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Short communication

A novel F-box protein represses endothecial secondary wall thickening for anther dehiscence in *Arabidopsis thaliana*

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ABSTRACT

In plants, the regulation of protein turnover by the ubiquitin proteasome system (UPS) is a key post-translational mechanism underlying diverse cellular processes. However, the participation of the UPS in cellular processes involved in anther dehiscence, especially endothecial secondary wall (ESW) thickening, has not been characterized. Here, we report that a novel F-box protein in arabidopsis, designated SAF1 (Secondary wall thickening-Associated F-box 1), negatively regulates ESW thickening in the anther. SAF1 is predominantly expressed in flower tissues and interacts with Arabidopsis-Skp1-like 19 (ASK19). *SAF1*-overexpressed (Ox) lines showed reduced fertility due to a lack or loss of ESW thickening in the anther and inhibition of the expression of relevant genes, such as *IRREGURAR XYLEMs* (*IRXs*) in flowers. These findings suggest that the novel Skp/Cul/F-box (SCF) complex consisting of SAF1 as an F-box protein and ASK19 as a Skp functions in secondary wall thickening of the anther endothecium.

Introduction

The very large number of F-box proteins encoded in the arabidopsis genome – nearly 700 identified to date – implies that Skp/Cullin/F-box (SCF) complexes, a major family of plant ubiquitin ligases, function in numerous cellular mechanisms during plant developmental and physiological processes. All F-box proteins contain a conserved F-box motif at their N-terminus, which interacts with the Arabidopsis-Skp1-like (ASK) subunit of the SCF complex, and most possess a protein-interaction domain, such as leucine-rich repeats (LRRs) or kelch repeats, which confers substrate specificity (Gagne et al., 2002; Jain et al., 2007). The F-box subunit of a specific SCF complex is responsible for appropriate target (substrate) recognition and delivery of this target to the complex. Although several arabidopsis F-box proteins are known to be involved in diverse aspects of plant growth and development, such as hormone signaling (reviewed by Santner and Estelle, 2010), floral development (Hepworth et al., 2006), leaf senescence (Woo et al., 2001), pollen development (Gusti et al., 2009), and tapetum degeneration (Kim et al., 2010), the functions of most F-box proteins remain largely unknown.

Anther dehiscence requires a series of sequentially programmed events in specific cell types, namely, degeneration of the middle layer and the tapetum, thickening of the endothecial layer, degradation of septum cells, and degeneration of stomium cells (Goldberg et al., 1993; Sanders et al., 1999). Secondary wall thickening of the anther endothecium provides the tensile force necessary for breakage of the stomium cells and, in turn, for release of the pollen grains, and is, therefore, indispensable for anther dehiscence. The significance of this process has been verified in studies with several knock-out mutant (Brown et al., 2005; Yang et al., 2007) or overexpressing (Jung et al., 2008; Villarreal et al., 2009) plants, all of which have defective endothecial secondary wall (ESW) thickening, resulting in anther indehiscence. Results from recent in-depth genetic analyses in arabidopsis suggest that large numbers of proteins possibly participate in flowering processes, including anther dehiscence (reviewed by Ma, 2005). A few F-box proteins have been reported to be involved with flower processes, such as unusual floral organs (UFO), which is required for floral organ identity (Hepworth et al., 2006), and reduced male fertility (RMF), which regulates degeneration of the tapetum and middle layer during anther development (Kim et al., 2010). To date, however, there has been no report of F-box proteins functioning in ESW thickening during anther dehiscence, even though this process is believed to be tightly regulated by the signaling circuitry underlying the control of programmed cell death.

Abbreviations: ASK, Arabidopsis-Skp1-like; ESW, endothecial secondary wall; MS medium, Murashige and Skoog medium; qRT-PCR, quantitative real time-PCR; RT-PCR, reverse transcription-PCR; SCF, Skp/Cul/F-box complex; SD medium, synthetic dropout medium; SEM, scanning electron microscopy; WT, wild-type.

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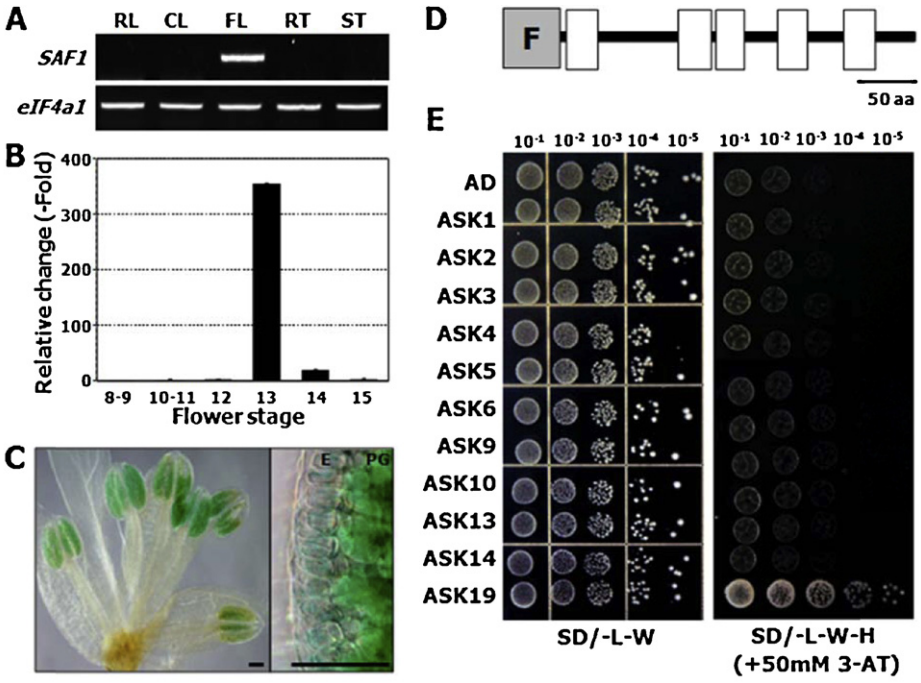

Fig. 1. Characterization of *SAF1*. (A) Spatial expression of *SAF1*. *eIF4a1* was used as a control. RL, rosette leave; CL, cauline leave; FL, flower; RT, root; ST, stem. (B) *SAF1* expression during flower development. Each bar represents the amount of the *SAF1* transcript relative to that of the *eIF4a1* gene as an internal control. The relative level of the *SAF1* expression at stages 8–9 was set at 1. The data represent the average value ± SD from three experiments. Relevant flower developmental stages were as defined by Smyth et al. (1990). (C) GUS expression of flower at stage 13. The enlarged view on the right side shows the representative anther walls. PG, pollen grains; E, endothecium. Scale bars, 100 μm. (D) Scheme of the predicted structure of the SAF1 protein. The F-box and the LRRs are noted by the gray box containing the letter F and by the white boxes, respectively. (E) Interaction assay of the SAF1 with the 11 representative ASKs in the yeast two-hybrid system. The growth of transformed cells was monitored on control SD/-L-W medium (left panel) or on selective SD/-L-W-H (+50 mM 3-AT) medium (right panel). Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) of cultured cells were spotted on the both plates. AD is an empty vector of pGADT7 that was used as the negative control.

We have identified a novel arabidopsis F-box protein, SAF1, which is predominantly expressed in flower tissues and interacts with ASK19 protein. Using the reverse genetic approach, we obtained experimental data supporting the role of SAF1 as a negative regulator of ESW thickening in the anther.

Materials and methods

Yeast two-hybrid interaction assay

Protein–protein interactions were analyzed using the yeast two-hybrid MATCHMAKER system (Clontech, Mountain View, CA) according to the manufacturer's protocols. This assay is described in detail in Supplementary Data.

Construction of the SAF1-Ox lines and identification of the T-DNA insertion line

SAF1-Ox transgenic arabidopsis plants were generated by first cloning the *SAF1* full-length open reading frame (ORF; 1244 bp) into the binary plant transformation vector pCAMBIA 2300 and then transforming the construct into *Agrobacterium tumefaciens* strain GV3101 using the freeze–thaw method as described by Xie et al. (2001). *A. tumefaciens*-mediated *in planta* transformation of *Arabidopsis thaliana* (Col-0) was performed according to the method of Clough and Bent (1998). Transgenic plants were screened on 1× MS (Murashige and Skoog, 1962) medium containing 1% (w/v) sucrose and 50 mg L^{-1} kanamycin, transferred to soil, and grown to maturity. Five independent T_3 homozygous lines were selected for further study. The T-DNA insertion line (SALK_08835) was obtained from the SALK Institute via NASC for mutant analysis. The insertion was identified by genomic PCR using primers LBa1 (5′-TGG TTC ACG TAG TGG GCC ATC G-3′), SAF1-S (5′-ATG GAT CGG ATC AGC AAT CTT C-3′), and SAF1-AS (5′-CTA GGA AAC GAC CTC GAA TTT GC-3′).

Morphological and cellular analyses

The protocols for sample preparation, β-glucuronidase activity, transverse section analysis, histochemical staining of lignin components, and microscopy analyses are described in detail in Supplementary Data.

Results and discussion

The SAF1, an F-box protein, is specifically expressed in flower tissues at anthesis and interacts with ASK19

The results of the reverse transcription-PCR (RT-PCR) expression analysis of SAF1 in various plant tissues, including the rosette leaf, cauline leaf, flower, root, and stem of 4-week-old plants, revealed that *SAF1* transcripts were predominantly expressed in flower tissue (Fig. 1A). A subsequent analysis of *SAF1* expression at stages 8–15 of flower development (classification according to Smyth et al., 1990) revealed that *SAF1* was expressed primarily from the point in time in stage 13 when anther dehiscence was just beginning through to stage 14 (Fig. 1B). Flowers at stage 13 possess anthers at one of three different stages, namely, stages 11–13 of anther development (Sanders et al., 1999). The events that occur in these stages include – in order of occurrence – expansion and thickening of the endothecium layer, degeneration of the tapetum and septum layers, and anther dehiscence caused by stomium cell breakage (Sanders et al., 1999). To confirm the precise spatial expression pattern of *SAF1*, we generated Pro_{SAF1}*:GUS* transgenic lines harboring the β-glucuronidase (GUS) reporter gene fused with