

## An $\alpha$ -expansin, *VfEXPA1*, is involved in regulation of stomatal movement in *Vicia faba* L.

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The wall loosening of guard cells differs from other types of plant cells. However, the regulation of wall loosening during stomatal movement is poorly understood. *VfEXPA1* is an  $\alpha$ -expansin gene cloned from *Vicia faba* epidermal strips. Expression of *VfEXPA1* is regulated by darkness and submergence, and is not affected by light and abscisic acid (ABA). *In situ* hybridization showed that *VfEXPA1* is expressed primarily in the guard cells. Overexpression of *VfEXPA1* in transgenic tobacco accelerated light-induced stomatal opening, and increased both transpiration and photosynthetic rates under favorable growth conditions. Our results indicate the guard cell-expressed expansin *VfEXPA1* plays an important role in regulation of stomatal opening.

**$\alpha$ -expansin, stomatal opening, guard cell wall, *VfEXPA1*, *Vicia faba***

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Plant stomatal pores are surrounded by guard cells in the epidermis of plant stems and leaves. Stomata are essential for oxygen-carbon dioxide exchange and release of water vapor [1]. The sensitivity of stomata is vital in the regulation of transpiration and photosynthesis. The uptake of water via osmosis is the result of  $K^+$  and  $Cl^-$  uptake,  $H^+$  extrusion and the production of organic solutes. This process leads to guard cell swelling and subsequent stomatal opening [2].

Compared to other epidermal cells or mesophyll cells, guard cell is a highly specialized cell type. In *Vicia faba* L., turgor pressure within guard cells increased by as much as 5.0 MPa [3], suggesting that the guard cell wall is strong enough to support 10 times the pressure of most other plant cell types. The shape and volume of guard cells during opening and closing can be dramatically altered in a matter

of minutes. Guard cells are reported to undergo a change in surface area of up to 40% [3,4]. Two opposing observations have been made regarding plant cell extension. It is widely accepted that internal turgor pressure drives cell extension; however, the cell wall restricts extension. Therefore, it is intriguing that such a rigid cell wall can undergo rapid and reversible adjustment in guard cells. The role of cell walls in the regulation of stomatal movement is poorly understood, but it is likely that a unique regulatory mechanism to complete a loosen-regeneration during a stomatal movement cycle in less than a few hours should exist. Several factors might accelerate primary wall loosening, but only the wall weakened by expansins does not gradually and progressively [5].

Expansins were identified as cell wall-loosening proteins that mediate pH-dependent plant cell wall extension and cell growth [6,7]. However, no enzymatic activity has been documented for expansin genes. A superfamily of expansin genes has been divided into four families, which consist of

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$\alpha$ -expansin (EXPA),  $\beta$ -expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) subfamilies [8]. Among these, only the  $\alpha$ -expansin and  $\beta$ -expansin families reportedly have cell wall-loosening properties [6,9]. Evidence suggests the known expansins disrupt noncovalent bonds between cellulose microfibrils and matrix glucans that adhere to microfibrils [10,11]. Therefore, expansin might be involved in adjusting guard cell wall loosening to meet the requirement of reversible and rapid movement of stomata in an energy-saving and nondestructive manner. A large number of expansins are involved in the growth of a specific cell type [12–14], while others are implicated in plant developmental processes such as fruit softening [15,16], abscission [17], and pollination [18]. Research on *Arabidopsis* indicates the expansin gene *AtEXPA1* might be guard cell-specific [19]. Recently, our results indicated *AtEXPA1* might be involved in the regulation of stomatal sensitivity [20]. It is intriguing whether the function of guard cell-expressed expansin is specific to *Arabidopsis* or is common to other plant species. In this study, *Vicia faba*, a model system for stomatal research, was chosen. An  $\alpha$ -expansin, *VfEXPA1*, was cloned from leaf epidermal strips. Our results on the expression pattern and functional analysis of *VfEXPA1* indicate *VfEXPA1* may play an important role in regulation of stomatal opening.

## 1 Materials and methods

### 1.1 Plant materials and growth conditions

*Vicia faba* seeds were sterilized in 0.1%  $\text{HgCl}_2$  for 10 min, soaked in water for 24 h, and incubated at 25°C for 2–3 d. Following germination, when the length of the root reached 0.5 cm, the seedlings were planted in a greenhouse under the following growth conditions: a 12 h/12 h (light/dark) cycle, illumination intensity of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , day/night temperatures of 22°C/17°C, and 70% relative humidity. Epidermal strips were removed from mature leaves for the extraction of RNA and protein.

Tobacco (*Nicotiana tabacum* L.) plants were grown in 3 L pots with ready-mixed soil. During transformation, tobacco plants were grown in 250 mL flasks that contained 100 mL agar medium. Growth conditions were 25°C/22°C (night/day) under a 12 h photoperiod with light intensity of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

### 1.2 Gene cloning

Following an analysis of known EXPA sequences, highly conserved sequence regions were selected to design primers: 5'-GTGATGCCCTCTGGCACAA-3' and 5'-GGAGGATTACACCAACCC-3'. Abaxial epidermal strips of mature *Vicia* leaves were harvested for reverse transcription. The first-strand cDNA was reverse transcribed from total RNA using the anchor primer T18GC. The resulting RT-PCR band was

cut and reclaimed, then cloned into the pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

The full-length cDNA was constructed in a two-step procedure using the SMART<sup>TM</sup> RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Following the specified protocol, 5'-RACE clones were generated. 5'-RACE-Ready cDNA was reverse transcribed from total RNA using a 5'-CDS primer and SMART II Oligonucleotide. PCR was then executed employing the Universal Primer A Mix (UPM; Clontech) and 5GSP1 (5'-ATTGAATGAACATCACAGCACC-3', designed according to the EST of *VfEXPA1*). The first PCR product was used as a template for nested PCR amplification, which was carried out using the Nested Universal Primer A (NUP; Clontech) and 5GSP2 (5'-GCAACCTTGGATCATCGTTGCATC-3', designed according to the EST of *VfEXPA1*). The amplified fragment was cloned into the pGEM-T vector and sequenced. In the second step, 3'-RACE-Ready cDNA was constructed using a 3'-CDS primer and PCR amplification was carried out using UPM and 3GSP1 (5'-CTTCTGGCACAATGGGTGGGGC-ATG-3', designed according to the EST of *VfEXPA1*). Nested PCR amplification was performed with NUP and 3GSP2 (5'-TCCATCAAAGGCTCAAGAACTTCATGGCAAC-3'). All gene-specific primers were designed according to the EST of *VfEXPA1*. The amplified product was cloned into pGEM-T and sequenced. Finally, the full-length cDNA was constructed.

### 1.3 Plasmid construction and plant transformation

The entire sequence of *VfEXPA1* was cloned by RT-PCR with primers 5'-CCTTCTAGAATGACTCAAAAGCTTAGAATG-3' and 5'-AATGAGCTCCTAAAATTGAGCTCCTTGAAAG-3'. The PCR fragment was digested with *Xba*I and *Sac*I and inserted into the binary vector pBI121 [21] under the control of the *Cauliflower mosaic virus* 35S promoter. The construct was confirmed by DNA sequencing and introduced into *Agrobacterium tumefaciens* strain LB4404 for tobacco plant transformation. Kanamycin-resistant seedlings were transferred to soil, and examined by genomic DNA PCR and Western blot analysis. For selection of T<sub>2</sub> transgenic plants, seeds were germinated on agar medium containing 50  $\mu\text{g/mL}$  kanamycin.

### 1.4 Measurement of stomatal aperture and density

Epidermal strips were removed carefully from the abaxial leaf surface of tobacco or *Vicia* and cut into 5 mm  $\times$  10 mm pieces. The strips were brushed to remove the mesophyll cells and destroy the epidermal cells. The efficacy of mesophyll cell removal and epidermal cell destruction was tested using Neutral Red staining. The staining showed only guard cells remained viable in the epidermal strip. Then, the strips were floated briefly in 10 mmol/L MES (pH adjusted to 5.8 with Tris base) with 50 mmol/L KCl and 100  $\mu\text{mol/L}$

CaCl<sub>2</sub>, and placed in the dark until the stomata were fully closed. The pieces were floated in the same buffer and treated with light (200 μmol m<sup>-2</sup> s<sup>-1</sup>) from a halogen cold source to induce stomatal opening. Five fields of view were randomly selected and 10 stomatal apertures were measured in each field. Each experiment was repeated three times and the average value ± SE was calculated. Stomatal density was determined following the standard protocol [22] by microscopic examination of nail-polished imprints from leaf surfaces.

### 1.5 Measurement of transpiration and photosynthesis

Both transpiration and photosynthetic rates (CO<sub>2</sub> uptake) were measured with a CI-510 hand-held photosynthesis system (CID, Camas, WA, USA). Measurements were recorded in the greenhouse between 9:00 and 10:00 (~180 μmol m<sup>-2</sup> s<sup>-1</sup>). All experiments were repeated three times.

### 1.6 *In situ* hybridization and immunohistochemical localization

Fixation, paraffin embedding, sectioning and hybridization were performed as described by Cho and Kende [23], except we used 12 μm sections. Probe hybridization was detected with antidigoxigenin antibodies conjugated to alkaline phosphatase, and visualized by color development according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Gene-specific 3'-untranslated regions were amplified with the primers 5'-TCCATCAAA-GGCTCAAGAAGTTCATGGCAACC-3' and 5'-CAATCA-TACTAATATCAATTGAAAAGA-3' from *Vicia faba* cDNA and cloned into pGEM-T. Sense and antisense *VfEXPA1*-specific probes were generated by *in vitro* transcription using a digoxigenin labeling kit (Roche Diagnostics). Probes were hydrolyzed to 100 nt by alkaline hydrolysis prior to hybridization. Sense or antisense probe hybridization was performed three times.

For immunochemical localization, tissues were prepared as described above for *in situ* hybridization and blocked for 2 h at room temperature in TBST (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% (v/v) Tween-20, pH 7.5) with 1% bovine serum albumin (BSA). Tissues were washed five times for 5 min each, and incubated for 2–3 h at 37°C with rabbit polyclonal antiserum against AtEXPA1 (1:200 dilution) [20]. After three washes in TBST, sections were incubated with anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO, USA) for 2 h and washed three times in TBST. The color reaction was carried out in the dark with NBT/BCIP.

### 1.7 Western blot analysis

Western blot analysis was achieved by protein transfer from polyacrylamide gels to a polyvinylidene difluoride filter

(Millipore, Bedford, MA, USA) using a gel blotter (Bio-Rad Laboratories, Richmond, CA, USA). The transfer was in 0.01 mol/L 3-cyclohexylamino-propanesulfonic acid (CAPS) and 10% (w/v) methanol at 100 V for 1 h at 4°C. After transfer, the PVDF membrane was blocked with 3% BSA and incubated with the primary polyclonal antibody against AtEXPA1 (1:200) for 2 h at room temperature. Cross-reaction with the antibody was revealed using an alkaline phosphatase conjugated with a secondary anti-rabbit antibody.

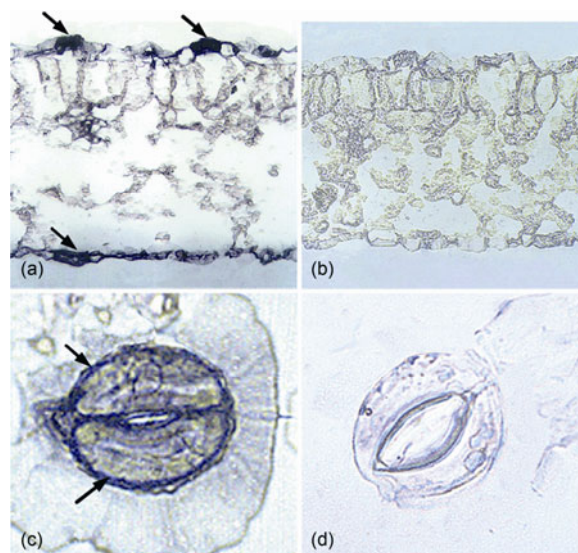
### 1.8 Northern blot analysis

For Northern blot, 20-d-old seedlings were treated with either abscisic acid (100 μmol/L), darkness (300 μmol m<sup>-2</sup> s<sup>-1</sup>, illumination intensity of 100 μmol m<sup>-2</sup> s<sup>-1</sup>), or submergence for 2 h. Total roots, stems, and mature leaves were collected from 4-week-old seedlings, and RNA isolation and gel blot analyses were performed as described by Cho and Kende [24].

## 2 Results

### 2.1 An abundance of expansin proteins were located in guard cell walls

Mature leaves of plants undergo few cell wall extension events. Thus, the function of expansins in mature leaves is puzzling. To elucidate the role of expansins in plants, immunohistochemistry staining was performed on mature leaves of *Vicia faba* using an antibody raised against the *Arabidopsis* expansin AtEXPA1 [20]. Most signals were detected in guard cells. The cells surrounding the vascular



**Figure 1** Immunolocalization of expansins in mature leaves of *Vicia faba*. Immunoblot with the (a), (c) AtEXPA1 antibody (1:200 dilution), and with (b), (d) preimmune serum (1:200 dilution) as the primary antibody, respectively. Arrows show the signals detected.

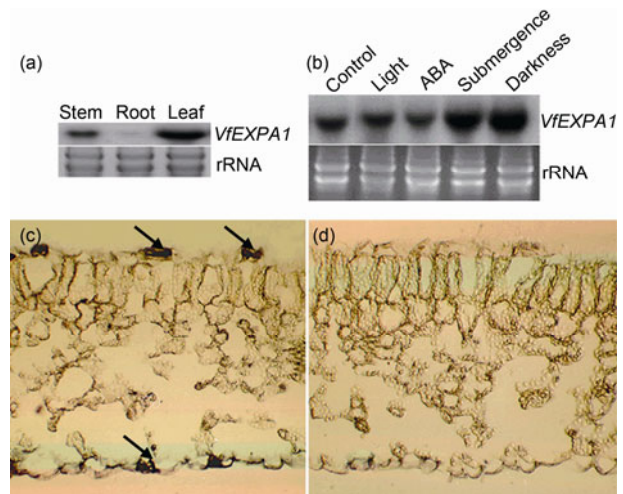
bundles also generated weak signals, but the signals were markedly weaker than those of guard cells (Figure 1(a) and (b)). Immunoassay of single stoma showed the proteins recognized by the antibody were mainly located on cell walls. However, the distribution of the immunostain signal was not uniform. Stronger signals were detected in the dorsal guard cell walls than in the ventral walls (Figure 1(c) and (d)).

## 2.2 Cloning and phylogenetic analysis of *VfEXPA1* from epidermal cells of *Vicia faba* mature leaves

To clone guard cell-expressed expansins, a highly conserved EXPA region, which encodes approximately 90 amino acids in the N-terminal region of mature proteins, was used to design RT-PCR primers. RT-PCR amplification was performed using total RNA extracted from epidermal strips of mature leaves. A 536-bp fragment was obtained and sequenced. The EST sequence of *Vicia faba* was submitted to GenBank (accession number AF464953). The full-length cDNA was then cloned using 5'- and 3'-RACE and subsequently named *VfEXPA1* (GenBank accession number EF190969). The *VfEXPA1* open reading frame was 765 bp and encoded an expansin with a molecular weight of approximately 27.5 kD. The cDNA contained a 45-bp 5'-untranslated region and a 318 bp 3'-untranslated region. The presence of a signal peptide was predicted by the SignalP program (<http://www.cbs.dtu.dk/services/>), and the most likely cleavage site was between amino acids 27 and 28. The mature protein was approximately 25 kD after excision of the signal peptide. A phylogenetic tree was constructed from the deduced amino acid sequence of *VfEXPA1* and all  $\alpha$ -expansins of *Arabidopsis*, which showed *VfEXPA1* belongs to the  $\alpha$ -subfamily and *AtEXPA8* has the highest sequence similarity (75%) to *VfEXPA1*. Alignment of the deduced amino acid sequence of *VfEXPA1* with typical known  $\alpha$ -expansins also indicated *VfEXPA1* was an  $\alpha$ -subfamily expansin (Figure S1). *VfEXPA1* showed a characteristic  $\alpha$ -insertion in front of a conserved HFD (His-Phe-Asp) motif with four highly conserved 'GWCN' residues at its 3' end [25] (Figure S1). In addition, two other conserved domains of expansins, the cellulose-binding-like domain (from amino acid 172 to 251) and the family-45 endoglucanase-like domain (from amino acid 50 to 162), were revealed in its structure.

## 2.3 *VfEXPA1* expression patterns

To investigate the tissue expression pattern of *VfEXPA1*, 20-d-old green seedlings of *Vicia* were dissected into roots, stems, and mature leaves for Northern blot analysis. *VfEXPA1* was expressed primarily in stems and leaves, especially at a higher level in leaves, and expression was not detected in the roots (Figure 2(a)). The expression of *VfEXPA1* was examined under treatment with light, dark, ABA, or sub-



**Figure 2** Expression patterns of *VfEXPA1* in *Vicia faba*. (a) Northern blot analysis of *VfEXPA1* mRNA in stems, roots and mature leaves. Total RNA (15  $\mu$ g) from each tissue was hybridized with 3'-UTR of *VfEXPA1* as the probe after gel electrophoresis. rRNA was used as the loading control. (b) Twenty-day-old *Vicia faba* plants were treated with light, abscisic acid, submergence, or darkness for 2 h, respectively; untreated leaves were collected as the control. Each lane represented 15  $\mu$ g of total RNA, and rRNA was used as the loading control. (c), (d) *In situ* localization of *VfEXPA1* mRNA in *Vicia faba* mature leaves. Transverse section probed with the antisense (c) and sense (d) *VfEXPA1* 3'-UTR RNA. Arrows indicate some of the hybridization signals. Note that the signals are found mainly in the guard cells.

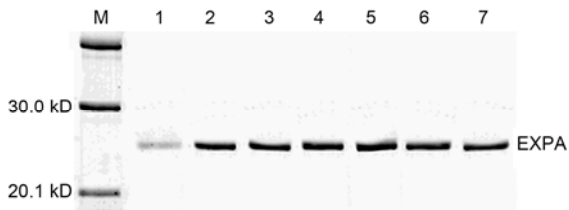
mergence in water for 2 h. Northern blot analysis revealed *VfEXPA1* was slightly upregulated by darkness and submergence, but was not affected by light and ABA (Figure 2(b)).

*In situ* hybridization in mature leaves with a gene-specific probe was used to analyze the cellular location of *VfEXPA1*. Transcripts of *VfEXPA1* accumulated in the guard cells. Weaker signals were observed in vascular bundles and some mesophyll cells (Figure 2(c), (d)).

## 2.4 Overexpression of *VfEXPA1* accelerated stomatal opening

To examine the function of *VfEXPA1*, the full length sequence was fused under the control of the CaMV 35S promoter and transformed into tobacco. Six  $T_2$  lines were selected from 60  $T_2$  kanamycin-resistant lines for further analysis. Proteins were isolated from epidermal strips of mature leaves of tobacco plants to perform Western blot analysis. A band with a molecular weight of 25 kD was detected in all lines, including the control plants, but was more abundant in the six transgenic lines (Figure 3). This result indicated *VfEXPA1* was overexpressed in the transgenic plants. Transgenic lines 2 and 34 were selected and designated OE1 and OE2 for further phenotype and physiological studies.

To verify the involvement of *VfEXPA1* in the regulation of stomatal movement, the rate of stomatal opening was



**Figure 3** Identification of expansins in *VfEXPA1* overexpression tobacco lines. Proteins (15  $\mu\text{g}/\text{sample}$ ) of epidermal strips were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The expansins were detected with a polyclonal antibody raised against recombinant AtEXPA1 expressed in *E. coli*. Lane 1, mature leaves of the wild type. Lanes 2–7, transgenic lines 2, 34, 19, 29, 40 and 43, respectively.

investigated in transgenic and wild-type plants. Abaxial epidermal strips of transgenic and wild plants were pretreated in the dark until stomata closure, and then subjected to cold light. *VfEXPA1* overexpression lines showed a much higher rate of light-induced stomatal opening (Figure 4(a)), especially in the early stage. However, the final stomatal aperture size was not altered by *VfEXPA1* overexpression.

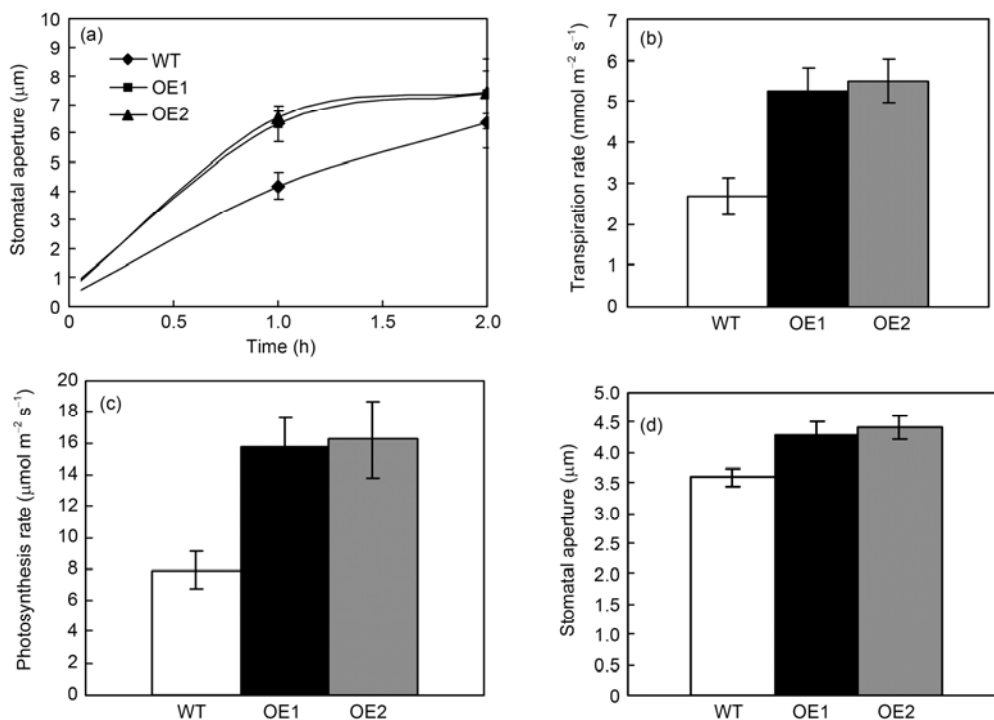
### 2.5 Overexpression of *VfEXPA1* enhanced transpiration and photosynthetic rates

Since *VfEXPA1* accelerates stomatal movement in epidermal strips *in vitro*, we wondered if this phenomenon has any

physiological effects and could be replicated *in vivo*. Therefore, we measured both transpiration and photosynthetic rates of transgenic and wild-type plants after 2 h exposure to light following pretreatment in the dark. When growing under favorable conditions, the transgenic plants showed significantly higher transpiration and photosynthetic rates than the control plants (Figure 4(b) and (c)). Because the stomatal number and sensitivity both determine the transpiration and photosynthetic rates, stomata density and stomatal aperture size of mature leaves were measured. Stomata density in transgenic lines was identical to that of the wild type (Table 1). However, the stomata of transgenic plants had larger apertures (Figure 4(d)), which indicated the increased stomatal sensitivity in *VfEXPA1* overexpression lines might induce higher transpiration and photosynthetic rates.

## 3 Discussion

Expansins usually function in growing regions of plants [26]. Developing leaves possess at least one expansin responsible for acid-growth properties [27,28]. Previous research found that most leaf expansins were expressed at leaf formation or in an elongation zone [14,28] and these specific expansins could be responsible for enhanced cell wall extensibility. However, several expansins are expressed in



**Figure 4** Effects of *VfEXPA1* overexpression on photosynthesis, transpiration rate and stomatal movement. (a) Epidermal strips from transgenic and wild-type plants were pretreated with darkness until all stomata closed, and then treated with cool light ( $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) to induce stomatal opening. Stomatal apertures were measured every 60 min. Error bars represent the standard error of the mean of at least 150 stomata per plant. Transpiration rate (b) and photosynthesis rate (c) of wild-type (white columns) and transgenic *VfEXPA1* (gray and black columns) lines in the greenhouse were measured at 11:00. Illumination intensity was  $180 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Concurrently, stomatal aperture (d) was measured on leaves at 9:00. White columns represent the wild type, gray and black columns represent transgenic plants. Error bars represent the standard error of the mean of at least five plants per line.

**Table 1** Stomatal density in epidermis of leaves of wild-type and *VfEXPA1* overexpression plants <sup>a)</sup>

Samples	Wild type plants	OE1	OE2
Upper leaf surface	58.6±2.9	60.3±1.8	56.7±3.8
Lower leaf surface	139.6±4.5	137.8±3.2	135.2±6.1

a) Average stomatal density values (number/mm<sup>2</sup> ± SE) were obtained from analysis of 60 microscopic fields selected randomly from leaves of three independent plants for each line.

mature leaves [29,30]. Our data also demonstrated the presence of expansin in mature leaves of *Vicia faba*. Moreover, immunoblot analysis showed expansins are present also in the mature leaves of aianthus, peach, dayflower, and wheat (Figure S2), which suggested expression in mature leaves is a common feature of certain types of expansin. Thus, it is interesting to elucidate the specific cell types in which expansins are expressed. In the present study, an immunoassay was performed to investigate the cellular location of expansin in mature leaves. Although the specificity of the anti-AtEXPA1 antibody has not been well investigated yet, the single band in the Western blot analysis of mature-leaf proteins of 5 plant species indicated at least it could specifically recognize a group of EXPA proteins with a 25 kD molecular weight. The immunoassay showed most expansins present in mature leaves were located in guard cells. In addition, we cloned an expansin gene, *VfEXPA1*, from epidermal strips from mature leaves of *V. faba*. Because guard cells are a major component of leaf epidermal strips, these results indicate *VfEXPA1* is a guard cell-expressed protein. Furthermore, *in situ* hybridization analysis proved *VfEXPA1* accumulated in guard cells. Together with previous findings of expansin specifically expressed in guard cells in *Arabidopsis* [19,20], these results imply in mature leaves expansin accumulates in guard cells.

The matrix of cellulose crosslinked by glycans, such as xyloglucan and glucuronarabinoxylan, is believed to be crucial for cell wall extensibility [31]. Congruent with a previous report [32], we found that cellulose hydrolytic enzymes, either cellulase or  $\beta$ -glucanase, have no detectable effect on stomatal movement (data not shown). However, modification of pectins might have an effect on stomatal movement. Overexpression of a polygalacturonase, which cleaves pectins, disable stomatal closure [33]. A pectin-degrading enzyme, arabinanase, can block guard cell movement while endopolygalacturonase (endoPGase), in concert with pectin methylesterase (PME), increased stomatal aperture [32,34]. However, it is important to note that the hydrolysis of pectin is slow and energy-dependent and, as a consequence of pectinase activity, the guard cells and neighboring epidermal cells are often separated by structural damage to the cell wall [33]. Therefore, enzyme-related pectin hydrolysis is unlikely to be the mechanism of the rapid and reversible cell wall loosening in guard cells.

Expansin is a potential candidate for a regulator of sto-

matal opening sensitivity, because it is known to catalyze the disruption of hydrogen bonds between cellulose and the load-bearing crosslinking glycans (such as xyloglucan in dicots) [10,35]. Expansins also induce cell expansion both *in vivo* and *in vitro* [6,36]. Thus expansins show characteristics for regulation of the rapid and non-destructive cell wall expansion required in stomatal movement. Our data indicate expansins in mature leaves are expressed predominantly in guard cells. Since cell growth has almost been completed in mature leaves, the accumulation of expansins in guard cells of mature leaves indicates expansin might have potential functions in stomatal movement. Furthermore, stomatal sensitivity is increased by overexpression of the guard cell-expressed expansin gene *VfEXPA1*. Although *VfEXPA1* in leaf tissue other than guard cells in overexpression lines might affect stomatal movement, mesophyll cells and epidermal cells were destroyed by careful brushing of epidermal strips used to test light-induced stomatal opening *in vitro*. In this case, the difference in stomatal sensitivity between *VfEXPA1* overexpression lines and the wild type is dependent on *VfEXPA1* expression in guard cells alone. Because stomatal sensitivity increased in both *in vitro* and *in vivo* tests on *VfEXPA1* overexpression lines, we believe *VfEXPA1* could regulate stomatal movement.

Immunohistochemical localization showed the expansin level increased in the dorsal wall compared with that of ventral walls in guard cells. It is interesting to note that in sugar beet (*Beta vulgaris* L.) leaves, the xyloglucan epitope recognized by the CCRC-M1 antibody was more abundant in the ventral wall [37]. Xyloglucan-crosslinked cellulose can decrease the rigidity and increase the extensibility of cell walls [38]. Expansin-facilitated relaxation of the cellulose-xyloglucan complex is typical in turgor-mediated cell expansion [39]. During stomatal opening, the dorsal wall extends more than the ventral wall, which borders the stomatal pore. This could be the reason for higher accumulation of expansins and a lower quantity of xyloglucan in the dorsal wall.

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## Supporting Information

**Figure S1** Amino acid sequence comparison of VfEXPA1 with other expansins.

**Figure S2** Expansins are present in the mature leaves of diverse plant species.

The supporting information is available online at [csb.scichina.com](http://csb.scichina.com) and [www.springerlink.com](http://www.springerlink.com). The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.