J Plant Growth Regul (2010) 29:385–393
DOI 10.1007/s00344-010-9150-3

ORIGINAL ARTICLES

Overexpression of the Brassinosteroid Biosynthetic Gene *AtDWF4* in *Arabidopsis* Seeds Overcomes Abscisic Acid-induced Inhibition of Germination and Increases Cold Tolerance in Transgenic Seedlings

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Received: 21 October 2009 / Accepted: 1 March 2010 / Published online: 3 June 2010

Abstract Brassinosteroids (BRs) are essential for proper plant growth and development and also protect plants from a variety of environmental stresses. Seeds contain relatively high levels of BRs, and BRs have been implicated in embryonic patterning and germination. How BR levels in seeds impact germination, growth, and stress tolerance in early seedlings is currently not known. To assess this, the BR biosynthetic gene *AtDWF4* was overexpressed in *Arabidopsis* under the control of a seed-specific oleosin promoter. The resulting transgenic seedlings could overcome inhibition of germination caused by exogenous abscisic acid (ABA) and the seedlings were more tolerant to cold stress compared to wild-type and vector control seedlings. Transcript levels of *COR15A*, a cold-responsive gene with an established function in cold tolerance, were approximately twofold higher in transgenic seedlings than in control seedlings under cold conditions. These results establish a role for BRs in opposing the inhibitory effects of ABA in seed germination and in promoting cold stress tolerance in early *Arabidopsis* seedlings.

Keywords Abscisic acid · *Arabidopsis* · *AtDWF4* · Brassinosteroid · Cold stress · Germination · Oleosin promoter · Seed

Introduction

Brassinosteroids (BRs) are a group of naturally occurring plant steroidal compounds with wide-ranging biological activity (Sasse 2002). BRs control important agronomic traits such as flowering time (Domagalska and others 2007; Yu and others 2008), plant architecture (Sakamoto and others 2006), seed yield (Choe and others 2001), and stress tolerance (Krishna 2003), which makes genetic manipulation of BR biosynthesis, conversion, or perception to increase crop yields through altering plant growth and to protect plants from environmental stresses an attractive concept (Divi and Krishna 2009a).

Several BR biosynthetic and signaling genes have been utilized in altering BR levels or perception in different plant species (Divi and Krishna 2009a). The *AtDWF4* gene encodes a cytochrome P450 enzyme (CYP90B1/C-22 hydroxylase) that mediates a rate-limiting step in BR biosynthesis (conversion of campestanol/6-oxocampastanol to 6-deoxocathasterone/cathasterone) (Kim and others 2006). Due to its important role in maintaining BR homeostasis, the *AtDWF4* gene has been a target for genetic manipulation of BR levels. Ectopic overexpression of *AtDWF4* resulted in a 59% increase in seed yield in transgenic *Arabidopsis* compared to wild type (WT) (Choe and others 2001). The increase in seed production was due to a greater number of seeds per plant. In another study, the C22 hydroxylation step of the BR pathway was manipulated in rice by expressing *CYPs* of different plant origins under the control of an *S-ADENOSYLMETHIONINE SYNTHASE* promoter, which is active in stems, leaves, and roots of rice plants (Wu and others 2008). Phenotypic changes resulting from *CYP* expression included increases in grain yield from 15 to 44%, in tiller and seed number per plant, and seed weight. Expression in cotton of another BR biosynthesis gene, *DET2*, which encodes steroid 5α-reductase (Noguchi and others 1999), under the control of a seed coat-specific promoter pFBP7 resulted in significant

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increases in fiber number and fiber length relative to control plants (Luo and others 2007).

In addition to their role in plant development, BRs protect plants from a variety of environmental stresses, including high and low temperatures, drought, salinity, and pathogen attack (Krishna 2003; Divi and Krishna 2009b). Studies involving treatment of plants with exogenous BR, as well as T-DNA knockout mutants of *OsGSK1*, a rice ortholog of the BR negative regulator *BIN2* (*BRASSINOSTEROID-INSENSITIVE 2*), have all provided evidence of a role of BR in stress tolerance (Dhaubhadel and others 1999, 2002; Kagale and others 2007; Koh and others 2007). Together, these studies demonstrate the potential of targeting endogenous BR levels or BR signaling for generating crops with increased yield and stress tolerance (reviewed in Divi and Krishna 2009a).

Several lines of evidence suggest a role for BR within plant seeds. Biologically active BRs brassinolide (BL) and castasterone (CS) are present at relatively high levels in rapidly growing pea seeds. Transcripts of biosynthesis, metabolism, and receptor genes of BRs are stored in mature seeds, and de novo synthesis of CS and its precursors can be detected in seedlings as early as 1 day old (Nomura and others 2007). BR-insensitive and BR-deficient mutants are more sensitive to inhibition of germination by abscisic acid (ABA) (Steber and McCourt 2001), and BR treatment rescues germination of seeds in gibberellin (GA) biosynthesis mutants (Steber and McCourt 2001). Consistent with the role of BR in cell expansion, it has been proposed that BR promotes seed germination by directly enhancing the growth potential of the emerging embryo (Leubner-Metzger 2001). Recently, a role for BR in embryonic patterning has emerged; BIM1 (BES INTERACTING MYC-LIKE PROTEIN 1), a protein involved in BR signaling, has been demonstrated to control embryonic patterning and to interact with DRN (DORNROESCHEN) and DRNL (DORNROESCHEN-LIKE), which also control embryonic patterning (Chandler and others 2009). Thus, although a role for BR in seed development and germination is becoming evident, there is as yet no evidence to suggest that BRs in seeds impact stress tolerance in early seedlings.

To assess if endogenous BRs in seeds can influence germination and stress tolerance of early seedlings, *AtDWF4* was overexpressed in *Arabidopsis* under the control of a seed-specific oleosin promoter. Oleosins are hydrophobic plant proteins that cover the surface of the oilbody and constitute about 2–3% of total seed protein in mature seeds (Huang 1992). The *Arabidopsis* oleosin gene promoter is active throughout seed maturation, including the late-cotyledonary stage of embryo development (Plant and others 1994). Here we demonstrate that overexpression of *AtDWF4* in *Arabidopsis* seeds causes seed germination to be more tolerant to the inhibitory effects of abscisic acid (ABA) and increases cold tolerance of transgenic seedlings compared to WT and vector control seedlings.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis (Col) seeds were surface sterilized (Kagale and others 2007) and sowed in pots containing autoclaved soil. The pots were kept at 4°C in the dark for 3 days and then transferred to a growth chamber set at 22°C, 16-h day length, and a light intensity of 80 μE m^{-2} s^{-1}. When the primary inflorescences reached 3-4 cm in height, they were decapitated to induce axillary bolts. Healthy plants that had many immature flower clusters were selected for transformation.

Plasmid Construction and Plant Transformation

The plasmid pSBS3062 containing the *Arabidopsis* oleosin promoter and terminator was obtained from SemBioSys Inc. (Calgary, AB, Canada). The *AtDWF4* cDNA was obtained from the *Arabidopsis* Biological Resource Center (clone No. U13551) and PCR amplified using forward (*NCOI-DWF4-F:* 5′ CGTTCCATGGATGTTCGAAACA GAGCATC 3′) and reverse (*NCOI-DWF4-R:* 5′ GCTGCC ATGGTTACAGAATACGAGAAACC 3′) primers that were designed to introduce *Nco*I restriction enzyme sites at both the 5′ and 3′ ends of the cDNA. The PCR product was initially cloned into the *Nco*I site of pSBS3062, placing the *AtDWF4* coding sequence between the oleosin promoter and terminator. The expression cassette containing the oleosin promoter, *AtDWF4* coding region in the right orientation, and the oleosin terminator were excised with *Pst*I and cloned into the binary vector pCAMBIA 2301 (CAMBIA, Canberra, Australia) to produce pCAMBIA-oleosin::*AtDWF4*. The sequence of the *AtDWF4* coding region was verified and the construct was introduced into the *Agrobacterium* strain GV3101 by electroporation. The *Agrobacterium* culture was used to transform plants according to Clough and Bent (1998). Seeds collected from transformed plants (T_0) were selected on 30 mg l^{-1} kanamycin. Homozygous lines were obtained by screening T_2 seeds for kanamycin resistance. Transgenic vector controls were generated similarly using the empty pCAMBIA 2301 vector for transformation.

Genomic DNA Isolation and PCR Analysis

Genomic DNA was isolated from transgenic and WT *Arabidopsis* seedlings as described by Doyle and Doyle

(1990). For identification of transgenic lines containing the desired insert, genomic DNA was PCR amplified using an oleosin-specific sequence as the forward primer (5′ AGC GGCTGCATGGTGACGC 3′) and *AtDWF4* cDNA-specific sequence as reverse primer (5′ GAATCAAGAACAA ACAAAGTATG 3′). PCR was performed using about 100 ng of DNA with an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation (30 s at 94°C), annealing (45 s at 56°C), and extension (1 min at 72°C). After the last cycle, a final extension was carried out for 5 min at 72°C.

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from frozen plant tissue using the SV Total RNA Isolation System (Promega, Madison, WI, USA). Five micrograms of total RNA was reverse transcribed using the oligo(dT)18 primer and SuperScript™ First Strand Synthesis System for RT–PCR (Invitrogen, Carlsbad, CA, USA). The cDNA was amplified using the *NCOI-DWF4-F* and *NCOI-DWF4-R* primers that were used for cloning the *AtDWF4* cDNA into pSBS3062. PCR was carried out with an initial denaturation step of 94°C for 4 min, followed by 32 cycles for *AtDWF4* and 21 cycles for *ACTIN* (internal control) of denaturation (30 s at 94°C), annealing (45 s at 56°C), and extension (1 min at 72°C). After the last cycle, a final extension was carried out for 5 min at 72°C. For quantitative RT-PCR (qRT-PCR) analysis of *COR15A*, primer pairs *COR15A-F* (5′ AAAG CTGCGGCGTATGTGGAG 3′) and *COR15A-R* (5′ CCTG CTTTACCCTCCGCGAAC 3′) were used. PCR reactions were performed using SYBR-Green I (Invitrogen) at 0.1× concentration and a Rotor Gene-3000 thermal cycler (Corbett Research, Sydney, Australia) with an initial denaturation step at 94°C for 4 min followed by various cycles of denaturation (15 s at 94°C), annealing (30 s at 56°C), and extension (30 s at 72°C and 15 s at 83°C). The following primers were used for *ACTIN* and *UBIQUITIN10* (*UBQ10*) that served as internal controls in RT-PCR and qRT-PCR, respectively: *ACTIN-F*: 5′ TGCTCTTCCTC ATGCTAT 3′*ACTIN-R*: 5′ ATCCTCCGATCCAGACA CTG 3′*UBQ10-F:* 5′ CAGAACTTTGGCCGACTAC 3′, *UBQ10-R:* 5′ ATGGTCTTTCCGGTGAGAG 3′.

In silico Gene Expression Analysis

The data for BL-responsive and cold-responsive expression of *COR15A* (At2g42540) were retrieved from AtGenExpress stress series data sets by performing e-Northerns using the web interface of the Botany Array Resource (BAR; Toufighi and others 2005; http://www.bar.utoronto.ca).

Inhibition of Germination by ABA

Seeds were surface sterilized and plated on 1× Murashige and Skoog (MS) medium supplemented with B5 vitamins, 1% Phytoblend (Caisson Laboratories, North Logan, UT, USA), and 1% sucrose. ABA treatment was done by including 1.5 μM ABA (Sigma-Aldrich, Co., St. Louis, MO, USA) in the medium, whereas the control plates contained 0.01% ethanol (solvent for ABA). The plates were kept in the dark at 4°C for 3 days and later transferred to a growth chamber set at 22°C to allow germination. Percent germination was determined 5 days after transferring the plates to 22°C. Seeds with emerging cotyledons were scored as germinated.

Cold Stress Treatment

Four independent homozygous transgenic lines expressing *AtDWF4* (OD1, OD10, OD11, and OD12), two vector controls (VC2 and VC4), and WT were analyzed for tolerance to cold stress. Seeds were surface sterilized and plated on 1× MS medium (Sigma) supplemented with B5 vitamins, 1% Phytoblend (Caisson Laboratories), and 1% sucrose. The plates were kept in the dark at 4°C for 3 days to encourage synchronized germination. Following this, the plates were transferred to a growth chamber and seeds were grown at 4°C for 10 days, followed by growth at 8°C for another 15 days. Plants were allowed to recover from cold stress at 22°C for 10 days. An 8/16 h (day/night) photoperiod and a light intensity of 80 μE m^{-2} s^{-1} were maintained during stress and recovery periods. Percent survival was calculated by counting the number of seedlings that showed green leaves with little to no bleaching or yellowing on the tenth day of recovery.

Results

Overexpression of *AtDWF4* in *Arabidopsis* Seeds

The *AtDWF4* gene is under tight transcriptional control and is in part responsible for the slow rate of the AtDWF4-mediated C22 hydroxylation step in the BR biosynthesis pathway (Kim and others 2006). Because ectopic and green tissue-specific overexpression of *AtDWF4* led to substantial increases in seed yield in *Arabidopsis* and rice, respectively (Choe and others 2001; Wu and others 2008), we wished to see if seed-specific overexpression of *AtDWF4* in *Arabidopsis* would positively impact germination and stress tolerance of early seedlings. A construct containing the *AtDWF4* coding sequence under the control of a seed-specific oleosin promoter (Fig. 1a) was cloned into pCAMBIA 2301 (Fig. 1b) and introduced into the genome

of *Arabidopsis*. Seventy-two transgenic plants were obtained, and 17 homozygous lines from these transgenic plants were selected in the T_2 generation. PCR analysis was carried out on genomic DNA of homozygous T_2 plants to determine the presence of the insert. Several *AtDWF4* transgenic lines (OD), two independent vector control transgenic lines (VC), and WT were screened for the presence of the oleosin::*AtDWF4* insert. Because one of the primers used in the analysis was oleosin-specific, no amplification was observed for WT and VC plants, whereas seven transgenic lines (OD1, OD10, OD11, OD12, OD14, OD15, and OD17) showed the presence of the insert (Fig. 2a). Lines OD1, OD10, OD11, and OD12 yielded larger quantities of seeds compared with other lines and were therefore used in further studies. The levels of *AtDWF4* transcripts were monitored by RT-PCR, using

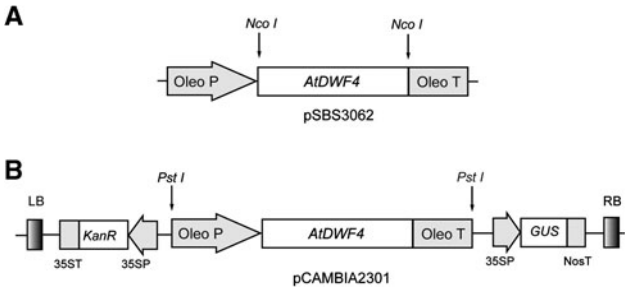

Fig. 1 Cloning strategy of *AtDWF4* for seed-specific expression. **a** cDNA sequence of *AtDWF4* was placed between the oleosin promoter (Oleo P) and oleosin terminator (Oleo T) by cloning at the *Nco*I site of pSBS3062. **b** The expression cassette was excised with *Pst*I and cloned into pCAMBIA 2301. LB, T-DNA left border; 35ST, cauliflower mosaic virus 35S terminator; *KanR*, kanamycin resistance gene; 35SP, cauliflower mosaic virus 35S promoter; *GUS*, *β*-glucoronidase gene; NosT, Noplaine synthase terminator; RB, T-DNA right border

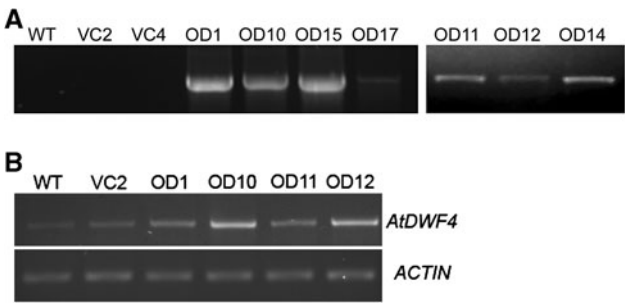

Fig. 2 Characterization of transgenic lines for the presence of *AtDWF4* insert and transcript abundance. **a** PCR amplification using genomic DNA of wild type (WT), vector controls (VC2 and VC4), and transgenic lines (OD1, OD10, OD15, OD17, OD11, OD12, and OD14) using an oleosin-specific forward primer and *AtDWF4*-specific reverse primer. **b** RT-PCR analysis of *AtDWF4* transcripts using RNA isolated from 5-day-old seedlings of WT, VC2, and transgenic lines (OD1, OD10, OD11 and OD12). *ACTIN* was included as a control for constitutive expression

RNA from 5-day-old transgenic (OD1, OD10, OD11, and OD12), VC2, and WT seedlings. A clear increase in the abundance of *AtDWF4* transcripts, relative to WT and VC2, was seen in OD10 and OD12 seedlings, whereas OD1 and OD11 showed minor increases over the control group (Fig. 2b).

Overexpression of *AtDWF4* in Seeds Helps to Overcome Inhibition of Germination by ABA

Several studies have pinpointed an antagonistic relationship between BR and ABA during seed germination, suggesting that a BR signal is needed to overcome inhibition of germination by ABA (Steber and McCourt 2001; Zhang and others 2009). Assuming that overexpression of *AtDWF4* in seeds leads to increased BR levels in seeds or increased potential of BR synthesis during germination, the germination rates of transgenic lines and the control group were compared in the presence of 1.5 μM ABA. In the absence of exogenous ABA there were no differences in the germination rates of different genotypes (Fig. 3a). However, in the presence of ABA, transgenic plants showed better germination efficiency compared to WT and vector controls (Fig. 3a, b). Under these conditions, WT, VC2, and VC4 had germination rates of 26, 35, and 48%, respectively. By contrast, transgenic lines OD1, OD10, OD11, and OD12 had germination rates of 55, 80, 90, and 91%, respectively. These results indicate that overexpression of a BR biosynthesis gene in seeds, leading to a potential increase in BR levels, can help to overcome inhibition of germination by ABA.

Overexpression of *AtDWF4* in Seeds Increases Cold Tolerance in Early Seedlings

BR effects on a plant's ability to cope with abiotic stresses have been evaluated in several studies (Krishna 2003; Divi and Krishna 2009b). Positive consequences of BR in combating cold stress were reported in *Arabidopsis*, maize, cucumber, tomato, and rice (He and others 1991; Kamuro and Takatsuto 1991; Katsumi 1991; Kagale and others 2007; Koh and others 2007; Xia and others 2009). To assess whether seeds of transgenic lines overexpressing *AtDWF4* could tolerate cold temperatures better than the control group (WT and VC) during germination and seedling growth, seeds of OD1, OD10, OD11, OD12, VC2, VC4, and WT were allowed to germinate at 4°C. No significant differences were seen between transgenic lines and the control group in seed germination at this temperature. Prolonged growth at 4°C resulted in cotyledons of all plants turning purple-red in color, which indicated severe stress. To reduce the severity of stress, the temperature was changed to 8°C after initial growth for 10 days at 4°C. No visible

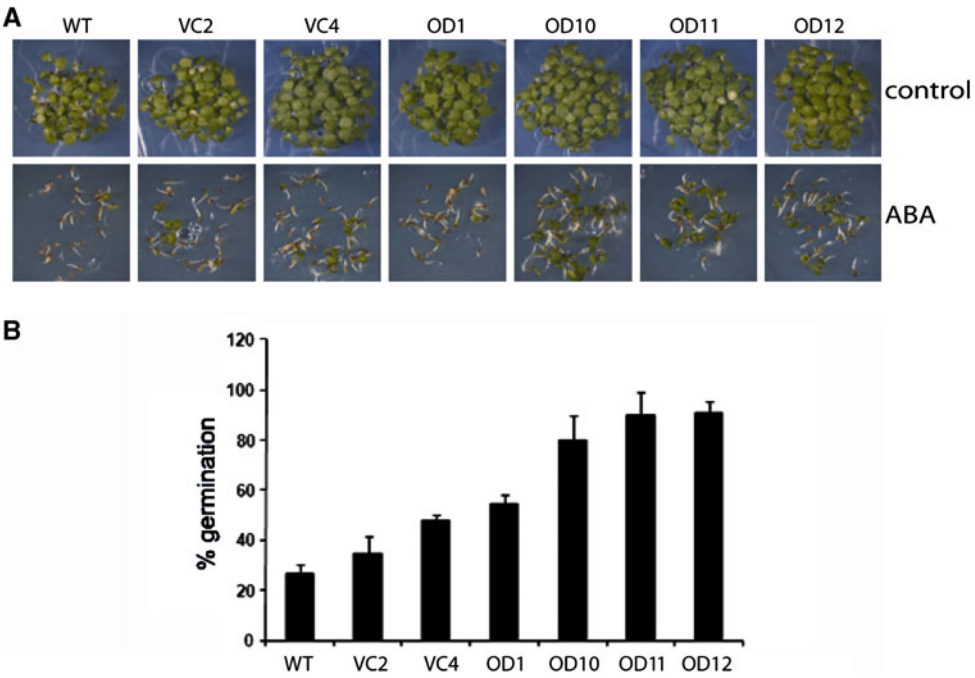

Fig. 3 Effect of seed-specific overexpression of *AtDWF4* on ABA-induced inhibition of germination. **a** WT, vector control (VC1 and VC2), and transgenic lines (OD1, OD10, OD11, and OD12) were germinated in the absence (control) or presence of 1.5 μM ABA (ABA). **b** Percent germination was determined after incubation of plates for 5 days at 22°C. Seeds with emerging cotyledons were scored as germinated. Data shown are averages of three replicates. Error bars represent standard error (SE) of mean for three replicates

morphological differences were observed between the different genotypes after 15 days under these conditions; all plants showed some bleaching of leaves. However, when the seedlings were allowed to recover for 10 days at 22°C, the transgenic lines remained green and healthy, whereas the majority of the control group seedlings became increasingly bleached and stopped growing (Fig. 4a). In accordance with these phenotypes, percentages of survival calculated as the number of green and healthy seedlings were 63.5, 83.5, 64, and 62% for OD1, OD10, OD11, and OD12, respectively, and only 36.5, 34.5, and 32.5% for VC2, VC4, and WT, respectively (Fig. 4b). These results demonstrate that transgenic lines overexpressing *AtDWF4* have a better ability to adapt to and survive cold temperatures during germination and early growth compared to the control group.

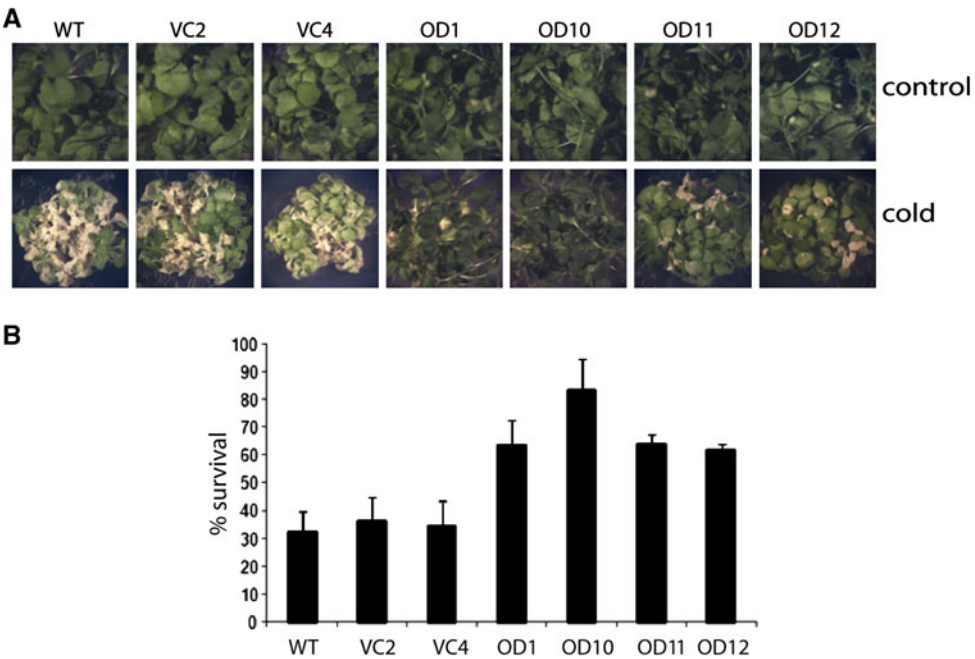

Fig. 4 Effect of seed-specific overexpression of *AtDWF4* on cold tolerance of *Arabidopsis* seedlings. **a** Seeds of WT, VC (VC1 and VC2), and transgenic lines (OD1, OD10, OD11, and OD12) were allowed to germinate and grow at 4°C for 10 days, followed by 15 days at 8°C. The seedlings were allowed to recover at 22°C for 10 days and photographs were taken at the end of the recovery period. **b** Percent survival was calculated by counting the number of green and healthy seedlings at the end of the recovery period. Data shown are averages of three replicates. Error bars represent standard error (SE) of mean for three replicates

Enhanced Expression of *COR15A* Transcripts in *AtDWF4* Transgenic Lines

Cold stress can remodel the plant transcriptome extensively. In *Arabidopsis*, cold stress results in the induction of CBFs/DREB1s (C-repeat binding factors or dehydration-responsive element-binding protein 1s), which can bind to *cis*-elements in the promoters of *COR* (*COLD-REGULATED*) genes and activate their expression (Stockinger and others 1997). Recently, it was shown that even moderate decreases in temperature could induce the expression of *COR15A* (*COLD-REGULATED 15A*) through the CBF signaling cascade and enhance freezing tolerance in *Arabidopsis* (Wang and Hua 2009). We performed in silico analysis of *COR15A* expression using BAR to see if BR had any effect on this gene. Treatment with BR at 22°C for 0.5 and 1 h increased *COR15A* expression in *Arabidopsis* seedlings by 1.23- and 1.5-fold, respectively (Fig. 5a), whereas treatment with cold (4°C) for 24 h increased *COR15A* transcript expression by 84-fold (Fig. 5b). To see if higher cold tolerance of transgenic plants could be correlated to higher expression of at least one concrete cold-stress marker gene, transcripts of *COR15A* were analyzed by qRT-PCR in leaf tissue of seedlings grown at 4°C and subsequently at 8°C (Fig. 5c). *COR15A* transcript levels in leaf tissue of transgenic lines OD1 and OD10 were at least twofold higher than the controls. This difference is substantial considering the significant upregulation of this gene first in response to cold. Slightly lower increases were observed for OD11 (1.5-fold) and OD12 (1.3-fold). Higher transcript levels of *COR15A* in transgenic lines suggest that other cold-responsive genes may display similar patterns of expression in *AtDWF4* transgenic lines, leading to increased cold adaptation/tolerance in these plants.

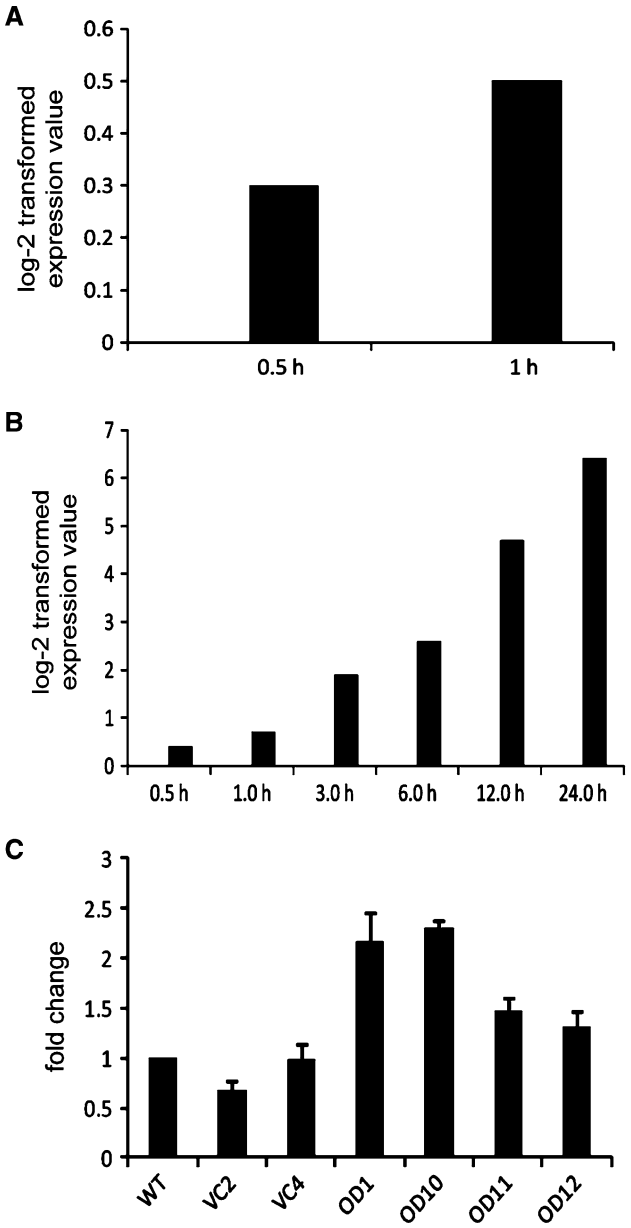

Fig. 5 In silico and qRT-PCR analysis of *COR15A* expression. **a** AtGenExpress data for *COR15A* expression in response to treatment with 10 nM brassinolide for 0.5 and 1 h. **b** AtGenExpress data for *COR15A* expression in response to cold (16-day-old seedlings subjected to cold stress for 0.5, 1, 3, 6, 12, and 24 h). **c** qRT-PCR analysis of *COR15A* transcripts in WT, vector control (VC2 and VC4), and *AtDWF4* transgenic lines (OD1, OD10, OD11, and OD12). RNA used in qRT-PCR analysis was isolated from leaf tissue of seedlings collected at the end of growth at 8°C

Discussion

BRs elicit a wide spectrum of physiological and molecular responses that are involved in growth, development, and stress tolerance (Khripach and others 2000; Sasse 2002; Krishna 2003; Divi and Krishna 2009b). Pollen and immature seeds are the richest sources of BRs, with levels ranging between 1 and 100 ng g^{-1} fresh weight, whereas shoots and leaves have lower amounts in the range of 0.01–0.1 ng g^{-1} fresh weight (Fujioka and others 1998; Bajguz and Tretyn 2003). In *Arabidopsis*, the highest BR levels and the highest expression of BR-related genes were detected in apical shoots followed by siliques, which contain actively developing embryos and seeds (Shimada and others 2003). Furthermore, *de novo* synthesis of BR was noted in as early as 1-day-old pea seedlings (Nomura and others 2007). BR-deficient *Arabidopsis* (*dwarf5*) and pea (*lk*) mutants have aberrations in seed shape (Choe and others 2000; Nomura and others 2004), and BR-deficient Faba bean (*Vicia faba*) has reduced seed size (Fukuta and others 2006). These observations, together with the recent implication of BR in embryonic patterning (Chandler and others 2009), make a case for a role of BR in seed

development; however, how endogenous BR levels in seeds impact stress tolerance in early seedlings is unknown. Earlier studies involving exogenous BR application have demonstrated that BR promotes seed germination and BR can rescue the retarded germination phenotype of biosynthetic and insensitive mutants of GA (Steber and McCourt 2001), as well as salt-induced inhibition of seed germination in *B. napus* (Kagale and others 2007). Because genetic modulation of endogenous BR levels and BR response has generally resulted in positive effects on seed yield (Divi and Krishna 2009a), we embarked on the question of how an increase in seed BR levels impacts germination in the presence of exogenous ABA and stress tolerance in young seedlings. The demonstration that seed-specific overexpression of a BR biosynthetic gene can produce seedlings that performed better than the control plants under both ABA inhibitory and cold stress conditions establishes the role of BRs in germination as well as cold stress tolerance in early seedlings and paves the way for engineering seedling establishment traits in susceptible plant species.

ABA plays important roles in seed maturation and dormancy as well as in adaptation to environmental stresses (Shinozaki and Yamaguchi-Shinozaki 2000; Finkelstein and others 2008). BR and ABA interact antagonistically in seed germination (Steber and McCourt 2001; Zhang and others 2009) and in some aspects of stress responses. For example, we have found that endogenous ABA suppresses BR-induced thermotolerance in *Arabidopsis* (Divi and others, unpublished results). The increased germination efficiency of *AtDWF4* transgenic seeds in the presence of ABA, compared to that of control seeds (Fig. 3), suggests that an increase in endogenous BR levels suppresses ABA-induced inhibition of germination. Because the *Arabidopsis* oleosin promoter is inducible by ABA (Plant and others 1994), it is possible that ABA treatment may have further impacted the expression of oleosin::*AtDWF4*, resulting in even higher endogenous BR levels and greater advantage in overriding inhibition of germination by ABA.

Earlier analysis of BRs in *Arabidopsis* transgenic plants ectopically overexpressing *AtDWF4* failed to detect brassinolide, the biologically most active BR, in those plants (Choe and others 2001). It was postulated that either brassinolide is rapidly degraded after being used by the signaling pathway, or, alternatively, BR biosynthetic intermediates preceding brassinolide are biologically active. Interestingly, some novel 22α-hydroxylated steroids and other compounds were detected in those transgenic plants (Choe and others 2001). We did not undertake BR metabolite measurement in the present study; however, in the future we plan to determine BR metabolite levels in the transgenic lines and attempt to correlate BR levels and their effects with the intensity and extent of cold stress. In the present study growth at 4°C did not produce any BR effects on a macroscopic level. The purple-red color of the cotyledons was most likely due to accumulation of anthocyanins, which is a stress response and usually precedes chlorophyll breakdown (Feild and others 2001; Chalker-Scott and Scott 2004). An increase in temperature to 8°C reduced such symptoms, but again produced no visible differences between the transgenic and control plants. However, when cold-stressed plants were allowed to recover at 22°C, a clear-cut difference in the survival of transgenic plants was seen compared to the control group (Fig. 4). These temperature conditions can be correlated with exposure of plants to fluctuating temperatures in the field, and the plant survival data to the differing abilities of plants to override stress and resume growth when conditions become favorable.

Several cold-inducible genes have been identified and characterized in plants (Chinnusamy and others 2007). Previously, we have shown that exogenous treatment with BR induces the expression of several stress-responsive genes, including those involved in cold tolerance (Kagale and others 2007). A correlation of *COR15A* transcript levels with cold tolerance is well established (Lin and Thomashow 1992; Wang and Hua 2009). Constitutive expression of *COR15A* in *Arabidopsis* significantly increased survival of isolated protoplasts frozen over −4.5 to −7°C (Steponkus and others 1998). The approximate twofold increase in *COR15A* transcript levels in transgenic seedlings compared to that in the control group under cold conditions is indicative of BR's effect on at least one cold-regulated gene. Interestingly, in addition to the in silico gene expression data showing BL responsiveness of *COR15A* (Fig. 5a), we have found in a microarray experiment that *Arabidopsis* seedlings grown in the presence of exogenous BR have up to a fivefold increase in *COR15A* transcript levels compared to untreated seedlings, under both no-stress and heat stress conditions (Divi and others, unpublished results). Thus, both exogenous BR treatment and possible endogenous overproduction of BR (present study) lead to higher expression of *COR15A* compared to control plants. Based on these results, we extrapolate that other cold-regulated genes may also be upregulated by the possible overproduction of BRs in seeds, which may contribute, at least in part, to the better recovery of transgenic plants from cold stress. Of the transgenic lines tested, OD10 had the greatest expression of *AtDWF4* and *COR15A* transcripts and the highest level of survival following cold stress. Such a correlation was not found with other transgenic lines, and realistically it is not expected. The genes may differ in their temporal expression patterns in different transgenic lines. It should be noted that the oleosin promoter is most active in developing seeds; thus, the oleosin::*AtDWF4* transcript levels in 5-day-old seedlings do not

necessarily reflect the maximum transgene expression capacity of the different transgenic lines. The detected oleosin::*AtDWF4* transcripts in 5-day-old seedlings may be indicative of either low-level promoter activity in germinating seedlings or stored transcripts in seeds.

Although seedlings grown on exogenously supplied BR are resistant to heat and salt (Kagale and others 2007), our preliminary experiments involving heat and salt stress did not result in any noticeable differences between the transgenic and control group plants. Because younger seedlings (5 days old) were employed in these experiments as opposed to 21-day-old seedlings used in exogenous treatment, it is possible that the BR effect on these stresses was missed in the present study. Additional standardization of the heat and salt stress assays are required to capture BR effects, if any, on these stresses arising from increased endogenous BR levels.

Acknowledgments We thank SemBioSys Inc. (Calgary, Alberta, Canada) for kindly providing the plasmid pSBS3062, and Harpreet Jammu and Debbie Dong for their help in screening the transgenic plants. This research was supported by grants to P.K. from the Natural Sciences and Engineering Research Council of Canada.

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