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Suppression of *ASKβ* (*AtSK32*), a Clade III *Arabidopsis GSK3*, Leads to the Pollen Defect during Late Pollen Development

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***Arabidopsis* Shaggy-like protein kinases (*ASKs*) are *Arabidopsis thaliana* homologs of glycogen synthase kinase 3/SHAGGY-like kinases (GSK3/SGG), which are comprised of 10 genes with diverse functions. To dissect the function of *ASKβ* (*AtSK32*), *ASKβ* antisense transgenic plants were generated, revealing the effects of *ASKβ* down-regulation in *Arabidopsis*. Suppression of *ASKβ* expression specifically interfered with pollen development and fertility without altering the plants' vegetative phenotypes, which differed from the phenotypes reported for *Arabidopsis* plants defective in other ASK members. The strength of these phenotypes showed an inverse correlation with the expression levels of *ASKβ* and its co-expressed genes. In the aborted pollen of *ASKβ* antisense plants, loss of nuclei and shrunken cytoplasm began to appear at the bicellular stage of microgametogenesis. The *in silico* analysis of promoter and the expression characteristics implicate *ASKβ* is associated with the expression of genes known to be involved in sperm cell differentiation. We speculate that *ASKβ* indirectly affects the transcription of its co-expressed genes through the phosphorylation of its target proteins during late pollen development.**

INTRODUCTION

Glycogen synthase kinase 3 (GSK3)/SHAGGY-like kinase (SGG) is a multifunctional non-receptor serine/threonine kinase found in all eukaryotes studied to date (Jope and Johnson, 2004; Kaidanovich-Beilin and Woodgett, 2011; Saidi et al., 2012). GSK3/SGG was originally characterized in the animal insulin signaling pathway and is considered to be a key regulator of many developmental processes, such as cell fate specification, cytoskeleton movements, and programmed cell death (reviewed by Saidi et al., 2012).

GSK3s/*SGGs* in Arabidopsis (*Arabidopsis thaliana)* are referred to as *ASKs* (*Arabidopsis Shaggy-like protein kinases*) or *AtSKs* (*Arabidopsis thaliana Shaggy-like kinases*). *Arabidopsis* contains ten *AtSKs*, which are grouped into four clades: clade I contains *AtSK11*/*ASKα*, *AtSK12*/*ASKγ* and *AtSK13*/*ASKε*; clade II contains *AtSK21*/*ASKη*/*BIN2* (*BRASSINOSTERIOD-INSENSITIVE 2*), *AtSK22*/*ASKι*/*BIL1* (*BIN 2-like 1*) and *AtSK23*/*ASKη*/*BIL2*; clade III contains *AtSK31*/*ASKθ* and *AtSK32*/*ASKβ* and clade IV contains *AtSK41*/*ASKκ* and *AtSK42*/*ASKδ* (Jonak and Hirt, 2002; Saidi et al., 2012).

ASKs are involved in growth, development and stress responses, and mounting evidence indicates that they function by integrating multiple hormonal signals (Saidi et al., 2012). All *ASKs* contain a tyrosine residue, which is located at a comparable position to that of the well-studied tyrosine in the activation loop of mitogen-activated protein kinase (Supplementary Fig. S1) (de la Fuente van Bentem et al., 2008). In *ASKα*/*AtSK11* and *ASKη*/*AtSK21*/*BIN2*, this tyrosine residue is phosphorylated and indispensable for its kinase activity (de la Fuente van Bentem et al., 2008; Kim et al., 2009).

ASKs appear to require "priming" phosphorylation of the substrate for their activity with some exceptions, such as BES1 (BRI1-EMS SUPPRESSOR 1) and BZR1 (Brassinazole resistant 1) (Zhao et al., 2002), as do their counterparts in animals: before being phosphorylated by GSK3/SGG, substrates are often phosphorylated by another kinase (Frame and Cohen, 2001). Once primed, the phosphorylated substrate interacts with a phosphate-binding pocket (arginine 142, arginine 226 and lysine 251 in the case of GSK-3β) and thereby partially substitutes T-loop phosphorylation, which is required for kinase activity (Doble and Woodgett, 2003). Among the ten proteins identified as putative substrates of ASKs, five of these (underlined) are phosphorylated at the potential priming sites: PEP carboxykinase PCK1, RanBP domain-containing protein, thioredoxin-regulated beta-amylase TR-BAMY, At1g72790, remorin family protein, At4g11270, nodulin family protein, RabGAP/TBC domain-containing protein AtGYPC1b, glycine-rich protein and At3g01160 (de la Fuente van Bentem et al., 2008).

All *ASKs* in clade II, along with other *ASKs*, are involved in brassinosteroid (BR) signaling pathways (Wang et al., 2013a; also reviewed in Saidi et al., 2012). Of the six *ASKs* (*ASKα*/*AtSK11*, *ASKγ*/*AtSK12*, *ASKη*/*AtSK21*/*BIN2*, *ASKι*/*AtSK22*/*BIL1*,

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ASKζ/AtSK23/BIL2, and *ASKθ/AtSK31*) implicated in brassinosteroid (BR) signaling pathways for their interaction with the BZR1 transcription factor (Kim et al. 2009), all three clade II *ASKs* have been functionally confirmed for their redundant roles in BR signaling (Yan et al., 2009). Although *ASKη (AtSK21/BIN2)* acts as a major negative regulator of BR signaling in *Arabidopsis*, other *ASKs* in clade II and III can also phosphorylate BES1. For example, the *bin2-1* gain-of-function mutation mimics BR deficiency, and BIN2 co-suppression can rescue BR signaling defects observed in a weak *bri1* allele (Li and Nam, 2002; Rozhon et al., 2010; Yan et al., 2009). Recently, it was reported that clade II ASKs positively modulate ABA signaling by phosphorylating Snf1-related kinase 2s (SnRK2s) (Cai et al., 2014). In addition, all clade II ASKs interact with tracheary element differentiation inhibitory factor (TDIF) receptor and repress xylem differentiation through BES1 (Kondo et al., 2014).

The functions of *ASKs* other than those in clade II are largely unknown. Two *ASKs* in clade I (ASKα/*AtSK11* and ASKγ/*AtSK12*) are up-regulated, especially in sepals and ovule primordia (Dornenals et al., 2000). While down-regulation of either gene using an antisense approach increased the number of sepals/petals and caused the splitting of styles/stigmas, these phenotypes could not be observed when T-DNA knock-out was used (Dal Santo et al., 2012; Dornenals et al., 2000). Additionally, introduction of a point mutation that reduced the primed activity of ASKθ/AtSK31 caused defective cell expansion (Claisse et al., 2007). *ASKθ/AtSK31* was subsequently shown to be an additional ASK that is negatively regulated by the BRI1 receptor and phosphorylates BZR1 and BES1 (Rozhon et al. 2010). Although the induction of some ASKs (*ASKε/ AtSK13*, *ASKθ/AtSK31* and *ASKδ/AtSK42*) by salt or osmotic stress conditions has been reported (Charrier et al., 2002), the role of ASK in the salt stress response has been experimentally determined only for another *ASK*, *ASKι/AtSK22/BIL1* (Piao et al., 2001).

In many crop species, male sterility is exploited for the production of F_1 seeds with desirable traits. As a result, much effort has focused on establishing male sterile lines without deleterious side effects. We recently identified *BrASKβ*, an *ASKβ* homolog showing differential expression in floral buds of genic male sterile plants, as a putative male fertility-related gene (Dong et al., 2013; "*BrASK2*" was the name used in the reference). In the current study, to gain insight into the role of *BrASKβ*, we transgenically suppressed the expression of *ASKβ* in *Arabidopsis*, the homolog of *BrASKβ*, using antisense technology, and examined the resulting phenotypes. The results of expression analysis of *ASKβ* and its co-expressed genes, together with the pollen phenotypes observed in transgenic plants, suggest that *ASKβ* is essential for pollen development in *Arabidopsis*.

MATERIAL AND METHODS

Plant materials and growth conditions

Transgenic plants were generated with the *Arabidopsis thaliana* ecotype Col-0. *Arabidopsis* plants were grown under long day (16 h light / 8 h dark) conditions with 140 ± 2 μmol/m 2/s light intensity at 22 ± 0.5°C. Seeds were stratified for 3 days under 4°C before sowing in 60 × 60 mm soil pots. To maintain the humidity high enough for seed germination, the pots were covered under transparent polyethylthene film for the first 5 or 6 days. For *in vitro* growth, seeds were sterilized with 0.1% Triton X-100 (Sigma, USA) and 30% bleach. After the stratification at 4°C for 3 days, seeds were planted on solid media containing half-strength MS medium (Duchefa Biochemie, The Netherlands), 1% sucrose, and 0.8% phyto agar.

Antisense constructs and plant transformation

The full-length coding sequence of *ASKβ* was cloned from the first-strand cDNA using primer set AKSβ-P2 (Supplementary Table S1) and inserted into T&A cloning vectors (RBC T&A Cloning kit, Real Biotech Corporation, Taiwan). After confirming its sequence and orientation, the *ASKβ* fragment was subcloned into the pCambina 3300-35S binary vector for plant transformation. *Agrobacterium tumefaciens* GV3101 carrying the above-mentioned binary plasmid was used for *Arabidopsis* transformation with floral dipping method (Clough and Bent, 1998). Transgenic plants (T1) were selected on half-strength MS media with 25 mg/ml gulfosinate (Sigma-Aldrich, USA) and further confirmed by gDNA PCR for the transgene. T1 plants were selfed and only the T1 lines, which showed the 3:1 segregation ratio for glufosinate resistant at T2 generation, were selected for the further experiment.

Reverse transcription (RT) PCR and qRT-PCR

The first-stand cDNA were synthesized following the Manufacturer's instructions for ReverTra Ace-α kit (Toybo, Japan). To be used as PCR template, cDNA was diluted to 12.5 ng/μl using NanoDrop ND-1000 (Thermo Scientific). Semi-quantitative RT-PCRs were carried out using a following protocol: denaturation at 94°C for 5 min, 25-cycle amplification (94°C, 30 s; 54°C, 30 s; 72°C, 1 min), and final extension at 72°C for 7 min. Reaction condition for quantitative RT-PCR (qRT-PCR) as follows: 95°C for 30 s and 40 cycles of three-step reaction (95°C for 5 s, 60°C for 20 s, and 72°C for 15 s). Sequences of primers used in this study are shown in Supplementary Table S1. Products from semi-quantitative PCR were separated on 1.5% agarose gels and visualized with ethidium bromide.

Pollen viability, semi-thin sections, and DAPI (4′,6-diamidino-2-phenylindole) staining

To determine the viability and developmental progression of pollen, flowers collected from wild-type and *ASKβS* antisense transgenic lines were fixed in Carnoy's solution (alcohol: chloroform: acetic acid = 6:3:1) for 2 h. Then, the anthers were dissected and stained with a solution containing Malachite green, acid fuchsin, and Orange G for 12 h as previously described (Peterson et al., 2010). Semi-thin sections were prepared as previously described (Javelle et al., 2011). Flower samples were cut and fixed in 4% paraformaldehybe overnight. Then, the samples were dehydrated gradually in an ethanol: Histochoice Clearing Agent (Sigma-Aldrich, USA) series and infiltrated with paraffin. Sections were sliced to an 8-micron thickness and mounted on slides. Paraffin was removed from the slides using Histo-clear and an ethanol series. For observation under a light microscope, samples were stained with 0.05% (W/V) toluidine blue O.

For DAPI staining, disrupted anthers were put on microscope slides with several drops of DAPI-staining solution [100 mm NaPO4, pH 7.5, 1 mM EDTA, 3 μg/ml DAPI (Sigma-Aldrich, high grade)]. The anthers on slides were inspected under a fluorescence microscope (Olympus, BX51) with a DAPI filter set, after incubated at room temperature for 5-10 min.

Phylogenetic tree construction

GSK/SGG-related amino acid sequences from *Arabidopsis* and Chinese cabbage (*Brassica rapa*) were identified by BLAST searches with the full-length amino acid sequence of *ASKβ* as a query and a cutoff value of 100 in the TAIR, and BRAD data-

bases (www.arabidopsis.org and http://brassicadb.org/brad/). The retrieved sequences were aligned with ClustalX2.0 (Larkin et al., 2007). The phylogenetic tree was constructed by the neighbor-joining method in Molecular Evolutionary Genetics Analysis (MEGA) software, using "pairwise deletion" option, "Poisson correction" model (Tamura et al., 2013). Bootstrap value for test was set for 1000 replications.

Co-expression and gene network analysis

"Expression Angler", which enables genome-wide co-expression analysis, was used to find genes that may have similar functions to that of *ASKβ* (Toufighi et al., 2005). Genes with a PCC (Pearson Correlation coefficient) value above 0.8 were chose for the additional analysis. The expression patterns of the identified genes were examined using *Arabidopsis* eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) (Winter et al., 2007). Cluster analysis for functional categorization and GO enrichment analysis were performed with the MeV_4_9 (http://www.tm4.org/mev.html) and agriGO (http://bioinfo.cau.edu.cn/agriGO/index.php), respectively (Du et al., 2010). The gene network was produced by GeneMANIA (Zuberi et al., 2013) and modified with Cytoscape (Version 3.1.0) (Shannon et al., 2003).

Motif analysis

Conserved DNA motifs for transcription factor binding in the promoter sequences of the *ASKβ* co-expressed genes were identified as follows. For each gene, conserved motifs were searched in the 500 bp upstream regions from the transcription start site (TAIR: www.arabidopsis.org) using MEME suite (version 4.9.1) (Bailey et al., 2009). Motifs with lengths of 6-50 nucleotides were searched on both strands using the "zero or one occurrence per sequence" option. Motifs with an E-value ≤ 1.00E + 02 were assessed for their similarity to known motifs using PLACE (Higo et al., 1999).

RESULTS

Shaggy-like kinase genes are conserved between Arabidopsis and Chinese cabbage

In the previous study, *BrASKβ* (*Bra003440*), a homolog of *ASKβ*, was shown to be specifically expressed in fertile buds after tetrad stage and prosed as a putative GMS gene (Dong et al., 2013). To gain further insight into the potential function(s) of *ASKβ* (and thus *BrASKβ*), we performed bioinformatics analyses. Using the Arabidopsis *ASKβ* full-length protein sequence as a query, we searched for the related genes in the Arabidopsis and *B. rapa* databases (www.arabidopsis.org and http://brassicadb.org/brad/). The results show that a large number of *ASKβ* relative genes are present in each plant genome, including 10, and 18 genes in Arabidopsis, and *B. rapa* respectively. An earlier study showed that the ten *shaggy-like kinases* in Arabidopsis (*AtSKs*) can be divided into four clades (I, II, III and IV) based on their sequence similarities (Jonak and Hirt, 2002). Phylogenetic analysis using all 28 sequences identified in our database search revealed that all *B. rapa* homologs are also clustered into four branches along with *Arabidopsis* sequences, and one to three *B. rapa* homologs are closely associated with each *ASK* sequence (Fig. 1).

The orthologue relationships between ASKs and BrSKs have been assinged using InParanoid Version 4.1 (Sonnhammer and Östlund, 2015). The results showed that *BrASKβ* (Bra003440) is the orthologues of *ASKβ* (AT3G61160) (Supplementary Table S2). Previously, it was shown that *BrASKβ* is differentially expressed between male fertile and sterile plants

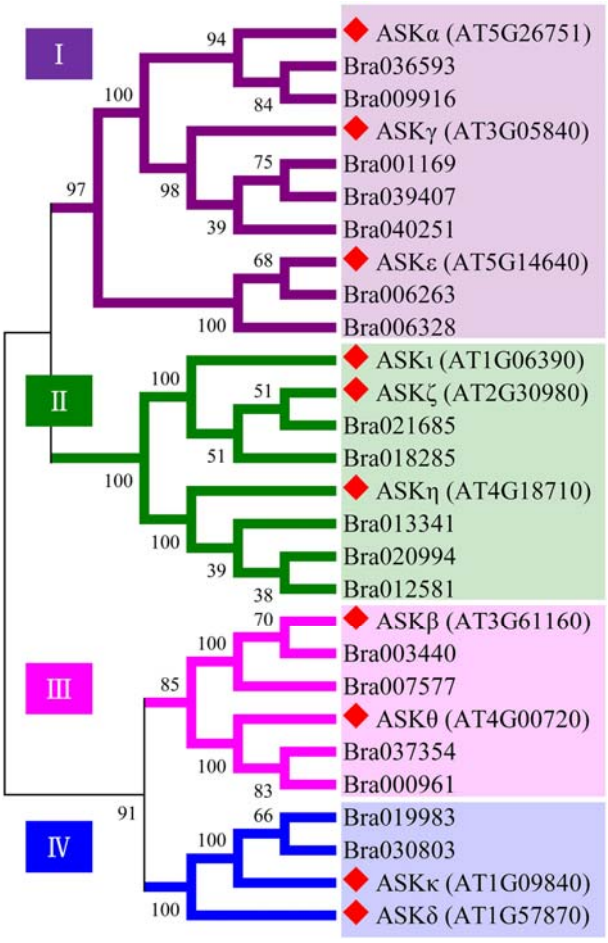

Fig. 1. Phylogenetic analysis of ten ASKs and their homologs. Sequences from *Arabidopsis thaliana*, and *Brassica rapa* were aligned and used to construct the phylogenetic tree using the Neighbor-joining method with MEGA software (versio 6.0). Diamonds indicate *Arabidopsis* ASKs. All proteins were clustered into clades I to IV, which is similar to previous results (Jonak and Hirt, 2002).

(Dong et al., 2013). Given the differential expression pattern of *BrASKβ* in sterile or fertile *B. rapa* floral buds and orthologuous relationship between *BrASKβ* and *ASKβ*, we suspected that *ASKβ* also plays a role during male gametogenesis in *Arabidopsis*.

ASKβ expression pattern in *Arabidopsis* suggests its putative function in pollen development

To infer the possible function of *ASKβ*, the expression patterns were examined in various wild-type Arabidopsis tissues using qRT-PCR and semi-quantitative RT-PCR (Fig. 2 and Supplementary Fig. S2). Relatively low levels of *ASKβ* expression were detected in roots, rosette leaves, stems, and siliques. By contrast, rather high levels of expression were detected in floral buds, especially later in "floral stage 13-16". Consistent with these results, two independent publicly available datasets indicate that *ASKβ* is predominantly expressed in floral tissues (Supplementary Figs. S2B and S2C). Based on the reported expression of group III *ASKs*, including *ASKβ*, in developing

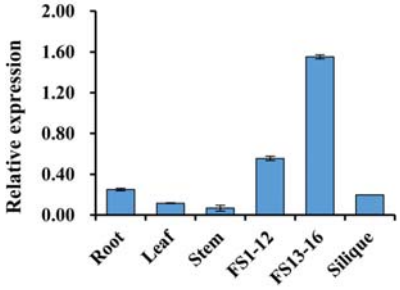

Fig. 2. Expression patterns of *ASKβ* in *Arabidopsis*. The qRT-PCR was carried out with wild-type *Arabidopsis*. FS1-12, flower stage 1 to stage 12; FS13-16, flower stage 13 to stage 16 (Smyth et al., 1990). Expression levels were normalized to that of *ACTIN7*. Data were obtained from two biological replicates and the error bar represents standard deviation.

pollen (Tichtinsky et al., 1998; Wellmer et al., 2004), we speculated that the major function of *ASKβ* is related to pollen development, with little or no role in other tissues or cell types.

Knock-down of *ASKβ* levels causes reduced fertility

We used antisense technology to monitor the phenotypes of plants in which *ASKβ* expression was reduced. No T-DNA mutant lines harboring insertions in the coding region are available for *ASKβ*. A line containing an insertion in the promoter region of *ASKβ* (SAIL_66_D05) did not show any reduction in *ASKβ* expression compare to WT plants (data not show). Therefore, we adopted antisense approach. The antisense construct included the entire *ASKβ* coding region driven by constitutive *CaMV 35S*, since identical nucleotide sequences longer than 21-nucleotide were rarely identified between *ASKβ* and other members (Fig. 3A). After glufosinate selection, the T1 transformants were further confirmed by genomic DNA PCR using the *AKSβ-P2* and *35S* primer sets listed in Table S1. In seven independent T2 transgenic lines, variable effects on *ASKβ* expression were detected (Figs. 3B and 3C): four lines (lines 4, 25, 29 and 33) showed over 70% reductions in *ASKβ* expression, while two lines (lines 2 and 20) showed 10-30% reductions and one line (line 24) showed no reduction. Unlike the changes in expression observed for *ASKβ*, the expression levels of *ASKθ* (another *ASK* in clade III, which is most closely related to *ASKβ*) only showed weak or no reductions (Supplementary Fig. S3B). In addition, the expression levels of other *ASK* genes in *Arabidopsis* have been determined for WT, antisense transgenic lines 2 and 4, and no significant reduction in gene expression was observed. We assume that the phenotype of *ASKβ* antisense plants is mainly due to the suppressed expression of *ASKβ*. During vegetative growth, no phenotypic

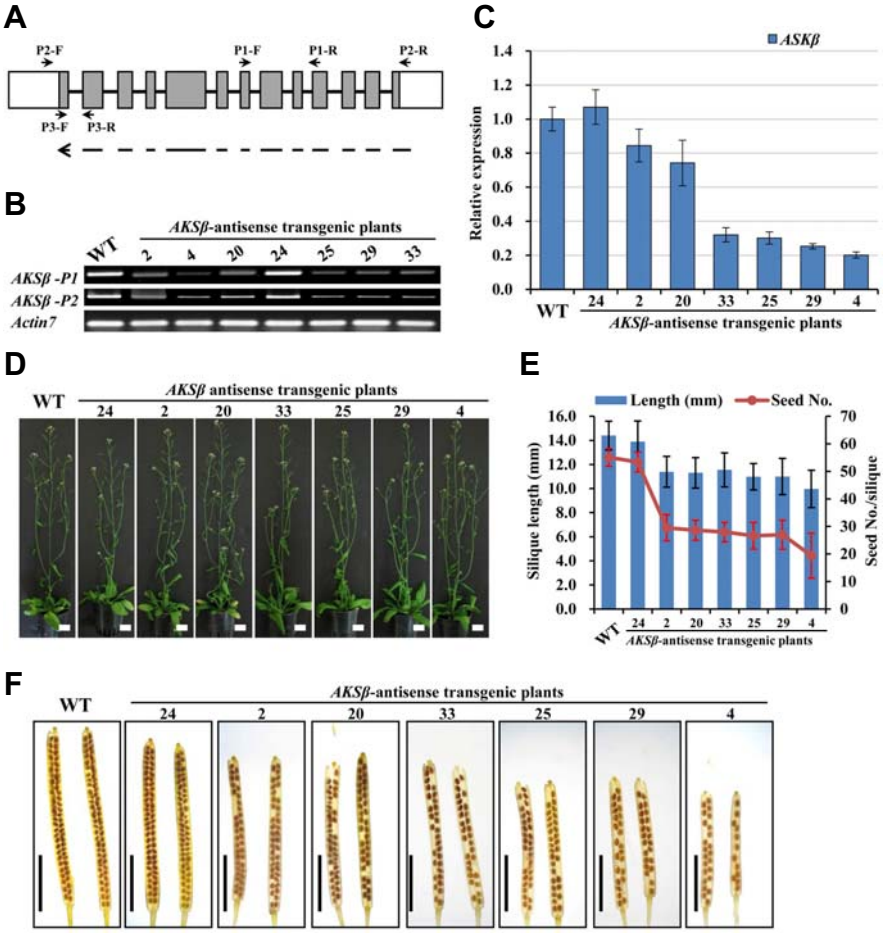

Fig. 3. Analysis of *ASKβ* antisense transgenic *Arabidopsis* plants. (A) Schematic representation of the *AKSβ* gene structure and DNA fragments for antisense constructs. Gray boxes indicate exons, while the line and white boxes represent introns and UTRs, respectively. The dotted arrow in the antisense orientation at the bottom shows the DNA region used in the constructs. Arrows with names starting with "P" indicate the positions and directions of primers used: P1 and P2 for Semi-RT-PCR, and P3 for qRT-PCR. (B) *AKSβ* transcript levels in floral buds of wild-type and antisense transgenic lines determined by semi-RT-PCR. *ACTIN7* PCR was performed to validate that equal amounts of cDNA were used in each reaction. (C) Transcript levels of *AKSβ* and *ASKθ* measured by qRT-PCR. Expression levels were normalized to that of *ACTIN7* and represented relative to wild-type expression levels. Error bars represent standard deviation (SD) of three biologically independent experiments. (D) Whole plant phenotypes showing no obvious differences between wild-type and *ASKβ* transgenic plants in vegetative growth. Bar = 20 mm. (E) Silique length and seed numbers. Mean values and SD were measured from three experiments using 18 plants per experiment. (F) Silique lengths and seed numbers in *ASKβ* antisense transgenic lines. Whole-mount images of mature siliques cleared with 0.2 NaOH and 1% SDS solution were used. Bar = 5 mm.

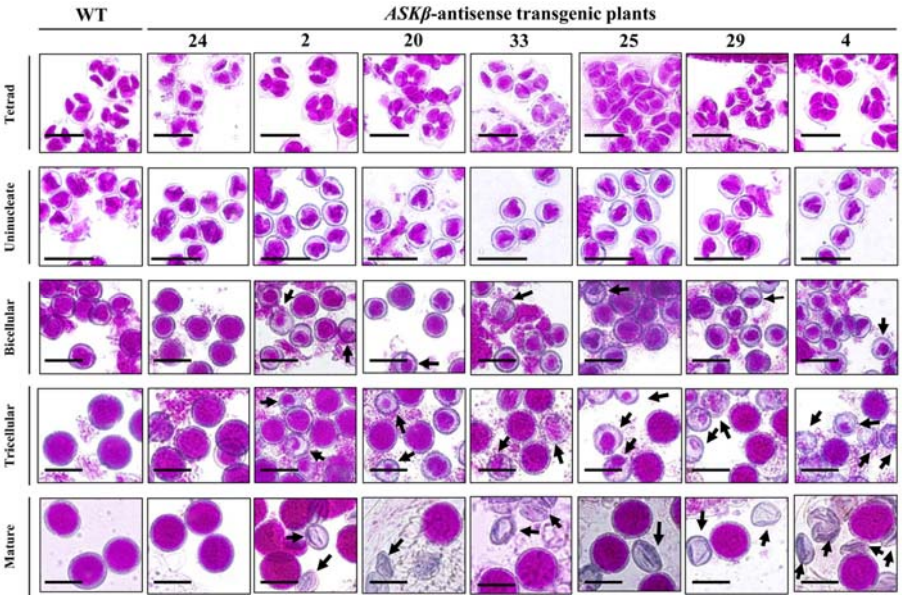

Fig. 4. Male gametophyte development in wild-type and *ASKβ* antisense transgenic lines. Male gametophytes were stained with modified Alexander's stain (Peterson et al., 2010). Tetrad, uninucleate, bicellular, tricellular and mature indicate the different stages of pollen development. Bar = 20 μm.

difference was observed between transgenic and wild-type plants (Fig. 3D). In contrast to vegetative phenotypes, reproductive traits including silique size and seed number per silique exhibited meaningful differences in the antisense transgenic lines. It should be noted that the seed number was greatly affected by the reduced expression of *ASKβ* but we could not find any correlation between *ASKθ* expression level and reduced fertility (Figs. 3D and 3E).

Down-regulation of *ASKβ* leads to defects in microspore development after the uninucleate stage

Because the predominant expression of *ASKβ* has been observed in pollen (Tichtinsky et al., 1998; Wellmer et al., 2004), we suspected disrupted pollen development in the antisense transgenic plants resulted in the reduced fertility. To test this, we first investigated the morphology of mature pollen grains with a modified Alexander's staining (Peterson et al., 2010). We found that significant amounts of pollen grains in the transgenic plants were aborted, even though overall anther morphology seemed to be normal (Supplementary Fig. S3A). The severity of the abortion phenotype showed a clear correlation with the strength of antisense effects on *ASKβ* expression (Fig. 3 and Supplementary Fig. S3A). As a first step in identifying the function of *ASKβ* in pollen development, the morphologies of developing male gametophytes at various developmental stages were systematically compared between wild-type and *ASKβ* antisense plants under light microscopy after Alexander staining (Fig. 4). Before tetrad stage, no differences were observed between wild-type and *ASKβ* antisense plants (data not show). As shown in Figure 4, the microspores were also very similar in the wild-type and transgenic plants at the tetrad and uninucleate stages. However, clear differences were detected after these stages. From the bicellular stage to the mature stage, wild-type microspores exhibited typical round, gradually enlarging forms. By contrast, some of the *ASKβ* microspores from the antisense transgenic lines displayed signs of cytosol shrinkage, which appeared to be responsible for the failure to develop intact pollen grains. The defects in *ASKβ* antisense pollen became more obvious at the tricellular stage. The loss of the inner contents of some mature pollen grains resulted in the production of smaller or shrunken pollen grains. To examine whether *ASKβ* knock-down also affects tapetal tissue development and differentiation and thereby indirectly causes defects in pollen development, semi-thin transverse sections were produced from anthers at stage 8 to 13 (Supplementary Fig. S4) (Sanders et al., 1999). Following the degradation of the callose wall, microspores are released at anther stage 8. From anther stage 9 to 12 (floral stage 10 to 12), microspores develop into pollen grains. Tapetum degradation occurs during stage 10 and 11, while pollen mitotic divisions are observed during anther stage 12, resulting in the formation of tricellular pollen grains. At stage 13 and 14, anther dehiscence and shrinkage of anther cells occurs, respectively (Ma, 2005). In *ASKβ* antisense transgenic plants producing defective pollen grains, tapetum differentiation seemed to be identical to that of wild-type plants. The normal anther morphology and tapetum differentiation observed in these lines (Supplementary Figs. S3A and S4) indicate that suppression of *ASKβ* levels specifically interferes with microspore development without affecting sporophytic tissues in the anther.

Some *ASKβ* co-expressed genes are involved in the late stage of pollen development

To help elucidate how *ASKβ* functions during pollen development, we analyzed genes that are co-expressed with *ASKβ* using 'Expression Angler' (Toufighi et al., 2005). A total of 641 co-expressed genes were isolated (Supplementary Table S3) and subjected to gene ontology (GO) annotation enrichment analysis using the singular enrichment analysis tool in agriGO (Du et al., 2010). Genes in the category "pollen development process (GO:0009555)" were highly represented (p value = 1.30E-11), which included 32 genes (Table 1 and Supplementary Table S4). The expression patterns of these 32 genes were reconstructed using *Arabidopsis* eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) (Winter et al., 2007) (Fig. 5A). All of these genes, as well as *ASKβ*, were highly expressed in tricellular and mature stage pollen grains, which is in

Table 1. List of pollen development-related genes that are co-expressed with *ASKβ*

At Locus	R-value to Bait	Gene name	Expression patterns	Phenotype of knock out	References
					Expression / Phenotype
At3g21180	0.9830	*ACA9*	Expressed primarily in pollen	Reduction in seed set; defects in pollen tube growth and fertilization	Schiott et al. (2004)
At1g24520	0.9770	*BCP1*	Diploid tapetum and haploid microspore	Pollen cytoplasmic degeneration and loss of cellular contents	Xu et al. (1995)
At4g05330	0.9670	*AGD13*	-	~5-10% two celled pollen grains	Reňák et al. (2012)
At3g57390	0.9650	*AGL18*	Male and female gametophyte, pollen	-	Kofuji et al. (2003)
At3g62230	0.9640	*DAF1*	Pollen sperm cells	-	Borg et al. (2011)
At5g64510	0.9580	*TIN1*	Highly expressed in pollen	Abnormal pollen surface morphology	Iwata et al. (2012)
At2g32460	0.9530	*MYB101*	-	Misarranged male sperm units	Reňák et al. (2012)
At3g01040	0.9470	*GAUT13*	Pollen grains and pollen tube	Defected in pollen tube growth	Wang et al. (2013b)
At2g02970	0.9450	*APY6*	Expressed in mature pollen grains	Minor change in pollen exine pattern	Yang et al. (2013)
At5g39400	0.9350	*ATPTEN1*	Expressed exclusively in pollen grains	Pollen cell death after mitosis	Gupta et al. (2002)
At3g47440	0.9340	*TIP5;1*	Pollen sperm cells	Defected in pollen tube in the absence of exogenous nitrogen	Borg et al. (2011) and Soto et al. (2010)
At4g35700	0.9340	*DAZ3*	Pollen sperm cells	-	Borg et al. (2011)
At1g53320	0.9160	*ATTLP7*	-	Two celled pollen	Reňák et al. (2012)
At2g35210	0.9040	*RPA*	-	No sperm cell in mature pollen grains and cytoplasmic degradation	Boavida et al. (2009)
At2g03060	0.9020	*AGL30*	Inflorescence tissues	Reduced pollen fertility	Adamczyk and Fernandez (2009)
At5g22000	0.8940	*RHF2A*	Stamens and carpels	Defective in the formation of male and female gametophytes	Liu et al. (2008)
At3g10470	0.8930	-	-	-	-
At1g68610	0.8890	*PCR11*	Pollen sperm cells	-	Borg et al. (2011)
At1g47270	0.8850	*ATTLP6*	-	Two celled pollen	Reňák et al. (2012)
At5g15470	0.8750	*GAUT14*	Pollen grains and pollen tube	Defected in pollen tube growth	Wang et al. (2013b)
At5g59030	0.8680	*COPT1*	Embryos, trichomes, pollen, and root	Displayed an enhanced nutritional induced pollen abnormalities	Sancenon et al. (2004)
At5g39650	0.8670	*DAU2*	Pollen sperm cells	-	Borg et al. (2011)
At5g23670	0.8490	*LCB2*	Ubiquitously expressed in *Arabidopsis*	Pollen contained aberrant endo-membrane and lacked an intine layer	Dietrich et al. (2008)
At3g50310	0.8480	*MKKK20*	Pollen sperm cells	-	Borg et al. (2011)
At5g02390	0.8380	*DAU1*	Pollen sperm cells	-	Borg et al. (2011)
At2g18080	0.8340	*EDA2*	-	-	-
At5g53520	0.8290	*ATOPT8*	Pollen sperm cells	-	Borg et al. (2011)
At1g18750	0.8290	*AGL65*	Inflorescence tissues	Reduced pollen fertility	Adamczyk and Fernandez (2009)
At1g22130	0.8270	*AGL104*	Inflorescence tissues	Reduced pollen fertility	Adamczyk and Fernandez (2009)
At1g77980	0.8240	*AGL66*	Inflorescence tissues	Reduced pollen fertility	Adamczyk and Fernandez (2009)
At2g42380	0.8180	*ATBZIP34*	Gametophytic and sporophytic tissues	Misshapen and misplaced nuclei; defected in exine shape	Gibalova et al. (2009)
At1g19890	0.8140	*ATMGH3*	Pollen sperm cells	-	Borg et al. (2011)

line with the time frame for the *ASKβ* antisense phenotypes (Fig. 4). The functions of the 32 genes mentioned above are summarized in Table 1; knock-outs of many of these genes have been reported to affect pollen development at later stages of sperm cell differentiation, pollen wall assembly, and pollen tube growth.

Since co-expressed genes tend to be regulated by the same or similar transcription factor(s) (Coppe et al., 2009), we analyzed the promoter sequences of these genes to gather information about the regulation of *ASKβ*. *Cis*-regulatory elements (CREs) are frequently distributed within a region 500 bp upstream of the transcription start site and their distributions largely determine the transcriptional activity of promoters (Wang et al., 2011). Analysis of the promoter regions of *ASKβ* and 32 co-expressed genes identified six conserved CREs at cutoff values of E≤1.00E + 02 (Supplementary Table S5). In particular, two CRE motifs (motif 1 and 3), which are annotated as *POLLEN1LELAT52* and *MYB1AT* in the PLACE database (Higo et al., 1999), were found in all of the promoter regions (Figs. 5B and 5C; Supplementary Fig. S5; Supplementary Tables S4 and S5). *POLLEN1LELAT52* specifies pollen-specific activation and was originally identified in the tomato

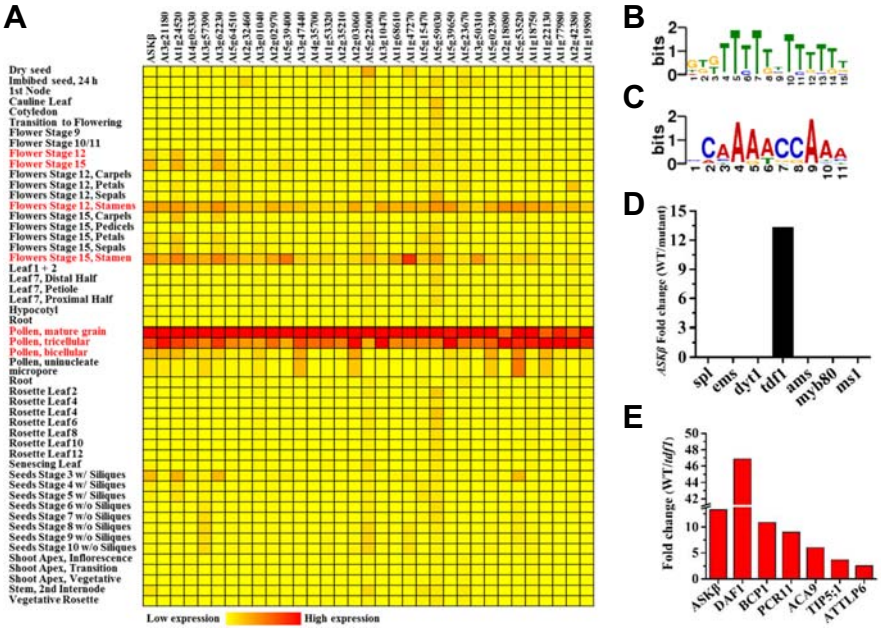

Fig. 5. Analysis of putative *ASKβ*-interacting genes. (A) Expression pattern analysis of *ASKβ* and co-expressed pollen development-related genes. (B) Sequence Logo of conserved Motif 1, which is annotated as POLLEN1LELAT 52 in PLACE database and is present in *ASKβ* and its 32 co-expressed genes. (C) Sequence Logo of the conserved Motif 3, which is annotated as MYB1AT in PLACE database and is present in all analyzed genes. (D) *ASKβ* expression levels in wild-type plants with respect to that in a corresponding mutant background (based on previous reports in Feng et al., 2012; Phan et al., 2011; Wijeratne et al., 2007; Xu et al., 2010; Yang et al., 2007; Zhu et al., 2008). (E) Expression levels of *ASKβ* and its six co-expressed genes in the *tdf1* mutant background, according to data in the above references.

lat52 gene (Abe et al., 2003; Bate and Twell, 1998), and *MYB1AT* is a CRE for MYB recognition (Filichkin et al., 2004).

TDF1 (TAPETAL DEVELOPMENT AND FUNCTION 1) and six pollen-expressed genes are a putative upstream regulator and downstream components of ASKβ, respectively

In *Arabidopsis*, functions of transcription factors controlling anther and pollen development have been well studied using mutant analysis, and many putative downstream genes have been isolated by microarray analysis (Feng et al., 2012; Phan et al., 2011; Wijeratne et al., 2007; Xu et al., 2010; Yang et al., 2007; Zhu et al., 2008). From these previously obtained data sets, we obtained the expression levels of *ASKβ* and its co-expressed genes, and replotted them as ratios between wild-type and mutants of interest (Figs. 5D and 5E). Among the mutant backgrounds of well-known transcription factors for male gametophyte development, *ASKβ* expression was strongly reduced only in the *tdf1* background (Fig. 5D), while another MYB transcription factor, MYB80 (also known as MYB103), did not show any effects. This result indicates that TDF1 is an upstream transcription factor necessary for *ASKβ* transcription, but other transcription factors used in the analysis are not. In addition, 6 out of 32 *ASKβ* co-expressed genes were down-regulated in the *tdf1* mutant (Fig. 5E), raising the possibility that they are expressed downstream of TDF1 and are also regulated by *ASKβ*: *At1g24520*/*BCP1* (Homolog of *Brassica campestris pollen protein 1*), *At3g62230*/*DAF1* (*DUO1-Activated F-box 1*), *At3g47440*/*TIP5;1* (putative *aquaporin TIP5-1*), *At1g68610*/*PCR11* (*plant cadmium resistance 11*), *At3g21180*/*ACA9* (*auto-inhibited calcium ATPase 9*), and *At1g47270*/*TLP6* (*Tubby-like protein 6*).

To test our hypotheses on the positions of upstream or downstream genes in the *ASKβ* transcriptional cascade, we performed qRT-PCR using floral buds of wild-type and *ASKβ* antisense transgenic lines (Fig. 6). In this analysis, we also included *MYB80*, which is a downstream gene of *TDF1* (Zhu et al., 2008), to narrow down the position of *ASKβ* in *TDF1*- and *MYB80*-dependent transcriptional regulation. Compared with wild-type plants, the expression levels of *TDF1* and *MYB80* did not change in the *ASKβ* antisense transgenic lines (Fig. 6A). Given that *ASKβ* expression is not affected by the mutation of *MYB80* (Fig. 5D), this result indicates that (1) *ASKβ* is transcriptionally regulated downstream of *TDF1*, and (2) *ASKβ* and *MYB80* are located in separate branches downstream of *TDF1*. Six *ASKβ* co-expressed and *TDF1*-regulated genes can be categorized by their putative functions or expression characteristic, as summarized in Table 1. Knock-out of *BCP1* and *ATTLP6* mutations leads to cytoplasmic degradation and defects in late pollen development, respectively (Reňák et al., 2012; Xu et al., 1995). While *DAF1*, *PCR11*, and *TIP5;1* are involved in sperm cell formation (Borg et al., 2011), *ACC9* is important for fertility and mutation in *ACC9* results in more than 80% reduction of seed set (Schiott et al., 2004). As expected for the downstream genes of *ASKβ*, the expression patterns of all six genes examined were quite similar to those of *ASKβ* in wild-type and antisense transgenic lines with variable *ASKβ* levels (Figs. 6B-6E).

ASKβ suppression affects sperm cell formation at the bicellular stage

Staining with DAPI allows easy visualization of the number, shapes, and positions of nuclei during late stages of pollen development (Fig. 7). In uninucleate microspores, DAPI-stained pollen from *ASKβ* antisense plants appeared to be normal. However, loss of cytoplasm and nuclei was observed from the bicellular stage pollen grains, which leads to the productions of slightly smaller and aberrant pollen grains. Defects in pollen grains were more clearly observed in tricellular stage and mature pollen grains (Fig. 7). Together with the results of co-expression and qRT-PCR analysis, these results lead to the conclusion that *ASKβ* plays an important role in *Arabidopsis* microgametogenesis through the activation of many important genes required for late pollen development: more specifically for pollen sperm nuclei formation.

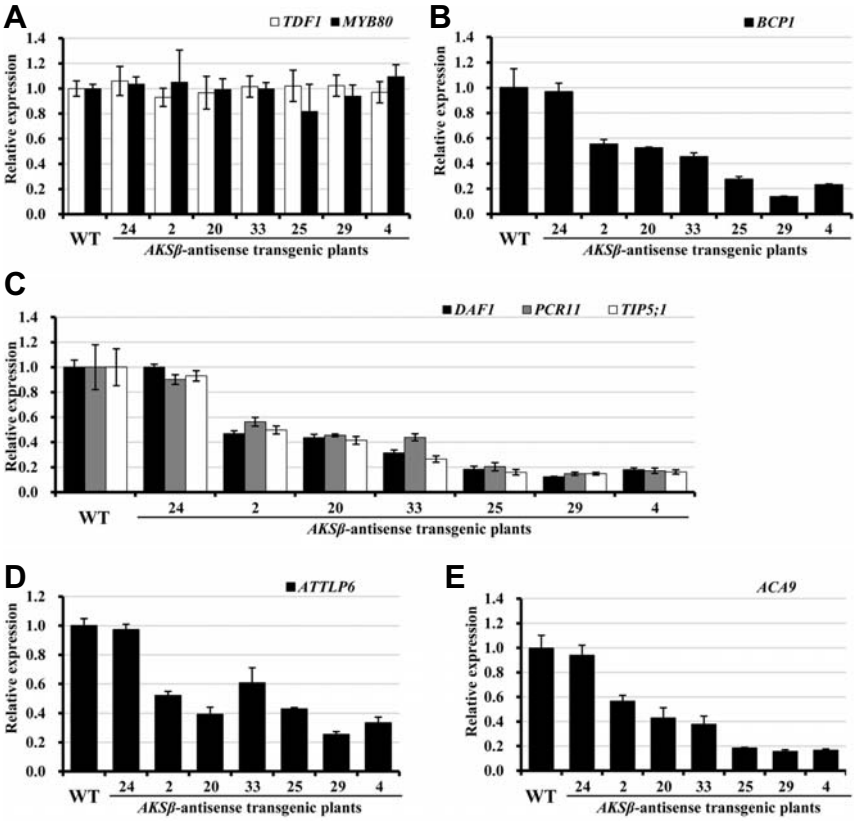

Fig. 6. Results of qRT-PCR analyses of putative upstream and downstream genes of *ASKβ*. Figures are grouped according to their putative functions or expression, as summarized in Table 1: *BCP1* for cytoplasm degradation; *DAF1*, *PCR11* and *TIP5;1* for sperm cell development; *ATTLP7* for two-celled pollen; *ACA9* for aborted fertilization. Relative transcript levels in wild-type and *ASKβ* antisense transgenic lines are shown as follows: (A) *TDF1* and *MYB80*; (B) *BCP1*; (C) *DAF1*, *PCR11* and *TIP5;1*; (D) *ATTLP6*; and (E) *ACA9*. Expression levels were normalized to that of *ACTIN7* and presented as the mean ± SD of three biologically independent experiments.

DISCUSSION

Phylogenetic and expression data strongly suggest an orthologous relationship between *ASKβ* (*At3g61160*) and *BrASKβ* (*Bra003440*)

BrASKβ, which is expressed in fertile floral buds but not in genic male sterile floral buds, was identified as a putative male fertility-related gene and was initially named based on its sequence similarity to *ASKβ* (Dong et al., 2013). The following observations support the notion that *ASKβ* is the *Arabidopsis* ortholog of *BrASKβ*: (1) these genes display a close association in genome-wide phylogenetic analysis (Fig. 1); (2) both genes are predominantly expressed in developing pollen (Dong et al., 2013; Tichtinsky et al., 1998; Figs. 2 and 5), and (3) their functions are linked to proper pollen development (Dong et al., 2013; Figs. 3 and 4; Supplementary Figs. S3A and S4). In the phylogenetic tree constructed using both *Arabidopsis* and *B. rapa ASK* genes, another *B. rapa* gene, *Bra007577*, was also found near *ASKβ* in a clade containing *ASKβ* and *ASKθ* (Fig. 1). *BrASKβ* and *Bra007577* may be produced from a common ancestor of *Arabidopsis ASKβ* by the genome triplication in *Brassica* lineage (Yang et al., 2005).

Putative function(s) of *ASKβ* appears to be restricted to pollen development

Most *ASKs* that have been functionally characterized to date are implicated in BR signaling and are found in clade I, II and III (Saidi et al., 2012). Although the roles of *ASKs* in flower development and osmotic stress responses have also been determined or proposed, seven of the ten *ASKs* in *Arabidopsis* are considered to be involved in BR signaling, including *ASKα*/*AtSK11*, *ASKγ*/*AtSK12*, *ASKδ*/*AtSK13*, *ASKη*/*AtSK21*/*BIN2*, *ASKι*/*AtSK22*/*BIL1*, *ASKζ*/*AtSK23*/*BIL2* and *ASKθ*/*AtSK31*. Among these, three *ASK* members play an additional role in flower development. *ASKα*/*AtSK11* and *ASKγ*/*AtSK12* are preferentially expressed in early floral meristems, and down-regulation of each gene via antisense technology results in similar phenotypes, *i.e.*, an increase in perianth organ number and defects in gynoecium development (Dornelas et al., 2000). Moreover, plants over-expressing the mutated version of *ASKθ*/*AtSK31* (R178A) produce flowers with smaller pedicels, petals, and sepals (Claise et al., 2007). Although Claise and colleagues did not observe any altered phenotypes when they over-expressed a wild-type version of *ASKθ*/*AtSK31* (Claise et al., 2007), a later study reported that over-expression of ASKθ/AtSK31-Myc fusion protein led to pleiotropic phenotypes in BR-deficient transgenic plants (Rozhon et al., 2010). The finding that BR and ABA can act antagonistically, which involves the action of clade II *ASKs* (*ASKη*/*BIN2*, *ASKι*/*BIL1*, and *ASKζ*/*BIL2*), as well as the observation that over-expression of a clade II ASK (ASKι/BIL1) can induce NaCl tolerance, suggest that ASKs function as integrators of plant hormones and environmental conditions (Cai et al., 2014; Piao et al., 2001; Zhang et al., 2009).

Unlike the phenotypes reported in previous studies of *ASKs*, our investigation of *ASKβ* function using antisense transgenic plants revealed that *ASKβ* is required for fertility in *Arabidopsis* (Figs. 3 and 4). The phenotypes observed in the current investigation seem to be caused by the selective suppression of the intended target, *ASKβ*. Most importantly, we found that reduced fertility, which can be explained by the presence of aborted

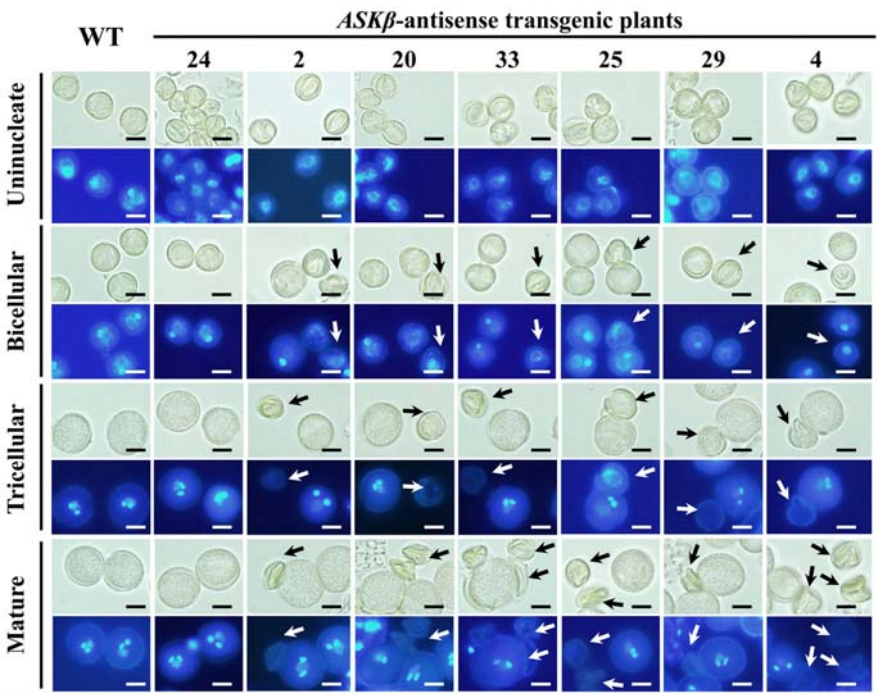

Fig. 7. Pollen grains of wild-type and *ASKβ* antisense transgenic plants stained with DAPI. For each developmental stage and phenotype, the upper and lower panel shows bright-field and fluorescence microscope images, respectively. Aberrant or no staining in pollen grains is indicated with arrows. Bar = 10 μm.

pollen, is not associated with the expression level of *ASKθ* (Fig. 3). Among *ASK* gene family members, *ASKθ* is most closely related to *ASKβ* (Fig. 1), and these two members (in clade III) are different from the other members, as they contain N-terminal extensions (Supplementary Fig. S1). Although *ASKβ* and *ASKθ* show rather high levels of sequence identity across the entire sequences at the nucleotide level, the length of identical regions without any mismatch are less than 21 nucleotides with a few exceptions (data not shown). The notion that antisense effect is specific to *ASKβ* is further supported by previous studies reporting no morphological defects in pollen development for plants with hypomorphic alleles of *ASKθ* or reduced expression of *ASKα*/*ASKγ* (Claise et al., 2007; Dornelas et al., 2000).

Various phenotypic outcomes reported for plants in which the functions of different *ASKs* were disrupted may be related to the discrete expression patterns and/or physical interactions of individual ASKs (Supplementary Fig. S6). According to the expression analysis using eFP browser tool, *ASKα* and *ASKγ* are widely expressed but are much more highly expressed in senescing leaves, while that of *ASKβ* is primarily limited to late stage pollen. *ASKθ*, the most closely related gene to *ASKβ*, was clearly induced in developing pollen, but only in the tricellular to mature pollen stage. By contrast, *ASKβ* was clearly expressed beginning at the bicellular stage. In addition, ASKθ strongly interacts with BR-responsive transcription factors (BEH2, BES1 and BZR1), but ASK*β* does not bind to any BR-responsive transcription factor (Rozhon et al., 2010).

ASKβ may regulate pollen development at late microsporogenesis

Previous studies have shown that *ASKβ* and some of the 32 co-expressed genes, such as *BCP1*, *TIP5;1*, *COPT1*, and *AGL65*, are expressed in a pollen-specific manner under the control of the MYB transcription factor TDF1 (Adamczyk and Fernandez, 2009; Sancenon et al., 2004; Soto et al., 2008; Wellmer et al., 2004; Xu et al., 1995; Zhu et al., 2008). Our results functionally confirm that *ASKβ* is expressed during late pollen development downstream of the TDF1 MYB transcription factor (Figs. 4-6; Supplementary Fig. S4). Furthermore, our expression profiling results indicate that *ASKβ* is an upstream component controlling the expression of *BCP1*, *DAF1*, *PCR11*, *TIP5;1*, *ATTLP6*, and *ACA9* (Fig. 6). Considering that *BCP1* antisense plants also display defects in pollen development and reduced fertility, and the first signs of pollen generation are evident at the bicellular stage (Xu et al., 1995), the phenotypes observed in the *ASKβ* antisense transgenic plants generated in the current study were at least partly associated with the down-regulation of *BCP1* (Fig. 6). Consistent with the current results, the expression of *DAF1*, *PCR11*, *TIP5;1*, *ATTLP6*, and *ACA9* is more pronounced after the tetrad stage when *ASKβ* expression is strong, and altering the expression of these genes also causes abnormal microgametogenesis (Borg et al., 2011; Reňák et al., 2012; Schiott et al., 2004; Soto et al., 2010). ASKβ may activate regulatory protein(s) that transcriptionally induce the expression of these genes.

ASKβ may function during sperm cell formation. Asymmetric division of the haploid microspore produces the male germline cells for double fertilization in flowering plants (Twell, 2011). In rice, knock-down of a glycosyltransferase, *OSGT1*, affects pollen intine formation, leading to the disappearance of nuclei and a reduction in pollen size (Moon et al., 2013). In addition, knock-down of Dynamin-related protein 2 (*DRP2*) results in the production of shrunken mature pollen grains with fewer or no nuclei (Backues et al., 2010). Numerous *ASKβ* co-expressed genes are involved in sperm cell differentiation (genes and their references are found in Table 1). Among these, mutants of *AGD13*, *ATTLP7*, *ATTLP6*, and *MYB101* exhibit two-celled pollen grains or misarranged male sperm units (Reňák et al., 2012), while mutants of *PDD6* have no sperm cells in their mature pollen grains and exhibit cytoplasmic degradation

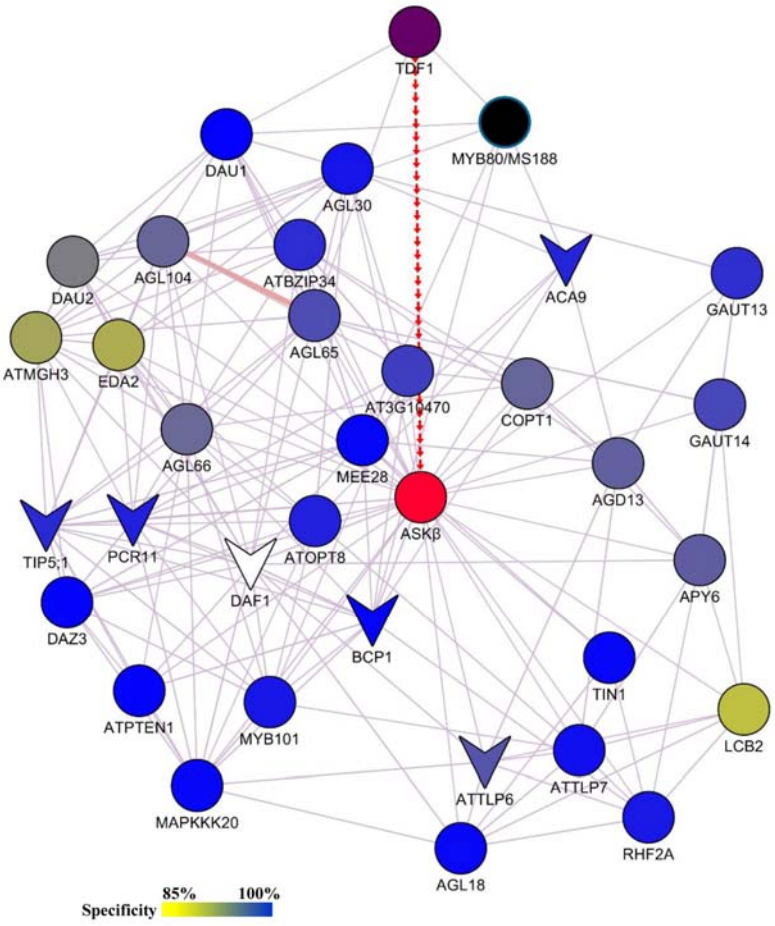

Fig. 8. A network composed of *ASKβ* and its 32 co-expressed genes that have the GO annotation "pollen development process". *ASKβ* is indicated in red, while *ASKβ* co-expressed genes are indicated with different colors (ranging from yellow to blue) based on their maximum specificity value for putative phosphorylation sites predicted by Musite (http://musite.sourceforge.net/). *DAF1* does not appear to have any putative phosphorylation sites at a cutoff of 85%. *TDF1* and *MYB80*, the upstream and independent gene of *ASKβ*, are indicated in dark purple and black, respectively. The genes indicated with a "V" shape are the putative downstream genes of *ASKβ* identified in this study. Light purple lines show the co-expression interactions, while the thick, light pink line indicates a reported physical interaction (de Folter et al., 2005; Adamczyk and Fernandez, 2009). The regulation of *ASKβ* by TDF1 confirmed in this study is indicated with red arrows.

(Boavida et al., 2009). Recently, *DAF1*, *PCR11*, and *TIP5;1* were reported to be target genes of the male germline-specific transcription factor *DUO POLLEN 1*, which is also required for germline nucleus development (Borg et al., 2014).

The putative roles of *ASKβ* in pollen development might also involve phosphorylation of co-expressed genes

In addition to dynamic changes in gene expression, post-translational modifications of regulatory proteins are also necessary for proper pollen development (Guan et al., 2014). Therefore, the disturbance of phosphorylation cascades in *ASKβ* antisense plants should be the primary factor contributing to the disappearance of pollen nuclei and the subsequent appearance of shrunken pollen. To extend our knowledge of the functions of ASKβ during pollen development, we built an expression network involving *ASKβ* and its 32 co-expressed genes (Fig. 8). For some co-expressed genes, ASKβ kinase is likely to phosphorylate upstream signaling components, resulting in the transcriptional activation of these co-expressed genes. However, it is possible that ASKβ-dependent phosphorylation cascades also directly modify the activities of these co-expressed genes at the protein level. Indeed, analysis using Musite (with a cutoff value of 85%) revealed that all *ASKβ* co-expressed gene products except *DAF1* contain at least one putative serine/threonine-specific phosphorylation site (Supplementary Table S6; Gao et al., 2010). GSK3/SGG kinases can use both priming-phosphorylated proteins and non-priming-phosphorylated proteins as substrates (de la Fuente van Bentem et al., 2008; Doble and Woodgett, 2003; Zhao et al., 2002). Many proteins encoded by *ASKβ* co-expressed genes may function as substrates of ASKβ and be directly regulated by ASKβ (Supplementary Table S6).

In summary, the current results demonstrate that *ASKβ* function is required for fertility of *Arabidopsis* plants downstream of *TDF1,* which is mediated through the coordinated regulation of many pollen development-related genes. Consistent with the expression patterns of *ASKβ*, we determined that the first signs of deteriorating male gametophytes begin to appear at the bicellular stage. Based on the finding that *ASKβ* co-expressed and -regulated genes play important roles in sperm cell differentiation, we propose that *ASKβ* plays a similar role. Identification of the target proteins of *ASKβ* kinase and investigation of the mechanism defining the distinct roles of *ASKβ* and *ASKθ* will further expand our knowledge of the pollen development process and GSK3/SGG-like kinases in plants.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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