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RESEARCH PAPER

Overexpression of the aspartic protease *ASPG1* gene confers drought avoidance in *Arabidopsis*

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Abstract

Drought is one of the most severe environmental stresses affecting plant growth and limiting crop production. Although many genes involved in adaptation to drought stress have been disclosed, the relevant molecular mechanisms are far from understood. This study describes an *Arabidopsis* gene, *ASPG1* (*ASPARTIC PROTEASE IN GUARD CELL 1*), that may function in drought avoidance through abscisic acid (ABA) signalling in guard cells. Overexpression of the *ASPG1* gene enhanced ABA sensitivity in guard cells and reduced water loss in ectopically overexpressing ASPG1 (*ASPG1-OE*) transgenic plants. In *ASPG1-OE* plants, some downstream targets in ABA and/or drought-signalling pathways were altered at various levels, suggesting the involvement of *ASPG1* in ABA-dependent drought avoidance in *Arabidopsis*. By analysing the activities of several antioxidases including superoxide dismutase and catalase in *ASPG1-OE* plants, the existence was demonstrated of an effective detoxification system for drought avoidance in these plants. Analysis of ProASPG1-GUS lines showed a predominant guard cell expression pattern in various aerial tissues. Moreover, the protease activity of ASPG1 was characterized *in vitro*, and two aspartic acid sites, D180 and D379, were found to be key residues for ASPG1 aspartic protease activity in response to ABA. In summary, these findings suggest that functional ASPG1 may be involved in ABA-dependent responsiveness and that overexpression of the *ASPG1* gene can confer drought avoidance in *Arabidopsis*.

Key words: ABA signalling, ASPG1, aspartic protease, drought avoidance, guard cell.

Introduction

Abiotic stress conditions such as drought, salinity, and extreme temperatures all have a negative impact on the growth and productivity of plants (Boyer, 1982). For example, dehydration can lead to inhibition of physiological processes; thus, plants have to initiate adaptive mechanisms to survive (Luan, 2002; Kwak *et al.*, 2008). Two pathways involved with drought stress adaptation have been characterized in *Arabidopsis*: (i) elevation of abscisic acid (ABA) levels can stimulate the activity of downstream targets; and (ii) an ABA-independent signal transduction pathway may direct counteraction against the dehydration (Yamaguchi-Shinozaki and Shinozaki, 2006). Numerous studies on ABA-regulated adaptation to drought stress have been reported (Schroeder *et al.*, 2001; Zhu, 2002; Shinozaki and Yamaguchi-Shinozaki, 2006). The increase in ABA biosynthesis caused by dehydration indicates the importance of ABA signalling in adaptation to drought stress in plants (Guerrero and Mullet, 1986). ABA may trigger oscillations of cytosolic calcium in guard cells (McAinsh *et al.*, 1990; Allan *et al.*, 1994). Subsequently, the two types (S-type and R-type) of anion channels in the plasma membrane of guard cells can be activated (Schroeder and Hagiwara, 1989; Hedrich *et al.*, 1990). Anion channels have been suggested to play a central role in stomatal closure. Genetic analysis has confirmed the regulatory role of SLAC1, the guard-cell S-type anion channel, in ABA-induced stomatal closure (Negi *et al.*, 2008;

Abbreviations: ABA, abscisic acid; ABRC, Arabidopsis Biological Resource Center; ABRE, abscisic acid-responsive element; ABF, ABRE-binding factor; amiRNA, artificial microRNA; CAT, catalase; CFP, cyan fluorescent protein; DCF, dichlorofluorescein; ER, endoplasmic reticulum; GCP, guard cell protoplast; GFP, green fluorescent protein; GUS, β-glucuronidase; H_2O_2, hydrogen peroxide; LUC, luciferase; MCP, mesophyll cell protoplast; ROS, reactive oxygen species; SOD, superoxide dismutase.

Vahisalu *et al.*, 2008). Under drought conditions, ABA triggers the production of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) in guard cells, in turn promoting stomatal closure (Pei *et al.*, 2000; Zhang *et al.*, 2001; Kwak *et al.*, 2003; Desikan *et al.*, 2004; Kwak *et al.*, 2008). ABA-induced elevation of cytosolic calcium can promote ROS production in guard cells through activation of NADPH oxidases (such as AtbohD and AtbohF), leading to stomatal closure (Kwak *et al.*, 2003). OST1 (a member of the protein kinase SnRK2 family; Yoshida *et al.*, 2002) acts upstream of ROS in guard cells in response to ABA (Mustilli *et al.*, 2002). A recent study demonstrated that the OST1 can regulate AtbohF activity through phosphorylation (Sirichandra *et al.*, 2009). In the primary ABA signal transduction pathway, the ABA receptor PYR/RCAR, protein phosphatase 2Cs (PP2Cs) ABI1, and protein kinase SnRK2 together act as essential components for initiating ABA signal transduction (Fujii *et al.*, 2009; Miyazono *et al.*, 2009; Nishimura *et al.*, 2009). Previous studies have also shown that H_2O_2 can inhibit the ABA-induced activities of two types of PP2Cs (ABI1 and ABI2) (Meinhard and Grill, 2001; Meinhard *et al.*, 2002). Thus, ROS can function as the second messenger in mediating ABA signal transduction in guard cells (Kwak *et al.*, 2008). Although ROS can act as a positive regulator in ABA signalling in guard cells, excessive accumulation of ROS during drought stress can be very toxic, killing the plant cells (Meinhard *et al.*, 2002; Kwak *et al.*, 2003). At least two regulatory mechanisms are required to balance the spatial–temporal dynamics of ROS production and scavenging: one to modulate low levels of ROS for signal transduction and another to detoxify excessive ROS in cells during stress (Dat *et al.*, 2000; Mittler, 2002). Hence, antioxidase activity is extreme important to scavenge the excessive amount of ROS in order to defend against oxidative damage in plants (Dat *et al.*, 2000; Mittler, 2002).

Although positive and negative regulators involved in ABA-dependent drought signal transduction have been reported, we are still far from understanding the molecular basis by how plants to adapt to drought stress. Extensive studies on characterization of the components involved in drought signalling in plants are essential. Aspartic proteases comprise a subfamily of proteolytic enzymes that have two highly conserved aspartates for catalysis of their peptide substrates (Szecsi, 1992). They are distributed among various organisms including viruses, bacteria, fungi, plants and animals (Davies, 1990; Rawlings and Barrett, 1995). The *Arabidopsis* genome contains at least 51 putative aspartic proteases, but their physiological and biochemical functions have remained elusive (Faro and Gal, 2005). A number of studies have shown important roles for aspartic proteases during *Arabidopsis* development (Xia *et al.*, 2004; Ge *et al.*, 2005). For example, PCS1 functions in cell fate determination during reproductive processes and embryonic development in *Arabidopsis*. The loss-of-function mutant *pcs1* results in excessive cell death in gametogenesis and embryogenesis, whereas overexpression of *PCS1* leads to male sterility by blocking anther dehiscence (Ge *et al.*, 2005). CDR1 is involved in salicylic acid-mediated disease resistance. Overexpression of *CDR1* produces dwarf plants and triggers resistance to virulent *Pseudomonas syringae* in *Arabidopsis* plants (Xia *et al.*, 2004). The *Oryza sativa* aspartic protease S5 can be functional in hybrid sterility, which acts as a major regulator for the reproductive barrier in *indica-japonica* (Chen *et al.*, 2008). Overall, it is clear that aspartic proteases are important for plant development. The involvement of aspartic proteases in abiotic stress, however, is poorly understood.

In this report, we characterized an *Arabidopsis* aspartic protease gene, named *ASPG1* (*ASPARTIC PROTEASE IN GUARD CELL 1*), which demonstrated preferential expression in guard cells of various aerial tissues in *Arabidopsis*. The data demonstrated that overexpression of *ASPG1* could confer drought avoidance via ABA-dependent signalling in *Arabidopsis*.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col) was used in this study and all transgenic plants were generated in a Col background. Both mutant alleles, *aspg1-1* (SALK_045354) and *aspg1-2* (SAIL_667_E02), were from the Arabidopsis Biological Resource Center (ABRC; http://www.arabidopsis.org/abrc/). Seeds were sown on MS medium (Murashige and Skoog, 1962) containing 0.8% (w/v) agar and 1% (w/v) sucrose. The sown seeds were stratified for 3 days in the dark at 4 °C before being transferred to a growth chamber for germination. All seeds were grown and stored under the same conditions. All plants were grown under a 16 h light/8 h dark photoperiod and 70% humidity at 23 °C.

Plasmids constructions

In order to generate ectopically overexpressing *ASPG1* (*ASPG1-OE*) transgenic plants, the pBA002-ASPG1 plasmid was constructed by cloning the full-length cDNA sequence (1503 bp) of the *ASPG1* gene into the pBA002 binary vector at the XmaI/SpeI cloning sites (Kost *et al.*, 1998). To make the plasmid ProASPG1-GUS containing the β-glucuronidase (GUS) gene, the *ASPG1* promoter fragment (1945 bp) was cloned into the binary vector pBI101-GUS at the SbfI/SmaI cloning sites. To analyse ASPG1 cellular localization, the plasmid pCFP-ASPG1 was constructed by a cloning cDNA fragment (1503 bp) of the *ASPG1* gene in the KpnI/SacI cloning sites of vector p35S-CFP, which was made by inserting a cyan fluorescent protein (CFP) fragment into vector p35S-MCS. To obtain the recombinant ASPG1 protein expressed in *Escherichia coli*, pET-30c-ASPG1 was made by inserting the 1503 bp *ASPG1* coding sequences with additional cloning sites (EcoRV and NotI) at the C terminus of a His tag into the pET-30c vector (Novagen, Germany). Site-directed mutations of pET-30c-$ASPG1_{D180N}$, pET-30c-$ASPG1_{D379N}$ and pET-30c-$ASPG1_{D180N/D379N}$ were constructed with a similar cloning strategy. For transient expression assays in mesophyll protoplasts, the 1503 bp cDNA fragments of *ASPG1*, $ASPG1_{D180N}$, $ASPG1_{D379N}$ and $ASPG1_{D180N/D379N}$ were subcloned into p35S-MCS at the KpnI/SacI cloning sites. All primers used for plasmid construction are listed in Supplementary Table S1 available at *JXB* online.

Generation of ASPG1-OE transgenic plants

To generate transgenic *ASPG1-OE* plants, plasmid pBA002-ASPG1 was introduced into the GV3101 strain of *Agrobacterium tumefaciens*. *Arabidopsis* wild-type (Col) plants were transformed

with the GV3101 *Agrobacterium* by floral dip-mediated infiltration (Clough and Bent, 1998). Transgenic plants were selected using BASTA (glufosinate ammonium; Sigma, USA) resistance. The homozygous T_3 transgenic lines were used for further analyses.

RT-PCR analyses

Semi-quantitative RT-PCR was performed to analyse the expression level of the *ASPG1* gene. Total RNA was isolated from 4-week-old seedlings using an RNAprep Pure Plant kit (Tiangen Biotech, China), following the manufacturer's instructions. RNA samples were reverse-transcribed with a ReverTra Ace-α-® kit (Toyobo, Japan). The expression level of the *ACTIN2* gene (AT3G18780) was used as a loading control. To assess gene expression levels, quantitative RT-PCR analysis was performed. The cDNA was amplified using a SYBR Green master mixture (Applied Biosystems, USA) with a Rotor-Gene 6000 (Corbett Research, Australia). The expression level of the *β-ACTIN8* gene (AT1G49240) was used as a loading control. The primer sequences for semi-quantitative and quantitative RT-PCR analyses are listed in Supplementary Table S2 available at *JXB* online.

Preparation of recombinant ASPG1 protein and assessment of ASPG1 protease activity

Plasmids pET-30c-ASPG1, pET-30c-ASPG1$_{D180N}$, pET-30c-ASPG1$_{D379N}$ and pET-30c-ASPG1$_{D180N/D379N}$ were transformed into the BL21(DE3) strain of *E. coli*. Bacteria harbouring expression plasmids were first incubated at 37°C until their exponential growth reached an optical density of 0.6 at 600 nm. Next, 0.5 mM isopropyl-β-D-thiogalactopyranoside (Sigma, USA) was added for 3 h at 20 °C to induce the recombinant protein expression. Purification of ASPG1 protein was performed using a TALON® Metal Affinity Resin column (Clontech, USA) following the manufacturer's instructions. The purity of the protein was determined by 10% SDS-PAGE and immunoblot analyses. An anti-His polyclonal antibody (Proteintech Group, China) was used for immunoblotting. Protease activity was assessed using a Protease Fluorescent Detection kit (Sigma, USA) followed the manufacturer's instructions.

Stomatal bioassay, water-loss quantification and ABA content measurement

A bioassay of stomatal apertures was carried out as described by Zhang *et al.* (2001). *Arabidopsis* leaves from 4-week-old plants were incubated in buffer containing 10 mM KCl, 50 μM $CaCl_2$, and 10 mM MES/KOH (pH 6.15). To induce stomatal opening, the leaves were first incubated in the light for 3 h and then treated with ABA for 3 h at 23°C. ROS production was detected in guard cells of the leaf epidermal peels using dichlorofluorescein (DCF) (Pei *et al.*, 2000). All images were taken with a TE-2000U inverted fluorescence microscope (Nikon, Tokyo, Japan) equipped with a cooled charged-coupled device camera (Cool SNAP HQ2; Roper Scientific, Houston, TX, USA). To detect the DCF fluorescent signal, a fluorescent filter set with excitation at 488 nm and emission at 525 nm was used. The sizes of the stomatal apertures were quantified using MetaMorph 7.5 software (Molecular Devices, USA). For water-loss quantification, detached rosette leaves from 4-week-old plants were placed in weighing dishes on the laboratory bench at room temperature. Fresh weight was measured throughout the time course of the experiment. Water loss was calculated as the percentage of initial fresh weight. The ABA content was measured using a method described previously (Chen *et al.*, 2011).

Determination of H_2O_2 content and assessment of antioxidant enzymes

To examine the response to drought stress, 2-week-old seedlings of wild-type Col *ASPG1-OE2* and *ASPG1-OE14* plants were grown without water for 14 days before re-watering. H_2O_2 content and activity of antioxidant enzymes were measured after drought treatment. Leaves (0.25 g) from Col and *ASPG1-OE* plants were extracted in 2 ml extraction buffer containing 50 mM potassium phosphate, 1 mM EDTA, 1% polyvinylpyrrolidone and 1 mM phenylmethylsulfonyl fluoride (pH 7.8). The protein content was normalized using the method of Bradford (1976). H_2O_2 content was determined as described by Bernt and Bergmeyer (1974). Superoxide dismutase (SOD) activity was analysed with a 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt assay following the method of Ukeda *et al.* (2002). Catalase (CAT) activity was measured as described by Aebi (1983).

Protoplasts preparation and transient expression assay

Arabidopsis guard cell protoplasts (GCPs) were prepared from rosette leaves of 4-week-old plants using a method established by Pandey *et al.* (2002). The yield of GCP obtained was 5×10^6 GCP per 100 leaves with 97.5–99% purity. The method for mesophyll cell protoplast (MCP) preparation was as reported by Yoo *et al.* (2007), and transient expression assays were performed with MCPs following this method. Plasmid ProRD29A-LUC containing the luciferase (LUC) gene was used as an ABA-responsive reporter and plasmid ProUBQ10-GUS was co-transfected as an internal control, and the relative LUC:GUS activity was scored (Wang *et al.*, 2011). In order to determine the cellular localization of ASPG1, a transient expression experiment was performed using a biolistic bombardment method (Sanford *et al.*, 1993). Images were taken with a laser-scanning confocal imaging system (FV1000; Olympus, Japan). CFP fluorescence was acquired with excitation at 435 nm and emission at 475 nm; green fluorescent protein (GFP) fluorescence was acquired with excitation at 488 nm and emission at 509 nm.

Results

Correlation of ASPG1 expression levels and ABA sensitivity

In order to understand better how ABA regulates many important cellular processes, this study aimed to identify new components involved in ABA signal transduction pathways. A putative ABA-insensitive mutant was screened from a LexA-VP16-estragon receptor (XVE)-tagged T-DNA insertion mutant pool (Zhang *et al.*, 2005) in a seed germination assay (Wang *et al.*, 2011). The original screened putative mutant showed ABA-insensitive seed germination that was not dependent on induction of estradiol (see Supplementary Fig. S1A and B available at *JXB* online). In analysis of this original mutant, the novel gene locus AT3G18490 was identified encoding a putative aspartic protease by thermal asymmetric interlaced-PCR (Liu *et al.*, 1995; Zuo *et al.*, 2000). The XVE T-DNA insert was found at nt 1275 in the putative aspartic protease domain of gene AT3G18490 (see Supplementary Fig. S1C).

By searching public available microarray databases, it was found that AT3G18490 is expressed preferentially in guard cells in *Arabidopsis* (Leonhardt *et al.*, 2004). This finding prompted further study of this gene. We named this gene *ASPG1* (*ASPARTIC PROTEASE IN GUARD CELL 1*), and obtained the loss-of-function mutant alleles SALK_045354 (*aspg1-1*) and SAIL_667_E02 (*aspg1-2*) (Fig. 1A, B), from ABRC.

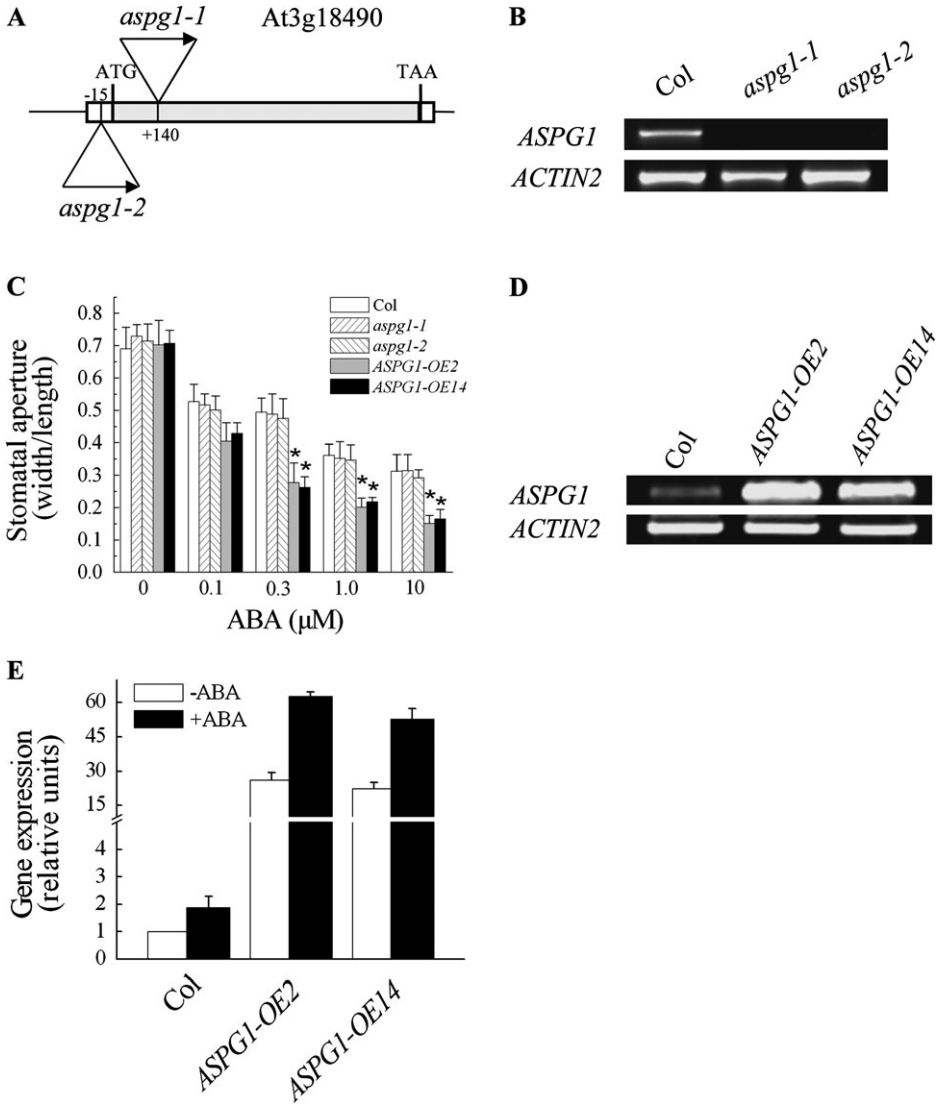

Fig. 1. Responses to ABA of *aspg1* mutant alleles and *ASPG1-OE* transgenic plants. (A) Schematic drawing (not to scale) showing the T-DNA insertion site in gene AT3G18490 revealed in the *aspg1-1* (SAIL_667_E02) and *aspg1-2* (SALK_045354) mutant alleles. (B) Semi-quantitative RT-PCR analysis of *ASPG1* gene expression in wild type (Col) and in the *aspg1-1* and *aspg1-2* mutant alleles. *ACTIN2* (AT3G18780) was used as the loading control. (C) Stomatal closure assay using ABA treatment. Values are means ±SE from three independent experiments (n=50). The epidermal peels of leaves from 4-week-old plants (Col, *aspg1-1* and *aspg1-2*, *ASPG1-OE2* and *ASPG1-OE14*) were first incubated in the light for 3 h to induce stomatal opening and then treated with ABA (0, 0.3, 1.0 and 10 μM) for 3 h. *, P<0.01 compared with wild-type Col in the same treatment. (D) *ASPG1* expression was elevated in the *ASPG1-OE2* and *ASPG1-OE14* transgenic lines. *ACTIN2* was used as a loading control. (E) Quantitative analysis of the expression level of *ASPG1* in Col, *ASPG1-OE2* and *ASPG1-OE14* lines after ABA treatment for 3 h. Total RNA was extracted from 4-week-old plants. (–ABA, treated with 0.05% ethanol; +ABA, treated with 50 μM ABA). *ASPG1* expression was analysed relative to the level of ABA-free treatment in Col plants, which was taken as 1. Results are shown as the mean ±SE of three independent experiments.

In scoring the stomatal closure with *aspg1* mutant alleles, we failed to find a significant difference compared with wild-type Col (Fig. 1C). It was thus speculated that functional redundancy might exist in guard cells. A previous microarray analysis showed that a homologue of aspartic protease, AT3G20015 appeared to have a very low expression level in both guard cells and mesophyll cells (Leonhardt *et al.*, 2004). Thus, an attempt was made to knock out/knock down the AT3G20015 gene (named *ASPG2*) in the *aspg1-1* mutant background using an artificial microRNA (amiRNA) strategy (Schwab *et al.*, 2006). The AT3G20015 gene was successfully knocked down in *aspg1-1* plants as follows. Gene expression reduction was examined in *aspg1-1 amiR-aspg2* lines by semi-quantitative and quantitative RT-PCR analyses, and 32 lines in which AT3G20015 gene expression was reduced were identified. The expression level of AT3G20015 gene in five lines was substantially reduced in comparison with that in wild-type Col. In line *aspg1-1 amiR-aspg2* #3, the expression level of the AT3G20015 gene was reduced

4.3-fold; in line #5, it was reduced 6.7-fold; in line #11, it was reduced 4.2-fold; in line #14, it was reduced 5.3-fold; and in line #23, it was reduced 4.8-fold (see Supplementary Fig. S2A available at *JXB* online). Because line #5 (expression reduced by 6.7-fold) and line #14 (reduced by 5.3-fold) both showed a stronger reduction in AT3G20015 gene expression in comparison with Col, the behaviour of stomatal closure in these lines was analysed further (see Supplementary Fig. S2); however, neither line showed a differential response in the ABA-induced stomatal closure bioassay (see Supplementary Fig. S2B). Therefore, we turned to gain-of-function analysis by generating *ASPG1-OE* transgenic plants in *Arabidopsis* (Col background) (Fig. 1D). Overexpression of *ASPG1* in these lines was found to significantly increase ABA sensitivity in guard cells (Fig. 1C). After 3 h of ABA treatment (0.1, 0.3, 1.0 and 10 μM), the *ASPG1-OE2* and *ASPG1-OE14* plants showed a significant hypersensitivity to ABA (Fig. 1C). These data suggested that *ASPG1* might play a role in ABA-induced guard-cell movement.

To test whether *ASPG1* expression was ABA inducible, the expression level of *ASPG1* was analysed without or with ABA treatment. A 1.89-fold change in *ASPG1* expression level was detected in *ASPG1-OE* and Col plants after treatment with 50 μM ABAfor 3 h (Fig. 1E), indicating that the *ASPG1* gene is ABA inducible in *Arabidopsis*.

As a secondary messenger, ROS could modulate ABA-induced closure of guard cells (Pei *et al.*, 2000; Zhang *et al.*, 2001; Kwak *et al.*, 2003; Desikan *et al.*, 2004; Kwak *et al.*, 2008). To investigate whether ABA-induced *ASPG1* gene expression is associated with ROS production in guard cells, the ROS level was compared in Col, *aspg1* and *ASPG1-OE* plants. An elevated ROS level was detected in the guard cells of *ASPG1-OE2* and *ASPG1-OE14* plants after 50 μM ABA treatment for 10 min (Fig. 2A). The ROS level increased by ~80% (80.0–81.7%) in guard cells of *ASPG1-OE* plants; however, there was only a 41.0% increase in ROS levels in Col guard cells, and an ~39% (39.4–39.6%) increase in ROS levels in the guard cells of *aspg1* mutant allele plants (Fig. 2B). Together, these quantitative data on ROS levels implicated that ASPG1 may be able to trigger ROS production in guard cells in response to ABA.

The response to drought stress in ASPG1-OE plants

As *ASPG1-OE* plants showed a remarkable increase in ABA sensitivity in the stomatal closure assay (Fig. 1C), the

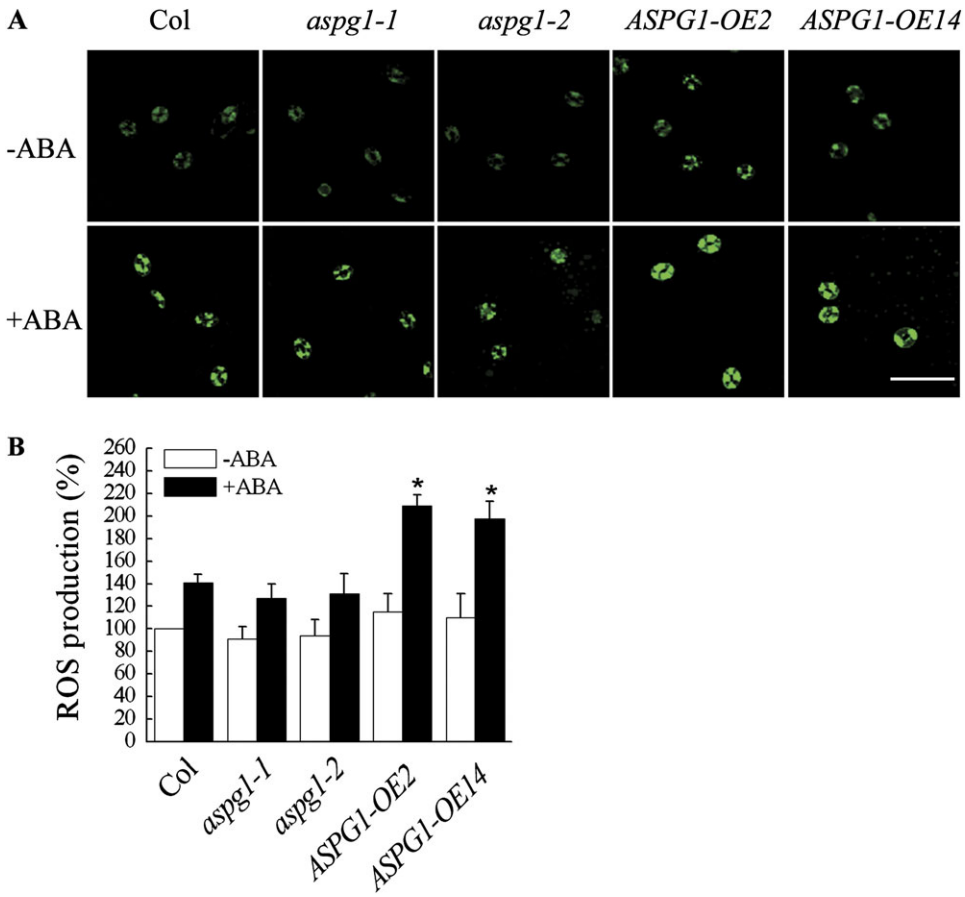

Fig. 2. Analysis of ROS production. (A) ROS production was detected using the fluorescent dye DCF. Epidermal peels were loaded with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 10 min before adding 50 μM ABA. Bar, 50 μm. (B) Quantification of ROS levels in guard cells of Col, *aspg1-1*, *aspg1-2*, *ASPG1-OE2* and *ASPG1-OE14* plants after 50 μM ABA treatment. Results are shown as the mean ±SE of three independent experiments (n=50, *, P<0.01 compared with Col after ABA treatment). The fluorescent intensity of guard cells in Col before ABA treatment was taken as 100%.

response to drought stress was examined in *ASPG1-OE* plants. Two-week-old plants were first grown without water for 14 days and were then rewatered. The results showed that *ASPG1-OE2* and *ASPG1-OE14* plants could recover from wilting by rehydration in contrast to Col plants (Fig. 3A). After rewatering for 24 h, on average, 90% (89.3–90.7%) of *ASPG1-OE* plants recovered (Fig. 3B). Transpirational water loss of detached rosette leaves was then measured from 4-week-old Col and *ASPG1-OE* plants, which were left on the laboratory bench at room temperature with a humidity of ~40–45%. Less water loss was detected in *ASPG1-OE* plants compared with Col plants (Fig. 3C). The endogenous ABA level was further analysed in 4-week-old Col and *ASPG1-OE2* plants exposed to the same stress condition for 6 h with a humidity of ~40–45% at 25 °C. The results showed that endogenous ABA content increased similarly in Col and *ASPG1-OE* plants (Fig. 3D). Taken together, these data suggested that the drought avoidance of *ASPG1-OE* plants was a consequence of ABA signalling, which might be modulated in guard cells (Figs 1C and 3C, D). To examine the expression level of *ASPG1* under drought conditions, *ASPG1* gene expression was characterized in Col and *ASPG1-OE* plants that were well watered (control) and in which water was withheld for 14 days (drought). *ASPG1* gene expression was increased up to 3-fold in the drought-stressed Col plants, and a 2.5-fold change in *ASPG1* expression was detected in drought-stressed *ASPG1-OE* lines (Fig. 3E). This further demonstrated that drought stress could also influence *ASPG1* expression. To validate the correlation between drought avoidance and guard cell density, guard-cell density was compared in 4-week-old *ASPG1-OE* plants and Col plants. The data showed that guard-cell development was not affected in *ASPG1-OE* plants (see Supplementary Fig. S3 available at *JXB* online). In summary, these results suggested that increased expression levels of *ASPG1* could confer drought avoidance of *Arabidopsis* plants by increasing ABA sensitivity in guard cells (Fig. 1C) accompanied by a reduction in transpirational water loss (Fig. 3C).

Drought stress is able to trigger ABA-induced ROS accumulation, and plants would thus need a ROS detoxification mechanism to achieve survival from drought stress (Smirnoff, 1993). To characterize whether ROS production accompanied the drought stress in *ASPG1-OE* plants, the level of H_2O_2 was analysed in 2-week-old *ASPG1-OE* plants under the conditions of drought (no water for 14 days). The H_2O_2 level decreased by 0.6- and 0.7-fold in *ASPG1-OE* plants on days and 14, respectively, after withholding water (Fig. 4A); thus, the reduction in H_2O_2 coincided with the mechanism of drought avoidance in *ASPG1-OE* plants (Fig. 3A, B). A previous study reported that ABA improves the drought adaptation of triploid bermudagrass by increases in the activities of the antioxidants SOD and CAT (Lu *et al.*, 2009). To test the correlation between the reduction of H_2O_2 and the activity of antioxidases in *ASPG1-OE* plants, the enzymatic activities of SOD and CAT were measured under the same drought conditions. The results showed that the levels of the SOD activity increased significantly by 1.6- and 1.5-fold in *ASPG1-OE* plants on days 10 and 14, respectively, after withholding water (Fig. 4B), and the levels of CAT activity increased significantly by 1.4-, 1.7- and 1.6-fold on days 6, 10 and 14, respectively, after withholding water (Fig. 4C). Results from the measurement of ascorbate peroxidase and glutathione reductase activity showed no differential activities of these enzymes in Col and *ASPG1-OE* plants treated under the same drought conditions (data not shown). Nevertheless, these analyses on antioxidases demonstrated that *ASPG1-OE* plants were capable of scavenging excessive ROS to prevent oxidative damage to cells through SOD activation, which could convert O_2^- to H_2O_2 at the first stage of defence; subsequently, CAT activation could detoxify H_2O_2 in the cells of *ASPG1-OE* plants, thus allowing *ASPG1-OE* plants to survive under drought conditions (Fig. 3A).

Transcriptional alterations of downstream targets in response to drought stress

To understand better the scenario of drought avoidance in *ASPG1-OE* plants, some of the downstream targets of drought stress were analysed in 4-week-old Col and *ASPG1-OE2* plants. Gene expression levels were analysed quantitatively after the plants had been subjected to drought stress for 6 h (Fig. 5). We analysed the expression of *ABF2*, a bZIP transcription factor that can bind to the ABA-responsive element (ABRE) (Kim *et al.*, 2004; Riera *et al.*, 2005; Shinozaki and Yamaguchi-Shinozaki, 2006). The level of *ABF2* gene expression was 2.1-fold higher in *ASPG1-OE2* plants than in Col plants after drought stress. Other drought- and/or ABA-inducible genes, such as *KIN1*, *KIN2*, *RAB18*, *RD20*, *RD22*, *RD26*, *RD29A* and *RD29B* (Riera *et al.*, 2005; Shinozaki and Yamaguchi-Shinozaki, 2006) were also upregulated in *ASPG1-OE2* plants in response to drought stress. The expression levels of these genes in *ASPG1-OE2* plants were increased at least 2-fold compared with levels in Col plants (Fig. 5). In contrast, the expression levels of the genes for two transcription factors, *MYB2* and *MYC2*, that are able to bind to the MYBR or MYCR element in drought- and ABA-inducible genes (Riera *et al.*, 2005; Shinozaki and Yamaguchi-Shinozaki, 2006) were similar in *ASPG1-OE2* and Col plants. Expression of the levels of primary ABA-responsive components such as *ABI1*, *ABI2*, *OST1*, *RbohD* and *RbohF* (Kwak *et al.*, 2008) was also analysed. However, these genes displayed similar expression patterns in Col and *ASPG1-OE2* plants (Fig. 5). Taken together, these data on gene expression suggested that *ASPG1-OE* plants are probably modulated by ABA-dependent signalling that involves bZIP transcription regulators (ABRE-binding factors or ABFs) such as ABF2. To clarify this notion, two AP2-type transcription factors, *DREB2A* and *DREB2B*, were analysed further and confirmed to be crucial for the ABA-independent drought response (Liu *et al.*, 1998; Riera *et al.*, 2005; Shinozaki and Yamaguchi-Shinozaki, 2006). Both *DREB2A* and *DREB2B* were maintained at similar

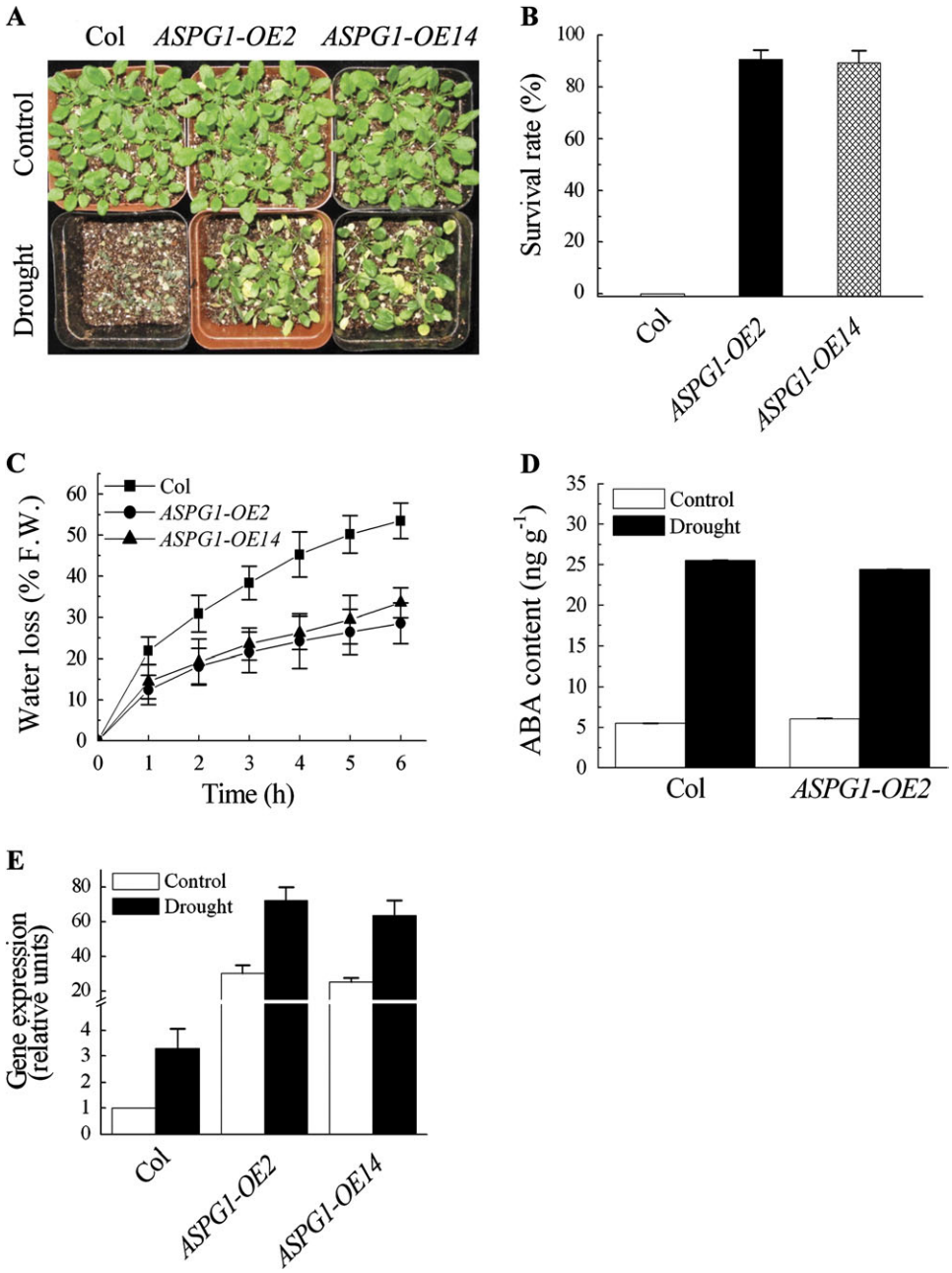

Fig. 3. Response of *ASPG1-OE* transgenic plants to drought stress. (A) Two-week-old Col and *ASPG1-OE* plants were well watered (Control) or deprived of water for 14 days and then rewatered (Drought). The photos were taken on day 1 after rewatering. (B) Survival rates of Col and *ASPG1-OE* plants on day 1 after rewatering. Values are means ±SE from three independent experiments (n=50). (C) Transpirational water loss from detached leaves of 4-week-old Col and *ASPG1-OE* plants at the indicated time points. Water loss rates are indicated as the percentage of the initial fresh weight (% FW). Results are shown as the mean ±SD from four replicated samples with five leaves used for each sample. (D) Endogenous ABA levels of 4-week-old Col and *ASPG1-OE2* plants without (Control) and with drought treatment for 6 h. Values are means ±SD of three independent experiments. (E) Expression levels of the *ASPG1* gene in Col and *ASPG1-OE* plants under drought conditions as described in (A). The expression level of *ASPG1* was analysed as relative to the level of the control treatment with Col plants, which was taken as 1. Results are shown as the mean ±SE of three independent experiments.

levels in Col and *ASPG1-OE2* plants after drought stress (Fig. 5), supporting the suggestion that the drought avoidance of *ASPG1-OE* plants was unlikely to involve an ABA-independent pathway. In addition, expression levels of *ABA3* and *NCED3* under drought treatment were determined to evaluate ABA biosynthesis. Both genes were induced at similar levels in Col and *ASPG1-OE2* plants (Fig. 5). Thus, the gene expression patterns of *ABA3* and *NCED3* in Col and *ASPG1-OE2* plants under conditions of drought stress reflected exactly the ABA levels in Col and

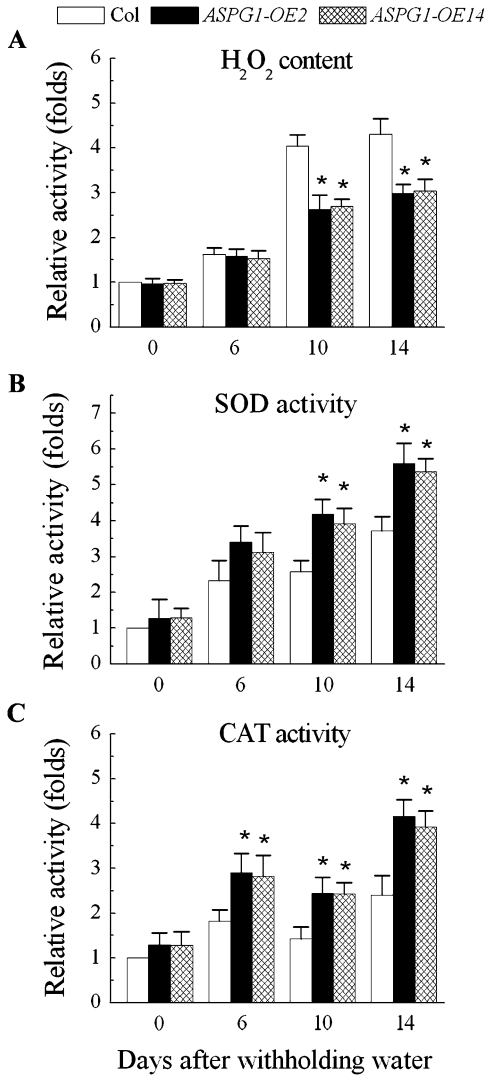

Fig. 4. Determination of H_2O_2 levels and antioxidant enzymes activities. (A) Comparison of H_2O_2 levels in Col and *ASPG1-OE* plants. (B, C) Comparisons of SOD (B) and CAT (C) activity in *ASPG1-OE* and Col plants. Two-week-old plants had water withheld for the indicated number of days. The relative content of H_2O_2 and the activity of SOD and CAT were quantified as fold change compared with the Col control. Results are shown as the mean ±SD (n=6; *, P<0.01).

ASPG1-OE2 plants (Fig. 3D). This finding indicated that the adaptation to drought stress in *ASPG1-OE* plants may be regulated by ABA signal transduction.

Preferential guard-cell localization of ASPG1

To understand the specificity and functionality of the *ASPG1* gene, its expression pattern was characterized in various tissues. *ASPG1* gene expression was detected in young seedlings, leaves, stems, flowers and siliques, but not in roots (Fig. 6A). Characterization of ProASPG1-GUS transgenic plants confirmed the tissue specificity of *ASPG1* expression (Fig. 6B). ASPG1-expressed GUS was predominantly in guard cells (Fig. 6B–D). The unique guard-cell localization of ASPG1-GUS might imply its specific function for adaptation to drought stress in *Arabidopsis*. The expression specificity of the *ASPG1* gene in guard cells was also quantified. *ASPG1*, remarkably, was found to be expressed in GCPs but not in MCPs (Fig. 6E). The purity of the GCP and MCP preparations was assessed by characterizing *KAT1* expression (a marker gene for guard cells) and *CBP* expression (a marker gene for mesophyll cells) (Fig. 6E).

Subcellular ER localization of ASPG1

The subcellular localization of the CFP–ASPG1 fusion protein was analysed in leaf epidermal cells of *Arabidopsis*. Fluorescence analysis of the expression of CFP-ASPG1 revealed a meshwork-like structure that appeared to shown endoplasmic reticulum (ER) localization. The ER localization was further confirmed by co-expressing GFP–KDEL, a marker of the ER (ER-gb *CD3-955*) in plant cells (Nelson *et al.*, 2007) (Fig. 7). The ER localization of the putative aspartic protease ASPG1 thus suggested its proteolytic characteristic under drought conditions.

Aspartic protease activity of ASPG1

ASPG1 is predicted to be an aspartic protease (PF00026) of 53 kDa belonging to the MEROPS peptidase family A1 (pepsin family) (http://arabidopsis.org/servlets/TairObject?id=40580&type=gene). ASPG1 does not have a signal peptide (http://smart.embl-heidelberg.de/smart/show_motifs.pl). Unlike other plant aspartic proteases, ASPG1 lacks a plant-specific sequence (Mutlu and Gal, 1999). By analysing the aligned sequences of ASPG1 and other aspartic proteases from different species, two putative aspartic acid residues (D180 and D379) were found to be conserved in the putative aspartic protease active sites of ASPG1 protein (see Supplementary Fig. S4 available at *JXB* online).

A previous study showed that the activity of aspartic proteases is pH dependent, and that pepstatin A is an inhibitor of aspartic proteases (Rawlings and Barrett, 1995). To determine the aspartic protease activity of ASPG1, the protease activity was assessed *in vitro* with a purified recombinant His–ASPG1 protein as well as the site-directed mutative proteins $ASPG1_{D180N}$, $ASPG1_{D379N}$ and $ASPG1_{D180N/D379N}$ (Fig. 8A). The protease activity of His–ASPG1 was measured under different pH conditions (pH 2.0–8.0). Despite there being a broad spectrum of aspartic protease activities, His–ASPG1 displayed a higher activity at pH 2.0 and 6.0 in this assay (data not shown). The protease activity of His–ASPG1 was validated further at pH 6.0 and confirmed by a reduction of ~70% activity when the protease inhibitor pepstatin A was added (Fig. 8B). In contrast, mutants His-$ASPG1_{D180N}$, His-$ASPG1_{D379N}$ and His-$ASPG1_{D180N/379N}$ did not show any obvious protease activity (Fig. 8B). These results from aspartic protease analyses demonstrated that ASPG1 has protease activity *in vitro* and that the two active aspartic acid residues, D180 and D379, are vital for ASPG1 activity.

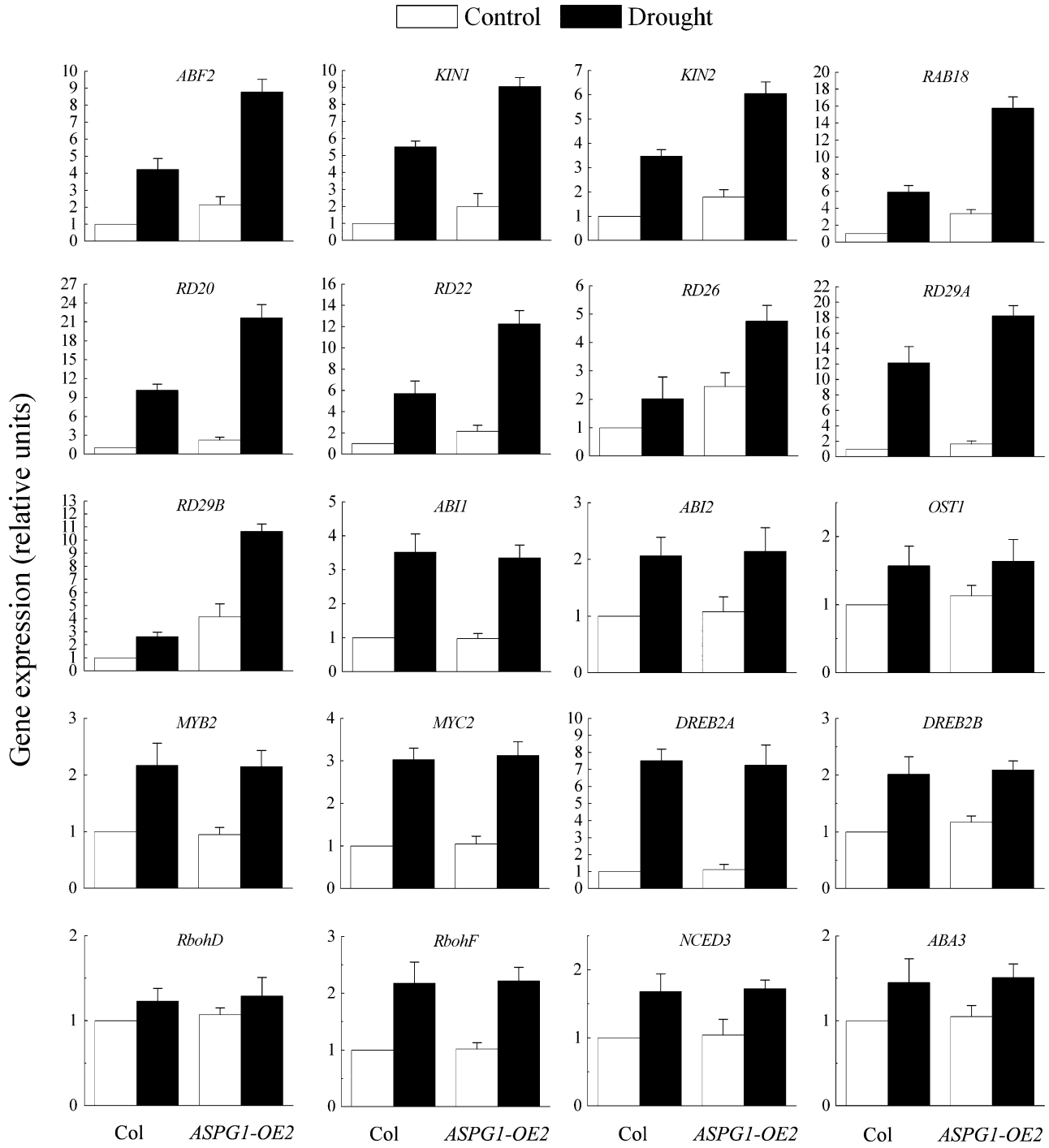

Fig. 5. Expression levels of drought- and ABA-responsive genes in *ASPG1-OE2* transgenic plants. The plants were subjected to drought conditions for 6 h; the control plants were not subjected to drought conditions. The expression levels of some drought- and ABA-responsive genes were analysed quantitatively by real-time RT-PCR with 4-week-old plants of Col and *ASPG1-OE2*. Gene expression levels were analysed relative to the expression levels in Col plants, which were taken as 1. Results are shown as the mean ±SE of three independent experiments.

As *ASPG1* might be involved in ABA-dependent drought signal transduction, further experiments were performed to investigate the correlation between ASPG1 and the ABA response. ABA responsiveness was analysed with the reporter RD29A–LUC using a transient expression assay. Plasmid ProRD29A-LUC with p35S-ASPG1, p35S-ASPG1$_{D180N}$, p35S-ASPG1$_{D379N}$ or p35S-ASPG1$_{D180N/D379N}$ were co-expressed in Col mesophyll protoplasts. ProRD29A-LUC was used as an ABA-responsive reporter and ProUBQ10-GUS was co-transfected as an internal control to monitor the transformation efficiency (Wang *et al.*, 2011). The relative LUC/GUS activity was quantified in the presence or absence of ABA. ProRD29A-LUC and ProUBQ10-GUS were transfected alone as negative

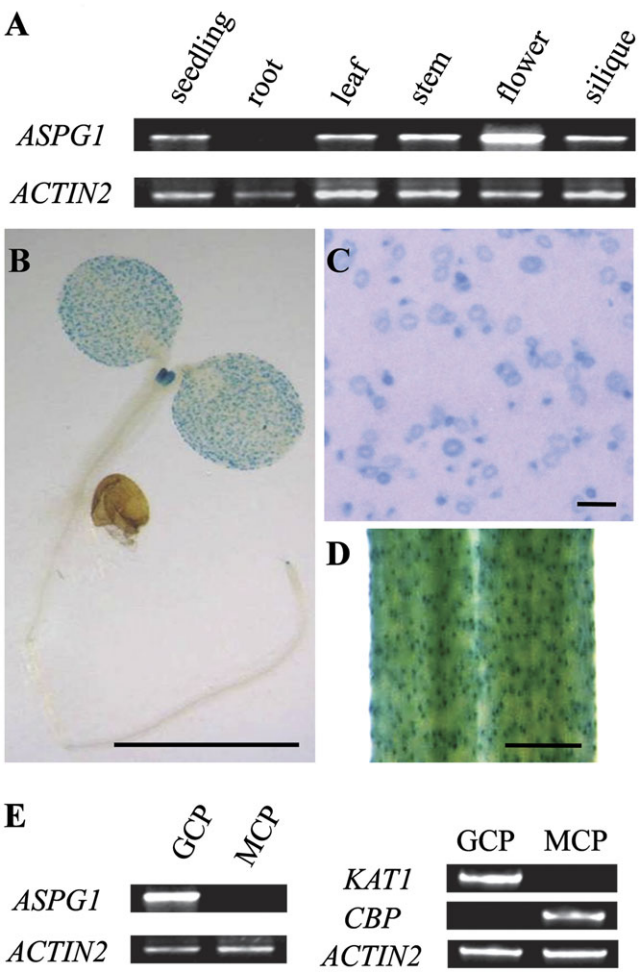

Fig. 6. The predominant guard-cell expression pattern of *ASPG1*. (A) Semi-quantitative analysis of *ASPG1* gene expression in various tissues. (B–D) The ProASPG1-GUS signal indicates the predominant guard-cell expression of *ASPG1* in 10-day-old seedlings. Bars, 1 mm (B, D); 0.1 mm (C). (E) Semi-quantitative analysis of *ASPG1* gene expression in GCPs and MCPs. Expression levels of the *KAT1* (AT5G46240, leaf guard-cell marker) and *CBP* (AT4G33050, mesophyll cell marker) genes were analysed to determine the purity of GCPs and MCPs. *ACTIN2* was used as a loading control.

controls. A significant increase in ProRD29A-LUC activity was obtained in the co-expressing plasmid p35S-ASPG1 with or without ABA (50 μM), but not when co-expressed with plasmid p35S-ASPG1$_{D180N}$, p35S-ASPG1$_{D379N}$ or p35S-ASPG1$_{D180N/D379N}$ (Fig. 8C). Therefore, these results indicated that the aspartic protease activity of ASPG1 is required for the ABA-induced activity of ProRD29A-LUC.

Discussion

Genes encoding plant aspartic proteases have been identified from different plant species (Mutlu and Gal, 1999; Murakamia *et al.*, 2000; Xia *et al.*, 2004; Ge *et al.*, 2005; Chen *et al.*, 2008). Although studies have revealed the functions of aspartic proteases in various physiological processes during plant development including seed germination (Belozersky *et al.*, 1989; Dunaevsky *et al.*, 1989), leaf senescence (Kato *et al.*, 2004), the immunity response (Xia *et al.*, 2004), cell death (Ge *et al.*, 2005) and reproduction (Chen *et al.*, 2008), little is known about aspartic proteases involving in abiotic stress. The findings in this study have shown that the overexpression of *ASPG1* can enhance ABA sensitivity in guard cells, in turn promoting adaptive drought avoidance in *Arabidopsis*.

ASPG1 confers drought avoidance through the ABA signal transduction pathway

Drought is one of the major abiotic stresses that can trigger severe damage to plants. It is imperative to search for genes that may be involved in plant adaptation to drought stress. In this study, it was found that an *Arabidopsis* aspartic protease, ASPG1, could play such a role. The findings demonstrated that overexpression of *ASPG1* enhanced ABA sensitivity in guard cells (Fig. 1C) and *ASPG1-OE* plants showed an improvement in drought avoidance of plants (Fig. 3). In *Arabidopsis*, drought stress may initiate responses to either ABA-dependent or ABA-independent signal transduction (Shinozaki and Yamaguchi-Shinozaki, 2006). In this report, although the expression levels of upstream components of ABA-signalling such as *ABI1*, *ABI2*, *OST1*, *RbohD* and *RbohF* (Kwak *et al.*, 2008) were all induced by drought stress, the expression patterns were not differentiated in Col and *ASPG1-OE2* plants. Therefore, ASPG1 is probably functional downstream of these components (Fig. 5). It should be noted that expression of *ABF2*, a member of the ABF family (Jakoby *et al.*, 2002), was significantly upregulated under drought conditions. ABFs are essential for ABA signal transduction (Fujii *et al.*, 2009), and are able to bind to the ABRE of downstream drought- and/or ABA-responsive genes to initiate stomatal closure while responding to drought stress (Riera *et al.*, 2005; Shinozaki and Yamaguchi-Shinozaki, 2006). In this study, the elevated expression level of *ABF2* by drought stress in *ASPG1-OE2* plants may suggest that ASPG1 involvement in drought adaptation may require ABF activity (at least that of ABF2); in turn, expression of downstream targets of ABA signalling could be stimulated under drought conditions (Fig. 5). This was confirmed by analysing ProRD29A-LUC activity (Fig. 8C). The *RD29A* promoter contains both an ABRE and a dehydration-responsive element (Yamaguchi-Shinozaki and Shinozaki, 2006). The data from the transient expression assay confirmed that ABA-induced activity of ProRD29A-LUC was significantly increased when co-expressed with plasmid p35S-ASPG1 (Fig. 8C). Thus, these results strongly suggested that ASPG1 may confer drought avoidance to plants via ABA signalling.

Drought stress causes endogenous ABA biosynthesis and in turn, ABA triggers ROS production to mediate downstream responses in guard cells (Pei *et al.*, 2000; Zhang *et al.*, 2001; Kwak *et al.*, 2003; Desikan *et al.*, 2004; Kwak *et al.*, 2008). In this study, we observed that the *ASPG1* gene was able to promote ROS (H_2O_2) production in guard cells after treatment with ABA (Fig. 2), indicating the

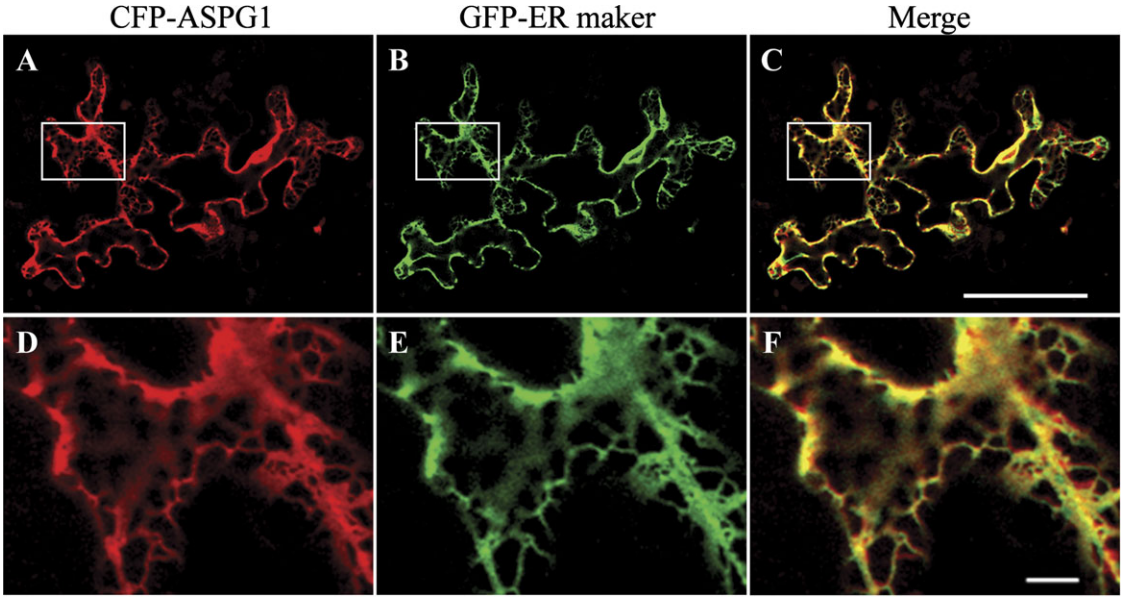

Fig. 7. ER localization of the CFP–ASPG1 fusion protein. (A–C) CFP–ASPG1 (red) and GFP–KDEL (ER-gb *CD3-955*, a well-known GFP–ER marker; green) were co-expressed in the leaves of 4-week-old Col plants following co-transformation of plasmids by biolistic bombardment. The overlay image (merge) shows co-localization of CFP–ASPG1 and GFP–KDEL (yellow). Bar, 60 μm. (D–F) Magnification of the areas outlined in (A)–(C). Bar, 5 μm.

involvement of ASPG1 in ROS-mediated ABA signal transduction. Accumulation of excessive ROS causes oxidative damage to cells and stimulates the activity of antioxidases (Mittler, 2002). Antioxidases SOD and CAT are two important enzymes that scavenge excessive ROS to prevent oxidative damage to cells. SOD could convert O_2^- into H_2O_2 and CAT could then detoxify the H_2O_2 to defend cells against oxidative damage (Mittler, 2002). We determined significant activity of SOD and CAT in 2-week-old *ASPG1-OE* plants after withholding water for 10–14 days (Fig. 4B, C), suggesting that *ASPG1-OE* plants may be able to scavenge excessive ROS under severe dehydration conditions.

The possible function of ASPG1 aspartic protease in plant cells

By analysing public available microarray data (Leonhardt *et al.*, 2004), it was noted that the *ASPG1* gene showed higher expression levels in *Arabidopsis* guard cells but was almost absent in mesophyll cells. This predominant guard-cell expression trait of the *ASPG1* gene was also observed by a histological assay in this study (Fig. 6). Coincidentally, *ASPG1-OE* plants were hypersensitive to ABA in stomatal closure assays (Fig. 1C). The appearance of drought avoidance in *ASPG1-OE* plants therefore may be relevant to the predominant guard-cell expression of *ASPG1*.

ASPG1 also showed proteolytic activity *in vitro*. Two aspartic residues D180 and D379 were identified within the highly conserved aspartic protease sites by a protease activity assay (Fig. 8A, B). The aspartic protease activity was found to be required for ASPG1 function in response to ABA (Fig. 8C). Nevertheless, the target proteins for ASPG1 remained unknown. Plant aspartic proteases have been demonstrated to play roles in pro-protein processing and protein degradation *in vitro* (Mutlu and Gal, 1999). A *Brassica napus* aspartic protease may process the polypeptide precursor of storage protein 2S albumins by cleaving within the N-terminal and internal pro-peptide linking the two subunits (D'Hondt *et al.*, 1993). Several aspartic proteases conduct storage protein degradation during seed germination (Runeberg-Roos *et al.*, 1994; Hiraiwa *et al.*, 1997) and the degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in tobacco senescent leaves (Kato *et al.*, 2004). The ER localization of CFP–ASPG1 in plant cells (Fig. 7) implies a possible role for ASPG1 in protein modification in the ER. Future analyses of the role of ASPG1 in protein processing and/or relevant protein modification in the ER would be interesting.

More than 50 putative aspartic proteases have been predicted in *Arabidopsis* (Faro and Gal, 2005). The functional redundancy of homologues of aspartic protease genes may complicate the scenario in guard cells when plants respond to drought stress, which may explain the insignificant phenotypes shown in the knockout *aspg1* mutant alleles compared with the wild-type Col (Fig. 1C). In this study, we failed to determine which homologue(s) has a similar function in response to drought stress in *Arabidopsis* (see Supplementary Fig. S2). Further studies to clarify this homologue redundancy are imperative.

In summary, the findings of this study demonstrated ASPG1 function in ABA-dependent drought signalling. Under drought conditions, ABA induced *ASPG1* gene expression. ASPG1 triggered stomatal closure to avoid water loss through the activation of antioxidases, thus preventing *Arabidopsis* plants from oxidative damage. As a consequence, the plant is able to adapt to drought stress.

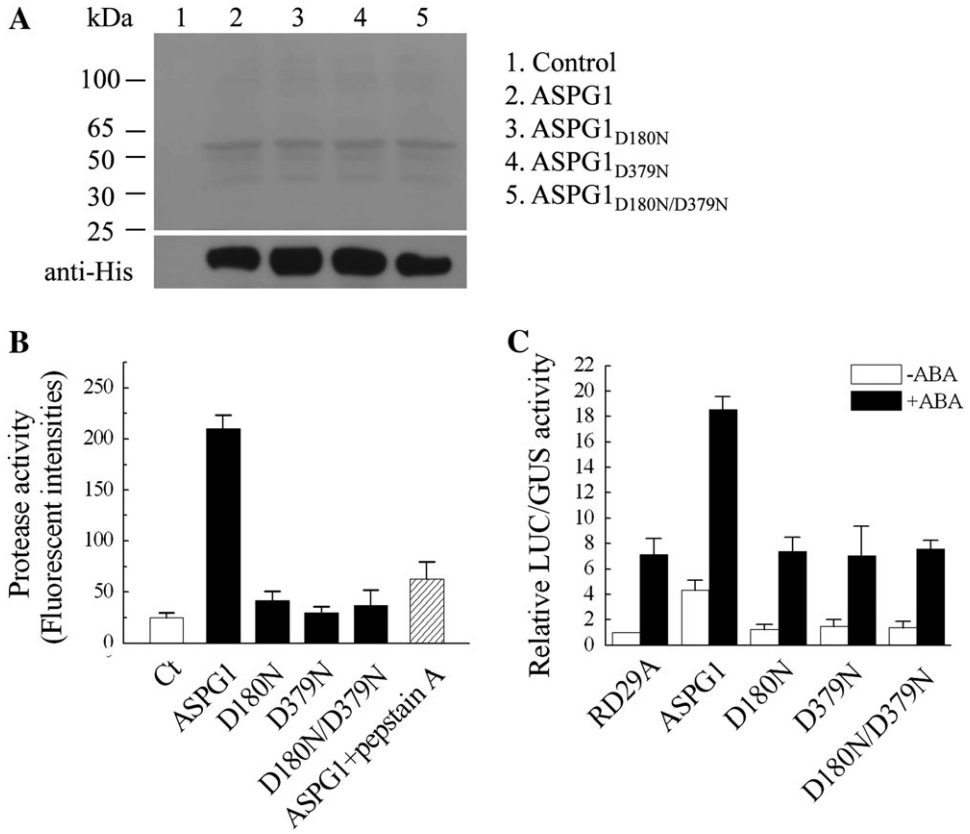

Fig. 8. *In vitro* assay of ASPG1 protease activity. (A) Coomassie blue staining (top panel) and western blotting (bottom panel) showing the recombinant proteins of ASPG1 and mutants, which all were expressed in and purified from *E. coli*. The His tag was detected as a control of loading levels. (B) *In vitro* assay to analyse the protease activity of ASPG1. Fluorescent intensities denote the protease activity of ASPG1. Casein was used as the substrate and pepstatin A was used to inhibit the protease activity. Ct, casein; D180N, $ASPG1_{D180N}$; D379N, $ASPG1_{D379N}$; D180N/D379N, $ASPG1_{D180N/D379N}$. Results are shown as means ±SD ($n = 6$). (C) Col MCPs were transfected with ProRD29A-LUC (RD29A), p35S-ASPG1 (ASPG1), p35S-$ASPG1_{D180N}$ (D180N), p35S-$ASPG1_{D379N}$ (D379N) or p35S-$ASPG1_{D180N/D379N}$ (D180N/D379N). The protoplasts were isolated from 4-week-old Col plants. ProRD29A-LUC was used as the ABA-responsive reporter and ProUBQ10-GUS was co-transfected as an internal control. After transfection, the protoplasts were incubated without ABA (–ABA) or with 50 μM ABA (+ABA) for 10 h in the dark at 23 °C. The relative LUC/GUS activity was quantified. Results are shown as mean ±SE of three independent experiments.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Original screening with the XVE-tagged T-DNA insertion mutant lines. (A) Seeds of XVE T-DNA-tagged lines were screened on an MS plate containing 1% sucrose, 0.8% (w/v) agar, with or without 10 μM 17-β-estradiol (E) and 1.5 μM ABA. Photographs were taken to show the germination phenotypes on the day 7 after stratification. (B) Germination rates (%) were analysed on day 7 after stratification by scoring the number of open green cotyledons. Values are means ±SE from three independent experiments (n=100). (C) Schematic drawing (not to scale) showing the T-DNA insertion site in gene AT3G18490 revealed in the original mutant screen. The putative aspartic protease domain at nt 481–1494 is shaded black. Arrows denote the orientation of gene transcription.

Supplementary Fig. S2. Response to ABA of the amiRNA lines. (A) Analyses of the gene expression of *ASPG2* (AT3G20015) in Col, *aspg1-1* and *aspg1-1 amiR-aspg2* lines (#3, #5, #11, #14 and #23). Expression levels were analysed relative to the level of *ASPG2* in Col plants, which was taken as 1. Results are shown as mean ±SE of three independent experiments. (B) ABA-induced stomatal closure. Values are means ±SE from three independent experiments (n=50). The leaves from 4-week-old plants of Col, *aspg1-1* and *aspg1-1 amiR-aspg2* lines (#5 and #14) were first incubated in the light for 3 h to induce stomatal opening and then treated with ABA (0, 5, and 10 μM) for 3 h.

Supplementary Fig. S3. Overexpression of *ASPG1* has no effect on the development of guard cells. (A) The epidermis of the abaxial surface of rosette leaves from Col and *ASPG1-OE2* plants, Bar, 80 μm. (B) Number of stomata mm^{-2} in the epidermis of the abaxial surface of rosette leaves of Col, *ASPG1-OE2*, and *ASPG1-OE14* lines were determined. Values are means ±SE from leaves of three individual plants of Col and *ASPG1-OE* lines. Three independent counts were performed on each leaf.

Supplementary Fig. S4. Two conserved putative aspartic activation sites in the ASPG1 protein. Alignments between predicted ASPG1 protein sequences containing two aspartic proteases in a number of organisms using ClustalX and GeneDoc3.2 tools. CDR1 and PCS1 are from *Arabidopsis thaliana*; CND41 is from *Nicotiana tobacum*. CNB-1 is from *Brachypodium sylvaticum*; S5 is from *Oryza sativa*; Q3UKT5 is from *Mus musculus*; Q9VLK3 is from *Drosophila melanogaster*; Q8WWD9 is from *Homo sapiens*. Arrows indicate the two catalytic aspartic acid residues in ASPG1.

Supplementary Table S1. Primer sequences used for plasmid constructions in this study.

Supplementary Table S2. Primer sequences used for semi-quantitative and quantitative RT-PCR experiments in this study.

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References

Aebi HE. 1983. Catalase. In: Bergmeyer HU, ed. *Methods of Enzymatic Analysis*, Vol. 3. Weinheim: Verlag Chemie, 273–286.

Allan AC, Fricker MD, Ward JL, Beale MH, Trewavas AJ. 1994. Two transduction pathways mediate rapid effects of abscisic acid in *Commelina* guard cells. *The Plant Cell* **6,** 1319–1328.

Belozersky MA, Sarbakanova ST, Dunaevsky YE. 1989. Aspartic proteinase from wheat seeds: isolation, properties and action on gliadin. *Planta* **177,** 321–326.

Bernt E, Bergmeyer HU. 1974. Inorganic peroxides. In: Bergmeyer HU, ed. *Methods of* enzymatic analysis, Vol. 14. New York: Academic Press, 2246–2248.

Boyer JS. 1982. Plant productivity and environment. *Science* **218,** 443–448.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248–254.

Chen M, Huang Y, Liu J, Yuan B, Feng Y. 2011. Highly sensitive profiling assay of acidic plant hormones using a novel mass probe by capillary electrophoresis-time of flight-mass spectrometry. *Journal of Chromatography B* **879,** 938–944.

Chen JJ, Ding JH, Ouyang YD, *et al*. 2008. A triallelic system of *S5* is a major regulator of the reproductive barrier and compatibility of indica-japonica hybrids in rice. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 11436–11441.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

Dat J, Vandenabeele S, Vranová E, Van Montagu M, Inzé D, van Breusegem F. 2000. Dual action of the active oxygen species during plant stress responses. *Cell Molecular Life Sciences* **57,** 779–795.

Davies DR. 1990. The structure and function of aspartic proteinases. *Annual Review of Biophysics and Biophysical*. *Chemistry* **19,** 189–215.

Desikan R, Cheung MK, Bright J, Henson D, Hancock JT, Neill SJ. 2004. ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. *Journal of Experimental Botany* **55,** 205–212.

D'Hondt K, Bosch D, van Damme J, Goethals M, Vandekerckhove J, Krebbers E. 1993. An aspartic proteinase present in seeds cleaves *Arabidopsis* 2S albumin precursors *in vitro*. *Journal of Biological Chemistry* **268,** 20884–20891.

Dunaevsky YE, Sarbakanova ST, Belozersky MA. 1989. Wheat seed carboxypeptidase and joint action on gliadin of proteases from dry and germinating seeds. *Journal of Experimental Botany* **40,** 1323–1329.

Faro C, Gal S. 2005. Aspartic proteinase content of the *Arabidopsis* genome. *Current Protein and Peptide Science* **6,** 493–500.

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cuter SR, Sheen J, Rodriguez PL, Zhu JK. 2009. *In vitro* reconstitution of an abscisic acid signalling pathway. *Nature* **462,** 660–664.

Ge XC, Dietrich C, Matsuno M, Li GJ, Berg H, Xia YJ. 2005. An *Arabidopsis* aspartic protease functions as an anti-cell-death component in reproduction and embryogenesis. *EMBO Reports* **6,** 282–288.

Guerrero F, Mullet JE. 1986. Increased abscisic acid biosynthesis during plant dehydration requires transcription. *Plant Physiology* **80,** 588–591.

Hedrich R, Busch H, Raschke K. 1990. Ca^{2+} and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. *EMBO Journal* **9,** 3889–3892.

Hiraiwa N, Kondo M, Nishimura M, Hara-Nishimura I. 1997. An aspartic endopeptidase is involved in the breakdown of propeptides of storage proteins in protein-storage vacuoles of plant. *European Journal of Biochemistry* **246,** 133–141.

Jakoby M, Weisshaar B, Droge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F. 2002. bZIP transcription factors in *Arabidopsis*. *Trends in Plant Science* **7,** 106–111.

Kato Y, Murakami S, Yamamoto Y, Chatani H, Kondo Y, Nakano T, Yokota A, Sato F. 2004. The DNA-binding protease, CND41, and the degradation of ribulose-1,5-bisphosphate

carboxylase/oxygenase in senescent leaves of tobacco. *Planta* **220,** 97–104.

Kim S, Kang JY, Cho DI, Park JH, Kim SY. 2004. ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. *The Plant Journal* **40,** 75–87.

Kost B, Spielhofer P, Chua NH. 1998. A GFP-mouse talin fusion protein labels plant actin filaments *in vivo* and visualizes the actin cytoskeleton in growing pollen tubes. *The Plant Journal* **16,** 393–401.

Kwak JM, Mäser P, Schroeder JI. 2008. The clickable guard cell, version II: interactive model of guard cell signal transduction mechanisms and pathways. In: *The Arabidopsis Book*. Washington, DC: American Society of Plant Biologists. http://www.aspb.org/publications/arabidopsis/.

Kwak J, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dang JL, Bloom RE, Bodde S, Jones JDG, Schroeder JI. 2003. NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. *EMBO Journal* **22,** 2623–2633.

Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, Schroeder JI. 2004. Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *The Plant Cell* **16,** 596–615.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *The Plant Cell* **10,** 1391–1406.

Liu YG, Mitsukawa N, Oosumi T, Whittier RF. 1995. Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *The Plant Journal* **8,** 457–463.

Luan S. 2002. Signalling drought in guard cells. *Plant, Cell and Environment* **25,** 229–237.

Lu SY, Su W, Li HH, Guo ZF. 2009. Abscisic acid improves drought tolerance of triploid bermudagrass and involves H_2O_2- and NO-induced antioxidant enzyme activities. *Plant Physiology Biochemistry* **47,** 132–138.

McAinsh MR, Brownlee C, Hetherington AM. 1990. Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. *Nature* **343,** 186–188.

Meinhard M, Grill E. 2001. Hydrogen peroxide is a regulator of ABI1. *a protein phosphatase 2C from Arabidopsis. FEBS Letters* **508,** 443–446.

Meinhard M, Rodriguez PL, Grill E. 2002. The sensitivity of AB12 to hydrogen peroxide links the abscisic acid-response regulator to redox signalling. *Planta* **214,** 775–782.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7,** 405–410.

Miyazono KI, Miyakawa T, Sawano Y, Kubota K, Kang HJ, Asano A, Miyauchi Y, Takahashi M, Zhi YH, Fujita Y, Yoshida T, Kodaira KS, Yamaguchi-Shinozaki K, Tanokura M. 2009. Structural basis of abscisic acid signalling. *Nature* **462,** 609–614.

Murakamia S, Kondob Y, Nakanoc T, Sato F. 2000. Protease activity of CND41, a chloroplast nucleoid DNA-binding protein, isolated from cultured tobacco cells. *FEBS Letters* **468,** 15–18.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J. 2002. *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *The Plant Cell* **14,** 3089–3099.

Mutlu A, Gal S. 1999. Plant aspartic proteinases: enzymes on the way to a function. *Physiologia Plantarum* **105,** 569–576.

Negi J, Matsuda O, Nagasawa T, Oba Y, Takahashi H, Kawai-Yamada M, Uchimiya H, Hashimoto M, Iba K. 2008. CO_2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature* **452,** 483–486.

Nelson BK, Cai X, Nebenfuhr A. 2007. A multicolored set of *in vivo* organelle markers for co-localization studies in *Arabidopsis* and other plants. *The Plant Journal* **51,** 1126–1136.

Nishimura N, Hitomi K, Arvai AS, Rambo RP, Hitomi C, Cutler SR, Schroeder JI, Getzoff ED. 2009. Structural mechanism of abscisic acid binding and signaling by dimeric PYR1. *Science* **326,** 1373–1379.

Pandey S, Wang XQ, Coursol SA, Assmann SM. 2002. Preparation and applications of *Arabidopsis thaliana* guard cell protoplasts. *New Phytologist* **153,** 517–526.

Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* **406,** 731–734.

Rawlings ND, Barrett AJ. 1995. Families of aspartic peptidases, and those of unknown catalytic mechanism. *Methods in Enzymology* **248,** 105–120.

Riera M, Valon C, Fenzi F, Giraudat J, Leung J. 2005. The genetics of adaptive responses to drought stress: abscisic acid-dependent and abscisic acid-independent signalling components. *Physiologia Plantarum* **123,** 111–119.

Runeberg-Roos P, Kervinen P, Kovaleva J, Raikel NV, Gal S. 1994. The aspartic proteinase of barley is a vacuolar enzyme that processes probarley lectin *in vitro*. *Plant Physiology* **105,** 321–329.

Sanford JC, Smith FD, Rushell JA. 1993. Optimizing the biolistic process for different biological application. *Methods in Enzymology* **217,** 483–509.

Schroeder JI, Hagiwara S. 1989. Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* **338,** 427–430.

Schroeder JI, Kwak JM, Allen GJ. 2001. Guard cell abscisic acid signalling and engineering drought hardiness in plants. *Nature* **410,** 327–330.

Schwab R, Ossowski R, Riester M, Warthmann N, Weigel D. 2006. Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. *The Plant Cell* **18,** 1121–1333.

Shinozaki K, Yamaguchi-Shinozaki K. 2006. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review of Plant Biology* **57,** 781–803.

Sirichandra C, Gu D, Hu HC, Davanture M, Lee S, Djaoui M, Valot B, Zivy M, Leung J, Merlot S, Kwak JM. 2009.

Phosphorylation of the *Arabidopsis* AtrbohF NADPH oxidase by OST1 protein kinase. *FEBS Letters* **583,** 2982–2986.

Smirnoff N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist* **125,** 27–58.

Szecsi PB. 1992. The aspartic protease. *Scandinavian Journal of Clinical and Laboratory Investigation Supplementum* **210,** 5–22.

Ukeda H, Shimamura T, Tsubouchi M, Harada Y, Nakai Y, Sawamura M. 2002. Spectrophotometric assay of superoxide anion formed in Maillard reaction based on highly water-soluble tetrazolium salt. *Analytical Science* **18,** 1151–1154.

Vahisalu T, Kollist H, Wang YF, *et al*. 2008. SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature* **452,** 487–491.

Wang YP, Li L, Ye TT, Zhao SJ, Liu Z, Feng YQ, Wu Y. 2011. Cytokinin antagonizes ABA suppression to seed germination of *Arabidopsis* by down-regulating ABI5 expression. *The Plant Journal* **68,** 249–261.

Xia YJ, Suzuki H, Borevitz J, Blount J, Guo ZJ, Patel K, Dixon RA, Lamb C. 2004. An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. *EMBO Journal* **23,** 980–988.

Yamaguchi-Shinozaki K, Shinozaki K. 2006. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual Review of plant Biology* **57,** 781–803.

Yoo SD, Cho YH, Sheen J. 2007. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocols* **2,** 1565–1572.

Yoshida R, Hobo T, Ichimura K, Mizoguchi T, Takahashi F, Aronso J, Ecker JR, Shinozaki K. 2002. ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis*. *Plant Cell Physiology* **43,** 1473–1483.

Zhang J, Xu JX, Kong YZ, *et al*. 2005. Generation of chemical-inducible activation tagging T-DNA insertion lines of *Arabidopsis thaliana*. *Acta Genetica Sinica* **32,** 1082–1088.

Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song CP. 2001. Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiology* **126,** 1438–1448.

Zhu JK. 2002. Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology* **53,** 247–273.

Zuo J, Niu QW, Chua NH. 2000. An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *The Plant Journal* **24,** 265–273.