Control of Abscisic Acid Catabolism and Abscisic Acid Homeostasis Is Important for Reproductive Stage Stress Tolerance in Cereals[1][W][OA]

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Drought stress at the reproductive stage causes pollen sterility and grain loss in wheat (*Triticum aestivum*). Drought stress induces abscisic acid (ABA) biosynthesis genes in anthers and ABA accumulation in spikes of drought-sensitive wheat varieties. In contrast, drought-tolerant wheat accumulates lower ABA levels, which correlates with lower ABA biosynthesis and higher ABA catabolic gene expression (ABA 8′-hydroxylase). Wheat *TaABA8′OH1* deletion lines accumulate higher spike ABA levels and are more drought sensitive. ABA treatment of the spike mimics the effect of drought, causing high levels of sterility. ABA treatment represses the anther cell wall invertase gene *TaIVR1*, and drought-tolerant lines appeared to be more sensitive to the effect of ABA. Drought-induced sterility shows similarity to cold-induced sterility in rice (*Oryza sativa*). In cold-stressed rice, the rate of ABA accumulation was similar in cold-sensitive and cold-tolerant lines during the first 8 h of cold treatment, but in the tolerant line, ABA catabolism reduced ABA levels between 8 and 16 h of cold treatment. The ABA biosynthesis gene encoding 9-cis-epoxycarotenoid dioxygenase in anthers is mainly expressed in parenchyma cells surrounding the vascular bundle of the anther. Transgenic rice lines expressing the wheat *TaABA8′OH1* gene under the control of the *OsG6B* tapetum-specific promoter resulted in reduced anther ABA levels under cold conditions. The transgenic lines showed that anther sink strength (*OsINV4*) was maintained under cold conditions and that this correlated with improved cold stress tolerance. Our data indicate that ABA and ABA 8′-hydroxylase play an important role in controlling anther ABA homeostasis and reproductive stage abiotic stress tolerance in cereals.

The world's most important staple crops depend on successful reproductive development for grain production. Abiotic stresses, such as drought, cold, heat, frost, and salinity, cause sterility and severe losses in grain yield. In maize (*Zea mays*), drought stress at anthesis causes ovary abortion and reduction in kernel number (Westgate and Boyer, 1985). In self-fertilizing cereals, such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and grain sorghum (*Sorghum bicolor*), successful pollen development is critical for grain production, and abiotic stresses interfering with the earliest stages of pollen formation lead to massive losses in grain number (Bingham, 1966; Satake and Hayase, 1970; Nishiyama, 1984; Saini et al., 1984; Briggs et al., 1999a, 1999b; Matsui and Omasa, 2002; Abiko et al., 2005; Jagadish et al., 2007; Jain et al., 2007; Endo et al., 2009). Stress-induced pollen sterility is not restricted to monocots, with reports that it also occurs in dicot plants (Aloni et al., 2001; Kim et al., 2001; Karni and Aloni, 2002; Pressman et al., 2002; Ghanem et al., 2009). In rice and wheat, irreversible abortion of pollen development is induced at the young microspore (YM) stage by cold and drought stress (Hayase et al., 1969; Ito et al., 1970; Satake and Hayase, 1970; Saini et al., 1984; Sheoran and Saini, 1996; Oliver et al., 2005; Ji et al., 2010; Zinn et al., 2010). Both cold and drought stresses were shown to trigger a premature cell death response in the tapetum (Oliver et al., 2005; Gothandam et al., 2007; Nguyen et al., 2009; Ji et al., 2010).

There are many similarities between cold- and drought-induced pollen sterility, suggesting that a common mechanism may trigger pollen abortion for both stresses. Induction of sterility is associated with the absence of starch accumulation in mature pollen (Satake and Hayase, 1970; Saini et al., 1984) and the accumulation of nonreducing sugars in anthers (Ito,

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1978; Dorion et al., 1996; Saini, 1997; Saini and Westgate, 1999; Koonjul et al., 2005; Oliver et al., 2005; Ji et al., 2010). While there is no starch accumulating in the pollen grains, abnormal starch accumulation was observed in the connective tissue of drought-stressed wheat anthers (Lalonde et al., 1997) and in the anther wall of cold-stressed anthers (Satake, 1976). Drought and cold stress both repress anther cell wall invertase activity and gene expression (Koonjul et al., 2005; Oliver et al., 2005; Ji et al., 2010), resulting in reduced anther sink strength. The cell wall invertase gene is expressed in the tapetum and around the vascular bundle of the anther (Goetz et al., 2001; Koonjul et al., 2005; Oliver et al., 2005). Repression of cell wall invertase was also observed in the case of heat stress in sorghum anthers (Jain et al., 2010). At the time of pollen meiosis, the tapetum is very active and the anther is the strongest sink organ in the flower (Clément et al., 1996; Castro and Clément, 2007). This may explain the higher vulnerability of pollen development to abiotic stresses.

Both cold and drought stresses cause abscisic acid (ABA) accumulation (for review, see Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Shinozaki et al., 2003; Chinnusamy et al., 2004). ABA plays a role in male sterility in tomato (*Solanum lycopersicum*; Santokh Singh and Sawhney, 1998) and is also involved in the response to heat stress (Toh et al., 2008). There is evidence of interactions between ABA and sugar signaling (Arenas-Huertero et al., 2000; Laby et al., 2000; Rook et al., 2001; Arroyo et al., 2003). In cold-stressed rice anthers, ABA repressed the cell wall invertase gene *OSINV4* (Oliver et al., 2007). Comparison of ABA biosynthesis in cold-sensitive and cold-tolerant lines indicated that cold-tolerant rice accumulated less ABA in response to cold treatment. This difference in ABA homeostasis was due to both lower ABA biosynthesis and faster turnover of ABA (Oliver et al., 2007). ABA levels in plants are the result of a balance between biosynthesis and catabolism (Nambara and Marion-Poll, 2005). ABA is synthesized from carotenoids, with the first committed step being catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED; Schwartz et al., 1997). The reaction catalyzed by NCED plays a regulatory role in many plant species (Qin and Zeevaart, 1999). However, the gene encoding zeaxanthin epoxidase (ZEP) was also shown to control ABA levels (Frey et al., 1999). A variety of pathways exist for ABA catabolism, but the 8′-hydroxylation of ABA to form phaseic acid appears to be the predominant pathway for ABA inactivation. This reaction is catalyzed by the cytochrome P450 8′-hydroxylase (CYP707A; Kushiro et al., 2004; Nambara and Marion-Poll, 2005). The delicate balance between ABA synthesis and catabolism determines ABA levels in plant tissues (Nambara and Marion-Poll, 2005).

In this article, we investigate the role of ABA in controlling abiotic stress tolerance in cereals. We show that ABA controls anther sink strength and abortion of pollen development under drought conditions in wheat. Transgenic approaches in rice to control ABA catabolism and reduce anther ABA levels under stress conditions were shown to improve spike fertility.

RESULTS

Drought-Tolerant Wheat Spikes Accumulate Less ABA

We studied the effect of drought stress at the YM stage on ABA accumulation in wheat ears. ABA levels were measured for control and drought-stressed spikes of the drought-sensitive varieties Sundor and Cranbrook and the drought-tolerant varieties Halberd and SYN604 (Ji et al., 2010). These contrasting varieties were chosen because of the availability of doubled haploid populations for quantitative trait locus mapping (Cranbrook × Halberd, Sundor × SYN604). There was no significant difference in the spike ABA levels for the four wheat lines under unstressed control conditions (Fig. 1A). After 5 d of drought stress, ABA levels were significantly increased in spikes of both drought-sensitive varieties, Sundor and Cranbrook. In the drought-tolerant lines Halberd and SYN604, ABA content increased in drought-stressed spikes, but levels remained significantly lower than in both drought-sensitive varieties Sundor and Cranbrook (64% for Halberd and 52% for SYN604; Fig. 1A). The differences in ABA accumulation following water stress treatment were less pronounced in the leaves. In unstressed leaves, there was no significant difference in endogenous ABA levels for any of the four varieties tested (Fig. 1B). In drought-stressed leaves of the drought-sensitive lines Sundor and Cranbrook, ABA levels increased 4.6- and 15-fold, respectively (Fig. 1B). In leaves of the drought-tolerant variety Halberd, ABA also accumulated to levels similar to Sundor and Cranbrook (17-fold increase), but in the variety SYN604, ABA levels remained significantly lower compared with all other varieties (Fig. 1B). Overall ABA accumulation in the leaves following drought treatment was on average four times higher than in the spike.

Drought Affects the Expression of ABA Metabolic Genes in Anthers

Wheat cDNA clones for the main ABA metabolic genes were identified using BLAST searches. We identified wheat EST clones with high sequence homology to the rice genes encoding ZEP (*TaZEP1* [AK332872]), NCED (*TaNCED1* [CA731387] and *TaNCED2* [CD884104]), and ABA 8′-hydroxylase (*TaABA8′0H1* [CN011303] and *TaABA8′0H2* [CD919420]). Sequence alignments and phylogenetic analyses of these clones are provided in Supplemental Figure S3. A full-length *TaABA8′OH1* gene was obtained from variety Sunstate using PCR primers designed for the barley *HvABA8′OH1* gene (see "Materials and Methods"; Millar et al., 2006). *TaABA8′OH1* is nearly identical to the CN011303 EST sequence and another full-length sequence available in the database (EU430344; Zhang et al., 2009), suggesting that these sequences are all

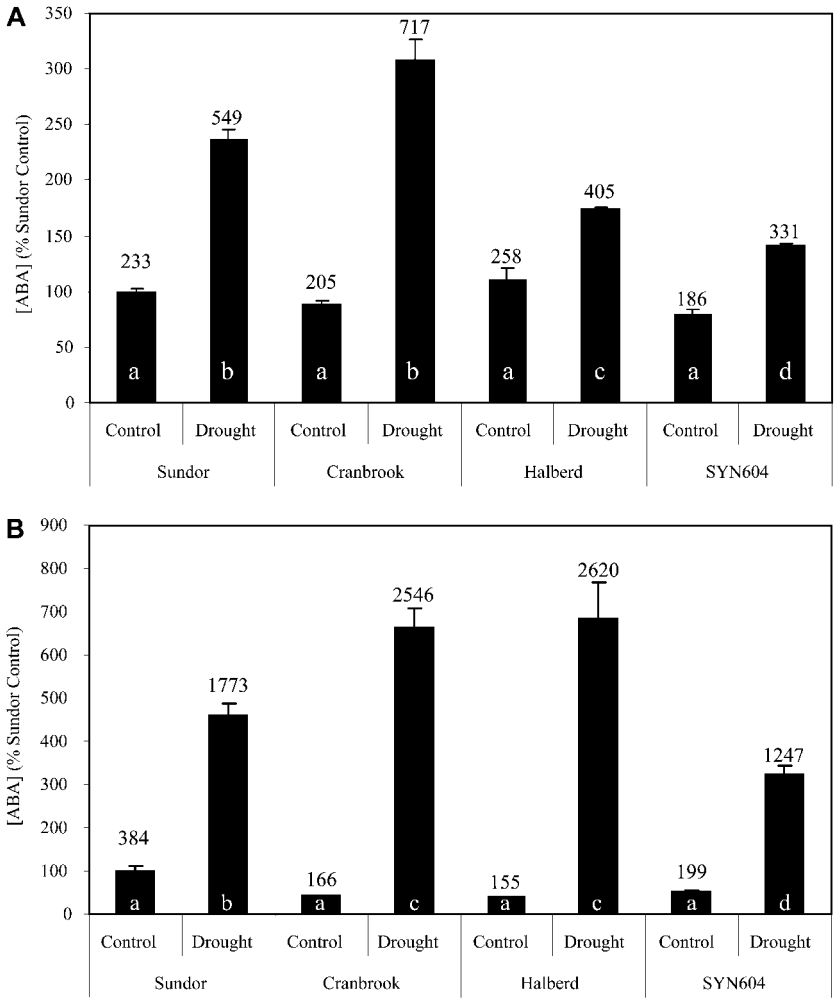

Figure 1. Measurement of ABA levels in drought-stressed wheat germplasm. ABA levels were measured in the spike (A) and leaves (B) of control and drought-stressed wheat plants. The values represent averages of four biological repeat measurements ($n = 4$), and error bars indicate SE. Bars with the same letter are not significantly different at the 1% level (*t* test; $P < 0.01$). The values above the bars represent average ABA concentrations for each sample in ng/g dry weight (DW).

derived from the same *TaABA8′OH1* gene. Analysis of deletion lines indicated that *TaABA8′OH1* is located on wheat chromosome 6D. The *TaABA8′OH2* cDNA sequence is derived from a different gene and is more homologous to barley *HvABA8′OH2* (Supplemental Fig. S3). *TaABA8′OH2* is identical to *Triticum boeoticum* and *Triticum monococcum ABA8′OH2* genes located on the long arm of chromosome $5A^m$ (Nakamura et al., 2010; GenBank identifiers AB455560 and AB455561).

Gene expression studies using semiquantitative reverse transcription (RT)-PCR indicate that the biosynthetic genes *TaZEP1*, *TaNCED1*, and *TaNCED2* are significantly induced (2- to 2.5-fold) by drought stress in wheat anthers of the drought-sensitive varieties Sundor and Cranbrook (Fig. 2). In the drought-tolerant varieties Halberd and SYN604, there was no significant induction of *TaZEP1* and *TaNCED1*. *TaNCED2* expression in SYN604 was nearly twice the expression level of Sundor under control conditions, but there was no induction by drought stress. *TaNCED2* induction by drought stress in Halberd was weaker compared with the sensitive variety Cranbrook (Fig. 2C).

The ABA catabolic gene *TaABA8′OH1* was not affected by drought stress treatment in anthers of the drought-sensitive varieties Sundor and Cranbrook (Fig. 3A). However, in both drought-tolerant varieties SYN604 and Halberd, expression under control conditions was significantly higher than in the drought-sensitive varieties. Water stress treatment caused a strong induction of *TaABA8′OH1* in SYN604, while expression in drought-stressed Halberd remained similar to control conditions (Fig. 3A). Expression levels between Sundor and Cranbrook did not differ significantly under control conditions (data not shown). In the drought-sensitive varieties, *TaABA8′OH2* was not affected by the stress treatment (Fig. 3B). In anthers of the tolerant line SYN604, *TaABA8′OH2* was again significantly higher under control conditions compared with Sundor, and the gene was also induced by drought stress (Fig. 3B). In Halberd, *TaABA8′OH2* expression under control conditions was similar to Cranbrook, but the gene was slightly repressed by drought treatment. These results indicate that ABA synthesis and catabolism are regulated differently in drought-tolerant wheat: ABA biosynthesis in tolerant lines is lower under drought conditions, and ABA catabolism is, except for *TaABA8′OH2* in Halberd, expressed at higher levels in the tolerant varieties.

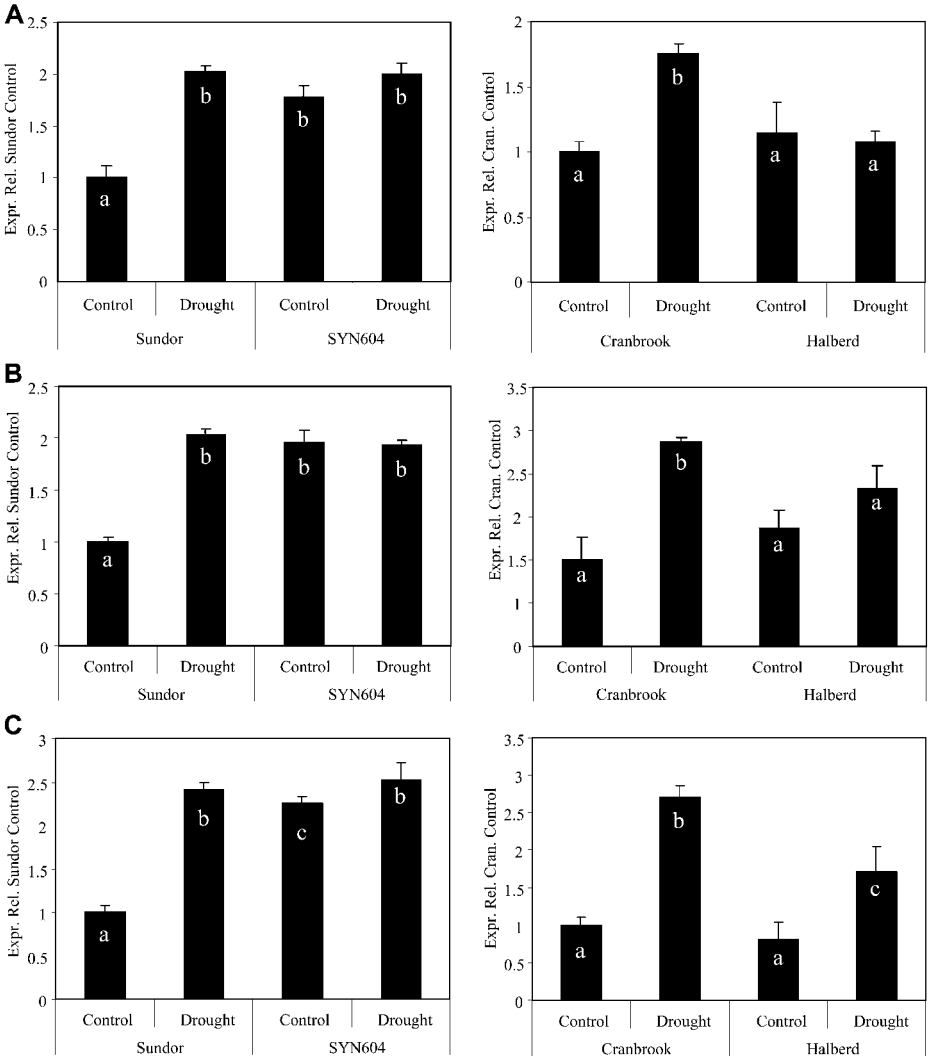

Figure 2. Expression of the wheat ABA biosynthetic genes *TaZEP1* (A), *TaNCED1* (B), and *TaNCED2* (C) in anthers. The semiquantitative RT-PCR expression data are relative to the drought-sensitive lines Sundor and Cranbrook. Bars with the same letter are not significantly different (*t* test; $P < 0.05$). The data represent averages of five biological repeats for Sundor and SYN604 ($n = 5$) and three repeats for Cranbrook and Halberd ($n = 3$).

TaABA8′OH1 Deletions Affect Drought Tolerance at the Reproductive Stage

A PCR approach was used to identify wheat lines with natural deletions in the *TaABA8′OH1* gene of the A, B, and D genome (see "Materials and Methods"; Supplemental Figs. S1 and S2). Genomic DNA was isolated from 1,650 different wheat accession lines from the Tamworth Winter Wheat Collection and screened by PCR for the presence or absence of the *TaABA8′OH1* gene region. Four *TaABA8′OH1* deletion lines were identified. Aus1731 (Zonk1) and Aus14510 (Gandum Garmah) have a deletion in the D genome, line Aus25138 (Shrike) in the A genome, and Aus26243 (KS92WGRC18) in the B genome copy of the *TaABA8′OH1* gene. The deletions were confirmed using Southern-blot hybridization (Supplemental Fig. S1). The deletion in the D genome of Zonk1 and Gandum Garmah was further characterized. The full-length *TaABA8′OH1* gene was amplified from the D genome and sequenced. The D genome gene from Zonk1 and Gandum Garmah contained an identical deletion between 1,349 and 1,600 bp of the Sunstate gene sequence (Supplemental Fig. S2). This deletion is predicted to lead to loss of function of the D genome allele of the *TaABA8′OH1* gene due to the loss of the region that spans across intron 3 and exon 4 of the gene. The deletion lines in the A, B, and D genome were backcrossed to variety Sunstate for five generations, and crosses were made between the deletion lines to combine the deletions in the three wheat genomes: Sunstate (+A+B−D), (+A−B+D), (+A−B−D). The triple deletion line of *TaABA8′OH1* could not be obtained, suggesting that it may be lethal.

ABA measurements in the spike of the *TaABA8′OH1* deletion lines show that ABA content was significantly increased compared with variety Sunstate (Fig. 4A). The Sunstate (+A+B−D) and (+A−B−D) deletion lines had significantly higher spike ABA levels under well-watered control conditions compared with the wild-type Sunstate line (Fig. 4A). Under drought conditions, spike ABA levels were further increased 2.2- and 3.1-fold above drought-stressed wild-type

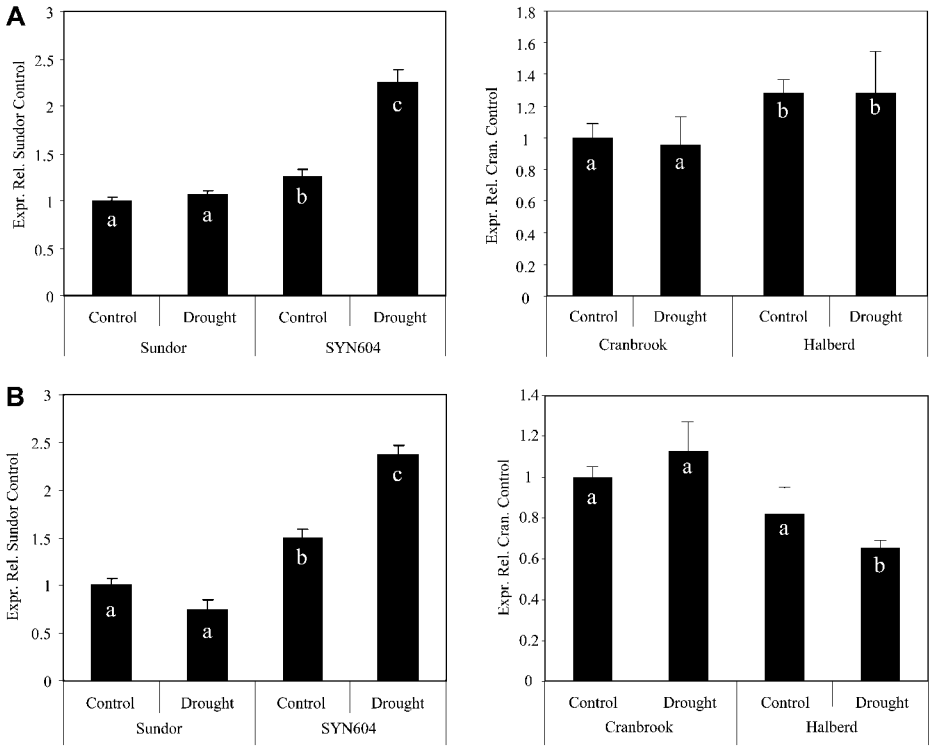

Figure 3. Expression analysis of the wheat ABA catabolic genes *TaABA8′OH1* (A) and *TaABA8′OH2* (B) in anthers. The semiquantitative RT-PCR expression data are relative to the drought-sensitive lines Sundor and Cranbrook and are averages of five and three biological repeats for Sundor/SYN604 and Cranbrook/Halberd, respectively. Bars with the same letter are not significantly different (*t* test; $P < 0.05$).

Sunstate levels in Sunstate (+A+B−D) and (+A−B−D) deletion lines, respectively (Fig. 4A). The effect of *TaABA8′OH1* deletions on spike ABA levels indicates that this gene is an important contributor to the total ABA 8′-hydroxylase activity in wheat.

Grain numbers under control conditions were slightly lower than in the wild-type Sunstate line for the Sunstate (+A+B−D) and (+A−B−D) deletion lines but did not differ significantly for the (+A−B+D) deletion line (Fig. 4B). Five days of drought treatment of the backcross parent line Sunstate caused a loss in grain number to 57% of control conditions (Fig. 4B). Deletions in the D (+A+B−D) or B (+A−B+D) genome, and especially the double-deletion line in both the B and D genomes (+A−B−D), caused a progressive loss in grain number to 15.7%, 11.6%, and 2.6% of control levels, respectively (Fig. 4B). These results indicate that endogenous ABA levels, expression of the ABA 8′-hydroxylase gene, and drought stress tolerance are correlated in wheat.

Drought-Sensitive and Drought-Tolerant Germplasm Have Different ABA Sensitivity

Injection at YM of an ABA solution (10^{-4} M) between the leaf sheaths containing the wheat spike caused significant grain loss (Fig. 5A). Grain number in the drought-sensitive lines Sundor and Cranbrook was on average 45% and 55% of control levels, respectively. ABA injection of the drought-tolerant lines SYN604 and Halberd caused significantly higher grain losses (28% and 44%, respectively), with the line with the highest drought tolerance sustaining the highest grain loss (Fig. 5A). ABA treatment of wheat spikes (10^{-4} M for 24 h) resulted in significant repression of the cell wall invertase gene *TaIVR1* (Koonjul et al., 2005; Fig. 5B). The repression by ABA treatment of *TaIVR1* expression was much stronger for both drought-tolerant lines (52% and 65% for SYN604 and Halberd, respectively) than for the drought-sensitive lines Sundor and Cranbrook (89% of control levels; Fig. 5B). ABA treatment did not have any significant effect on expression of the ABA biosynthetic genes (data not shown) or on expression of the *TaABA8′OH1* catabolic gene (Fig. 5C). But the *TaABA8′OH2* gene was significantly induced by ABA (Fig. 5D). The induction levels of *TaABA8′OH2* of the drought-tolerant lines Halberd and SYN604 were significantly higher than those of the drought-sensitive lines Sundor and Cranbrook (Fig. 5D). These data indicate that drought-sensitive and drought-tolerant wheats have different sensitivities to ABA, and this is reflected in differential regulation of the catabolic gene *TaABA8′OH2* to ABA application.

Different Kinetics of ABA Accumulation in Stress-Sensitive and -Tolerant Germplasm

As a more convenient replacement for applying drought stress, we used a hydroponic system to impose osmotic stress on wheat plants. This enabled us to better control and synchronize the onset of stress conditions for a number of plants. In wheat tissues, NaCl is excluded from uptake, thereby acting as osmoticum, causing a water stress phenotype (Munns

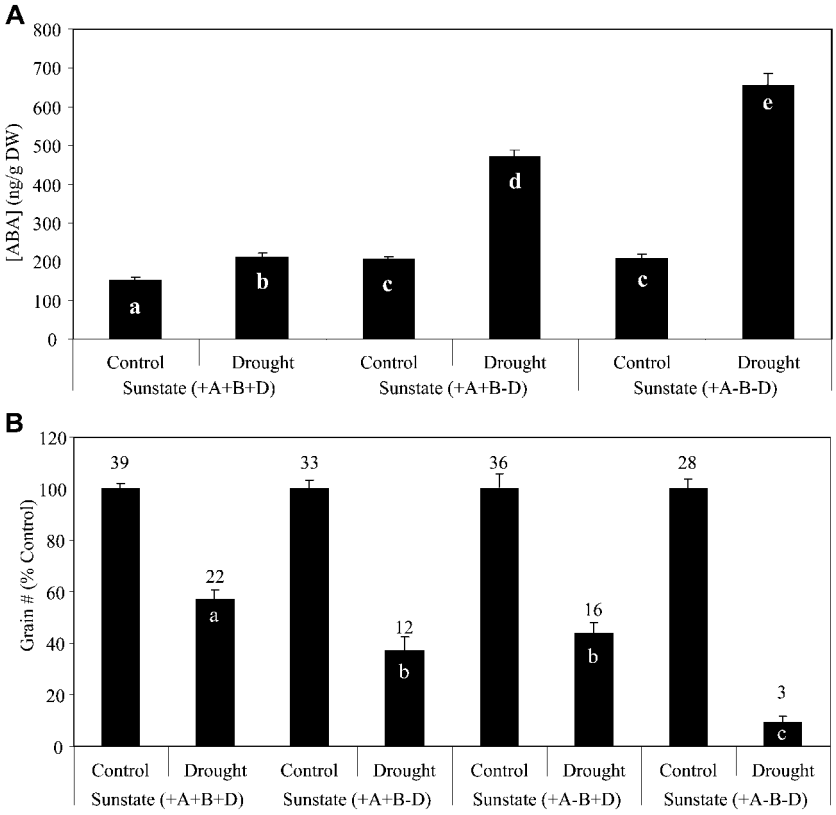

Figure 4. Effect of drought stress on grain number for ABA 8′-hydroxylase deletion lines. A, Effect of drought stress on endogenous ABA levels in leaves and spikes of Sunstate (+A+B+D), the D genome deletion line Sunstate (+A+B−D), and the B and D genome double-deletion line Sunstate (+A−B−D). DW, Dry weight. B, Effect of drought stress on grain number for Sunstate (+A+B+D), Sunstate D genome deletion line (+A+B−D), Sunstate G genome deletion line (+A−B+D), and Sunstate double-deletion line (+A−B−D). The grain number data are averages of 15 to 30 spikes (*n*) collected over two biological repeat experiments, and the same letter in the bars of the drought-stressed samples indicates that they are not significantly different (*t* test; $P < 0.01$). The numbers above the bars represent average grain number for each of the samples.

et al., 2000). Measurements of ABA levels in wheat spikes show that in both Sundor and SYN604, ABA levels increased 2-fold by 4 h of stress treatment, but from 8 h onward, ABA levels in the drought-tolerant line SYN604 were significantly lower than in Sundor. At the end of a 4-d osmotic stress treatment, ABA levels in SYN604 were 2.3-fold lower than in Sundor (Fig. 6A). To compare these results in wheat with rice, we carried out a time-course experiment for cold stress in rice. In spikelets of cold-sensitive cv Doongara, cold treatment caused ABA levels to quickly increase by 2.9- and 4.2-fold after 4 and 8 h, respectively. ABA levels continued to increase and maintain a high level throughout the 5-d stress period (Fig. 6B). ABA levels increased in spikelets of the cold-tolerant cv R31 up to 8 h of cold treatment, but from 8 h onward, they decreased again and continued to decrease until the end of the treatment (Fig. 6B). At the end of the 5-d cold treatment, ABA levels were 5.2-fold higher in Doongara compared with R31 (Fig. 6B).

We studied the expression of the three *OsNCED* and *OsABAOX* genes in rice anthers at the 0-, 8-, and 16-h time points of cold treatment. These time points were chosen to correlate the decline in ABA levels observed for R31 (starting at 16 h) with changes in the expression of ABA metabolic genes between 8 and 16 h of cold treatment. In Doongara anthers, *OsNCED1* and -2, but not *OsNCED3*, expression was increased by 16 h of cold treatment (Fig. 6C). However, in the cold-tolerant line R31, *OsNCED1* and especially *OsNCED2* expression was decreased, while *OsNCED3* expression was slightly increased after 16 h of cold treatment (Fig. 6C). Anther expression levels of the three ABA 8′-hydroxylase genes were decreased in Doongara after 16 h of cold treatment, but in the cold-tolerant line R31, all three *ABA8′OH* genes, especially *OsABA8OX2*, were up-regulated after 16 h of cold treatment (Fig. 6C). Cold-tolerant anthers switch from ABA biosynthesis to ABA catabolism between 8 and 16 h of cold treatment.

ABA Biosynthesis Occurs in Anther Vascular Parenchyma Cells

To assess the tissue and cell specificity of ABA biosynthesis, we analyzed the expression pattern of *OsNCED3*-GUS and *OsNCED3*-ECFP constructs in transgenic rice. The *OsNCED3* gene was chosen because this gene (together with *OsNCED1*) is induced by cold stress in rice anthers (Oliver et al., 2007). Expression of the *OsNCED3* promoter was confined mainly to the vascular bundles in roots and leaves and to the seeds. Expression was present in all the embryo tissues but not in the endosperm of the mature seed (radicle and coleoptile; Supplemental Fig. S4, A–C). In YM-stage anthers, *OsNCED3* expression was largely confined to the vascular system of the anther and was highest at the base, tapering off toward the tip (Fig. 7A). Expression was particularly high in the filament, just below the point where it attaches to the anther

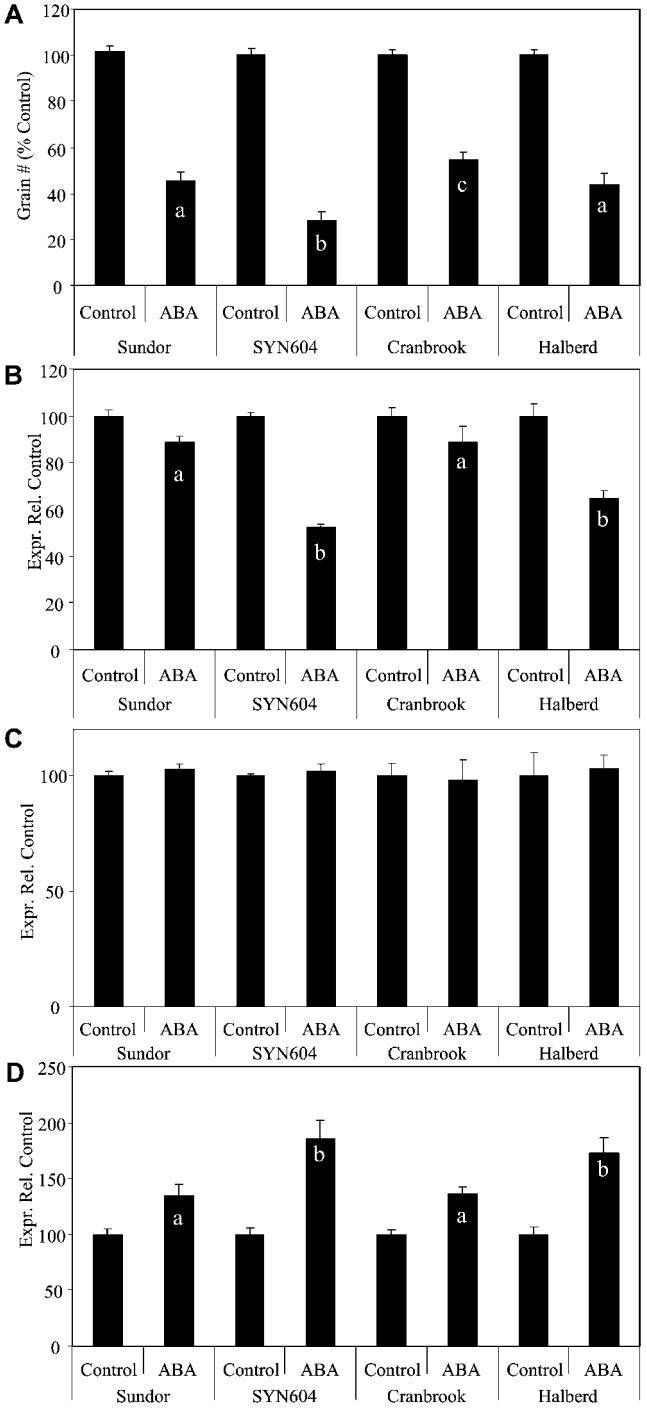

Figure 5. Effect of ABA treatments (10^{-4} M) of wheat spikes on grain number (A), *TaIVR1* cell wall invertase (B), *TaABA8′OH1* (C), and *TaABA8′OH2* (D) gene expression. For each variety, the ABA treatment was evaluated relative to its control using *t* tests. For the effect on grain number, between 43 and 75 ears (*n*) were analyzed ($P < 0.01$). The ABA-treated samples were also compared between varieties. Bars with the same letter are not significantly different ($n = 3$ for A and B; $P < 0.05$). There were no significant differences between the samples shown in C.

(Fig. 7, A and B). Throughout the anther, *OsNCED3* expression was predominantly associated with the xylem tissue (Fig. 7, B–D). In cold-stressed rice anthers, expression was also found in guard cells of stomata on the anther connective tissue (Fig. 7C; Supplemental Fig. S4D). In wheat anthers, in situ hybridization also revealed expression of *TaNCED2* in the cells surrounding the vascular bundles (Fig. 7, E and F). In situ hybridization showed a similar expression pattern for *TaNCED1* in wheat anthers (Supplemental Fig. S5).

The specific expression of *OsNCED3* in vascular tissue, especially in the region of the filament close to attachment to the anther, led us to investigate anther anatomy at the YM stage in more detail (Fig. 8). The four lobes of the anther (two large and two shorter, smaller lobes; Fig. 8, A and B) are attached by connective tissue containing a central vascular bundle (Fig. 8, C–E). In the filament, the vascular bundle comprises a single xylem element and three phloem sieve tubes (position 1; Fig. 8, D and E). Closer to the anther lobes, the xylem in the filament branches into two or three xylem elements (position 2; Fig. 8, D and E), reaching a maximum of three to five elements in the basal connective tissue (Fig. 7B, asterisk). Farther up the anther, the xylem again comprises two to three xylem elements (positions 3 and 4; Fig. 8, C–E), occasionally branching into two separate strands of xylem (Fig. 8E). The region of highest *OsNCED3* expression at the top of the filament (Fig. 7, A and B) may be correlated with the branching of the xylem elements (position 2; Fig. 8, D and E).

Overexpression of *TaABA8′OH1* Improves Cold Tolerance in Rice

To test the role of ABA 8′-hydroxylase in controlling ABA levels and stress tolerance in anthers, we made transgenic rice plants expressing the wheat *TaABA8′OH1* gene. As a promoter to drive *TaABA8′OH1* expression, we used the rice *OsNCED3* promoter. However, none of the transgenic plants produced seeds. Anthers of the T1 transgenic plants developed normally, but at anthesis, they appeared smaller, shriveled, and irregular in shape. Although overall there were fewer pollen grains, they contained starch and appeared fertile (Supplemental Fig. S6, A–C). We concluded that the infertility of the *OsNCED3-TaABA8′OH1* transgenic lines may have been due to pollen indehiscence at anthesis.

We also used the promoter of the *OsG6B* gene (Os11g37280) to drive the expression of *TaABA8′OH1* in transgenic rice. *OsG6B* is abundantly expressed in the tapetum at the YM stage (Tsuchiya et al., 1994). Random sequencing of clones from a YM-stage rice anther cDNA library indicated that 2.96% of the total number of clones sequenced were *OsG6B* (data not shown). Expression of *OsG6B* is highly abundant but is repressed by cold and ABA treatment to about 62% of control expression (Fig. 9A). All three transgenic lines tested (T8-2, T10-18, and T10-19) had a normal, fertile phenotype (Supplemental Fig. S6, D–F) and expressed

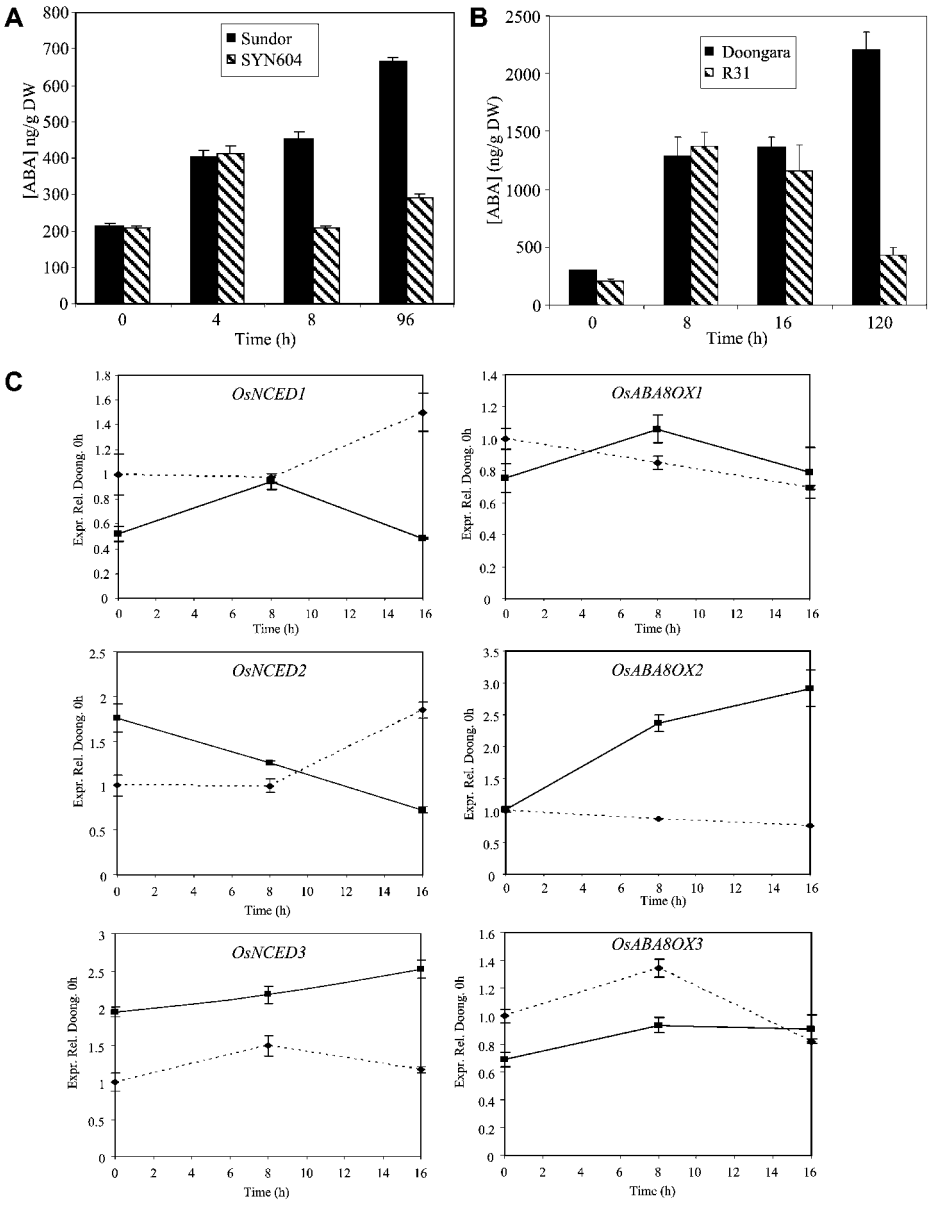

Figure 6. Changes in ABA levels during stress treatment in tolerant and sensitive germplasm of wheat and rice. A, Use of osmotic stress treatment using NaCl as osmoticum in wheat (hydroponic system). ABA accumulates to the same level in drought-sensitive variety Sundor and tolerant variety SYN604. From 8 h onward, ABA levels decrease again in SYN604, while they continue to increase in Sundor. Error bars show the SE for three repeat measurements. B, Time course showing the effect of cold treatment (12°C, 5 d) at the YM stage on ABA accumulation for cold-sensitive rice variety Doongara and cold-tolerant variety R31. Error bars show the SE for four repeat measurements. DW, Dry weight. C, Gene expression studies for the time points of 0, 8, and 16 h showing differences in gene expression for *OsNCED1*, *-2*, and *-3* and *OsABA8OX1*, *-2*, and *-3* for the cold-sensitive rice variety Doongara (dotted lines) and the cold-tolerant variety R31 (solid lines). Error bars show SE for four repeat measurements.

the wheat *TaABA8′OH1* gene in the spike (Fig. 9B). In contrast to the endogenous *OsG6B* gene in untransformed lines (Fig. 9A), the three *OsG6B-TaABA8OH1* transgenic lines did not show a repression but an induction of the *TaABA8′OH1* transcript by cold stress (Fig. 9B). Endogenous ABA levels in cold-stressed spikelets of the transgenic lines were significantly reduced compared with levels found in their respective null-segregant lines that were grown at the same time under the same conditions: spikelet ABA levels were 58.4% to 75.9% of those in the null-segregant lines (Fig. 9D). The induction of the *TaABA8′OH1* transgene in the cold-stressed transgenic lines is likely due to the fact that endogenous ABA levels in the transgenic lines are reduced. This was confirmed by the fact that the endogenous *OsG6B* gene is not repressed in the transgenic lines but, in the case of two lines (T8-2 and T10-19), was even up-regulated (Fig. 9C). The fact that expression of the endogenous *OsG6B* gene is lower in the transgenic lines suggests that the endogenous gene may be partially suppressed as a result of sequence homology with the promoter of the transgene (translational fusion). We found that *OsINV4* cell wall invertase gene expression is not repressed by cold in any of the *TaABA8′OH1* transgenic rice lines, while it is significantly repressed by cold stress in the null-segregant lines (Fig. 9E). This clearly illustrates that the *OsG6B-TaABA8′OH1* construct works: spike ABA accumulation is reduced under cold conditions, and these lower ABA levels result in the maintenance of *OSINV4* expression. Analysis of cold tolerance showed that sterility for

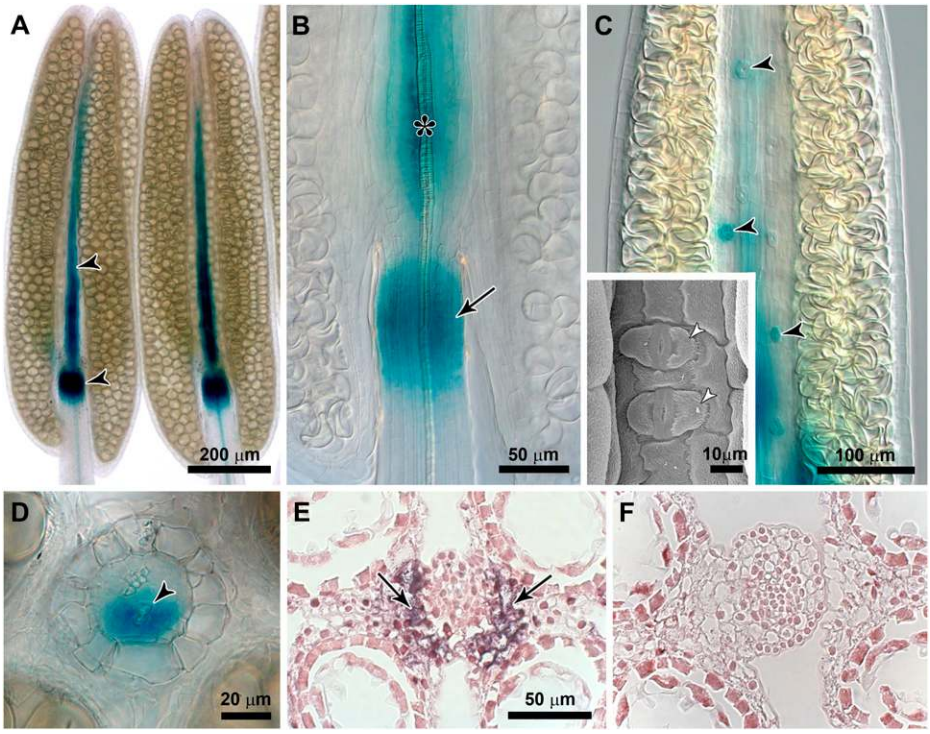

Figure 7. Expression pattern of the *NCED* promoter in rice and wheat anthers. A, GUS staining of *OsNCED3*-GUS transgenic rice anthers showing expression around the central vascular bundle of the anther (arrowheads). B, Higher magnification of another anther showing high *OsNCED3* expression in the vascular parenchyma in the connective tissue (asterisk) and where the base of the anther is connected to the filament (arrow). C, Expression is also associated with stomata (arrowheads) present within the epidermis of the connective tissue between the two large lobes. The inset shows a scanning electron micrograph of an anther, showing stomata in the connective tissue epidermis (arrowheads). D, Hand cross section, just below position 3 in Figure 8B, showing expression predominantly associated with the xylem tissue (arrowhead). E and F, In situ hybridization of *TaNCED2* in wheat anthers reveals expression around the vascular bundle in the center of the anther (arrows). E, Hybridization using antisense probe. F, Control hybridization using sense probe. The scale is the same in E and F.

the three rice transgenic lines was significantly reduced over their respective null-segregant lines (Fig. 9F). The percentage sterility in the overexpressing lines was reduced by 30% to 63% compared with the null-segregant lines (Fig. 9F). Grain numbers under control conditions did not show any significant differences. These results indicate that overexpression of wheat *TaABA8′OH1* gene activity in the tapetum results in reduced ABA accumulation and improvement of cold stress tolerance in rice.

DISCUSSION

ABA is a "stress hormone." Its most conspicuous function is in controlling water relations by regulating stomatal conductance and CO_2 uptake (Kim et al., 2010). ABA, response to abiotic stress, regulation of plant metabolism, and development are intertwined (Hey et al., 2010). It has been shown that ABA and sugar signaling overlap and that ABA is involved in controlling sink-source relationships and sugar metabolism (Arenas-Huertero et al., 2000; Laby et al., 2000; Rook et al., 2001, 2006; Arroyo et al., 2003). Exogenous ABA improves tolerance to a number of abiotic stresses (Chen et al., 1983; Larosa et al., 1985; Robertson et al., 1994; Kato-Noguchi, 2000; Hsu and Kao, 2003; Lu et al., 2009), and transgenic approaches increasing tissue ABA levels by ZEP and NCED overexpression resulted in improved drought tolerance (Nambara and Marion-Poll, 2005).

In studying the role of ABA in abiotic stress tolerance, the focus has mainly been on the vegetative parts of the plant. However, at the reproductive stage, there appears to be a negative correlation between ABA levels and abiotic stress tolerance. Cold-induced pollen sterility in cold-sensitive rice lines is aggravated by ABA accumulation in anthers (Oliver et al., 2007), and we show in this article that there is a similar correlation for drought-induced sterility in wheat. ABA treatment of YM-stage spikelets induces sterility as a result of pollen abortion and mimics the effect of cold and drought treatment. ABA application represses cell wall invertase gene expression (*OsINV4*) and reduces anther sink strength in rice (Oliver et al., 2005, 2007). The fact that ABA regulates *OSINV4* expression in rice is also confirmed using transgenic lines that have reduced anther ABA levels (see below). We show here

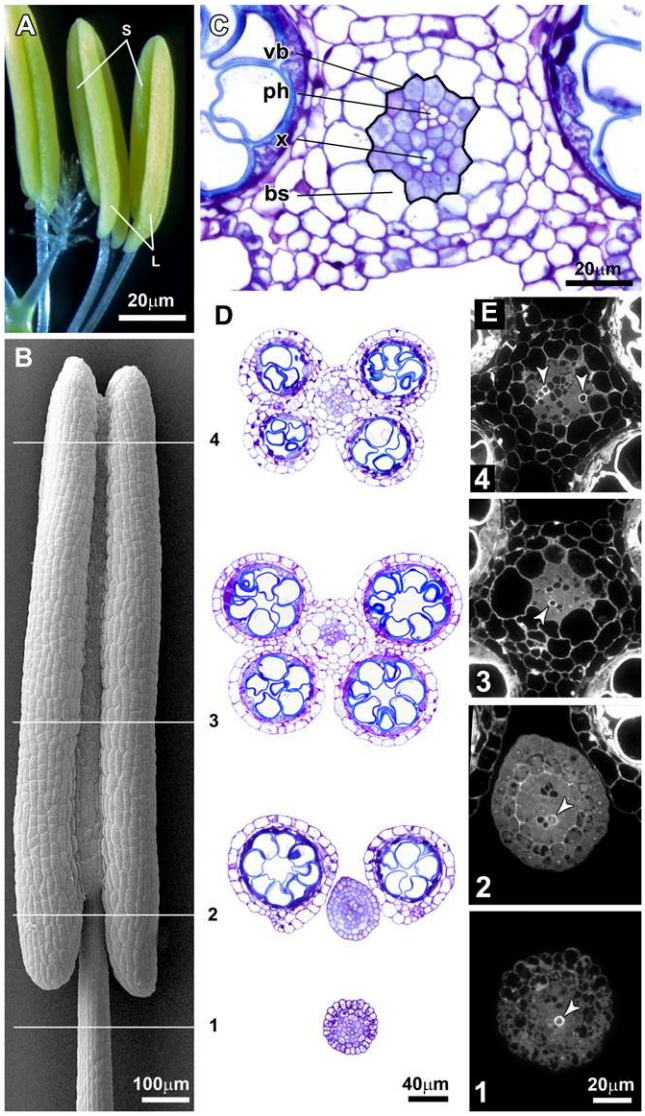

Figure 8. Anatomy of rice anthers. A, Rice anthers showing two small (S) and two large (L) pollen-containing lobes. B, Scanning electron micrograph of an anther showing the two large lobes that conceal the two small lobes in this image. C, Higher magnification of the connective tissue (similar to position 2 in B) showing details of vascular tissue. bs, Bundle sheath; ph, phloem; vb, vascular bundle (outlined in black); x, xylem. D, Series of toluidine blue-stained resin sections taken at positions (1–4) similar to those indicated in B. Sections are oriented with the large lobes facing the top of the image. E, UV autofluorescence images of resin sections adjacent to those shown in D. Arrowheads indicate xylem.

that ABA treatment also induces sterility and represses the wheat *TaIVR1* cell wall invertase gene. Analysis of wheat *TaABA8′OH1* deletion lines provides genetic evidence that higher ABA levels in these lines cause increased sensitivity to drought stress. Maintaining low ABA levels, therefore, is important for stress tolerance and pollen fertility. One important downstream effect of ABA is the repression of sugar transport. Maintenance of anther sink strength is critical for the development of fertile pollen, but ABA is likely to affect many other anther genes.

Drought-tolerant wheat germplasm, like cold-tolerant rice lines, prevents the accumulation of spike ABA in response to stress treatment. This lower ABA accumulation in drought-tolerant wheat is the combined result of both lower expression of ABA biosynthesis genes (*ZEP* and *NCED*) and higher expression of the *ABA8′OH* catabolism genes. We do not have knowledge about the number of wheat *ZEP*, *NCED*, and *ABA8′OH* genes, but the three wheat ABA biosynthesis genes and two ABA catabolic genes we identified show a similar trend under drought conditions in anthers as their rice counterparts under cold conditions (Oliver et al., 2007). In addition, anther ABA levels in the tolerant wheat lines are lower than in the sensitive lines. ABA homeostasis, therefore, is important for controlling abiotic stress tolerance and pollen fertility in wheat and rice.

The rates of ABA biosynthesis and catabolism and the precise balance between the two are important for controlling ABA accumulation in plant tissues (Nambara and Marion-Poll, 2005). ABA biosynthesis and catabolism genes are regulated by a variety of stimuli, including drought, cold, high temperature, light, sugars, and mRNA stability (Nambara and Marion-Poll, 2005; Gubler et al., 2008; Argyris et al., 2011). Some evidence suggests that ABA catabolism plays an important role in controlling tissue ABA levels and ABA homeostasis (Chono et al., 2006; Millar et al., 2006; Okamoto et al., 2006; Umezawa et al., 2006; Yang and Zeevaart, 2006; Ren et al., 2007; Zhu et al., 2009). The wheat *TaABA8′OH1* deletion lines certainly indicate that deletion of one of the wheat *ABA8′OH* genes leads to ABA accumulation and the increased reproductive drought sensitivity of wheat. ABA in these deletion lines accumulates to high levels, and there is no effect on expression of the *TaNCED* biosynthetic genes, suggesting that there is no feedback regulation system restricting ABA biosynthesis in response to environmental stimuli like drought stress. This is confirmed by ABA application experiments, which show that there is no effect of ABA treatment on *TaNCED1* and *TaNCED2* expression, but we did find that ABA affects the expression of *TaABA8′OH2*, one of the two *TaABA8′OH* genes we tested. This induction is also stronger for the drought-tolerant wheat lines SYN604 and Halberd. But reducing tissue ABA levels also requires the repression of ABA biosynthesis. Time-course experiments indicate that ABA biosynthesis is very similar in sensitive and tolerant wheat and rice lines during the first hours of stress treatment; but then the tolerant lines reduce ABA levels while they continue to increase in the sensitive lines. In cold-stressed rice, we investigated what happens with the expression of the ABA metabolic genes between 8 and 16 h of cold treatment before the decline in ABA levels occurs in the tolerant line R31. We found that ABA 8′-hydroxylase gene expression increases in the tolerant line by 16 h, and this is combined with a reduction in *NCED* gene expression between 8 and 16 h

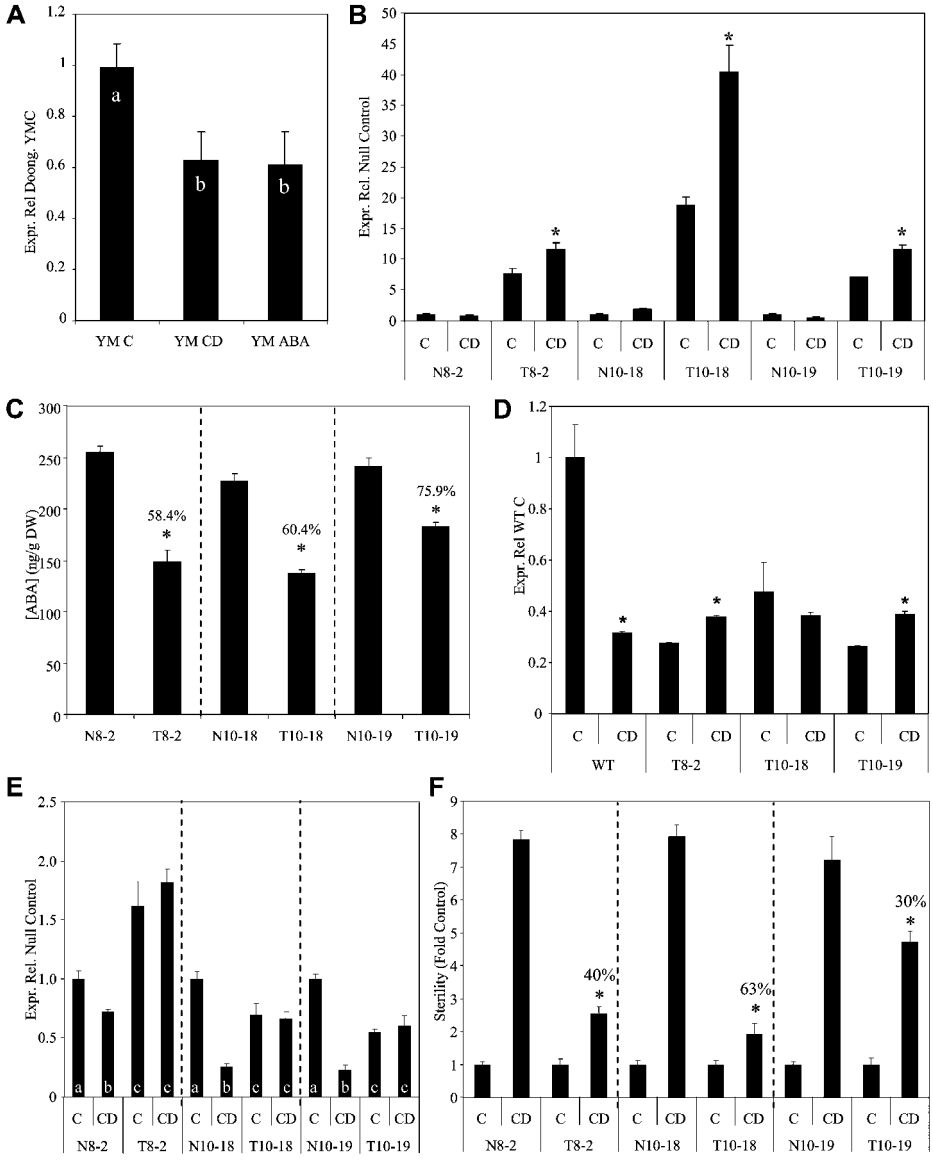

Figure 9. Analysis of three transgenic rice lines (T8-2, T10-18, T10-19) transformed with the *OsG6B-TaABA8′ OH1* construct and their respective null-segregant lines (N8-2, N10-18, N10-19). A, Semiquantitative RT-PCR, showing the expression of the endogenous *OsG6B* gene in rice YM-stage anthers under control (C), cold (CD), and ABA treatment (10^{-4} M) conditions. Error bars indicate SE, and bars with the same letter are not significantly different ($n = 6$; $P < 0.01$). B, Real-time PCR showing the expression of the wheat *TaABA8′ OH1* transgene in three positive transgenic rice lines and their respective null-segregant lines. Error bars indicate SE, and asterisks in the transgenic samples indicate that the effect of the cold treatment versus the control for each line is significant ($n = 3$; $P < 0.05$). C, ABA measurements in cold-stressed samples of the three *TaABA8′ OH1* transgenic lines and their respective null-segregant lines. Asterisks indicate significant differences compared with the null-segregant line ($n = 6$; $P < 0.01$), and the percentages above the bars indicate the average percentage decrease in ABA content in the transgenic lines versus the null-segregant line. DW, Dry weight. D, Real-time PCR showing the expression of the endogenous *OsG6B* gene in the three positive transgenic lines and compared with the wild type (WT). Error bars indicate SE, and asterisks indicate that the effect of the cold treatment versus the control for each line is significant ($n = 3$; $P < 0.01$). E, Real-time PCR showing the expression of the *OsINV4* cell wall invertase gene in the three transgenic lines and their respective null-segregant lines (N8-2, N10-18, N10-19). Error bars indicate SE, and bars with the same letter are not significantly different ($n = 3$; $P < 0.01$). F, Cold tolerance testing results of the three transgenic lines and their respective null-segregant lines. Error bars indicate SE. The percentages above the cold treatment bars of the transgenic lines indicate the reduction in sterility compared with the cold treatment of the corresponding null-segregant line. Asterisks indicate that these differences are significant for all three transgenic lines ($n = 18$–26; $P < 0.01$).

of cold treatment. ABA 8′-hydroxylase gene expression is higher at the start and end of a 5-d stress treatment in both wheat (Fig. 3) and rice (Oliver et al., 2007).

ABA treatment of drought-tolerant and drought-sensitive wheat indicates that the tolerant lines are more sensitive to ABA than the drought-sensitive lines. This observation is similar to what we previously observed for cold-tolerant rice lines (Oliver et al., 2007). ABA catabolism is not induced in drought-sensitive wheat lines, even though endogenous ABA levels are higher than in tolerant lines. In contrast, in tolerant wheat lines, *TaABA8′OH2* is induced by drought treatment, and this induction is significantly stronger in the drought-tolerant compared with the drought-sensitive wheat lines. There is no effect of ABA treatment on the expression of the two *TaNCED* genes, and only *TaABA8′OH2*, and not *TaABA8′OH1*, was responsive to ABA. Tolerant lines appear to react quicker to changes in endogenous ABA levels than sensitive varieties. The higher sensitivity to ABA treatment of drought-tolerant wheat is also reflected by the significantly stronger repression of the wheat *TaIVR1* gene compared with drought-sensitive wheat lines. The difference in ABA sensitivity may point to a regulatory mechanism of ABA catabolism that is tuned differently in tolerant lines. Expression profiling studies using DNA microarrays may provide an opportunity to identify genes that are differentially expressed between 8 and 16 h of cold treatment in anthers of tolerant and sensitive rice lines.

Localization of ABA biosynthesis in anthers using *OsNCED3*-GUS transgenic plants indicated that under normal conditions at the YM stage, ABA biosynthesis is expressed at high levels in the vascular parenchyma cells at the base of the anther, where the filament connects to the anther, and at lower levels around the vascular bundle leading to the top of the anther. In situ hybridization experiments on anther cross sections showed that the wheat *TaNCED* genes are also expressed in the anther vascular parenchyma cells. The rice and wheat *NCED* genes are expressed in the same cell types in anthers as the Arabidopsis (*Arabidopsis thaliana*) *NCED* genes in leaves (Endo et al., 2008a, 2008b), suggesting that vascular parenchyma cells play an important role in ABA signaling and function. Localization studies of ABA pools using ABA-responsive promoters fused to a reporter gene have shown that, while ABA treatment results in uniform distribution of reporter gene expression in the leaves, drought stress only resulted in expression around the vascular bundles and the stomata in leaves (Christmann et al., 2005). This suggests that the primary site of ABA action under drought conditions is confined to the tissues where ABA is synthesized. Similarly, expression of the Arabidopsis *CYP707A1* and *CYP707A3* ABA 8′-hydroxylase genes was also confined to the regions around the vascular tissues and the guard cells of leaves (Okamoto et al., 2009). In cold-stressed rice anthers, expression was found in guard cells that are located on the connective tissue of rice anthers. Stomata in anthers could play a role in pollen dehiscence in entomophilous plants, where they are associated with nectaries and play a role in sugar secretion (Keijzer et al., 1987, 1996; Varassin et al., 2008). The role of these stomata in the self-fertilizing plant species rice is unclear, but also Arabidopsis was shown to have functional nectaries (Kram and Carter, 2009). In older anthers, ABA synthesis appears in the tip of the anther only. Pollen dehiscence is a process that involves ethylene and programmed cell death.

We tested the effect of reducing anther ABA levels on cold tolerance in rice by manipulating expression levels of the ABA catabolic gene ABA 8′-hydroxylase using the wheat *TaABA8′OH1* gene. We could not obtain fertile T1 plants when the *OsNCED3* promoter was used to drive *TaABA8′OH1* expression. This suggests that exact synchronization of both the timing and location of ABA catabolism and biosynthesis in anthers leads to sterility. Anthers of transgenic plants appeared smaller and shriveled in the *OsNCED3-TaABA8′OH1* lines, and there were fewer starch-containing pollen grains. Baseline ABA levels are required for normal anther development and function (van Bergen et al., 1999; Wang et al., 1999). Sterility in the *OsNCED3-TaABA8′OH1* transgenic lines may have been due to the absence of pollen dehiscence. ABA may be involved in the desiccation response that is required for pollen dehiscence. This is compatible with the expression pattern close to anthesis of the *OsNCED3*-GUS lines, showing expression near the tip of the anther, where pollen dehiscence starts in rice (Matsui et al., 1999). When a strong *OsG6B* tapetum-specific promoter was used, transgenic plants were fertile and anther ABA levels following stress treatment were significantly lower. The *OsG6B* promoter is itself regulated by ABA, providing a self-regulating mechanism to drive ABA catabolism: in the *OsG6B-TaABA8′OH1* transgenic lines, overexpression of the transgene leads to reduced ABA levels, which maintains expression of the *OsG6B* promoter. We observed that fertility of the *OsG6B-TaABA8′OH1* lines was improved significantly compared with plants that did not have the transgene. This indicates that ABA 8′-hydroxylase expression in the tapetum is capable of reducing ABA levels under cold stress conditions, thereby partially maintaining fertility of the pollen grains. Manipulation of ABA 8′-hydroxylase activity was also shown to affect seed dormancy (Millar et al., 2006).

In rice and wheat anthers, cell wall invertase gene expression occurs around the vascular bundles and in the tapetum (Koonjul et al., 2005; Oliver et al., 2005). The fact that ABA down-regulates cell wall invertase expression suggests that repression of cell wall invertase expression in the vascular parenchyma cells may be the main reason for the abortion of pollen development. However, the improved cold tolerance of the *OsG6B-TaABA8′OH1* transgenic lines suggests that ABA must diffuse from the vascular parenchyma throughout the anther and into the tapetum. Therefore, cold-induced accumulation of ABA, resulting in

the repression of cell wall invertase expression in the tapetum, may account for pollen abortion. *OsG6B-TaABA8′OH1* transgenic lines did not show repression of anther *OSINV4* expression, further confirming that maintenance of sink strength in the tapetum is important for pollen development and fertility. It remains to be established whether cold tolerance in rice can be further improved to the level observed in cold-tolerant rice lines by using other tapetum-specific promoters. In addition, we will investigate whether the same *OsG6B-TaABA8′OH1* construct can improve the drought tolerance of transgenic wheat lines.

MATERIALS AND METHODS

Germplasm, Plant Growing Conditions, and Stress Treatments

The wheat (*Triticum aestivum*) and rice (*Oryza sativa*) varieties used in this study and the growing conditions were as described previously (Oliver et al., 2005; Ji et al., 2010). Briefly, wheat plants were grown in the glasshouse under natural lighting conditions (24°C) and drought stressed for 5 d at the YM stage under controlled environment conditions (16 h of light, 24°C; 8 h of dark, 15°C; light intensity of 200 μmol m^{-2} s^{-1}). An auricle distance (AD) measurement scale was established to estimate the pollen developmental stage; the AD is the distance between the auricles of the flag leaf and the penultimate leaf and is a measure of spikelet development (Oliver et al., 2005; Ji et al., 2010). To obtain drought conditions around YM, plants started the drought treatment about 4 cm (about 4 d) ahead of the AD where YM is reached (AD 0 to +2 cm). This AD differed for different varieties (Ji et al., 2010).

Rice grains were sown in 15-cm pots containing a mix of 25% compost, 75% vermiculite, and approximately 5 g of osmocote fertilizer. Plants were grown in the greenhouse (light, 28°C; dark, 24°C) with the pots and root system submerged in water. Tillers were tagged at the YM stage and transferred to a controlled growth chamber for cold treatment at a constant temperature of 12°C for 5 d (13 h of light/11 h of dark; light intensity of 400 μmol m^{-2} s^{-1}). Time-course experiments were carried out under continuous light conditions (400 μmol m^{-2} s^{-1}) to avoid diurnal fluctuations in hormone levels. For evaluation of sterility, stressed wheat and rice plants were returned to normal growth conditions and the grain number was scored at maturity. For gene expression studies, spikes or anthers (dissecting microscope) were harvested in the afternoon (middle of the light cycle) to minimize diurnal variation effects. Upon harvesting, plant material was immediately frozen in liquid nitrogen and stored at −80°C.

A hydroponic system was established for carrying out osmotic stress treatments in wheat. Sundor and SYN604 were grown in pots containing fine gravel through which Hoagland medium was circulated as described by Munns et al. (2000). The salt concentration of the Hoagland medium was increased daily in 50 mM steps from 100 to 250 mM when the plants were about 4 cm (about 4 d) ahead of the AD where YM is reached (AD 0 to +2 cm). Supplemental calcium ($CaCl_2$) was added to achieve a Na^+-to-Ca^{2+} ratio of 15:1. To compare the ABA response of the two varieties, panicles were collected at different time points from the start of the salt treatment.

ABA Treatment of Wheat Ears and Rice Panicles

ABA was injected through the leaf sheaths into the space containing the inflorescence as described before (Oliver et al., 2007). ABA injections were carried out using a 10^{-4} M ABA (2-cis, 4-trans-ABA; Sigma) solution (in distilled water with one drop of Tween 20 per 50 mL). Control mock-injected plants were treated with the same solution lacking ABA. Plants were injected at a fixed time in the afternoon to minimize diurnal variation effects. For gene expression studies, ears were collected for RNA extraction 2 d after ABA injection. Control ears were collected from mock-injected plants. To determine the effect of ABA treatment on grain number, plants were grown until maturity, and the grain number for ears that were treated at the YM stage was counted, together with mock-injected control ears that were treated at the same time.

ABA Measurements

ABA concentrations in spikes were measured using the Phytodetek competitive ELISA kit (Agdia). Plants were drought or cold stressed at the YM stage, and spikes were then collected and frozen immediately in liquid nitrogen. Control untreated spikes were collected at the same time. The collected samples were ground in liquid nitrogen, the powder was freeze dried, and the dry weight was determined. The powder was extracted overnight at 4°C in cold 80% methanol. The mixture was then centrifuged at 5,000 rpm for 5 min, and the supernatant was collected. The overnight extraction was repeated on the pellet, and the supernatants were pooled. The pellet was washed a further three times in cold 80% methanol by vortexing and spinning, and the supernatant for each sample was pooled and dried down in a SpeedVac until approximately 50 μL of liquid remained. Then, Tris-buffered saline buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, and 3 mM NaN_3) was added to a final volume of 1 mL. These extracts were diluted 10-fold in Tris-buffered saline and used in the ELISA according to the Phytodetek protocol. The final concentrations were calculated as nanograms per gram dry weight. Each measurement was replicated three times using different panicle samples and in replicate experiments (biological repeats) using different batches of plants, as indicated in the figure legends.

Identification of Wheat Genes for Gene Expression Studies

Wheat genes encoding the ABA biosynthesis enzymes NCED and ZEP and the ABA catabolic enzyme ABA 8′-hydroxylase were identified using BLASTN and tBLASTN searches of the GenBank (http://www.ncbi.nlm.nih.gov/) and GrainGenes (http://wheat.pw.usda.gov/) databases with the rice homologs as query sequences (Oliver et al., 2007). We focused on ESTs that were derived from cDNA libraries constructed from wheat reproductive tissue RNA. The full-length wheat *TaABA8′OH1* gene was isolated by PCR (95°C for 5 min; 35 cycles at 95°C for 15 s and 55°C for 15 s; and 72°C for 2 min) using primers based on the 5′ and 3′ untranslated regions (5′-GTTGCAGGTTGCAGGTAACAGAAC-3′ and 5′-GTCGCCTCTATCGTGCAGTTG-3′) of the barley (*Hordeum vulgare*) *HvABA8′OH1* gene (Millar et al., 2006). A genomic fragment containing the complete *TaABA8′OH1* gene from the D genome was amplified from wheat variety Sunstate genomic DNA. Sequence alignments were investigated using ClustalW using Vector NTI Advance version 11.0 software (Invitrogen), and phylogenetic analysis was conducted using ClustalW and MEGA version 4.0 software (Tamura et al., 2007).

Identification of Wheat *TaABA8′OH1* Deletion Lines

To identify small deletions in the *TaABA8′OH1* gene in the A, B, and D genomes in wheat accession lines, we analyzed A, B, and D genome homologs of the *TaABA8′OH1* gene for DNA polymorphisms that could be used for generating genome-specific PCR primers. A 751-bp fragment of the *TaABA8′OH1* gene, encompassing the three introns of the gene, was amplified by PCR from wheat line Bob White 26 genomic DNA (primers 5′-GCTCACGTGGATGGTCAAGTTCC-3′ and 5′-TTCCCGAACGGCATGAACGTGTTG-3′; one cycle at 95°C for 5 min; 35 cycles of 95°C for 15 s, 62°C for 15 s, and 72°C for 30 s; one cycle at 72°C for 3 min). The products were cloned into pGEMT-Easy and sequenced. Three very similar but different groups of sequences were identified and are predicted to represent *TaABA8′OH1* sequences from the A, B, and D genomes of wheat (Supplemental Fig. S1) Three primer pairs were designed to selectively amplify a DNA fragment from each sequence type (Supplemental Fig. S1) using the following conditions: one cycle at 95°C for 5 min; 35 cycles at 95°C for 15 s, 62°C for 15 s, and 72°C for 30 s; and one cycle at 72°C for 3 min. To identify whether the primer pairs were specific for different *TaABA8′OH1* genes on the A, B, and D genomes, the primer pairs were tested on nullisomic-tetrasomic lines of wheat variety Chinese Spring. One primer pair was identified that was specific for the *TaABA8′OH1* gene present on chromosome 6 of the A, B, and D genomes (Supplemental Fig. S1). These genome-specific primer pairs were then used to screen for mutations and deletions in the *TaABA8′OH1* gene in wheat populations.

RNA Extraction and Semiquantitative RT-PCR

Total RNA was extracted from wheat anthers and ovaries using the RNeasy Plant Kit (Qiagen). Genomic DNA traces were removed using RQ1 DNase

treatment (Promega) and precipitation using 2 M LiCl. RNA concentrations were measured spectrophotometrically, and an RNA gel was run from each batch of RNA samples in order to check the quality of the RNA and the accuracy of the concentrations. Gene expression studies were carried out using semiquantitative and quantitative real-time RT-PCR as indicated in "Results." Semiquantitative RT-PCRs were carried out by RT of 0.5 μg of total anther RNA using Thermoscript reverse transcriptase (Invitrogen). The PCR step was carried out using Immolase DNA polymerase (Bioline) with gene-specific primers. The sequences of the primers used for wheat genes are shown in Supplemental Table S1. Primers for rice ABA metabolic genes were as described previously (Oliver et al., 2007). Wheat β-actin1 was used as a control in all wheat RT-PCRs, and the β-actin1 results were used to normalize the gene expression results. Rice RT-PCRs used the α-tubulin gene as a control (Oliver et al., 2005). RT-PCR products were analyzed on 1.5% agarose gels and quantified by image-scanning software (Multigauge; Fujifilm). A Rotor-Gene 3000 quantitative PCR machine (Corbett Research) and SYBR Green I (Macroprobe) fluorescent nucleotide were used for real-time RT-PCR. The results were analyzed with RG3000 version 6.0 software (Gene Company). Each sample was run in triplicate. The deviations of crossing point values were less than 1.0 cycle for independent mRNA preparations and less than 0.5 cycles for replicates of the same mRNA preparation. The two-sample *t* test of the means was used for significance testing, and the *P* value as well as the number of repeat RNA samples analyzed (*n*) are indicated in "Results."

Reporter Gene Constructs and Rice Transformation

A 1-kb *Bam*HI/*Nco*I *OsNCED3* promoter fragment was generated by PCR of rice genomic DNA and fused to the ATG of the GUS-3′Nos cassette plasmid pHW9 (Dolferus et al., 1994) to make the *OsNCED3*-GUS construct. The same *OsNCED3* promoter was also fused to an ECFP-3′CaMV reporter gene (for enhanced cyan fluorescent protein fused to the cauliflower mosaic virus transcriptional terminator). Both constructs were transferred to binary vector pCAMBIA1300 (GenBank accession no. AF234296; Hajdukiewicz et al., 1994) for transformation to rice. The wheat *TaABA8′OH1* gene was transformed to rice under the control of the tapetum-specific promoter of the rice *OsG6B* gene (Os11g37280; LTPL68 protease inhibitor/seed storage/LTP family; Tsuchiya et al., 1994). A 1,703-bp *Eco*RI-*Nco*I fragment of the *OsG6B* promoter, including its translation start site, was used to make translational fusions with the GUS reporter gene of pHW9. A 1.7-kb full-length cDNA of the wheat *TaABA80X1* gene (EU430344) was generated by PCR and cloned as a *Nco*I/*Sac*I fragment in a pGEMT-Easy plasmid containing the *OsG6B* promoter and the cauliflower mosaic virus 3′ terminator sequence. The chimeric gene construct was then transferred to binary vector pCAMBIA1300 for transformation to rice. *Agrobacterium tumefaciens*-mediated transformation of Nipponbare rice callus was carried out according to Upadhyaya et al. (2000). Nipponbare is a cold-sensitive rice variety with sensitivity levels similar to Doongara (data not shown). Biochemical, molecular, and cold tolerance analyses of the transgenic lines were carried out in the T2 and T3 generations.

RNA in Situ Hybridization

Wheat florets were dissected at the YM stage and fixed immediately for 2 h at 4°C in 4% freshly prepared paraformaldehyde (Sigma) containing 0.25% glutaraldehyde (Sigma) and 1 mM dithiothreitol (DTT) in 25 mM phosphate buffer, pH 7.0. After vacuum infiltration for 20 min, the fixative was replaced and the samples were incubated overnight in fixative at 4°C. After fixation, the tissues were washed twice for 10 min each in 0.05 M phosphate buffer, pH 7.2, containing 1 mM DTT on ice. Dehydration was done on ice in ascending concentrations of ethanol: 30 min each treatment in 10%, 30%, 50%, and 70% ethanol containing 1 mM DTT and stored in 70% ethanol at −20°C. After dehydration, fixed tissues were embedded in paraffin (Paraplast Plus; Sherwood Medical). Ribbons of 10-μm sections were cut, mounted on poly-Lys-coated glass slides (Esco; Biolab Scientific), and left to dry overnight. A 260-bp *TaNCED2* probe was generated by PCR amplification from wheat anther cDNA and subcloned into pGEMT-Easy vector. Sense and antisense RNA probes were generated using a digoxygenin RNA labeling kit (Roche Diagnostics Australia) according to the manufacturer's instructions. Probe hydrolysis was carried out according to Jackson (1991). The hybridization mixture consisted of 50% deionized formamide, 1.25× hybridization buffer (3 M NaCl, 0.1 M Tris-HCl, pH 6.8, 0.1 M $NaPO_4$ buffer, 50 mM EDTA, 12.5% dextran sulfate, 1.25 mg mL^{-1} yeast tRNA, and 1.25× Denhardt's solution), and 2 μL hydrolyzed and denatured digoxygenin-labeled RNA probe. About 125 μL of hybridization mixture was distributed over the tissue and covered with Hybri-slips (Sigma), and hybridization was carried out in a humidified box at 50°C overnight. Posthybridization washes were as described in the Roche protocol manual. For detection of the hybridization probe, a digoxygenin nucleic acid detection kit (Roche Diagnostics Australia) was used. Photographs were taken under bright-field illumination after overnight staining using a Zeiss AxioImager microscope and camera.

Reporter Gene Localization, Anther Anatomy, and Scanning Electron Microscopy

For the in situ determination of GUS activity, plant tissues were collected in ice-cold 90% acetone, rinsed briefly in sterile water, vacuum infiltrated for 3 min in 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid, 20% methanol, and 50 mM Na_2HPO_4, pH 7.0, and incubated overnight at 37°C in the dark. After destaining overnight in 70% ethanol, anthers were cleared in saturated chloral hydrate, and the GUS reporter was localized in whole anthers with a Zeiss AxioImager compound microscope. Fresh florets of CFP-expressing plants were examined using a Leica TCS SP2 confocal microscope. Untreated florets were fixed overnight in 3% glutaraldehyde in 25 mM phosphate buffer, pH 7.0, dehydrated in an ethanol series, and embedded in LR White resin for detailed anatomical analysis of the attachment zone of anthers to the filament, where NCED transcript and protein expression were highest. Sections were either imaged unstained using UV autofluorescence or stained with 0.05% toluidine blue. For scanning electron microscopy, flowers were cut and immersed in 70% ethanol for 2 h. Flowers were then dehydrated to 100% ethanol, critical point dried, and viewed with a Zeiss EVO LS15 scanning electron microscope.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Alignment of the three types of PCR fragment sequences obtained for the *TaABA8′OH1* gene, and specific primers designed for PCR of the A, B, and D genome copies of *TaABA8′OH1*.

Supplemental Figure S2. Full-length gene sequence of *TaABA8′OH1* (D genome sequence).

Supplemental Figure S3. Amino acid sequence alignment and phylogenetic analysis of candidate wheat genes encoding NCED, ZEP, and ABA 8′-hydroxylase.

Supplemental Figure S4. Expression pattern of the *OsNCED3* promoter using the GUS (A–C) and CFP (D–F) reporter genes.

Supplemental Figure S5. In situ hybridization using the wheat *TaNCED1* gene.

Supplemental Figure S6. Staining of starch in transgenic rice plants expressing the wheat *TaABA8′OH1* gene under the control of the rice *OsNCED3* promoter (A–C) and the rice *OsG6B* promoter (D–F).

Supplemental Table S1. Primers used in RT-PCR gene expression studies.

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