A Putative Hydroxysteroid Dehydrogenase Involved in Regulating Plant Growth and Development[1]

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We have functionally characterized an Arabidopsis (*Arabidopsis thaliana*) gene At*HSD1* (At5g50600) that encodes a protein with homology to animal 11-β-hydroxysteroid dehydrogenase (HSD). Transgenic Arabidopsis plants overexpressing At*HSD1* (designated AOHSD plants) under the control of the cauliflower mosaic virus 35S promoter showed increased growth and seed yield as well as increased tolerance of saline stress and reduced seed dormancy. In canola (*Brassica napus*), transgenic plants overexpressing At*HSD1* also outgrew wild-type plants. AOHSD phenotypes were similar to those of plants that overproduced brassinosteroids (BRs) or overexpressed the BR receptor gene *BRI1*. A loss-of-function *hsd* mutant produced by RNA interference displayed a semidwarfed phenotype with reduced sensitivity to BRs. In contrast, AOHSD plants were hypersensitive to BRs and exhibited increased catabolism of abscisic acid (ABA). Germination of AOHSD seeds was less sensitive to ABA, while *hsd* seed was more sensitive to ABA during germination. At*HSD* transcription was rapidly induced by BR treatment in wild type and was expressed widely in aerial plant parts, especially vascular tissues. This study demonstrates that At*HSD1* is involved in regulating growth and development in plants and is likely to promote or mediate BR effects. The gene has significant potential for improving growth and yield of canola and other agricultural crops.

Glucocorticoids are a class of animal steroid hormones involved in metabolic, inflammatory, cardiovascular, and behavioral processes as well as response to stress (Tomlinson et al., 2004). The enzyme 11-β-hydroxysteroid dehydrogenase (HSD) is a key regulator of the level of glucocorticoids and catalyzes the interconversion of biologically active glucocorticoid (cortisol in human and corticosterone in rats and mice) and inactive glucocorticoid (cortisone and 11-dehydrocorticosterone; Kallberg et al., 2002; McCormick et al., 2006). Although 11-hydroxy steroids have not (to our knowledge) been identified in plants, genome sequence annotation has identified eight HSD-like genes in Arabidopsis (*Arabidopsis thaliana*) whose functions have not been fully characterized.

We initially identified canola (*Brassica napus*) *HSD* as being highly expressed in nongerminating, abscisic acid (ABA) analog-treated seed relative to germinating seed (Li et al., 2005). It has been established that *HSD*s are minor components of oil bodies in the oilseed Brassicaceae and in sesame (*Sesamum indicum*; Jolivet et al., 2004; d'Andrea et al., 2007). More recently, the protein encoded by *HSD1* has been shown to exhibit the properties of an NADP-dependent 11β-, 17β-HSD/17β-ketosteroid reductase (d'Andrea et al., 2007). However, their true substrates and physiological role in oil bodies remain unclear.

Brassinosteroids (BRs) are powerful plant hormones involved in vascular differentiation, seed germination, and vegetative growth (Sasse, 2003). They regulate some growth-specific processes including photomorphogenesis and skotomorphogenesis as well as cell expansion in the presence of a potentially growth-limiting cell wall (Clouse, 2002), and BR-deficient mutants display extreme dwarf phenotypes (Choe et al., 1999; Noguchi et al., 1999). BR biosynthetic pathways have been characterized and many of the genes and proteins involved in the known pathways have been identified (Fujioka and Yokota, 2003).

The use of Arabidopsis mutants that are insensitive to BR resulted in the identification of several components of the BR signaling pathway. The BR receptor *BR-INSENSITIVE1* (*BRI1*) encodes a plasma membrane localized Leu-rich repeat kinase with an extracellular domain that binds brassinolide (BL), the most physiologically active BR. Binding of BL to BRI1 results in phosphorylation of the kinase domain that activates the BRI1 protein leading to BR responses (Friedrichsen et al., 2000; Wang et al., 2001). *BRI1* is expressed in various tissues and cells involved in cell elongation (Caño-Delgado

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et al., 2004). Recent studies have revealed another Leu-rich repeat receptor-like kinase *BRI1-ASSOCIATED RECEPTOR KINASE1* (*BAK1*) that interacts with *BRI1* and regulates BR signaling (Li et al., 2002; Nam and Li, 2002).

Loss-of-function genetic screens have identified additional BR signaling components downstream of *BRI1* and *BAK1*. These include *bri2*, a negative regulator of the BR pathway, which displays *bri1*-like phenotypes including dwarfism and BR-insensitive and ABA-hypersensitive responses (Li et al., 2001). Both *bri1 ems suppressor1-D* (*bes1*) and *brassinazole resistant1-1D* can rescue the phenotypes of weak *bri1* mutants, and are insensitive to the BR biosynthesis inhibitor brassinazole (BRZ; Asami et al., 2000).

Evidence has accumulated of cross talk between BRs and other hormones including auxin, ABA, jasmonic acid, and ethylene (Krishna, 2003; Sasse, 2003). Of these, the antagonistic interaction between ABA and BR has been explored in a couple of systems at the level of gene expression (Sasse, 2003). The opposing actions of the two hormones can be rationalized insofar as many of the overall effects of both hormones are opposite. In general, BR effects lead to increased growth whereas ABA effects result in slower growth. However, there is an important exception to these opposing effects since both ABA and BR enhance abiotic stress tolerance (Krishna, 2003). Therefore the interaction between these two hormones is complex: at times synergistic and at other times antagonistic. In this context, it is of interest to see if the effects of BRs include causing changes in ABA metabolism.

In this study, we report on an *HSD*-like gene from Arabidopsis referred to as At*HSD1*. Plants overexpressing At*HSD1* constitutively expressed BR response genes and displayed similar phenotypes to those overproducing BRs or *BRI1*.

RESULTS

Structural Analysis of AtHSD1

We studied an Arabidopsis gene that is annotated by the Munich Information Centre for Protein Sequences Arabidopsis Genome Database as a homolog of human and animal 11β-HSD, an enzyme that plays an important role in steroid metabolism (Tomlinson et al., 2004; McCormick et al., 2006). We provisionally refer to this gene as At*HSD1*. A BLASTN search against the Arabidopsis genome database showed that there are two copies of At*HSD1* in the genome (Arabidopsis Genome Initiative locus At5g50600 and At5g50700, respectively). The AtHSD1 protein belongs to the short chain dehydrogenase/reductase family most of which are known to be NAD- or NADP-dependent oxidoreductases (Kallberg et al., 2002). The At*HSD1* gene consists of six exons and five introns and encodes a protein of 389 amino acid residues (Fig. 1A) with a calculated molecular mass of 39.0 kD and an pI of 6.15. Structural analysis revealed the presence of an N-terminal transmembrane region (amino acids 7–29). The sequence of the N-terminal region of the AtHSD1 protein to amino acid 233 shows high homology to human and mouse HSD proteins (Fig. 1B), whereas the C-terminal sequence (amino acids 234–349) diverges from that of the animal HSDs. In total, there are two identical copies of At*HSD1* and six other homologs (At*HSD2–7*): At3g47350, At3g47360, At5g50590, At4g10020, At5g50770, and At5g50690 that, respectively, show 46%, 45%, 49%, 45%, 56%, and 46% sequence identity with At*HSD1*.

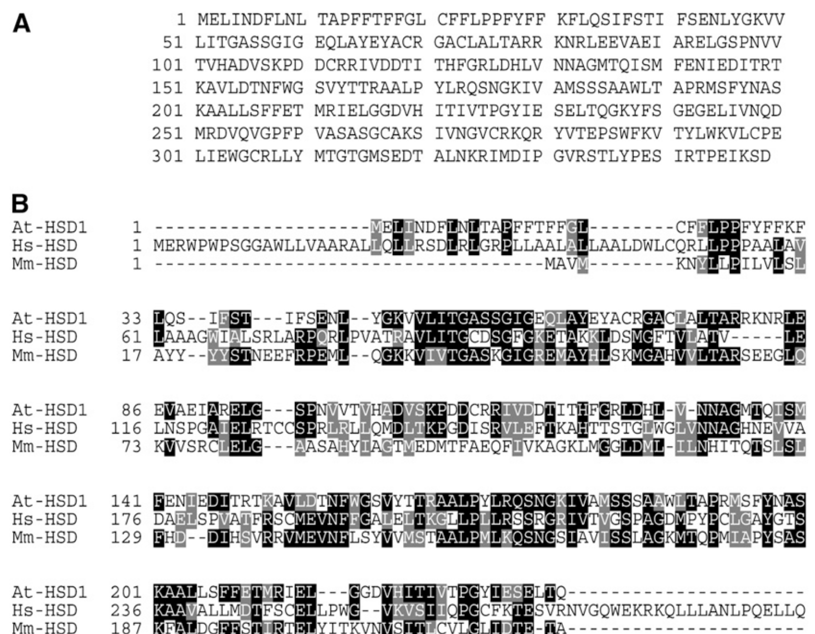

Figure 1. Sequence analysis of At*HSD1*. A, AtHSD1 amino acid sequence. B, Sequence alignments of Arabidopsis HSD-like (AtHSD1), human HSD (Hs-HSD), and mouse HSD (Ms-HSD) amino acid sequences.

Phenotypes Resulting from Constitutive Overexpression of At*HSD1* Are Similar to Those Produced by Overproducing BRs or *BRI* Genes

The At*HSD1* cDNA was fused to the cauliflower mosaic virus 35S promoter to drive high levels of gene expression in vegetative tissues. After transformation and selection, 42 kanamycin-resistant T1 transgenic plants were obtained. Reverse transcription (RT)-PCR analysis confirmed higher At*HSD1* mRNA level in T3 plants of four transgenic lines overexpressing At*HSD* (AOHSD lines) relative to wild type (Fig. 2A).

The effects of At*HSD1* overexpression on growth of AOHSD plants were monitored during plant development. The number of rosette leaves, flowering time, and flower morphology were not significantly different between wild-type and transgenic plants. However, as shown in Figure 2, B and C, the AOHSD lines outgrew the wild type and mature AOHSD plants were reproducibly approximately 20% taller (43.0 ± 1.4 cm) than wild-type plants (35.8 ± 1.0 cm). Stem diameter was consistently larger in transgenics than in wild type, and the number of branches and siliques were also greater in transgenics than in wild-type plants (Fig. 2C), leading to a doubling of seed weight per plant in two transgenic lines relative to wild type (Fig. 2F). The higher seed yield was due to an increased number of siliques per plant and seed size was not significantly increased (data not shown). The roots of AOHSD plants grown on Murashige and Skoog medium for 7 d were 23% longer (4.8 ± 0.5 cm) than those of wild-type plants (3.9 ± 0.4 cm). Similarly, plants overexpressing At*HSD1* in canola (BOHSD plants) displayed increased growth from the seedling stage to flowering (Fig. 2, D and E). Increased stem diameter was also observed in BOHSD plants relative to wild type. In three transgenic lines (T1 generation), average diameters after 68 d of plant growth (measured just above soil level) were 113%, 127%, and 152% of stems in untransformed controls (average diameter of controls was 0.82 cm).

Overall, these results are similar to the phenotypes produced by overexpression of *DWARF4*, which encodes a steroid 22α hydroxylase involved in BR biosynthesis (Choe et al., 2001) and overexpression of *BRI1* (Nam and Li, 2002). Similar phenotypes were produced by treating untransformed Arabidopsis plants with exogenous BL, whereas treatment with exogenous gibberellin (GA) produced elongated internodes, leading to a quite different leggy morphology (data not shown) similar to that produced by transgenic GA overproduction (Coles et al., 1999).

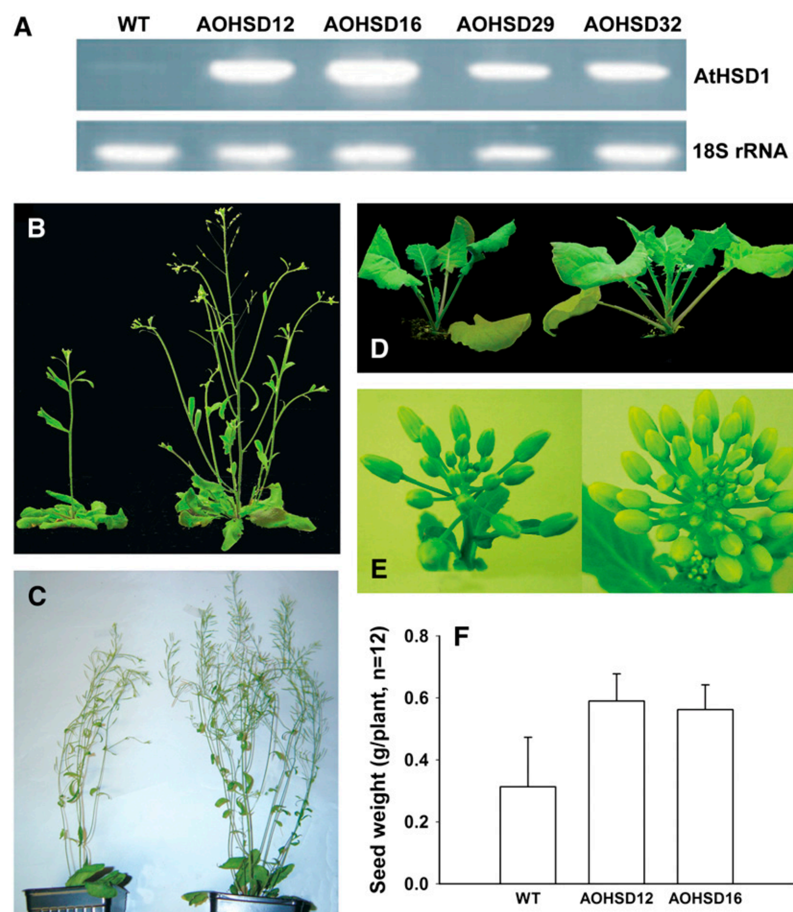

Figure 2. Expression of At*HSD1* and visible phenotypes in wild-type, AOHSD, and BOHSD plants. A, RT-PCR analysis of At*HSD1* transcript levels in Arabidopsis wild-type and overexpression lines AOHSD12, 16, 29, and 32. B, Arabidopsis plants after 1 month: AOHSD (right) and wild type (left). C, Arabidopsis plants after 2 months: AOHSD (right) and wild type (left). D, Canola plants after 1 month: wild type (left) and BOHSD (right). E, Comparison of 6-week-old wild-type (left) and BOHSD2 (right) inflorescences. F, Seed weights per plant of wild type and AOHSD16, $n = 12$.

Seed Dormancy Is Reduced in Overexpression Lines

Arabidopsis seeds exhibit primary dormancy (Bentsink and Koornneef, 2002), which means that mature seeds are unable to germinate under the appropriate, permissive environmental conditions. To test whether At*HSD1* overexpression affected seed dormancy, we compared the germination of seeds under different conditions. Overall, transgenic seeds exhibited reduced dormancy compared with the wild type. In the absence of stratification at 4°C, the germination of AOHSD seeds was faster than that of wild type, at 2 d after imbibition reaching 80% to 90% compared to 30% germination of wild type (Fig. 3A). Primary dormancy of wild-type seeds is highest at harvest and decreases during storage (Fig. 3C). The difference between transgenic and wild-type seeds was even more striking when freshly harvested seeds were employed, reaching more than 95% germination in AOHSD seeds compared to less than 10% of wild-type seeds (Fig. 3, B and C). However, the germination of freshly harvested seeds is dependent on light and when seeds were incubated in darkness, almost no germination occurred either in wild-type or transgenic seeds. A 4-d cold treatment (stratification) eliminated primary dormancy in wild-type seeds and the requirement for light. A brief (1-d) cold treatment revealed that transgenic seeds are more sensitive to stratification than wild-type seeds. The cold treatment made little difference to germination of wild-type seeds but significantly increased germination in both transgenic lines (Fig. 3D). Steber and McCourt (2001) reported that BR promotes seed germination and is needed for normal germination in Arabidopsis. Overexpression of At*HSD1* promoted seed germination, possibly by enhancing the effects of endogenous BRs or by leading to elevated BR concentrations.

BRZ blocks BR biosynthesis at the C-22 hydroxylation step (Asami et al., 2000) and was used to test whether At*HSD1* effects require BRs. Figure 4 showed that both AOHSD and wild-type seedlings were sensitive to BRZ under both light and dark. Therefore, At*HSD1* effects depend on the presence of BRs and At*HSD1* overexpression appears to increase the sensitivity of plants to BRs.

Loss-of-Function Plants Show Semidwarfed Phenotype and Insensitivity to BL

There were no apparent phenotypes in At*HSD1* T-DNA knockout lines, probably because phenotypic effects were masked by gene redundancy. To produce plants with reduced At*HSD* gene expression, an At*HSD1* RNA interference (RNAi) transformation was conducted. Six out of 15 transgenic lines displayed a semidwarf phenotype with slightly darker green, wider leaves, and shorter petioles than wild-type plants and typical examples are shown in Figure 5, A and B. Semiquantitative PCR showed At*HSD1* expression was substantially reduced in a representative RNAi plant relative to wild type (Fig. 5C), and transcripts of other gene family members were also reduced in RNAi plants (data not shown). The phenotypes produced by the RNAi lines are similar to those exhibited by *bes1*, which contains a defective transcription factor mediating

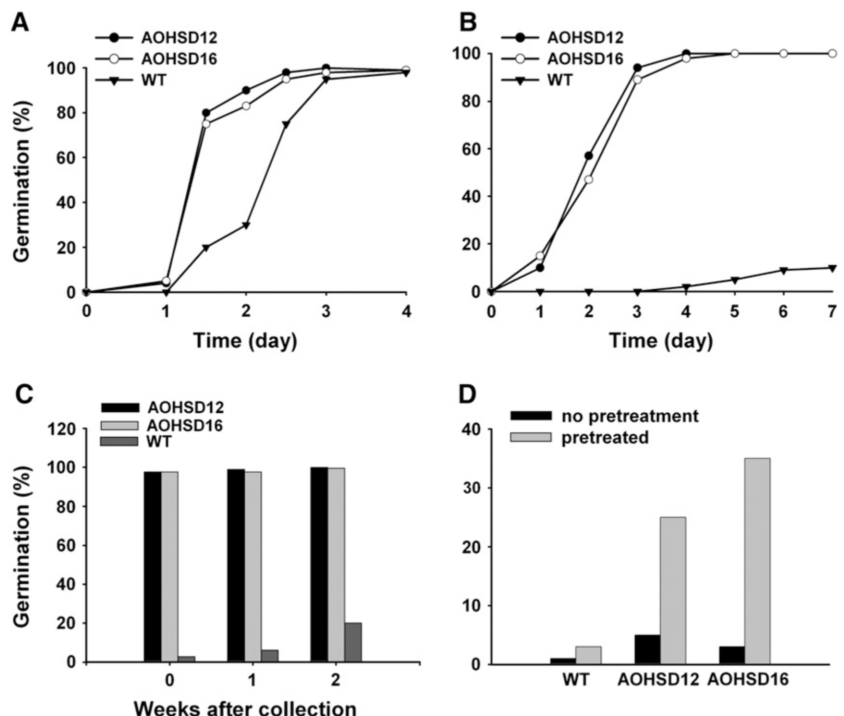

Figure 3. Reduced dormancy in AOHSD plants. A, Germination time course for seeds of two AOHSD lines and wild type stored for 4 weeks at room temperature without stratification at 4°C. Germination was scored after 7 d of incubation. B, Germination time course for freshly harvested seed of two AOHSD lines and wild type. Germination was scored after 7 d incubation. C, Germination of seeds from two AOHSD lines and wild type after varying times of storage. Germination was scored after 7 d incubation. D, Germination of two AOHSD lines and wild-type freshly harvested seeds in darkness with or without a 1 d pretreatment at 4°C. Germination was scored after 7 d of incubation.

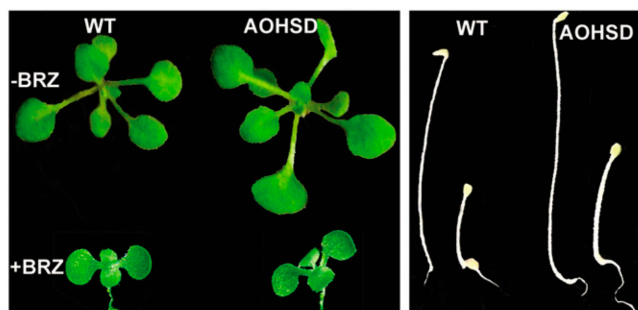

Figure 4. Transgenic plants expressing P_{35S}:At*HSD1* are sensitive to the BR biosynthesis inhibitor BRZ under light (left) and dark (right). Three-week-old, light-grown seedlings of wild type and transgenics on Murashige and Skoog medium with or without 1 μM BRZ, and 4-d-old dark-grown seedlings of wild type and transgene with (right) or without (left) 1 μM BRZ.

BR-regulated gene expressions (Yin et al., 2005). The responsiveness of various genotypes to exogenously applied BR is shown in Figure 5D. A representative AOHSD line exhibited enhanced BR sensitivity relative to wild type, whereas a typical At*HSD1* RNAi line (*hsd*) exhibited reduced BR sensitivity compared with wild-type plants. For example, although primary roots of untreated AOHSD plants were longer than those of wild-type plants, they were shorter than wild-type plants at BL concentrations of 10 nM and above. Therefore, expression of At*HSD* is directly related to the responsiveness of plants to BR.

At*HSD* Gene Expression Affects Sensitivity to ABA and ABA Metabolism

ABA and BRs have been shown to act antagonistically (Mandava, 1988). Therefore, to determine the relationship between At*HSD* gene expression and ABA, we measured the germination of seeds on media containing increasing concentrations of (+)-ABA. The results indicated that AOHSD seeds have greatly reduced sensitivity to ABA in germination compared with wild-type Columbia (Col). Wild-type seed germination was almost completely inhibited at 0.5 μM (+)-ABA, whereas transgenic seed germination was not totally blocked even at 100 μM (+)-ABA (Fig. 6A). After germination, AOHSD seedlings grew well on medium containing up to 10 μM (+)-ABA, whereas wild-type Col seedlings failed to grow on medium containing 0.5 μM (+)-ABA (data not shown). Seeds from a typical *hsd* (RNAi) line were more sensitive to ABA than wild-type seeds; *hsd* seed germination was strongly inhibited at 0.05 μM (+)-ABA, whereas wild-type seed germination reached 100% (Fig. 6A). The germination of both the BR biosynthetic mutant *det2-1* and the BR-insensitive mutant *bri1-1* is more sensitive to ABA than the wild type (Steber and McCourt, 2001).

To investigate the relationship between ABA metabolism and the ABA insensitivity of AOHSD lines, we profiled ABA and its metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry (Feurtado et al., 2004). There are several pathways of ABA catabolism involving either hydroxylation or conjugation. In most cases, the predominant pathway of ABA catabolism is via hydroxylation at the 8′ position to yield phaseic acid (PA), which is then reduced to dihydrophaseic acid (Zaharia et al., 2005).

As expected (Fig. 6B), the ABA content of freshly harvested wild-type seeds (292 ng/g dry weight) is more than that of seeds stored 4 weeks at room temperature (168 ng/g dry weight), consistent with increased germination after storage (Fig. 3C). The ABA contents of both freshly harvested and stored seeds of AOHSD lines (326 and 344 ng/g dry weight, respectively) are a little higher than in wild-type seeds. After 2 d treatment of stored wild-type seeds with 2 and 10 μM (+)-ABA, the tissue ABA levels (1,360 and 6,570 ng/g dry weight,

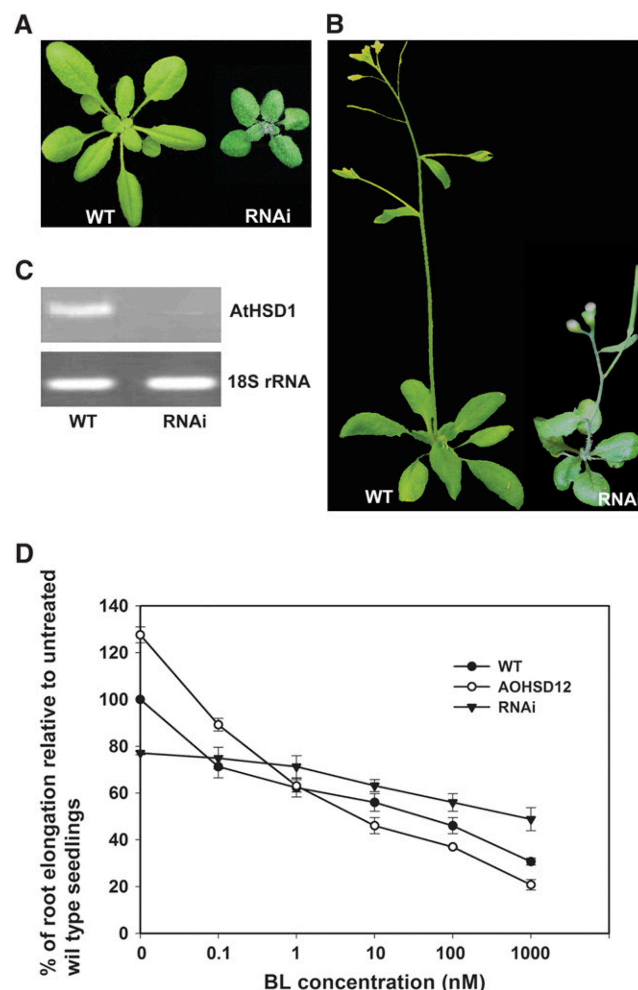

Figure 5. A representative plant expressing At*HSD1* RNAi (*hsd*) shows a semidwarfed phenotype and reduced sensitivity to BRs. A, Three-week-old wild-type and *hsd* plants grown in soil. B, Five-week-old wild-type and *hsd* plants grown in soil. C, RT-PCR analysis of At*HSD1* expression in wild-type and *hsd* plants. D, Root length grown on media containing different BL concentration. Each measurement is the average of 15 roots. The measurements were taken 7 d after germination.

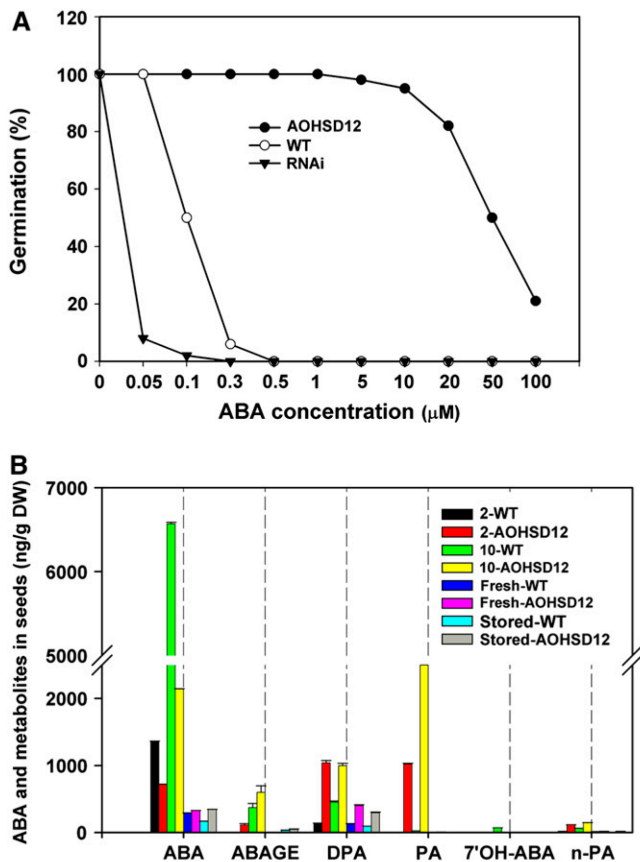

Figure 6. Effects of (+)-ABA treatments on germination of Arabidopsis seeds and ABA contents under different conditions. A, Comparison of germination in Col wild-type (○), AOHSD (●), and *hsd* (RNAi; ▼) seeds. Percent germination was determined from a minimum of 120 seeds. B, Endogenous ABA and its metabolites in AOHSD and wild-type seeds under different conditions. Fresh, Freshly isolated seed; Stored, seed stored for 4 weeks at room temperature; 2-, stored seed treated with 2 μM (+)-ABA for 2 d; 10-, stored seed treated with 10 μM (+)-ABA for 2 d; ABA-GE, ABA-Glc ester; DPA, dihydrophaseic acid; 7′-OH-ABA, 7′-hydroxy ABA; n-PA, neo-PA.

respectively) were much higher than in untreated wild-type seeds (168 ng/g dry weight). In contrast, the ABA levels in AOHSD seeds were lower after ABA treatments (717 ng/g dry weight in the 2 μM treatment and 2,140 ng/g dry weight in the 10 μM ABA treatment) than in wild-type seeds, but the levels of all ABA metabolites were higher than in wild-type seeds (Fig. 6B). For example, PA only significantly accumulated in ABA-treated AOHSD seed. The elevated presence of ABA catabolites and reduced ABA content in AOHSD seed relative to wild type are consistent with increased flux through the ABA metabolic pathway.

Wild-type seeds did not germinate after ABA treatment (Fig. 6A) or if they were untreated but freshly harvested (Fig. 3B). However, AOHSD seeds can germinate and grow well despite the presence of high concentrations of applied ABA (Fig. 6A). The results described above suggest that AOHSD seeds have a higher capacity for ABA metabolism that is manifested in greater catabolism of exogenously applied ABA. However, despite increased ABA catabolism in AOHSD seeds, it is noteworthy that ABA nonetheless accumulates to high levels (e.g. to 2,140 ng/g dry weight in AOHSD seeds treated with 10 μM ABA) but the seeds nonetheless germinate readily. Therefore, the transgenic seeds demonstrate markedly reduced sensitivity to ABA.

At*HSD1* Is Induced by BR

To determine if At*HSD1* is responsive to BL, the most active BR, a time course of BL treatment was performed. Induction of At*HSD1* occurred rapidly with a maximum around 2 h. Treatment with other plant hormones, such as ABA and GA_3, did not significantly induce gene expression (Fig. 7A). On the other hand, At*HSD1* expression was severely reduced in all BR-deficient mutants tested, such as *cbb1*, *cbb3*, and *det2* (Fig. 7B), suggesting that At*HSD1* was induced specifically by BRs during plant growth. To further test the genetic relationship between At*HSD1* and BR perception, we treated *bri1* seedlings with BL. The results showed that the induction of At*HSD1* was reduced in *bri1* (Fig. 7C), suggesting that At*HSD1* transcription is dependent on BR perception.

Differential Expression of Genes in Overexpression Plants

To examine the effects of At*HSD1* gene expression on other genes, expression profiling was conducted using Arabidopsis cDNA microarrays. Comparisons were made between 4-week-old seedlings of wild-type (Col) and AOHSD lines (Table I) and the experiments were repeated four times. Statistical analysis using Significant

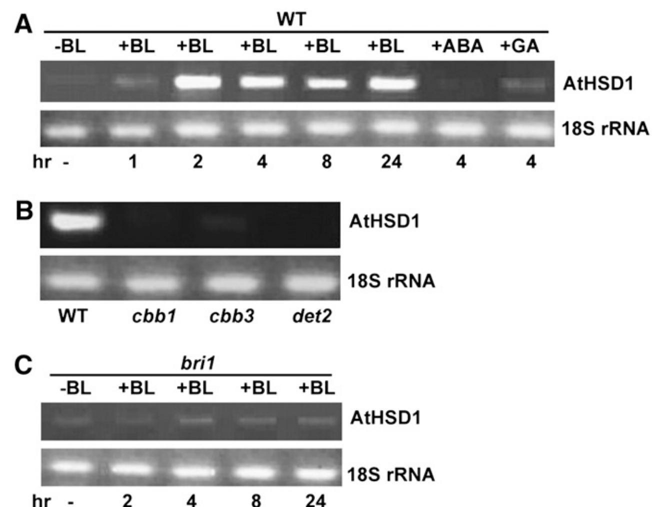

Figure 7. At*HSD1* expression is induced by BL. A, BL-induced accumulation of At*HSD1* mRNA in wild-type plants. B, Low expression of At*HSD1* in BR-deficient mutants *cbb1*, *cbb3*, and *det2*. C, BL-induced accumulation of At*HSD1* mRNA is reduced in the *bri1* background.

Table I. *Genes up-regulated in AOHSD transgenic plants relative to wild type*

Locus	Annotation	GenBank Accession
At1g12080	Expressed protein	AY140106
At5g13420	Transaldolase, putative	NM_121345
At2g45180	Protease inhibitor/seed storage/lipid transfer protein family	NM_129813
At2g42520	DEAD box RNA helicase	NM_001035807
At4g30960	CBL-interacting protein	AF436831
At2g34430	Chlorophyll A-B binding protein/LHCII type I	AF339687
At3g47470	Chlorophyll A-B binding protein 4, LHCI type III CAB-4	AF325012
At5g54270	Chlorophyll A-B binding protein/LHCII type III (LHCB3)	AF361858
At1g29910	Chlorophyll A-B binding protein 2, LHCII type I CAB-2	AY065165
At5g54280	Myosin heavy chain	NM_124808
At3g08640	Alphavirus core protein family	AY081327
At2g30820	Expressed protein	NM_128635
At3g56510	TBP-binding protein	NM_115509
At2g34420	Chlorophyll A-B binding protein/LHCII type I	AF419587
At3g33530	Transducin family protein	NM_114071
At4g27440	Protochlorophyllide reductase B	AY081465
At1g15820	Chlorophyll A-B binding protein, chloroplast (LHCB6)	AF332425
At5g02160	Expressed protein	AF325034
At2g45010	Expressed protein	AF327424
At1g45201	Lipase class 3 family protein	NM_202246
At5g05070	Chlorophyll A-B binding protein/LHCII type II (LHCB2.2)	NM_120589
At4g38770	Proline-rich family protein	AY092992
At4g39660	Alanine-glyoxylate aminotransferase, putative	NM_120126
At5g35735	Auxin-induced protein	AW784033
At4g24050	Short-chain dehydrogenase/reductase (SDR) family protein	AF439829
At4g35160	*O*-methyltransferase family 2 protein	AY099803
At1g03400	Ethylene synthesis regulatory protein E8, putative	AY133559
At1g48690	Auxin-responsive GH3	NM_103764
At1g04240	Auxin-responsive protein	AF332393
At5g04960	Pectinesterase family protein	NM_120578
At3g16770	AP2 domain-containing protein	AY142562
At1g74420	Xyloglucan fucosyltransferase, putative (FUT3)	AF417473
At5g19510	Elongation factor 1B	AF360304
At5g50700	Short-chain dehydrogenase/reductase (SDR) family protein	AF446888
At2g14900	GA-regulated protein 1 (GASA1)	AW985337
At2g40890	Cytochrome P450 98A3, putative	NM_180006
At1g20620	Catalase 3 (SEN2)	NM_001035995
At5g19770	Tubulin α-3/α-5 chain (TUA3)	NM_121982

Analysis of Microarrays (SAM) showed 127 genes to be significantly differentially expressed by more than 2-fold, including 38 up-regulated genes and 89 down-regulated. Among the 38 induced genes, there were several with functions similar to those of known BL-induced genes encoding putative cell elongation or expansion-associated proteins such as pectinesterase and xyloglucan fucosyltransferase. BRs are known to increase expression of many genes involved in cell wall biosynthesis and modification, consistent with their effects on increasing cell expansion and division (Haubrick and Assmann, 2006). Other growth-related genes included elongation factor 1B as well as auxin or GA induced genes (Goda et al., 2002). The auxin-responsive gene *SHY2/IAA3* (At1g04240) regulates auxin responses and such genes are known to also mediate BR responses (Nakamura et al., 2006). There were seven up-regulated genes involved in photosynthesis, which is also consistent with the increased growth of the plants. In addition, a Pro-rich family protein (At4g38770) playing an active role in plant defense reactions (Fowler et al., 1999) was also contained in this group. Some of the up-regulated genes were involved in lipid metabolism and other conversions of primary metabolism. The results suggest that the transgenic plant phenotype of increased growth is consistent with the observed changes in gene expression.

The *BR ENHANCED EXPRESSION3* (*BEE3*) gene encodes a basic helix-loop-helix transcription factor that is induced by BR and is a positive regulator of BR responses. The *bri1 SUPPRESSOR gene* (*BRS*) encodes a secreted Ser carboxypeptidase that acts as a negative regulator of BR action, although its true mode of action is unknown (Haubrick and Assmann, 2006). Expression of both of these genes is significantly reduced in genotypes with defects in BR synthesis or responses (Fig. 8). However, expression of these genes is slightly increased in AOHSD plants and this result, together

with the increased expression of BR-related genes in Table I, suggests elevated BR signaling and responses in plants overexpressing *HSD*.

At*HSD1* Expression Is Tissue Specific

An At*HSD1* promoter-reporter gene fusion was employed to study the tissue specificity of At*HSD1* expression. The promoter region of At*HSD1* (approximately 1.5 kb) was isolated from Arabidopsis genomic DNA and fused to the GUS protein coding sequence (P_{HSD1}:*GUS*). Histochemical analysis of GUS activity in transgenic plants harboring P_{HSD1}:*GUS* showed a high level of expression in the above-ground parts of seedlings, and weak expression in root tissues in both light and darkness (Figs. 9, A–D and 8H). P_{HSD1}:*GUS* was strongly expressed in vascular tissues (Fig. 9E) and this is consistent with the known involvement of BRs in vascular differentiation (Sasse, 2003). The vascular localization of expression may be related to our observation (noted earlier) that stem diameter was consistently higher in transgenic plants than in wild type. GUS activity was also observed in the bud and silique pedicels (Fig. 9, F and G).

Increased Stress Tolerance of AOHSD and BOHSD Plants

The abiotic stress tolerance of plants overexpressing P_{35S}:*HSD* were assessed by their ability to tolerate a saline growth medium. Wild-type Arabidopsis plants died after application of 300 mM NaCl, whereas transgenic plants were healthy and unbleached at 400 mM (Fig. 10A), although their rate of growth was reduced relative to untreated material. The effect of salt stress on seedling vigor was also assessed. Both wild-type and transgenic seeds of both Arabidopsis and canola were germinated on medium containing 100 mM NaCl and after 2 weeks a significant number of wild-type seedlings had died, whereas only a few of the transgenic seedlings failed to grow. Furthermore, wild-type seedlings were stunted and discolored, whereas transgenic seeds appeared healthy (Fig. 10B).

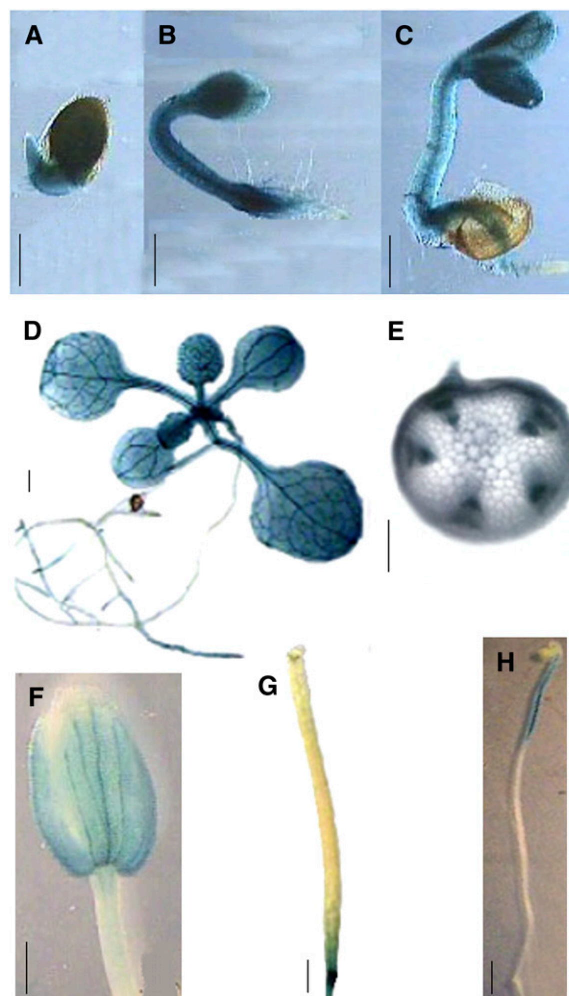

Figure 9. Expression of P_{HSD1}:*GUS* in various tissues of transgenic plants. GUS activity was detected in germinating seeds at 1 (A), 2 (B), and 4 d (C) poststratification, Roots, cotyledons, and apical meristem (D), vascular tissues (E), petal (F), base of silique (G), and seedling growing under darkness (H).

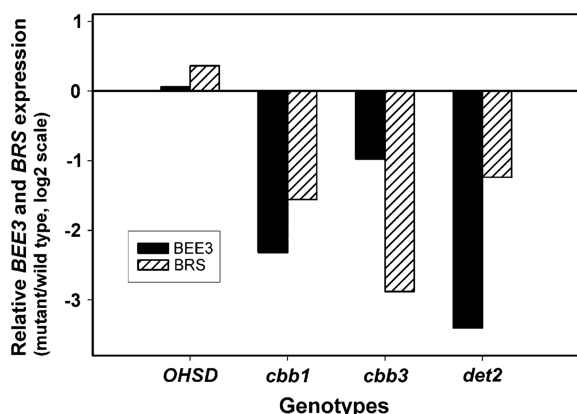

Figure 8. Expression of *BEE3* and *BRS* in various genotypes. Real-time PCR was used to compare the expression of *BEE3* and *BRS* in OHSD plants and in mutants defective in BR biosynthesis or signaling.

DISCUSSION

We have characterized the functional effects of a gene with homology to animal 11β-HSD. Although 11-hydroxysteroids are important in regulating growth and development in animals, they have not been found in plants so the possible enzymatic functions of plant HSDs are uncertain. Enzyme assays of AtHSD have demonstrated that it possesses 11β- and 17β-HSD activity (d'Andrea et al., 2007) but its true substrate(s) is/are unknown. Our data suggest that At*HSD* produces BR-like effects such as increased growth, branching, and flower production as well as thicker stems and

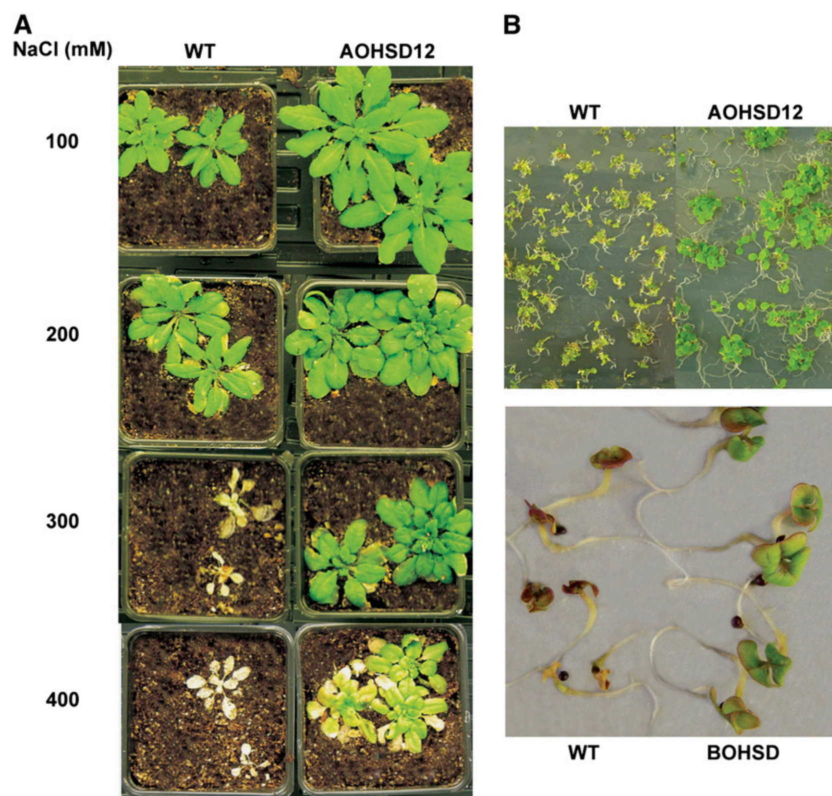

Figure 10. Effect of salt stress on survival of plants. A, Two-week-old plants were flooded once a week for 4 weeks with 100, 200, 300, and 400 mM of NaCl. B, Seedlings of Arabidopsis and canola were germinated and grown for 2 weeks on agar medium supplemented with 100 mM NaCl.

increased stress tolerance. In addition, expression of the native *HSD* gene was induced by BL. Although GAs are also associated with rapid growth and increased height (e.g. Coles et al., 1999), we observed that exogenous application of GA produced a more elongated type of growth than that produced in AOHSD and BOHSD plants or by application of BL to control plants. Furthermore, treatment of plants with GAs or transgenic overproduction of GAs has not, to our knowledge, been associated with increased stress tolerance. On the other hand, the increased ABA catabolism and reduced sensitivity to ABA that we measured in AOHSD plants cannot account for the increased growth observed in OHSD plants and for their enhanced stress tolerance. Typically, ABA-deficient and ABA-insensitive mutants grow no better than wild type and are susceptible to water loss (e.g. Leon-Kloosterziel et al., 1996). Conversely, although ABA-hypersensitive mutants are more tolerant of drought stress, they typically grow slowly (e.g. Pei et al., 1998).

However, the role of HSD in relation to BR action has not been established. One possibility is that AtHSD is responsible for catalyzing a step in the biosynthesis of these hormones. However, the possibility that AtHSD functions in BR signaling cannot be excluded. For example, the fact that OHSD plants are hypersensitive to BR and that *hsd* (RNAi) plants are relatively insensitive to BR (Fig. 5) is more consistent with a role in mediating responses to BR than in controlling levels of BR.

Previous reports have suggested a reciprocal relationship between BR and ABA. The experiments reported here also provide examples of this phenomenon. AOHSD plants are hypersensitive to BR and insensitive to ABA and conversely, RNAi plants are insensitive to BR. We also show that enhanced BR effects in AOHSD seeds are associated with a greater ability to catabolize exogenous ABA, suggesting either faster ABA metabolic flux or at least a higher capacity for ABA metabolism. The antagonistic relationship between ABA and BR raises the puzzling question of how BR-like effects of *HSD* can be associated with enhanced stress tolerance (which is promoted by ABA) as observed in this study (Fig. 9) and in previous ones (Krishna, 2003). In this context, we have noted previously that a canola homolog of *HSD* was highly expressed in nongerminating, ABA analog-treated seed relative to germinating seed (Li et al., 2005), which is consistent with its known role as a component of oil bodies (Jolivet et al., 2004; d'Andrea et al., 2007) that are degraded during germination. However, these facts are difficult to reconcile with our observation that overexpression of At*HSD* promotes germination. Indeed, there is no apparent relationship between the involvement of *HSD* in oil bodies and its role in promoting BR-mediated vegetative growth.

The interaction between ABA and BR is clearly complex: partially antagonistic and partially synergistic. A full understanding of the role that *HSD* plays in this hormonal interaction will require further experiments to define its mode of action. For example, analysis of the effects of *HSD* expression on active BR levels will be required to characterize the relationship of *HSD* to

BR biosynthesis. Genetic experiments to determine whether *HSD* overexpression complements BR-deficient and/or BR-insensitive mutants are also required. However, irrespective of its precise mode of action, At*HSD* is clearly an important contributor to plant growth and development and provides the potential for producing increased yield and stress tolerance in crop plants.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 was used as the wild type, and Arabidopsis transgenic plants were transformed with *Agrobacterium tumefaciens* GV3101 using the floral dip method (Clough and Bent, 1998). Canola (*Brassica napus*) was transformed by the method of Cardoza and Stewart (2003). To examine the growth of seedlings, Arabidopsis and canola seeds were surface sterilized with 10% bleach solution for 5 min and washed three times with sterile water. The sterilized seeds were sown in 9 cm petri dishes on Murashige and Skoog medium containing 1% (w/v) agar. Ten days after germination, seedlings with similar sizes were transferred to soil and grown in a growth chamber under long-day growth conditions (16-h light followed by 8-h darkness) at 20°C ± 2°C. To measure germination, sterilized seeds were incubated on Murashige and Skoog medium under continuous light at 23°C. Germination was scored after 7 d of incubation unless otherwise noted.

The *cbb1* and *cbb3* mutant seeds were kindly provided by Dr. Carsten Müssig, and the *det2* mutant seeds by Dr. Jianming Li. *bri1* (*cbb2*, CS292) mutant seeds were obtained from the Arabidopsis Biological Resource Center. For the mRNA analysis following various hormone treatments, 4-week-old wild-type or mutant plants were sprayed with 1 μM BL (OlChemIm Ltd., http://www.olchemim.cz/INDEX_e.HTM), 100 μM (+)-ABA, or 5 μM GA (GA_{4+7}), respectively, and incubated for varying times. For (+)-ABA dose response curves, seeds (>120) were germinated and grown in light on Murashige and Skoog medium containing varying concentrations of (+)-ABA and germination was scored after 7 d. The seeds were treated for 2 d on Murashige and Skoog medium containing 2 and 10 μM (+)-ABA, respectively, then collected, washed, and frozen in liquid nitrogen.

Isolation of At*HSD1* cDNA and Constructs

A genome database search resulted in the identification of a genomic sequence encoding a protein that shares high homology with human and animal HSD, especially in the N-terminal region. We named this gene At*HSD1*. The coding region of At*HSD1* gene was generated by PCR amplification of plasmid Uvi51 containing full coding sequence of this gene, the primers 5′-**GGGGACAAGTTTGTACAAAAAAGCAGGCTATGG**AGTTGATAAACGACTTTCTC-3′ and 5′-**GGGGACCACTTTGTACAAGAAAGCTGGGT**CTAATCCGACTTGATTTCTGGAGT-3′ (attB sites for recombination cloning are shown in bold, and the sequence corresponding to AtHSD1 is underlined) were used for PCR. For generating At*HSD1* overexpression lines, the PCR product was introduced into the binary vector pK7WG2 (Karimi et al., 2002). The insert in the construct was sequenced to confirm the orientation and sequence and then transformed to Arabidopsis and canola. For At*HSD1* RNAi, an At*HSD1* full-length cDNA was amplified and cloned into pK7GWIWG2(I) in antisense/sense (Karimi et al., 2002).

Construction and Expression of Promoter-Reporter Gene

The region of At*HSD1* from −1,534 to +75 bp was generated by PCR amplification of genomic DNA using primers 5′-**GGGGACAAGTTTGTACAAAAAAGCAGGCTATGG**CAATGGAACCGAAAGCCTAA-3′ and 5′-**GGGGACCACTTTGTACAAGAAAGCTGGGT**AGGCAAGAAGAAGCAGAGACC-3′ (attB sites for recombination cloning are shown in bold, and the sequence corresponding to At*HSD1* is underlined). The resultant 1,609-bp fragment was subcloned into binary vector pMDC163 (Curtis and Grossniklaus, 2003), and the resulting P_{HSD1}:*GUS* construct was transferred into Arabidopsis (Col).

For GUS staining, seedlings were immersed in 50 mM sodium phosphate buffer (pH 7.2) with 1 mM 5-bromo-4-chloro-3indolyl-D-GlcUA and incubated at 37°C for 12 h. Chlorophyll was extracted by passing through increasing concentrations of ethanol. Nineteen independent T2 transgenic lines were analyzed and commonly observed staining patterns were recorded.

RT-PCR Analysis

Total RNA (2.5 μg) from each sample, treated with RNase-free DNase (Promega), was used for reverse transcriptase reactions. First-strand cDNA was synthesized with random hexamers using a SuperScript first-strand synthesis system according to the manufacturer's instructions (Invitrogen Life Technologies), and QuantumRNA 18S internal standards (Ambion) were used as a positive control for quantification of the relative amounts of cDNA. One microliter of RT reaction mixture was used as a template in a 20 μL PCR. For At*HSD* amplification, the primers HSD1-1 (5′-TGCCAAGTCGATAGTGAACG-3′) and -2 (5′-CAGTAACCGACAACCCCACT-3′) were used for PCR. For amplification of *BRS1*, the primers BRS1-1 (5′-CCAACCAAAAGTGGCATTCT-3′) and BRS-2 (5′-TGTTGATACGAAACGGTCCA-3′) were employed. For amplification of *BEE3*, the primers BEE3-1 (5′-CGACGAGGGAAAATAAACGA-3′) and BEE3-2 (5′-CATGGATTCCACAGCATCAG-3′) were employed. The amplification conditions were 94°C (30 s), 56°C (30 s), and 72°C (30 s) and 28 cycles. RT-PCR was repeated twice.

HPLC-Mass Spectrometry Analyses

HPLC was performed using a Waters 2695 separation module (Waters). The extraction and purification of ABA and its metabolites, HPLC conditions, addition of internal standards, mass spectrometry, and quantification of endogenous levels of compounds were performed as described by Feurtado et al. (2004). Each analysis was performed in triplicate.

Microarray Analysis of Gene Expression Profiling in AOHSD Plants

Four-week-old seedlings of AOHSD and wild-type plants were harvested and frozen quickly in liquid nitrogen. Total RNA was extracted using RNeasy mini kits (Qiagen). Each total RNA sample (50 μg) was converted to cDNA and labeled using the Cyscribe Post-Labeling kit (Amersham Bioscience, RPN5660) following the manufacturer's instructions. The CyScribe GFX Purification kit (Amersham Bioscience, RPN5660X) was used to purify the fluorescently labeled cDNA probe by removing free nucleotides and unincorporated CyDye molecules. Arabidopsis 12 K cDNA microarrays (average length approximately 300 bases) from the Keck Foundation Biotechnology Resource Laboratory at Yale University School of Medicine were used. Hybridized slides were scanned sequentially for Cy3- and Cy5-labeled probes with a ScanArray 4000 laser scanner at a resolution of 10 Am. The experiments were repeated four times. Image analysis and signal quantification were performed using Quantarray (GSI Lumonics). Clones showing a signal value <800 in both Cy3 and Cy5 channels were eliminated from the analysis. The average of the resulting total Cy5 and Cy3 signals were used to calculate the ratios that were used for normalization. Data storage, preliminary data processing, and Lowess normalization were performed with the Bioarray Software Environment (Saal et al., 2002). Background-subtracted clone signals were used to calculate Cy5/Cy3 ratios. For each gene, the statistical significance of differences in expression between the control and AOHSD plants was calculated using SAM (P value ≤ 0.005; Tusher et al., 2001). SAM is a statistical technique for finding significant genes in a set of microarray experiments. SAM computes a statistic for each gene, measuring the strength of the relationship between gene expression and the response variable. It uses repeated permutations of the data to determine if the expression of any genes are significantly related to the response. The threshold for significance is determined by a tuning parameter delta, chosen by the user based on the false-positive rate. Users also have an option to choose a fold-change parameter, to ensure that called genes change at least a prespecified amount. Genes with a ratio of transgenic to wild-type plants of greater than 2.0 or less than 0.5 were selected.

Salt Stress Tolerance

The salt sensitivity of plants was evaluated by growth in pots in a controlled environment chamber. After 2 weeks of growth under normal conditions,

plants were flooded once a week with solutions containing varying NaCl concentrations.

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