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Abscisic acid regulates gene expression in cortical fiber cells and silica cells of rice shoots

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Received: *1 November 2007*
Accepted: *19 November 2007*

Summary

• Drought-induced growth arrest is a major cause of yield loss in crops and is mediated in part by abscisic acid (ABA). The aim of this study was to identify the cell types targeted by ABA during arrest.

• As transcription factors ABI3 and ABI5 are essential for ABA-induced growth arrest in Arabidopsis, BLAST was used to identify OsVP1 and OsABF1 as their structural orthologues in rice (*Oryza sativa*), and employed RNA *in situ* hybridization to reveal the cell types accumulating the corresponding transcripts in response to ABA.

• Exogenous ABA arrested the growth of leaves 1, 2 and 3 in young rice shoots and inhibited secondary cell-wall formation in sclerenchyma, including expression of the cellulose synthase gene *OsCesA9*. Transcripts for *OsVP1*, *OsABF1* and of the putative target genes *OsEm*, *OsLEA3* and *WSI18*, increased under ABA, accumulating principally in the cytosol of the major support cells (sclerenchymatous cortical fiber cells and epidermal silica cells) of slowly growing leaf 1. Rapidly growing immature tissues in leaves 2 and 3 accumulated *OsABF1*, *OsEm* and *WSI18* transcripts in the nuclei of all cells, irrespective of ABA treatment.

• It is concluded that during arrest of leaf growth, ABA targets support cells in maturing tissues. Target cells in immature tissues remain to be identified.

Key words: abscisic acid (ABA), growth arrest, *Oryza sativa*, sclerenchyma, silica cells, transcription factors.

New Phytologist (2008) **178**: 68–79

doi: 10.1111/j.1469-8137.2007.02365.x

Introduction

Abscisic acid (ABA) is a sesquiterpenoid hormone that plays central roles in seed maturation, germination and stress responsiveness (Finkelstein *et al.*, 2002). The agricultural importance of ABA lies in its impact on processes such as seed dormancy (Koornneef *et al.*, 1989), stomatal closure (Leung & Giraudat, 1998), leaf rolling (You *et al.*, 2006) and growth arrest (Lopez-Molina *et al.*, 2001, 2002).

Growth arrest is relevant to the impact of drought stress on crop establishment, biomass production, harvest index and yield (O'Toole & Namuco, 1983; Lopez-Molina *et al.*, 2001) and is a potential target in breeding programs designed to enhance yield under stress. Abscisic acid-induced growth arrest has been most intensively studied in *Arabidopsis thaliana* seedlings (Finkelstein *et al.*, 2002). Arabidopsis mutants that fail to show ABA-induced growth arrest have helped to define specific steps in this particular signal transduction pathway. Six *AB*A-*i*nsensitive (*abi*) mutant genes have been cloned. *ABI1* and *ABI2* encode PP2C protein phosphatases (Leung *et al.*, 1997; Rodriguez *et al.*, 1998). *ABI3*, *ABI4* and *ABI5* encode transcription factors belonging to the B3 domain family (Giraudat *et al.*, 1992), the APETALA2 (AP2) domain family (Finkelstein *et al.*, 1998) and the basic leucine zipper

(bZIP) domain family (Finkelstein & Lynch, 2000), respectively. *ABI8* encodes a novel protein of unknown function (Brocard-Gifford *et al.*, 2004). Many other mutants of ABA function and metabolism also affect seed germination and shoot growth (Nambara & Marion-Poll, 2005; Yoshida *et al.*, 2006).

ABI3 and ABI5 bind to promoters either separately or as a complex (Nakashima *et al.*, 2006). ABI3 itself recognizes Sph *cis*-elements (Monke *et al.*, 2004), whereas ABI5 binds to ABRE *cis*-elements (Hobo *et al.*, 1999a,b; Hattori *et al.*, 2002; Kim *et al.*, 2002; Nakashima *et al.*, 2006). One of the best studied target genes for the ABI3–ABI5 complex is *AtEm6*, which belongs to the late embryogenesis abundant (LEA) protein family. The complex between ABI3 and ABI5 is more effective than either protein alone in transcribing the *AtEm6* gene (Nakamura *et al.*, 2001; Gampala *et al.*, 2002).

The orthologues of ABI3 in maize and rice are believed to be the transcription factors VIVIPAROUS1 (VP1) and OsVP1, respectively, which are required in establishing seed dormancy (Hattori *et al.*, 1995). The rice orthologue of ABI5 has not been identified, but TRAB1, a rice bZIP protein that forms a transcription complex with OsVP1 on the *OsEm* promoter (Hobo *et al.*, 1999a), has been considered a strong candidate. Complex formation is also seen between HvVP1 and HvABI5 on the barley *HVA1* promoter (Casaretto & Ho, 2003; Shen *et al.*, 2004). *HVA1* is an ABA-inducible *LEA* gene (Hong *et al.*, 1988) and its closest rice homologues are *WSI18* and *OsLEA3*. Expression of *WSI18* is induced by drought (Joshee *et al.*, 1998), and expression of *OsLEA3* is induced by ABA (Moons *et al.*, 1997).

Abscisic acid-induced growth arrest was examined in rice seedlings. BLAST analysis and reverse transcription–polymerase chain reaction (RT-PCR) amplification confirmed that the rice orthologue of *ABI3* is *OsVP1* (Hattori *et al.*, 1995) and it was shown that the structural orthologue of *ABI5* is *OsABF1*, a gene not previously studied. Furthermore, RNA *in situ* hybridization established that *OsVP1*, *OsABF1*, *TRAB1*, *OsEm*, *OsLEA3* and *WSI18* are highly expressed in the cytosol of the support cells in shoot tissues that are mature enough to possess them. However, in immature tissues the targets of ABA-induced growth arrest remain to be identified.

Materials and Methods

Plant material

Seeds of rice (*Oryza sativa* L. cv. IR64) were obtained from the International Rice Genebank at IRRI (accession number IRGC 66970). Seed dormancy was broken by incubating the rice seeds in an oven at 50°C for 48 h. Dehulled and sterile IR64 seeds were germinated on Petri plates, containing MS medium (Murashige & Skoog, 1962), for 2 d in a lit room (30°C, continuous cool white light, 100 μmol m^{-2} s^{-1}). For treatment (±)-ABA or gibberellin (GA_3) (Sigma Chemical Co., St Louis, MO, USA), 2-d-old seedlings were transferred to MS medium supplemented with 0, 2, 5 or 10 μM ABA and/or 5 μM GA_3. Seedling samples were taken for RNA extraction at the indicated times, frozen in liquid nitrogen and stored at −80°C until processed.

Gene family detection, phylogenetic trees and promoter analysis

The protein sequences of Arabidopsis ABI3 and ABI5 were used as TBLASTN queries (http://www.ncbi.nlm.nih.gov/blast/) against the rice and Arabidopsis genomes. Sequence alignments and tree drawings were conducted by CLUSTALW analysis (http://align.genome.jp/) and the TREEVIEW program for tree construction (Page, 1996). Table S1 (Supplementary material) lists accession numbers for the rice genes examined in this study. The accession numbers for retrieved proteins of Arabidopsis and other species are given in the legends to Supplementary material Figs S1 and S2. OsVP1 and OsABF1 were established as the structural orthologues of ABI3 and ABI5 (see the Supplementary material).

RNA extraction and RT-PCR

Total RNA was extracted from rice tissues using the TRIzol protocol, according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). Three biological replicates were examined. After removal of DNA by treatment with RNase-free DNase I (Promega Corporation, Madison, WI, USA), total RNA levels were normalized, based on 28S and 18S rRNA contents. For gene-specific amplification of each gene by RT-PCR, a primer pair was designed based on the predicted exon–intron structure of the gene (Supplementary material, Table S1). Where possible, the forward primer was derived from the second-last or third-last exon and the reverse primer was derived from the 3′ untranslated (UTR) region. The optimum number of cycles was selected to reveal any differences in gene expression among treatments. Two technical RT-PCR replicates were performed for each of the three biological replicates.

RNA *in situ* hybridization

Gene-specific primer pairs were designed to give a probe of *c.* 400 nucleotides (Supplementary material, Table S2). The forward primer was near the end of the coding region or the 3′-UTR, and the reverse primer was further downstream in the 3′-UTR. Each RT-PCR amplification product was cloned into a pGEM-T Easy vector (Promega). Sense and antisense riboprobes were labeled with digoxigenin-11-UTP using the DIG RNA labelling mix (Roche Molecular Systems, Pleasanton, CA, USA) and SP6 and T7 RNA polymerases. The orientation of the insert in the vector was determined by sequencing (Macrogen, Seoul, Korea).

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Table 1 Growth arrest by abscisic acid (ABA) in young leaves of rice (*Oryza sativa* cv. IR64)

	Leaf length (mm)		
Treatment	Leaf 1	Leaf 2	Leaf 3
2 d –ABA	8 ± 2b	9 ± 2g	2 ± 1k
4 d –ABA	12 ± 1a	44 ± 2d	34 ± 3h
2 d –ABA, 2 d + 1 μM ABA	10 ± 1b	30 ± 2e	17 ± 2i
2 d –ABA, 2 d + 10 μM ABA	9 ± 1b	18 ± 2f	6 ± 1j
Growth without ABA, days 3 + 4	4 ± 3q	35 ± 4p	32 ± 4p
Growth arrest by 10 μM ABA (%)	75	74	87

Lowercase letters after the values indicate groupings according to the Multiple Range Test of Duncan (1955): a–k, within-column comparisons; p–q, within-row comparison. Values are means ± SD (n = 10).

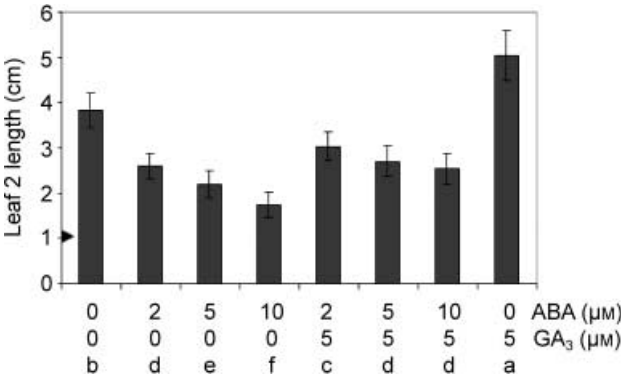

Fig. 1 Impact of abscisic acid (ABA) and the synthetic gibberellin, GA_3, on shoot growth in rice (*Oryza sativa*) cv. IR64, measured in terms of the length of leaf 2 after 4 d of cultivation. Seedlings were cultured on MS medium for 2 d and then transferred to MS medium containing ABA (0, 2, 5 or 10 μM) and/or GA_3 (5 μM), for a further 2 d. Arrow: growth achieved after the first 2 d of growth on MS medium. The letters below the histogram indicate groupings according to the Multiple Range Test of Duncan (1955). Error bars show SD (n = 20).

Tissues were fixed, embedded and sectioned, and sections on slides were dewaxed, rehydrated and washed, as described by Ji *et al.* (2005), except that the sections were 10 μm thick and were baked at 45°C for 48 h. The sections on the slides were hybridized with digoxigenin-11-UTP-labeled sense and antisense probes and subjected to posthybridization work-up, including visualization of hybrids, as described previously (Ji *et al.*, 2005). The color reaction was terminated by dipping the slides in 50 mM Tris–HCl (pH 8.0) containing 1 mM EDTA. The slides were air-dried, mounted and examined under bright-field microscopy using an Axioplan2 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany), supported by IMAGE-PRO PLUS 5.1 software (Media Cybernetics, Singapore). Two to four biological replicates were examined for RNA *in situ* hybridization.

Additional microscopy

Shoots were fixed overnight in 10% (v/v) formaldehyde, 50% (v/v) absolute ethanol, 5% (v/v) acetic acid (FAA solution), dehydrated through a graded ethanol series and embedded using paraffin (Paraplast Plus; Sigma Chemical Co.). Serial sections of 10 μm thickness were placed on Superforst Plus microscope slides (Fisher Scientific, Hampton, NH, USA) and incubated at 45°C for 48 h. Sections were dewaxed in xylene, rehydrated through a graded ethanol series, stained with acridine orange (Sigma; 330 μg ml^{-1} in 0.1 M potassium phosphate buffer, pH 7.0) for cell structures, or with 4′-6-diamidino-2-phenylindole (DAPI) (1 μg ml^{-1} in 137 mM NaCl, 10 mM potassium phosphate, pH 7.4) for nuclei. Sections were viewed under epifluorescence using an Axioplan 2 microscope (Zeiss), supported by IMAGE-PRO PLUS 5.1 software.

Results

ABA inhibits shoot growth in rice

Seeds of rice cv. IR64 were germinated on MS agar for 48 h and then transferred to MS agar containing 0, 0.3, 1, 3 or 10 μM ABA for another 48 h. During the second 48 h period and in the absence of ABA, leaves 1, 2 and 3 grew 4, 35 and 32 mm, respectively, whereas 10 μM ABA inhibited the growth of these leaves by 75, 74 and 87%, respectively (Table 1). The growth observed in the presence of 10 μM ABA occurred mainly during the first 12 h. Lower ABA concentrations were less inhibitory, with 1 μM ABA inhibiting growth by approx. 50%.

IR64 seedlings are an order of magnitude less sensitive to ABA than Arabidopsis seedlings but the reason for this is unclear. It may reflect a lower sensitivity of the rice signal transduction pathway to ABA or a higher abundance of antagonistic gibberellins, which are released from imbibing rice embryos to promote endosperm breakdown. We compared the impacts of ABA and the synthetic gibberellin GA_3 on the growth of leaf 2 (Fig. 1). In contrast to ABA, 5 μM GA_3 stimulated leaf growth significantly and partly reversed the inhibition seen with 2, 5 or 10 μM ABA. Higher concentrations of GA_3 did not reverse the inhibition further (data not shown). As leaf growth may be driven, in part, by sugars and amino acids released from the endosperm, the higher ABA requirement for growth arrest in rice may reflect the need to inhibit not only leaf processes but also mobilization of materials stored in the endosperm. In subsequent studies, we used 10 μM ABA in MS agar to arrest the growth of rice shoots.

ABA increases transcript levels of rice structural orthologues of *ABI3* and *ABI5* genes

Although ABA affects many aspects of leaf metabolism and growth, the ABA-regulated genes most specifically associated

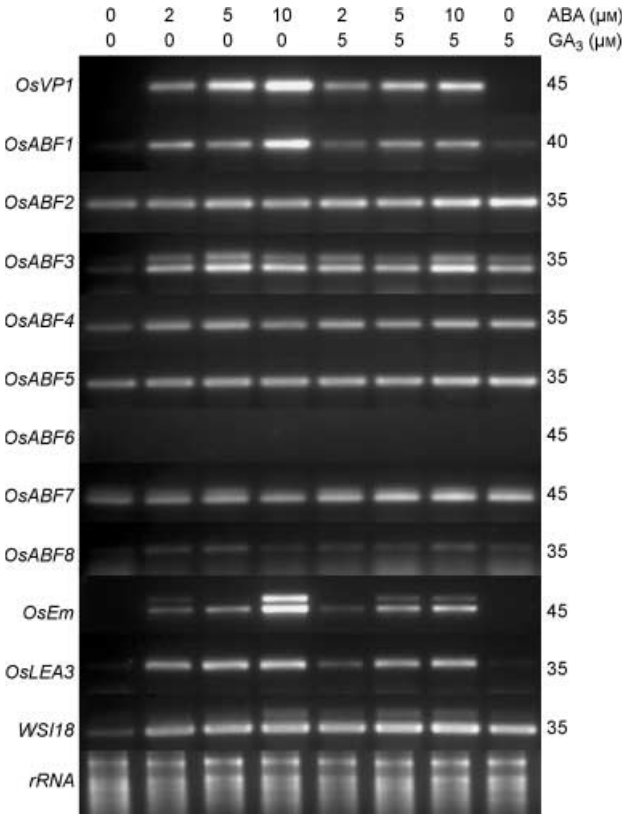

Fig. 2 Effect of abscisic acid (ABA) and gibberellin (GA_3) on gene expression in rice (*Oryza sativa*) cv. IR64. Transcript levels were estimated by the reverse transcription–polymerase chain reaction (RT-PCR). The genes are indicated on the left and the numbers of RT-PCR cycles are indicated on the right. rRNA, loading control of DNAse-treated rRNA.

with growth arrest in Arabidopsis seedlings are the *ABI* genes. We focused on two of these genes – *ABI3* and *ABI5* – which encode a B3 domain and a bZIP domain transcription factor, respectively. The availability of the complete genome sequences of Arabidopsis and rice facilitates the identification of structural orthologues (see the Supplementary material). BLAST and phylogentic analysis confirmed the long-held view (Hattori *et al.*, 1995) that OsVP1 is the structural orthologue of ABI3 in rice (Supplementary material, Fig. S1) and established that a bZIP protein, which we denoted as OsABF1, is the structural orthologue of ABI5 in rice (Supplementary material, Fig. S2).

The question of the orthology of OsABF1 and ABI5 is complicated by a gene duplication that generated both *ABI5* and *AtDPBF2* in Arabidopsis. The function of the latter is poorly understood (Kim *et al.*, 2002). However, a detailed comparison of bZIP domains (the basic DNA-binding domain and the adjacent leucine zipper domain) established that the sequence similarity between OsABF1 and ABI3 in these domains is greater than that between OsABF1 and AtDPBF2 (Supplementary material, Fig. S3).

Further support for the orthology of ABI5 and OsABF1 comes from the regulation of transcription. ABI5 transcript levels, like those of ABI3, are increased by the treatment of seedlings with ABA (Söderman *et al.*, 1996; Kim *et al.*, 2002; Arroyo *et al.*, 2003), while AtDPBF2 transcript levels are not increased (Kim *et al.*, 2002). The RT-PCR analysis showed that transcript levels for *OsVP1* and *OsABF1* are enhanced by ABA (Fig. 2). The gibberellin GA_3 antagonized these responses to a small extent and had no inductive effect when used alone.

Abscisic acid responsiveness is not a general feature of the *OsABF* subfamily of bZIP genes. When we conducted RT-PCR on transcripts of the seven bZIP genes most closely related to *OsABF1* (i.e. *OsABF2*–*OsABF8*), only *OsABF3* also responded to ABA (Fig. 2). The nonresponsive members included *OsABF5*, which is also known as *TRAB1*, the only *OsABF* gene to have been previously studied (Hobo *et al.*, 1999a). We were unable to detect transcripts of *OsABF6* when we used primers based on the annotated sequence (Supplementary material, Table S1). When primers consistent with an alternative splicing pattern were used (Supplementary material, Table S1), *OsABF6* transcripts were detected but proved unresponsive to ABA (data not shown). We conclude that, although the eight *OsABF* genes encode proteins with identical or almost identical DNA-binding domains (Supplementary material, Fig. S3), only *OsABF1* and *OsABF3* show ABA responsiveness at the level of transcript abundance in seedlings.

We studied the ABA and GA_3 responsiveness of three additional rice genes (*OsEm*, *OsLEA3* and *WSI18*). *OsEm* is known to be a target of TRAB1 (Hobo *et al.*, 1999a), while *OsLEA3* and *WSI18* are structural orthologues of *HVA1*, a target of HvABI5 (Casaretto & Ho, 2003). The transcript levels of *OsEm* and *OsLEA3* increased in response to ABA, and GA_3 antagonized these responses to a small extent without having any detectable effect when used alone (Fig. 2). The transcript levels of *WSI18*, however, increased in response to ABA and GA_3, indicating that its transcriptional control differed from that of its close paralogue, *OsLEA3*.

Several genes shown in Fig. 2 produced dual bands (*OsABF3*, *OsEm* and *WSI18*). To avoid confusing RT-PCR products of the transcripts with PCR products of contaminating genomic DNA, we designed primers to flank introns whenever possible. We also digested RNA with DNAse. In each case of dual bands in Fig. 2, the lower band was the expected band and the upper band corresponded to incompletely spliced transcripts. It is possible that incomplete splicing arises from immature tissue where some transcripts are retained in the nucleus (Fig. 3).

Sites of transcript accumulation in the shoot base

We used RNA *in situ* hybridization to determine where the transcripts of *OsVP1*, *OsABF1*, *TRAB1*, *OsEm*, *OsLEA3* and *WSI18* accumulate in shoots. Both sense and antisense probes

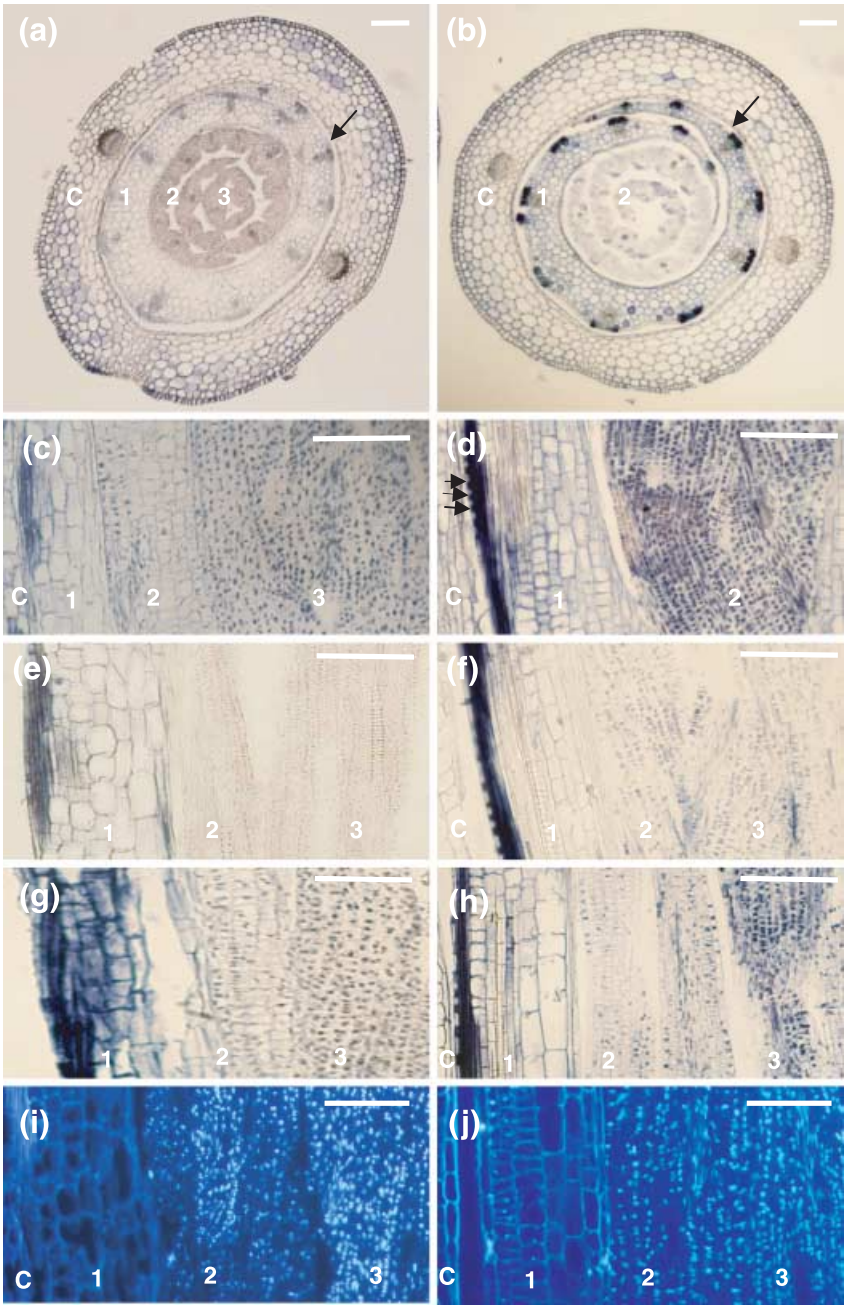

Fig. 3 Sites of transcript accumulation for *OsABF1*, *OsVP1* and *TRAB1* in shoots of rice (*Oryza sativa*) cv. IR64. Left column: shoots from seedlings grown in the absence of abscisic acid (–ABA). Right column: shoots from seedlings grown in the presence of abscisic acid (+ABA). (a–h) RNA *in situ* hybridization conducted with antisense probes on basal sections of rice shoots; mRNA–cRNA hybrids were detected using alkaline phosphatase-linked anti-DIG IgG immunoglobulin. Sections: (a) and (b), transverse; (c)–(j), longitudinal. Genes: (a)–(d), *OsABF1*; (e) and (f), *OsVP1*; (g) and (h), *TRAB1*. Sections shown in (i) and (j) were stained with 4′-6-diamidino-2-phenylindole (DAPI). C, coleoptile; 1,2,3, leaf number; long arrows, abaxial sclerenchyma; short arrows, epidermal cells accumulating transcripts. Bars, 100 μm.

were used, but hybridization signals were obtained only with antisense probes. Transverse and longitudinal sections of the shoots were examined near the apex and also near the base, where the leaves are surrounded by the coleoptile.

Cellular differentiation was most active and most ABA-sensitive near the base (Fig. 3). In the transverse section for *OsABF1* transcripts, all three leaves were visible in the shoot not treated with ABA (–ABA) (Fig. 3a), whereas in the shoot treated with ABA (+ABA) (Fig. 3b), growth of leaf 3 was sufficiently inhibited to be absent from the section. The antisense probe for *OsABF1* transcripts showed weak hybridization to all three leaves of the transverse section of the –ABA shoot, except for a significant level of hybridization in the abaxial sclerenchyma (long, thin arrow) of leaf 1. In the +ABA shoot, hybridization of *OsABF1* was very strong in the abaxial sclerenchyma of leaf 1. Longitudinal sections (Fig. 3c,d) confirmed these hybridization patterns for *OsABF1* transcripts and established three additional points. First, in the sclerenchyma, hybridization was clearly present throughout the cytosol of the cortical fiber cells. Second, in the epidermis, there was a file of cells in which cells containing transcripts (small arrows) alternated with cells lacking transcripts. In

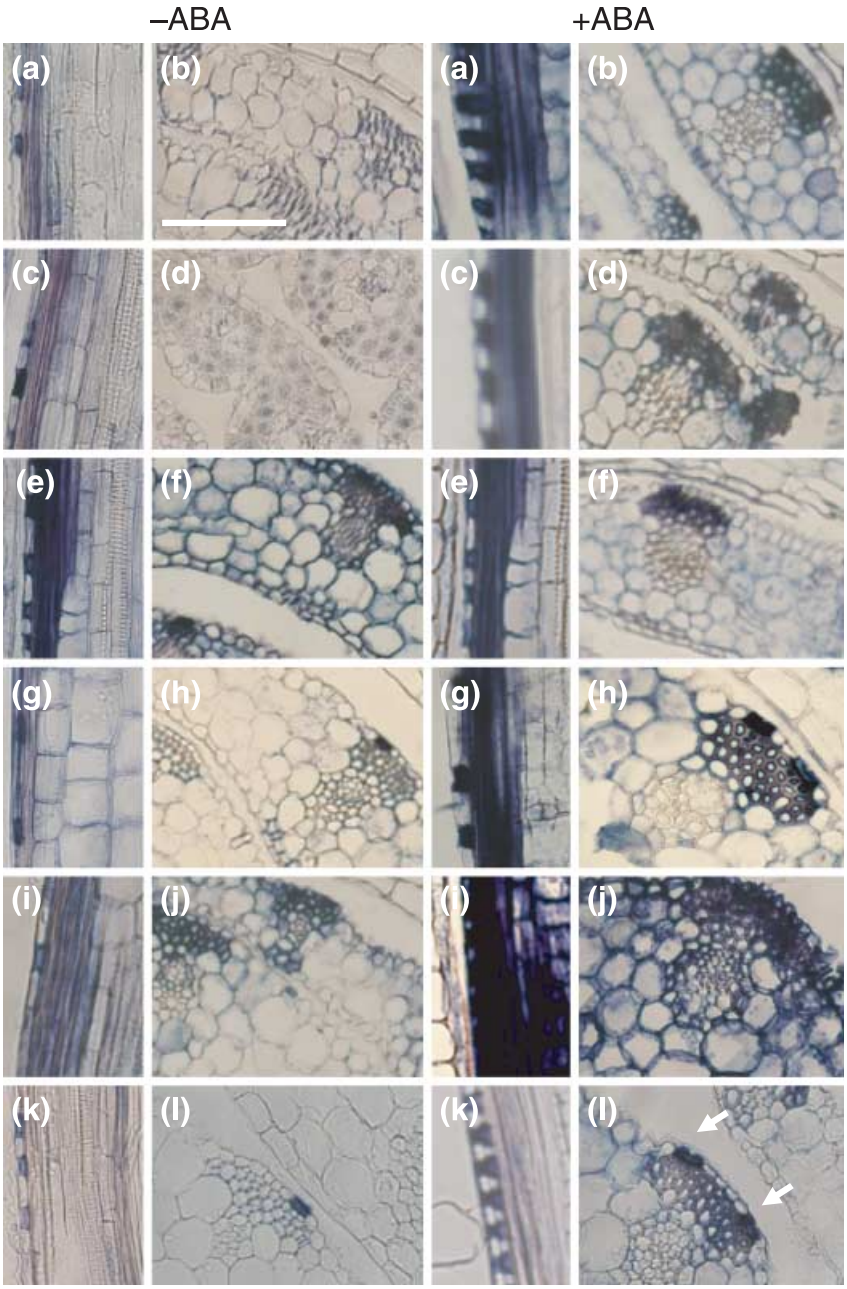

Fig. 4 Sites of transcript accumulation for six genes in leaf 1 of IR64 rice (*Oryza sativa*) seedlings grown in the absence (left columns) and presence (right columns) of 10 μM abscisic acid (ABA). Narrow images, longitudinal sections; wide images, transverse sections. RNA *in situ* hybridization was conducted on basal sections using antisense probes for the indicated genes; mRNA–cRNA hybrids were detected with alkaline phosphatase-linked anti-DIG IgG immunoglobulin. Genes: (a) and (b), *OsABF1*; (c) and (d), *OsVP1*; (e) and (f), *TRAB1*; (g) and (h), *OsEm*; (i) and (j), *WSI18*; (k) and (l), *OsLEA3*. The same magnification was used for all images (bar, 100 μm). Leaf thickness decreased with distance from the mid-vein.

the following paragraphs we show that these two cell types are silica cells and pavement cells, respectively. Third, in immature leaves of –ABA and +ABA shoots, hybridization was localized in spots that resembled DAPI-stained nuclei (Fig. 3i,j) in their size and general distribution throughout the tissue.

Transcripts of *OsVP1* (Fig. 3e,f) and *TRAB1* (Fig. 3g,h) showed broadly similar results to those for *OsABF1* transcripts, but with two differences. First, *OsVP1* transcripts were not clearly visible in immature leaves of –ABA or +ABA shoots, presumably reflecting low abundance. Second, *TRAB1* transcripts were much more abundant than *OsABF1* or *OsVP1* transcripts in leaf 1 of –ABA shoots, a result consistent with the RT-PCR data (Fig. 2). Like transcripts of *OsABF1*, *TRAB1* transcripts appeared to be localized in nuclei in the immature leaves of –ABA and +ABA shoots. Nuclear localization was also observed for *OsEm* and *WSI18* transcripts (results not shown); like OsVP1 transcripts, *OsLEA3* transcripts were not sufficiently abundant in immature leaves to allow their location to be seen clearly.

Figure 4 shows RNA *in situ* hybridization in leaf 1 of –ABA and +ABA shoots for transcripts of all six genes. For both

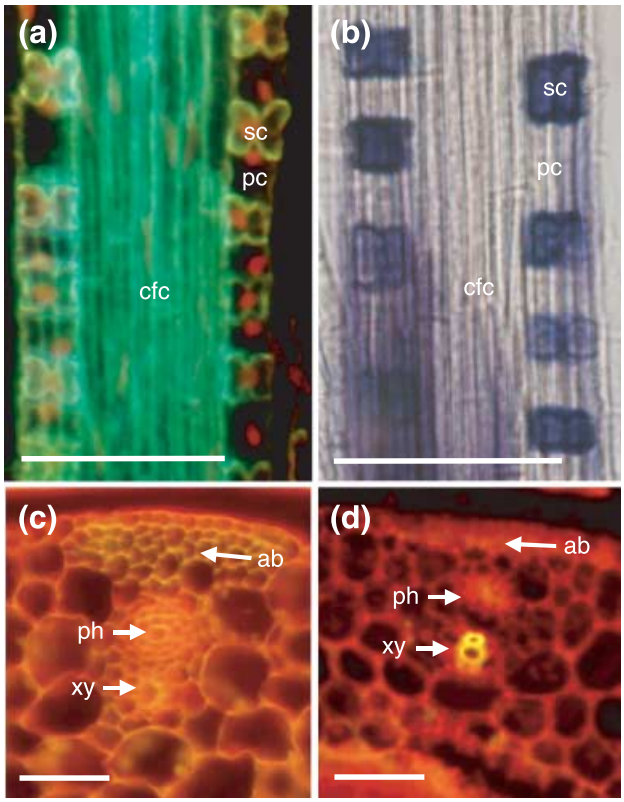

Fig. 5 Anatomical features of IR64 rice (*Oryza sativa*) shoots during abscisic acid (ABA)-induced growth arrest. (a) and (b) Identification of silica cells as sites of accumulation of *OsVP1* transcripts in a basal longitudinal section of leaf 1 in shoots grown in the presence of ABA (+ABA). (a) Leaves stained with acridine orange. (b) RNA *in situ* hybridization using an antisense probe for *OsVP1*; mRNA–cRNA hybrids were detected using alkaline phosphatase-linked anti-DIG IgG immunoglobulin. cfc, cortical fiber cells; pc, epidermal pavement cells; sc, silica cells. (c) and (d) Acridine orange staining of leaf 1 in basal transverse sections of shoots grown in the absence (–ABA) (c) and presence (+ABA) (d) of ABA. ab, abaxial sclerenchyma; ph, phloem sclerenchyma; xy, xylem sclerenchyma. Bars, 50 μm.

treatments, longitudinal sections are on the left and show cytosolic hybridization within the epidermal silica cells and the cortical fiber cells of the sclerenchyma. Transverse sections are on the right and demonstrate that the files of silica cells (arrowed for *OsLEA3*) are separated from one another by two to four nonhybridizing epidermal cell files and are separated by the abaxial sclerenchyma from the subtending vascular bundles. In the –ABA shoots, strong hybridization was seen only for *TRAB1* transcripts. By contrast, in the +ABA shoots, all six genes showed strong hybridization to cortical fiber cells and silica cells. Both of these cell types are known to be major support cells of the leaf (Savant *et al.*, 1997; Tanaka *et al.*, 2003; Ma *et al.*, 2004). *OsABF1*, *OsVP1*, *OsEm* and *OsLEA3* transcripts accumulated mainly in these support cells, whereas *TRAB1* and *WSI18* transcripts were abundant also in other cell types in leaf 1.

We examined in more detail the epidermal cell files in which alternate cells possessed or lacked the six transcripts. In Fig. 5, acridine orange staining was compared with RNA *in situ* hybridization for the antisense probe of *OsVP1*. The hybridizing cells are easily recognized as silica cells by their dumbbell structure (Kaufman *et al.*, 1985), and we confirmed the presence of silica in these cells by staining with methyl red (data not shown). Acridine orange staining elicited a brilliant red fluorescence from the nuclei of both the silica cells and the epidermal pavement cells that alternated with them along the file. The presence of nuclei in the pavement cells suggested that they were active but unable to accumulate transcripts of the six genes studied here. The files themselves occurred in pairs that were not immediately adjacent but separated by several files of other epidermal cells (compare with arrows in Fig. 4d).

Comparison of the bases of leaf 1 in –ABA shoots (Fig. 5c) and +ABA shoots (Fig. 5d) revealed that ABA inhibited sclerenchyma development. In –ABA shoots, the abaxial sclerenchyma and the sclerenchyma of the xylem and phloem were well developed. In +ABA shoots, all three regions of sclerenchyma were poorly developed and the cortical fiber cells lacked their characteristic thick walls.

The cortical fiber cells of the sclerenchyma are thick-walled because of the formation of the secondary walls, a major component of which is cellulose. Rice contains at least 10 genes encoding cellulose synthase subunit A. Mutant analysis indicated that three of these genes (*OsCesA4*, *OsCesA7* and *OsCesA9*) are essential for secondary cell-wall formation and for the strength of the stem (Tanaka *et al.*, 2003). CesA4, CesA7 and CesA9 subunits do not have entirely redundant functions, because knocking out the gene for any one of these enzymes reduced the thickness of the sclerenchyma, weakened the stem and caused dwarfing. We compared the transcript levels of these three genes with those of *OsVP1* and *OsABF1* (Fig. 6). Seedlings were grown on MS agar without ABA; after 2 or 4 d, some seedlings were transferred to agar containing 10 μM ABA for 1 or 2 d, and then some were returned to MS agar without ABA for 1 d. Transcript levels for *OsVP1* and *OsABF1* were low on –ABA medium, increased in response to ABA and then decreased on return to –ABA medium. High transcript levels were seen for *OsCesA4* and *OsCesA7* throughout these treatments. By contrast, transcript levels for *OsCesA9*, which decreased slowly when seedlings were grown on –ABA medium, decreased rapidly when seedlings were grown on +ABA medium and then increased again when seedlings were returned to –ABA medium. The loss of *OsCesA9* transcripts in response to ABA treatment may contribute to growth arrest in general and to failure of sclerenchyma development in particular.

Sites of accumulation of transcripts in the shoot apex

We conducted RNA *in situ* hybridization for *OsABF1*, *OsVP1*, *TRAB1*, *OsEm*, *WSI18* and *OsLEA3* on transverse sections of

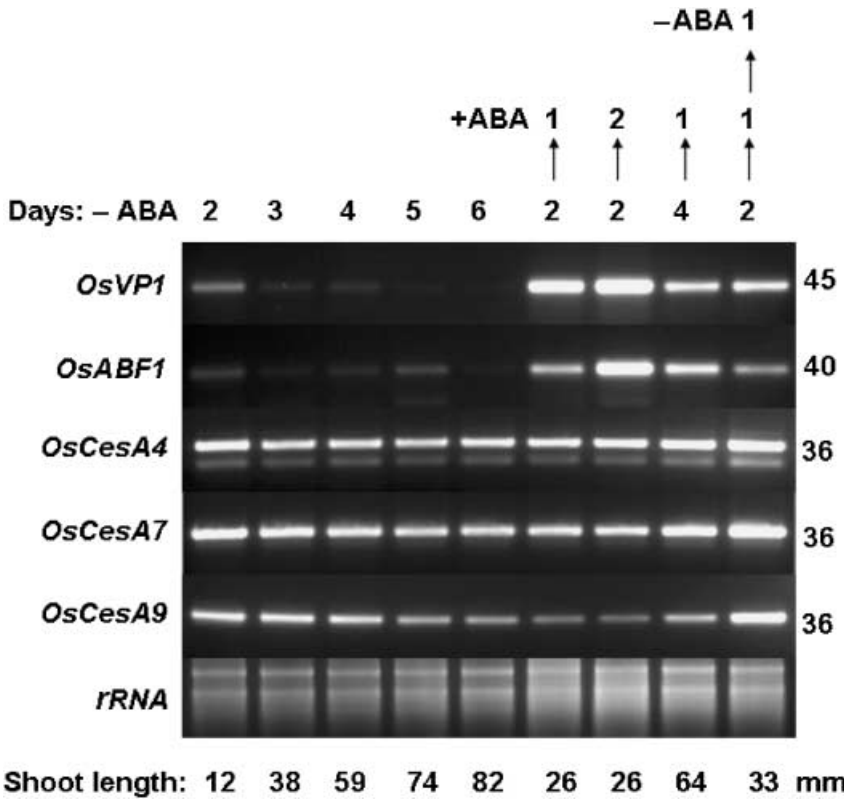

Fig. 6 Effect of 10 μM abscisic acid (ABA) on the transcript levels of *OsVP1*, *OsABF1* and three *OsCesA* genes required for secondary cell-wall formation. RNA was extracted from IR64 rice (*Oryza sativa*) seedlings grown under the indicated conditions. Transcript levels were visualized by reverse transcription–polymerase chain reaction (RT-PCR). The genes are indicated on the left and the numbers of RT-PCR cycles are indicated on the right. rRNA, loading control of DNAse-treated rRNA. The shoot length is the length of the longest leaf at each time point.

the more mature apical region of –ABA and +ABA shoots (Fig. 7). In general, the patterns for the apex were similar to those recorded in Fig. 4 for the base, but a clear difference was the presence in the apex of leaf 1 of both abaxial and adaxial sclerenchyma, with significant hybridization in both, especially *OsABF1* and *OsLEA3*.

Discussion

Our objective was to clarify the mechanism of ABA-induced growth arrest in rice seedlings through transcriptional analysis of the rice orthologues of ABI3 and ABI5, two transcription factors essential for ABA-induced growth arrest in Arabidopsis. We confirmed that the structural orthologue of ABI3 is OsVP1 and established that the structural orthologue of ABI5 is a previously unstudied protein, which we termed OsABF1 (see the Supplementary material for a full discussion).

ABA-induced growth arrest was achieved by transferring 2-d-old rice seedlings to MS agar containing 10 μM ABA. Over the next 2 d in the absence of ABA, leaf 1 grew 4 mm, whereas leaves 2 and 3 grew *c.* 34 mm. In the presence of ABA, growth of all three leaves ceased within 12 h. We used RNA *in situ* hybridization to examine the transcript levels of *OsVP1*, *OsABF1* and four other genes (*TRAB1*, *OsEm*, *WSI18* and *OsLEA3*) in these leaves in the presence and absence of ABA. *TRAB1* is the only previously studied member of the *OsABF* family in rice but it is clearly not the structural orthologue of ABI5 (see the Supplementary material). However, TRAB1 and six other OsABFs share an essentially invariant DNA-binding domain with OsABF1, raising the possibility that they recognize similar, if not identical, targets in the rice genome. *OsEm*, *WSI18* and *OsLEA3* are putative targets of OsVP1 and the OsABFs (Hobo *et al.*, 1999a; Casaretto & Ho, 2003).

Near the base of the rice shoot, two patterns of transcript accumulation were observed for most of the six genes. One pattern was observed in leaf 1, while a second pattern was observed in leaves 2 and 3, which were less mature and elongated more rapidly than leaf 1. In leaf 1, transcripts accumulated principally in the cytosol of two types of support cells: cortical fiber cells of the abaxial sclerenchyma and epidermal silica cells. Transcript levels were enhanced by ABA, except for *TRAB1*, results supported by RT-PCR assays. By contrast, in leaves 2 and 3, transcripts of *OsABF1*, *TRAB1*, *OsEm* and *WSI18* accumulated significantly in the nuclei of all cells in the presence and absence of ABA, indicating at least partial disruption of mRNA transport from the nucleus to the cytosol. Transcripts of *OsVP1* and *OsLEA3* were insufficiently abundant to be clearly localized in leaves 2 and 3.

A third pattern of transcript accumulation was observed in the apex of the shoot, which contained the tips of leaves 1 and 2: in +ABA shoots, transcripts accumulated in both the

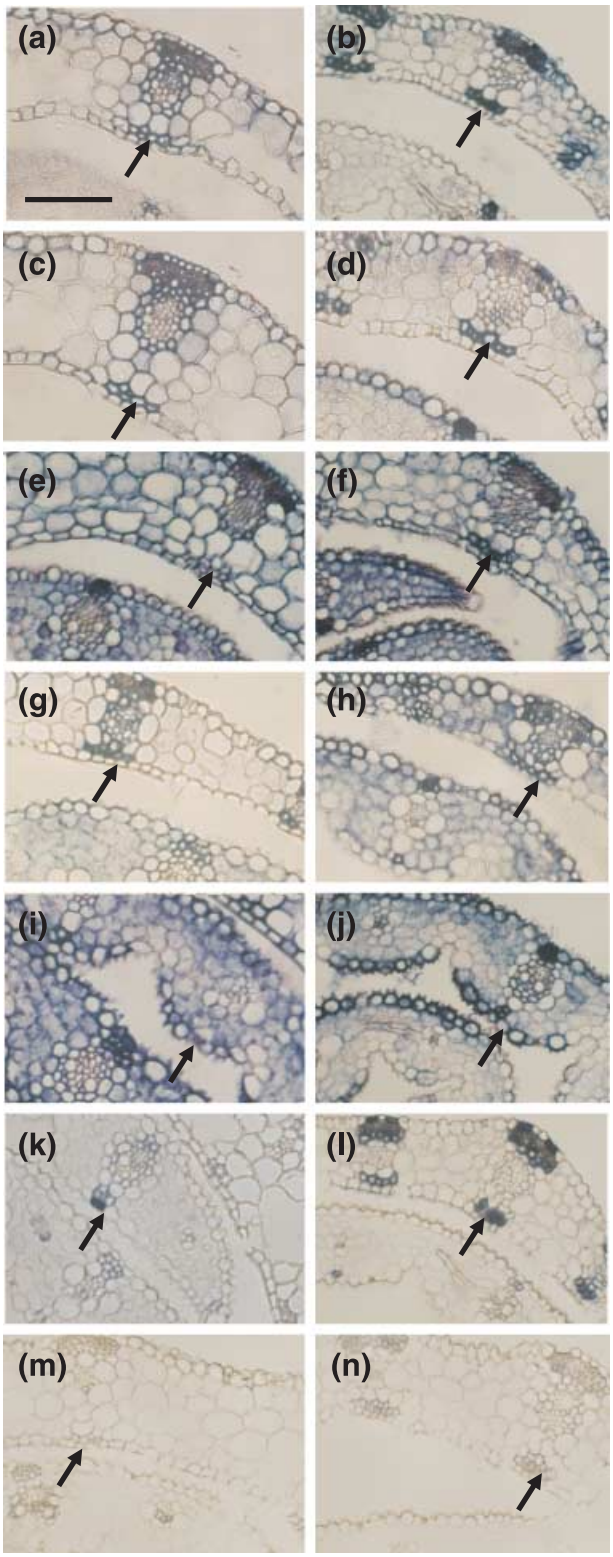

Fig. 7 Sites of transcript accumulation for six genes in the shoot apex of IR64 rice (*Oryza sativa*) seedlings grown in the absence (left column) and presence (right column) of 10 μM abscisic acid (ABA). RNA *in situ* hybridization was conducted on transverse sections using antisense probes (a–l), no probe (m) and sense probe (n); mRNA–cRNA hybrids were detected using alkaline phosphatase-linked anti-DIG IgG immunoglobulin. Genes: (a) and (b), *OsABF1*; (c) and (d), *OsVP1*; (e) and (f), *TRAB1*; (g) and (h), *OsEm*; (i) and (j), *WSI18*; (k), (l) and (n), *OsLEA3*; (m), without probe. Arrows, adaxial sclerenchyma. The same magnification was used for all images (bar, 100 μm). Leaf thickness decreased with distance from the mid-vein.

abaxial and adaxial sclerenchyma and the silica cells. We discuss these three transcript patterns and their possible significance in the following sections.

ABA-induced rise in transcript levels in sclerenchyma cells

Leaves exhibit a gradient of support cell abundance, which is highest at the apex and lowest at the base. At the stage of development observed here, the apex of leaf 1 contains both abaxial and adaxial sclerenchyma and the base of the same leaf contains only abaxial sclerenchyma, whereas the apex of leaf 2 contains only abaxial sclerenchyma and the base lacks sclerenchyma, like the whole of leaf 3. ABA inhibits the growth of abaxial, adaxial and vascular sclerenchyma and promotes cytosolic accumulation of transcripts of *OsABF1*, *OsVP1*, *OsEm* and *OsLEA3* in the remaining abaxial and adaxial sclerenchyma. *TRAB1* and *WSI18* transcripts accumulate not only in these support cells but also in other cell types.

OsEm, *OsLEA3* and *WSI18* encode members of the LEA family, which are believed to have the capacity to protect cells against water deficit. This property of LEAs has been studied in detail for *Typha latifolia* pollen (Wolkers *et al.*, 2001) and transgenic plants, including rice (Xu *et al.*, 1996). The characteristic thick walls of sclerenchyma cells are the result of secondary cell-wall formation. Mutation in any one of the three Arabidopsis *CesA* genes involved in secondary cell-wall formation (*AtCesA4*, *AtCesA7* and *AtCesA8*) elicits a defense response that involves ABA accumulation and enhanced resistance to drought, osmotic stress and bacterial infection (Chen *et al.*, 2005; Hernandez-Blanco *et al.*, 2007). Among the defense products are LEA proteins. A different response pathway, involving ethylene and jasmonic acid, operates in response to damage to primary cell walls (Hernandez-Blanco *et al.*, 2007). Thus, plants have evolved a broad protective mechanism linking the health and growth of the secondary cell wall with resistance to abiotic and biotic stresses, and ABA is a mediator of the mechanism. Our data suggest that expression of *OsVP1*, *OsABF1*, *TRAB1*, *OsEm*, *OsLEA3* and *WSI18* contribute to a similar protective mechanism in rice.

Three *CesA* genes (*OsCesA4*, *OsCesA7* and *OsCesA9*) are also required in rice for secondary cell-wall formation in sclerenchyma (Tanaka *et al.*, 2003). We showed that one of these genes, *OsCesA9*, was markedly down-regulated during growth arrest by ABA in seedlings. On return of these seedlings to ABA-free medium, the transcript level for *OsCes9A* recovered and leaf growth resumed. Tanaka *et al.* (2003) showed that a mutation in any of these three genes is sufficient

to prevent secondary cell-wall formation and greatly weaken leaf support in rice. Loss of *CesA9* transcripts may therefore contribute to ABA-induced growth arrest in leaves 1 and 2.

The conservation of DNA-binding domains among OsABFs suggests that these paralogues might compete with one another to bind to promoters when they are expressed in the same cell. Competition for promoters has been demonstrated in Arabidopsis for ABI5, EEL, ABF1 and ABF3 (Bensmihen *et al.*, 2002; Finkelstein *et al.*, 2005). Competition between OsABF1 and TRAB1 or other OsABFs for binding to target promoters may allow fine-tuning of stress-responsive pathways because OsABFs, by virtue of sequence divergence outside the DNA-binding domain, can differ in the proteins that they attract to promoters, including regulatory protein kinases. Specifically, OsABF1 lacks the serine that in the C2 domain of TRAB1 is phosphorylated to provide ABA-mediated post-translational activation (Kagaya *et al.*, 2002) by protein kinases SAPK8, SAPK9 and SAPK10 (Kobayashi *et al.*, 2005). Conversely, *TRAB1* lacks the ABA inducibility at the transcript level that is shown by *OsABF1*.

ABA-induced rise in transcript levels in silica cells

Like formation of secondary cell walls, deposition of silica within cell walls makes an important contribution to leaf strength in those species that accumulate the mineral. Drooping leaves are a feature of silicon accumulators that are grown without silicon nutrition (Savant *et al.*, 1997) and of a mutant of rice defective in silicon uptake (Ma *et al.*, 2004). By contrast, adequate silicon nutrition allows leaves to become erect. Erectness is associated with higher yields in densely planted fields (Sinclair & Sheehy, 1999) and with reduced photoinhibition under high light intensities (Murchie *et al.*, 1999). Silicon also reduces transpiration and provides protection against compression stress, chewing insects and diseases (Epstein, 1999).

Among flowering plants, the Poales are the family with the largest number of silicon-rich species. Silicon is deposited as phytoliths ($(SiO_2)_m \cdot nH_2O$) in the root endoderm, around aerial sites of water evaporation such as stomata and seed hulls, and in silica cells of the leaf epidermis (Kaufman *et al.*, 1985). Silicon can account for 10% or more of the total dry weight of rice leaves. The frequency of silica cells per unit leaf area in rice is three-fold higher than in C_4 plants and 60-fold higher than in dryland C_3 grasses (Kaufman *et al.*, 1985).

The ABA-stimulated accumulation of transcripts of *OsVP1*, *OsABF1*, *OsEm*, *OsLEA3* and *WSI18* in silica cells is a novel observation and suggests that silica cells are in greater need than pavement cells of special protection during stress. The deposition of silica around stomata and in husks occurs passively as a result of transpiration, but deposition in silica cells occurs before the onset of transpiration (Motomura *et al.*, 2006) and may require specific expression of silica transporter genes of the type discussed by Ma *et al.* (2004, 2006). ABA-induced changes of gene expression in silica cells may prevent the premature deposition of silicon during growth arrest.

Nuclear localization of transcripts in immature leaves

In immature leaves 2 and 3 of –ABA and +ABA shoots, transcripts of *OsABF1*, *TRAB1*, *OsEm* and *WSI18* accumulated in the nuclei of all cells. This contrasts with mature leaf 1, where transcripts for the above genes are cytosolic, cell-type specific (mainly support cells) and ABA responsive, rather than nuclear, ubiquitous and constitutive (except for *TRAB1*). The results suggest that the mRNA transport pathway for these genes is adversely affected in early leaf development.

In several Arabidopsis mutants, defects in mRNA export and metabolism cause ABA hypersensitivity (Xiong *et al.*, 2001; Gong *et al.*, 2005; Parry *et al.*, 2006; Verslues *et al.*, 2006). In mutants involving DEAD RNA helicase (Gong *et al.*, 2005) and nucleoporin AtNUP160 (Parry *et al.*, 2006), mRNAs are known to accumulate in nuclei but they have not yet been identified. Xiong *et al.* (2001) suggested that ABA hypersensitivity may be caused by the absence of a negative regulator of the ABA response pathway. Several negative regulators of ABA action are known, including microRNA miR159 (Reyes & Chua, 2007). In Arabidopsis, ABA induces miR159 via a mechanism requiring ABI3. Rice produces miR159 (Axtell & Bartel, 2005) and if this production requires OsVP1, the low level of *OsVP1* transcripts in immature leaves may cause hypersensitivity to ABA and contribute to ABA-induced growth arrest in leaves 2 and 3.

Concluding remarks

Our results establish that ABA-induced growth arrest in rice shoots involves the regulation of gene expression in support cells (sclerenchyma and silica cells) of those tissues sufficiently mature to contain them. ABA exerts both inhibitory and protective actions. The former are illustrated by the inhibition of both sclerenchyma formation and expression of *OsCesA9*, while the latter are illustrated by enhanced cytosolic transcript levels for *OsVP1*, *OsABF1* and three putative target genes (*OsEm*, *WSI18* and *OsLEA3*). In less mature tissues of the shoot, *OsVP1* and *OsLEA3* are poorly expressed, whereas *OsABF1*, *OsEm*, *WSI18* and *TRAB1* transcripts are found in the nuclei of all cells, irrespective of ABA treatment, suggesting altered regulation of gene expression, including inefficient processing and/or transport of the mRNA. Thus, the target cells for ABA-induced growth arrest remain to be identified in immature leaves.

Acknowledgements

This research was funded by the grant 'Applying Genetic Diversity and Genomic Tools to Benefit Rice Farmers at Risk

from Drought' from the Bundesministerium für wirtschaftliche Zusammenarbeit und Entwicklung (BMZ, Germany), the grant 'Identifying Genes Responsible for Failure of Grain Formation in Rice and Wheat under Drought' from the Generation Challenge Program, and a grant from the Iran-IRRI Collaborative Project. We thank Dr Philippe Hervé for sharing facilities and Leonardo Estenor, Blesilda Albano-Enriquez and Gina Borja for assistance.

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Supplementary Material

The following supplementary material is available for this article online:

1. Primers used in this research

Table S1 Reverse transcription–polymerase chain reaction (RT-PCR) primers used in this research.

Table S2 Primers for amplification and cloning of probes for RNA *in situ* hybridization.

2. Identification of structural orthologues of ABI3 and ABI5 in rice.

Text S1 Discussion of orthology in relation to ABI3 and ABI5.

Fig. S1 OsVP1 is the rice orthologue of ABI3.

Fig. S2 OsABI5 is the rice orthologue of ABI5.

Fig. S3 Alignment of C2 domains, basic DNA-binding domains and leucine zipper domains of ABI5 and similar Arabidopsis, rice, wheat and barley proteins.

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