit of Cruciferae family is silique, a two-valved dry capsule and usually dehiscent. However, the fruit of woad is indehiscent and is named as silicle owing to the short, flat and elliptic shape. Moreover, each silicle contains only one seed and fruiting wing exists around the pericarp, which is distinctly different from the dehiscent silique of Arabidopsis containing a lot of seeds. These morphological variations suggest that woad and Arabidopsis possess different features in floral transition and in reproductive growth, although both are cruciferous plant species. Therefore, it is valuable to investigate the functions of the flowering regulating genes in woad. To study the early steps of flower initiation and development in woad, the orthologous gene of Arabidopsis *FUL* was isolated and was designated as *IiFUL* in the present work. Then the expression patterns of *IiFUL* during vegetative and reproductive developments were analyzed by real-time quantitative PCR. To determine the function of *IiFUL*, transgenic Arabidopsis plants overexpressing *IiFUL* were prepared by floral dip method mediated by Agrobacterium. Detailed observation to the phenotypes of the transgenic Arabidopsis plants indicated that *IiFUL* could affect the development of leaves, inflorescence meristems, flowers and fruits obviously.

2. Materials and methods

2.1. Plant materials and bacterial strains

Flowers of the biennial woad plants growing in greenhouse were used as materials. pMD 18-T and pRI 101-AN were used in cloning of cDNA sequences and in construction of the overexpressing vector, respectively. *Escherichia coli* strain DH5α and *Agrobacterium tumefaciens* strain LBA4404 were used as host cells in construction of vectors and in transformation of Arabidopsis, respectively.

2.2. Identification of the full-lengh cDNA sequences of IiFUL

The previously reported degenerate primers were used to amplify the conserved segments encoding the M domain of MADS-box proteins in woad (Zong et al., 2007; Li et al., 2011). Gene specific primers were designed according to sequences of the conserved segments. In details, IiFUL-5′RACE-Primer (5′-GCATCGCAGAGAACAGAGATCTCATGAGC-3′) and IiFUL-3′RACE-Primer (5′-TTTCGAGGAGAGAGAGATGGGAAG-GGGT-3′) were used to amplify the 5′-end and the 3′-end unknown fragment of *IiFUL* cDNA, respectively.

Total RNA was isolated from woad flowers with Trizol reagent and residual genomic DNA was eliminated with RNase-free DNase I (Takara). The samples were analyzed with formaldehyde/MOPS gel. SMARTer RACE cDNA Amplification kit (Takara) was used in amplification of the unknown cDNA sequences. The first-strand cDNA was synthesized with SMARTScribe reverse transcriptase. In preparation of 5′-RACE cDNA with 5'–$(T)_{25}$VN–3′, the SMARTScribe reverse transcriptase can add several cytosine nucleotides to the 3′ end of the first-strand cDNA with its terminal transferase activity. The SMARTer II A Oligonucleotide (5′–AAGCAGTGGTATCAACGCAGAGTACXXXXX–3′) provided by the kit contains primer sequences at 5′ end and several guanine nucleotides at 3′ end, and can form 5′-protruding terminus in complementary base-pairing with the 3′ end of the first-strand cDNA. SMARTScribe reverse transcriptase can use the SMARTer oligonucleotide as template and introduces the adapter sequences into the 3′ end of the first-strand cDNA. The 5′-end fragment of *IiFUL* cDNA was amplified with adapter primer UPM (5′–CTAATACGACTCACT ATAGGGCAAGCAGTGGTATCAACGCAGAGT–3′, 5′–CTAATACGACTCA CTATAGGGC–3′) and the reverse gene-specific primer (IiFUL-5′RACE-Primer) by touchdown PCR.

3′-RACE CDS Primer A (5′–AAGCAGTGGTATCAAC GCAGAGTAC$(T)_{30}$VN–3′) provided by the kit was used in preparation of the first-strand cDNA of 3′-RACE. Because the 5′-end of the 3′-RACE