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RESEARCH PAPER

Misregulation of the LOB domain gene *DDA1* suggests possible functions in auxin signalling and photomorphogenesis

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Abstract

The *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*) gene family encodes plant-specific transcription factors. In this report, the *LBD* gene *DOWN IN DARK AND AUXIN1* (*DDA1*), which is closely related to *LATERAL ORGAN BOUNDARIES* (*LOB*) and *ASYMMETRIC LEAVES2* (*AS2*), was characterized. *DDA1* is expressed primarily in vascular tissues and its transcript levels were reduced by exposure to exogenous indole-3-acetic acid (IAA or auxin) and in response to dark exposure. Analysis of a T-DNA insertion line, *dda1-1*, in which the insertion resulted in misregulation of *DDA1* transcripts in the presence of IAA and in the dark revealed possible functions in auxin response and photomorphogenesis. *dda1-1* plants exhibited reduced sensitivity to auxin, produced fewer lateral roots, and displayed aberrant hypocotyl elongation in the dark. Phenotypes resulting from fusion of a transcriptional repression domain to DDA1 suggest that DDA1 may act as both a transcriptional activator and a transcriptional repressor depending on the context. These results indicate that *DDA1* may function in both the auxin signalling and photomorphogenesis pathways.

Key words: Auxin, Aux/IAA, HY5, *LBD* genes, photomorphogenesis.

Introduction

The plant-specific *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*) gene family comprises 43 members in *Arabidopsis* (Shuai *et al.*, 2002). Members of this family share the conserved LOB domain, which has recently been shown to have DNA-binding activity (Husbands *et al.*, 2007). While the functions of the majority of *LBD* genes are unknown, members of this family have been implicated in a number of developmental processes including leaf polarity establishment (Lin *et al.*, 2003; Xu *et al.*, 2003), lateral root formation (Inukai *et al.*, 2005; Liu *et al.*, 2005; Okushima *et al.*, 2007), tracheary element development (Soyano *et al.*, 2008), boundary delimitation (Shuai *et al.*, 2002; Borghi *et al.*, 2007; Lin *et al.*, unpublished results), cytokinin signalling (Naito, 2007), inflorescence branch formation (Bortiri *et al.*, 2006), female gametophyte development (Evans, 2007), and *KNOX* gene regulation (Ori *et al.*, 2000; Semiarti *et al.*, 2001; Chalfun-Junior *et al.*, 2005; Borghi *et al.*, 2007). The founding member of this family, *LATERAL ORGAN BOUNDARIES* (*LOB*) was isolated from an enhancer-trap screen, based on its expression on the adaxial side of lateral organ boundaries (Shuai *et al.*, 2002). In *Arabidopsis*, *LOB* defines a subgroup of *LBD* genes that also includes *LBD10/ASL2*, *LBD25/ASL3*, *LBD36/ASL1*, and *AS2/LBD6* (Iwakawa *et al.*, 2002). Among this

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subgroup, *AS2* (*ASYMMETRIC LEAVES2*) is the only gene with clearly defined functions. *AS2* is required to prevent expression of the class I *KNOX* homeobox genes *BREVIPEDICELLUS* (*BP*), *KNAT2*, and *KNAT6* in the leaf (Ori *et al.*, 2000; Semiarti *et al.*, 2001; Lin *et al.*, 2003). *AS2* is expressed on the adaxial side of lateral organs (Iwakawa *et al.*, 2002, 2007; Wu *et al.*, 2008) and misexpression leads to the formation of adaxialized leaves (Lin *et al.*, 2003), implicating *AS2* in adaxial cell fate specification. *LBD36/ASL1* is expressed primarily in the vasculature and, when misexpressed, also results in repression of *BP* (Chalfun-Junior *et al.*, 2005). *LBD36/ASL1* may have limited redundancy with *AS2* to control cell fate determination in petals (Chalfun-Junior *et al.*, 2005). Functions have not been ascribed to the two remaining members of this subgroup, *LBD10/ASL2* and *LBD25/ASL3*.

The hormone auxin has been implicated in multiple developmental responses in plants (reviewed in Vanneste and Friml, 2009). Auxin signalling is regulated through proteolysis of the Aux/IAA proteins, which act as transcriptional repressors (Worley *et al.*, 2000; Gray *et al.*, 2001; Reed, 2001; Dharmasiri and Estelle, 2002). Aux/IAA proteins form heterodimers with auxin response factors (ARFs), negatively regulating their activity to repress downstream auxin responses (Dharmasiri and Estelle, 2002, 2004; Liscum and Reed, 2002). The F-box protein TIR1, which acts as part of the SCF complex, is an auxin receptor (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005) that, upon interaction with auxin, targets Aux/IAA proteins for degradation. Degradation of Aux/IAA proteins frees the ARFs to regulate gene expression through auxin response elements (AuxREs) present in the promoters of auxin-regulated genes (Worley *et al.*, 2000; Dharmasiri and Estelle, 2002, 2004; Liscum and Reed, 2002). Despite the extensive body of knowledge about auxin signalling that has been amassed, several components of this pathway await characterization.

Recent data have implicated several *LBD* genes in various aspects of auxin signalling. Microarray experiments identified a number of *Arabidopsis LBD* genes that are regulated by auxin (Nemhauser *et al.*, 2004; Paponov *et al.*, 2008). *Crown rootless1* (*Crl1*)/*Adventitious rootless1* (*Arl1*), which is required for formation of crown and lateral roots in rice, is a direct target of the ARF protein OsARF1 (Inukai *et al.*, 2005; Liu *et al.*, 2005). In *Arabidopsis*, the three genes most closely related to rice *Crl1*, *LBD16*, *LBD18*, and *LBD29*, are also regulated by auxin (Okushima *et al.*, 2005; Lee *et al.*, 2009). All three of these genes function in lateral root formation downstream of ARF7 and ARF19 (Okushima *et al.*, 2007; Lee *et al.*, 2009). Furthermore, *LBD16* and *LBD29* are directly regulated by ARF7 and ARF19 (Okushima *et al.*, 2005, 2007). The maize gene *rootless concerning crown and seminal roots* (*RTCS*) is also a presumptive *Crl1* orthologue involved in lateral root formation (Taramino *et al.*, 2007). Additionally, the *Arabidopsis LBD* gene *JAGGED LATERAL ORGANS* (*JLO*) regulates the expression of the auxin efflux carrier *PIN*, suggesting a role in auxin signalling (Borghi *et al.*, 2007).

The phenotypes observed in some auxin mutants suggest that there is an interaction between the auxin and light signalling pathways (Reed, 2001; Liscum and Reed, 2002). In fact, both pathways involve protein degradation via the proteasome (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005). In the dark, the light-inactivatable repressor of photomorphogenesis COP1 is translocated to the nucleus (von Arnim and Deng, 1994). In the nucleus, COP1 binds directly and specifically to HY5 (Ang *et al.*, 1998), a bZIP transcription factor that promotes photomorphogenesis by mediating light-controlled gene expression (Chattopadhyay *et al.*, 1998). The interaction of HY5 and COP1 targets HY5 for proteasome-mediated proteolysis (Osterlund *et al.*, 2000), resulting in the inhibition of light-regulated gene expression in the dark (Yadav *et al.*, 2002). Analyses of the *hy5* mutant indicate that HY5 might also be involved in auxin signalling, further supporting the idea that the auxin and light pathways intersect (Cluis *et al.*, 2004).

In this study, it was shown that the *Arabidopsis LBD* gene *DOWN IN DARK AND AUXIN1* (*DDA1*), formerly *LBD25/ASL3*, functions in both auxin signalling and aspects of photomorphogenesis. *DDA1* transcript levels were reduced following treatment with exogenous indole-3-acetic acid (IAA) or exposure to dark conditions. The *dda1-1* mutant, which behaves as a conditional gain-of-function semi-dominant allele, had a diminished auxin response and displayed aberrant hypocotyl elongation in the dark, indicative of defects in some aspects of auxin response and photomorphogenesis, respectively.

Material and methods

Plant materials and growth conditions

Arabidopsis thaliana plants were grown in soil or on 1× Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) as described previously (Shuai *et al.*, 2002). All genotypes were in the Col-0 ecotype, with the exception of *hy5-1*, which is in L*er*. The *dda1-1* T-DNA mutant, SALK_033840, was isolated from the Salk Institute Genomic Analysis Laboratory collection (Alonso *et al.*, 2003). Homozygous mutants were isolated by genomic DNA gel blot analysis and PCR-based genotyping using gene-specific primers *DDA1*-H (5′-CTTGGGAAATTGA-GAATAATCCATAC-3′) and *DDA1*-F (5′-CCAACC-CATGTCTCCTCTTTATCTC-3′) in combination with the T-DNA primer LBA1 (5′-TGGTTCACGTAGTGGGCC-ATCG-3′).

Plasmid constructs

The *DDA1* promoter region (from –3201 bp upstream of the ATG to +18) was amplified from Col genomic DNA using Ex-Taq Polymerase (Takara, Shiga, Japan) with the primers p*DDA1*-F (5′-TCTAGAGATTCGGGTTGATATCTGAT-3′) and p*DDA1*-R (5′-GGATCCTGTTTCTCTCTTGGG-CATTA-3′), which contained introduced *Xba*I and *Bam*HI sites. PCR products were cloned into pCR-II TOPO

(Invitrogen, Carlsbad, CA, USA) and sequenced to confirm their integrity, then subcloned into the *Xba*I and *Bam*HI sites of pCB308 (Xiang *et al.*, 1999) to create an in-frame translational fusion of the first six amino acids of DDA1 to β-glucuronidase (GUS).

To generate fusions to the hormone-binding domain of the rat glucocorticoid receptor (GR) (Picard *et al.*, 1988), a Gateway destination vector was constructed. pBI-ΔGR (Lloyd *et al.*, 1994) was digested with *Bam*HI and the overhangs filled in using Klenow polymerase. The resulting DNA was ligated to Gateway conversion Cassette C (Invitrogen), to create the destination vector pBI-ΔGR-GW, which allows the generation of in-frame fusions to the hormone-binding domain of GR. The *DDA1-GR* construct was generated using a Gateway recombination with pBI-ΔGR-GW and PYAT3G27650, which contains the *DDA1* coding sequence in a Gateway entry vector (Gong *et al.*, 2004), according to the manufacturer's instructions (Invitrogen).

The *DDA1-EAR* construct was generated using a Gateway recombination reaction between entry clone PYAT3G27650 (Gong *et al.*, 2004) and destination vector pDNG, kindly provided by Rüdiger Simon. pDNG contains the *alcA* promoter (Roslan *et al.*, 2001) and a synthetic EAR (ERF-associated amphiphilic repression) domain (Hiratsu *et al.*, 2003) flanking the *ccdB* cassette. The resulting *DDA1-EAR* construct contained the *AlcA* promoter driving an in-frame fusion of DDA1 to the EAR domain. The *35S:AlcR* construct, pJH0022, was kindly provided by Syngenta.

All binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 and subsequently transformed into Col wild-type plants using the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998).

GUS *expression analyses*

Single-copy homozygous p*DDA1*:*GUS* plants were grown on MS medium with or without supplementation with 10 μM IAA or 85 nM 2,4-D for 7 d under a 16 h light/8 h dark photoperiod or in total darkness. Histochemical analyses and microscopy were performed as previously described (Shuai *et al.*, 2002).

Phenotypic characterization

To determine lateral root numbers, seedlings were grown vertically for 4 d on unsupplemented MS medium, then transferred to medium supplemented with 85 nM 2,4-D, or to unsupplemented control medium, and grown for an additional 4 d. Visible lateral roots formed on the primary root were counted. Hypocotyl measurements were determined for 7-day-old seedlings grown on MS medium in total darkness or under a 16 h light/8 h dark photoperiod. To increase the level of endogenous auxin, seedlings were grown at 28 °C as previously described (Gray *et al.*, 1998). Root growth sensitivity to auxin was determined as previously described (Lincoln *et al.*, 1990). A standard table-top scanner was used to obtain images of seedlings on plates, and measurements were obtained using MCID Elite 7.0 software (Imaging Research Inc., Ontario, Canada).

Ethanol induction

F_1 plants derived from a cross between a homozygous single-copy *pAlcA*:*DDA1-EAR* plant and a homozygous single-copy *35S:AlcR* plant were termed *35S>>DDA1-EAR* and were used in all ethanol induction experiments. Seedlings were grown on MS medium in closed transparent containers. Seedlings were induced by exposure to ethanol vapour—two 1.5 ml tubes containing 1 ml of 50% ethanol each were placed inside the containers for 2 h d^{-1} for 4 d. Control-treated plants were maintained in a closed container in a separate growth chamber.

Expression analyses

For expression analyses, seedlings were grown for 6 d on MS solid medium (Murashige and Skoog, 1962), then transferred to MS liquid medium (Murashige and Skoog, 1962), and maintained overnight to equilibrate. Auxin or dark exposure treatments were done the following day by the addition of 20 μM IAA or by wrapping the plates in aluminium foil. RNA extraction and cDNA syntheses were performed as previously described (Lin *et al.*, 2003). PCR conditions for *DDA1* and *ACTIN2* (*ACT2*) amplification were: denaturation at 94 °C for 10 min, followed by 15 cycles (*DDA1*) or 10 cycles (*ACT2*) of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and one final cycle of 72 °C for 10 min using the primers *DDA1*-F (5′-GAATTCATGCCCAAGAGAGAAAC-3′) and *DDA1*-R (5′-GCGGCCGCACCCCTCCGACCACC-3′) for *DDA1*, and ACT2-N (5′-AAAATGGCCGATGGTGAGG-3′) and ACT-C2 (5′-ACTCACCACCACGAACCAG-3′) for *ACT2*. The blotting and hybridization were performed as previously described (Lin *et al.*, 2003). RT-PCR analyses of *DDA1* and *ACT2* transcript levels using different amounts of cDNA template demonstrated that the PCRs were quantitative under these conditions (see Supplementary Fig. S1 available at *JXB* online).

Results

LBD25 (At3g27650, also known as *ASL3*) is a member of the *LBD* gene family and belongs to a subclade of *LBD* genes that includes *LOB*, *AS2*, *LBD36*/*ASL1*, and *LBD10*/*ASL2* (Iwakawa *et al.*, 2002; Shuai *et al.*, 2002). *lob* loss-of-function mutants did not display conspicuous phenotypes, therefore it was suspected that other *LBD* genes might have functions overlapping those of *LOB*. Phylogenetic analyses indicated that *LBD25* was a likely candidate, as it is more closely related to *LOB* than any other *LBD* gene (Iwakawa *et al.*, 2002; Shuai *et al.*, 2002). Based on the observed down-regulation of *LBD25* expression by auxin and dark conditions (see below), *LBD25* was named *DOWN IN DARK AND AUXIN1* (*DDA1*).

DDA1 *is transcriptionally regulated in response to auxin and dark*

Previous expression analyses using RT-PCR showed that *DDA1* was expressed more broadly than *LOB* (Shuai *et al.*, 2002), but a detailed characterization of the *DDA1* expression pattern has not previously been reported. To examine *DDA1* expression, a promoter–reporter gene construct containing a 3.2 kb region upstream of the translation start site and including the first six *DDA1* codons fused, in-frame, to *uidA* (*GUS*) was introduced into *Arabidopsis*. More than 30 independent transgenic plants were analysed and all showed a similar *GUS* expression pattern (data not shown). Several single-copy p*DDA1:GUS* transgenic lines were identified, one of which was used for detailed expression analyses. In p*DDA1:GUS* seedlings, *GUS* expression was detected in the vasculature of cotyledons, at the base of the hypocotyl, and in the root, but was excluded from the root tip (Fig. 1A, C). *GUS* expression was also observed in the vasculature of rosette leaves (Fig. 1B) and cauline leaves, although GUS activity was weaker in the latter (data not shown). In the flower, *GUS* expression was detectable in the vasculature of sepals but not petals, in the stigma, in the placenta, in pollen grains, and at the base of floral organs (Fig. 1D). As some promoter:*GUS* fusions have been reported to result in artefactual GUS activity in pollen (Mascarenhas and Hamilton, 1992), it was confirmed that *DDA1* transcripts were detectable in anthers using RT-PCR (data not shown). After pollination, GUS activity was observed at the base of the silique, in the placenta, and in the degenerating stigma (Fig. 1E), similar to the pattern observed in flowers.

Examination of publicly available microarray data revealed that *DDA1* transcript levels were reduced by treatment with auxin (Nemhauser *et al.*, 2004) and exposure to dark (www.arabidopsis.org). To investigate *DDA1* regulation further, GUS activity was compared in 7-day-old p*DDA1*:*GUS* seedlings grown in the presence or absence of exogenous auxin and in seedlings grown under a long-day light–dark cycle or in complete darkness. Growth on 10 μM IAA resulted in a substantial decrease in GUS activity in cotyledon vasculature (Fig. 1G) compared with seedlings grown on unsupplemented medium (Fig. 1F), while GUS activity was nearly abolished in dark-grown seedlings (Fig. 1H). GUS activity was also reduced in the roots of p*DDA1*:*GUS* plants that were grown on 85 nM 2,4-D (compare Fig. 1I and J). These observations indicate that the regulation of *DDA1* in response to auxin and dark exposure is likely to be, at least in part, at the transcriptional level.

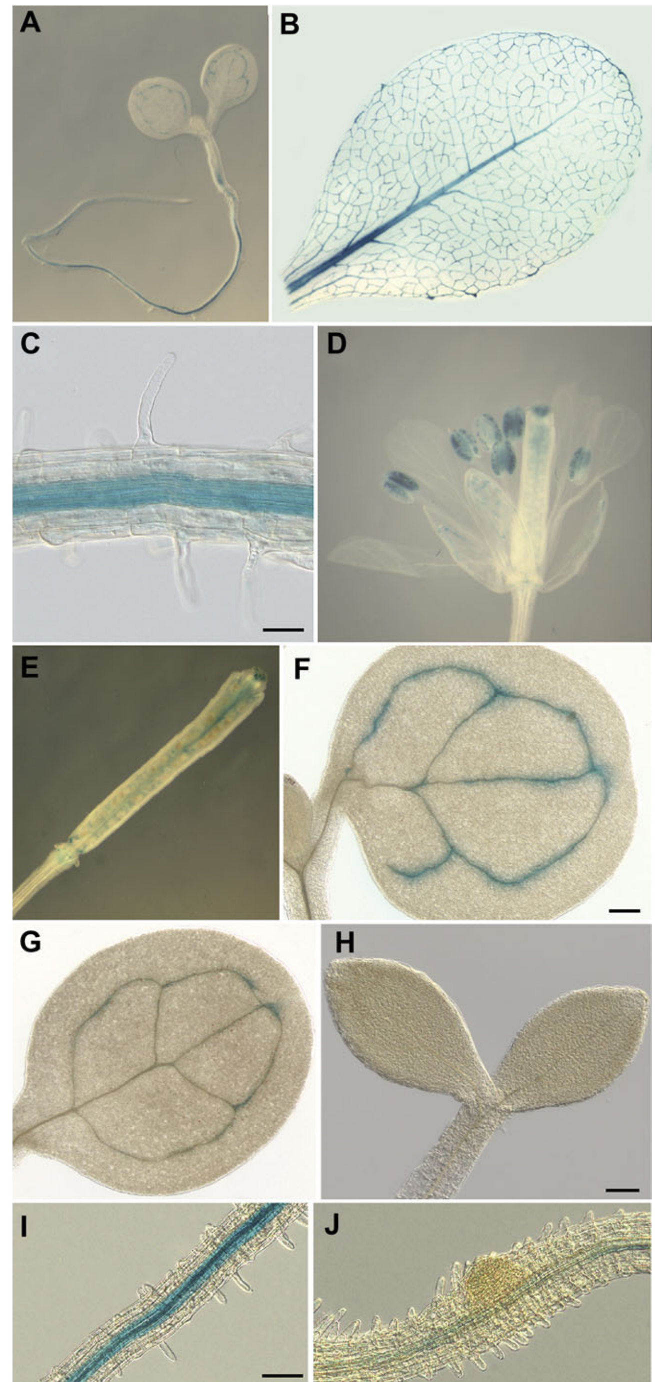

Fig. 1. *DDA1* is expressed in the vasculature and is transcriptionally regulated by auxin and dark exposure. Histochemical GUS analysis of p*DDA1*:*GUS* transgenic plants. (A) Seven-day-old seedling. (B) Mature rosette leaf. (C) Root of 7-day-old seedling. (D) Open flower. (E) Silique. (F) Cotyledon of 7-day-old seedling grown under standard conditions. (G) Cotyledon of 7-day-old seedling grown in 10 μM IAA. (H) Seven-day-old seedling grown in constant dark. (I) Root of 7-day-old seedling grown on unsupplemented medium. (J) Seven-day-old seedling root grown on medium supplemented with 85 nM 2,4-D. Size bar in (C) = 50 μm, in (F) = 200 μm, and in (H) and (I) = 100 μm. The magnification in (F) and (G) is the same; the magnification in (I) and (J) is the same.

dda1-1 *mutants exhibit reduced auxin responses*

To better understand the function of *DDA1*, a T-DNA insertion line, SALK_033840 (Alonso *et al.*, 2003), was identified which was designated *dda1-1*. This line contained an insertion in the sole intron of the *DDA1* gene (Fig. 2A). To determine whether the T-DNA insertion affected *DDA1*

transcript accumulation, RT-PCR was used to amplify the coding region of *DDA1* transcripts in homozygous *dda1-1* seedlings. Reduced transcript levels were detected in *dda1-1* homozygotes compared with the Col wild type, suggesting it is a hypomorphic allele (Fig. 2B). Sequencing of RT-PCR products demonstrated that transcripts produced in *dda1-1* were accurately spliced and therefore apparently functional (data not shown).

Based on the observed regulation of *DDA1* in the presence of auxin, the *dda1-1* mutant was examined for auxin responses. Whether the hypocotyl of *dda1-1* responded normally to increased auxin concentrations was first examined. *dda1-1* plants were grown at 28 °C, a condition that increases endogenous auxin levels (Gray *et al.*, 1998). *dda1-1* seedlings did not show a significant difference in hypocotyl length compared with the Col wild type when grown at either 22 °C or 28 °C (Fig. 3A), indicating that auxin signalling is not perturbed in the hypocotyl of the *dda1-1* mutant.

Auxin sensitivity assays were performed to determine whether auxin responses were affected in *dda1-1* roots. Four-day-old seedlings were transferred to medium containing 2,4-D or to unsupplemented control medium, and root growth in a 3 d period was determined. Sensitivity to a range of 2,4-D concentrations was examined. The most significant difference between *dda1-1* and wild-type Col plants was observed using 10 nM 2,4-D (see Supplementary Fig. S2 at *JXB* online), therefore subsequent experiments used 10 nM 2,4-D. Wild-type Col plants exhibited an ~40% inhibition in root growth in response to auxin treatment. *dda1-1* mutants displayed reduced sensitivity to auxin compared with Col, showing ~32% inhibition (Fig. 3B).

Lateral root formation in *dda1-1* seedlings was also examined as an additional indicator of auxin responsiveness. Compared with the wild type, *dda1-1* mutants did not

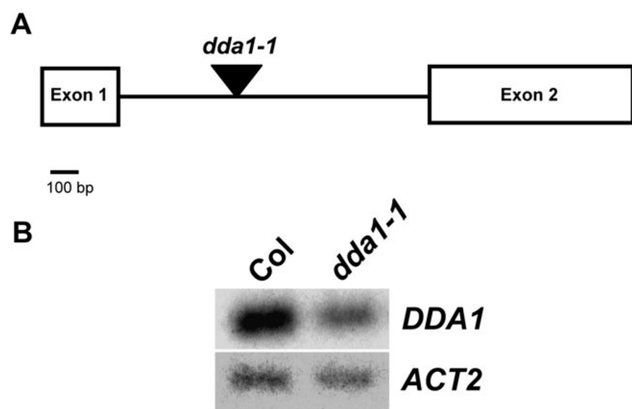

Fig. 2. Location and consequences of T-DNA insertion in the *dda1-1* mutant. (A) The genomic structure of *DDA1* indicating the position of the T-DNA insertion in *dda1-1* (triangle). (B) RT-PCR analysis of *DDA1* transcript levels in 7-day-old seedlings of Col and *dda1-1*. RT-PCR products were detected by blotting and probing with gene-specific probes, following either 15 cycles (*DDA1*) or 10 cycles (*ACT2*) of amplification. The primers used for *DDA1* amplification span the entire coding region.

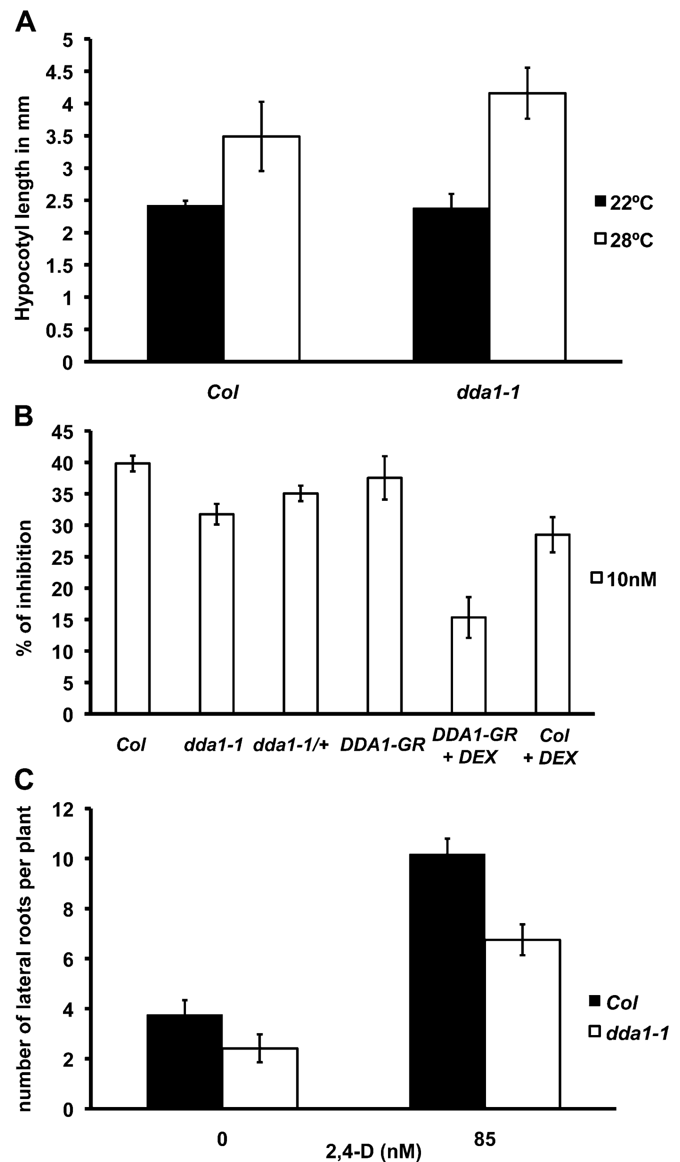

Fig. 3. *dda1-1* mutant seedlings show reduced sensitivity to auxin. (A) Hypocotyl measurements of 7-day-old Col and *dda1-1* seedlings grown at 22 °C or 28 °C. A minimum of 10 seedlings was assayed for each background and temperature. Error bars represent the standard error. *t*-test indicates that the values between genotypes are not significantly different. (B) Reduction in root growth resulting from 2,4-D exposure. Seedlings were grown on unsupplemented medium for 4 d, then transferred to 2,4-D-supplemented medium. After 3 d, root length was measured. Inhibition of root growth is calculated from growth on 2,4-D relative to growth on unsupplemented medium. A minimum of 10 seedlings was assayed for each background. Error bars represent the standard error. *t*-test P <0.01 (Col×*ddal1-1*); P< 0.05 (Col×*dda1-1/+*); P <0.01 (Col+DEX×*DDA1-GR*+DEX). (C) The number of lateral roots per 8-day-old seedling following transfer to unsupplemented or 2,4-D-supplemented medium after 4 d growth. A minimum of 12 seedlings was assayed for each background and treatment. Error bars represent the standard error. *t*-test for 0 nM indicates that the values are not significantly different and for 85 nM, P <0.001.

show a significant difference in lateral root number when grown on unsupplemented medium (Fig. 3C). To examine auxin-induced lateral root production, 4-day-old plants were transferred to medium containing 85 nM 2,4-D and lateral root numbers were determined after 4 d of growth. *dda1-1* mutants produced ~35% fewer lateral roots than the wild type. These data are consistent with reduced auxin sensitivity in *dda1-1* roots.

The axr3-1 *mutant disrupts* DDA1 *regulation by auxin*

In order to gain insight into the regulation of *DDA1*, *DDA1* transcript levels were examined in the *axr3-1* mutant background. *AXR3* encodes the Aux/IAA protein IAA17 (Leyser *et al.*, 1996), a repressor of auxin responses that is targeted to the proteasome for degradation in the presence of auxin. The *axr3-1* mutation results in protein stabilization and a resulting alteration in auxin responses (Leyser *et al.*, 1996; Rouse *et al.*, 1998). In *axr3-1* seedlings, *DDA1* transcript abundance was not altered by treatment with IAA (Fig. 4A), indicating that AXR3 degradation is required for the reduction of *DDA1* transcripts following exposure to exogenous auxin. This places down-regulation of *DDA1* downstream of auxin-mediated proteolysis of Aux/IAA proteins. Given that there are many Aux/IAA proteins in *Arabidopsis* and *axr3-1* is a gain-of-function mutant, it is possible that AXR3 does not normally participate in *DDA1* regulation in wild-type plants, where this role might be performed by other related Aux/IAA proteins.

dda1-1 *displays aberrant response to dark growth conditions*

As *DDA1* transcript levels were modulated by exposure to dark conditions, dark-grown *dda1-1* seedlings were examined for etiolation characteristics such as hypocotyl elongation, apical hook formation, and closed cotyledons (McNellis *et al.*, 1994). Dark-grown *dda1-1* seedlings exhibited lack of chlorophyll pigmentation, an apical hook, and closed cotyledons, similar to the wild type (data not shown). However, dark-grown *dda1-1* hypocotyls were significantly shorter than those of the wild type (Fig. 5A). In contrast, *dda1-1* hypocotyls were slightly longer than those of the wild type when grown under a 16 h light/8 h dark photoperiod (Fig. 5A), indicating that they exhibited an aberrant response

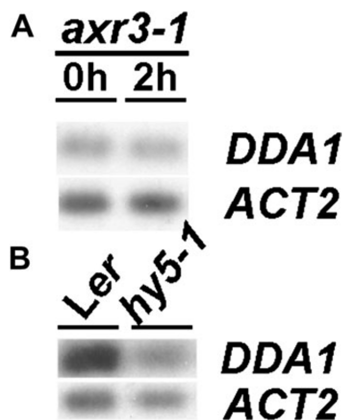

Fig. 4. *DDA1* transcript levels are regulated by AXR3 and HY5. (A) RT-PCR analysis of *DDA1* transcript levels in 7-day-old *axr3-1* mutant seedlings following 0 h or 2 h exposure to 20 μM IAA. (B) RT-PCR analysis of *DDA1* transcript levels in 7-day-old L*er* and *hy5-1* seedlings. RT-PCR products were detected by blotting and probing with gene-specific probes, following either 15 cycles (*DDA1*) or 10 cycles (*ACT2*) of amplification.

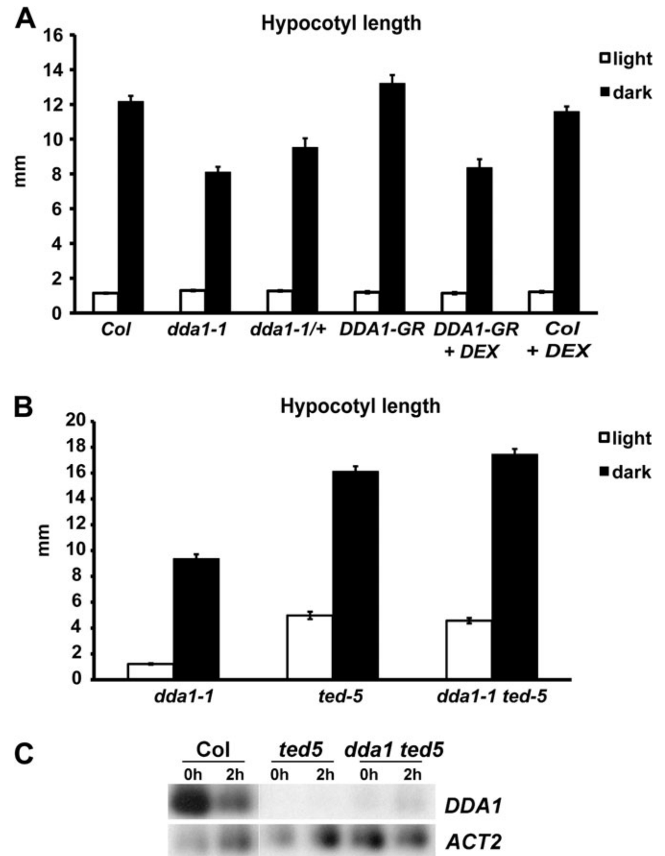

Fig. 5. *dda1-1* mutants display shorter hypocotyls in the dark. (A) Hypocotyl length of 7-day-old Col, *dda1-1*, and *DDA1-GR* seedlings grown under a 16 h light/8 h dark photoperiod (white columns) or in the dark (black columns). A minimum of 12 seedlings was assayed for each background and growth condition. Error bars represent the standard error. *t*-test for dark treatment, P <0.0001 (Col×*dda1-1*); P <0.001 (Col×*dda1-1/+*); P <0.0001 (Col+DEX×*DDA1-GR*+DEX). (B) The same experiment as in (A) but using *dda1-1*, *ted5-1*, and *dda1-1 ted5-1* seedlings. *t*-test for light treatment, P <0.0001 (*dda1-1*×*ted5-1*); P <0.0001 (*dda1-1*×*dda1-1 ted5-1*). *ted5-1* and *dda1-1 ted5-1* were not significantly different (P <0.2). *t*-test for dark treatment, P <0.0001 (*dda1-1*×*ted5-1*); P <0.0001 (*dda1-1*×*dda1-1 ted5-1*); P <0.05 (*ted5-1*×*dda1-1 ted5-1*). (C) RT-PCR analysis of *DDA1* transcript levels in 7-day-old Col, *ted5-1*, and *dda1-1 ted5-1* seedlings following 0 h or 2 h exposure to dark conditions. RT-PCR products were detected by blotting and probing with gene-specific probes, following either 15 cycles (*DDA1*) or 10 cycles (*ACT2*) of amplification.

to dark growth conditions. As other etiolation responses were normal, *DDA1* appears to function in only one aspect of etiolation—hypocotyl elongation. *dda1-1* mutant hypocotyls responded normally to auxin (Fig. 3A), therefore the aberrant hypocotyl elongation observed in dark-grown plants does not appear to be the result of disturbed auxin responses.

A major factor in the promotion of photomorphogenesis is the bZIP transcription factor HY5, which is targeted to the proteasome for degradation in dark conditions (Ang *et al.*, 1998; Chattopadhyay *et al.*, 1998; Osterlund *et al.*, 2000; Yadav *et al.*, 2002). To investigate the relationship between *DDA1* and *HY5*, steady-state levels of *DDA1* transcripts were examined in the *hy5-1* mutant background. *DDA1* transcript levels were significantly reduced in *hy5-1* seedlings compared with the wild type (Fig. 4B), indicating that HY5 activity contributes to *DDA1* regulation. To investigate this relationship further, double mutants were generated between *dda1-1* and the *HY5* mutant allele *ted5-1* (Pepper and Chory, 1997). In both light- and dark-grown conditions, *ted5-1* mutant hypocotyls were longer than *dda1-1* hypocotyls (Fig. 5B). *dda1-1 ted5-1* double-mutant hypocotyls were similar to those of *ted5-1* single mutants (Fig. 5B). The restoration of dark-induced hypocotyl elongation in the double mutant, relative to the *dda1-1* single mutant, indicates that *ted5-1* is epistatic to *dda1-1*. To investigate the molecular nature of this epistasis, *DDA1* transcript abundance was examined in *dda1-1 ted5-1* double mutants. *DDA1* transcript levels were reduced in *dda1-1 ted5-1* seedlings, similar to the levels observed in *ted5-1* (Fig. 5C). Further, there was no apparent dark-induced transcript regulation in the double mutants (Fig. 5C). These data are consistent with the hypothesis that *DDA1* negatively regulates hypocotyl elongation during photomorphogenesis.

The dda1-1 *mutation affects* DDA1 *transcript accumulation in the presence of auxin and in the dark*

As *DDA1* transcript levels were reduced in response to auxin or dark exposure, the phenotypes observed in the *dda1-1* mutant—reduced sensitivity to auxin and aberrant response to dark—were inconsistent with its apparent hypomorphic nature. Because of this contradiction, *DDA1* steady-state transcript levels were analysed in both wild-type and *dda1-1* seedlings following treatment with auxin or exposure to dark conditions. Exposure to 20 μM IAA for 2 h resulted in a reduction in the abundance of *DDA1* transcripts in wild-type seedlings (Fig. 6A), in agreement with the behaviour of the p*DDA1:GUS* reporter line. However, while *dda1-1* seedlings showed reduced steady-state transcript levels prior to auxin treatment, an increase in transcript abundance was observed following the induction (Fig. 6A). Exposure to total darkness for 2 h resulted in a small increase in transcript abundance in *dda1-1* seedlings (Fig. 6B), in contrast to the reduction observed in wild-type seedlings. Thus, although *dda1-1* seedlings had reduced transcript levels under standard growth conditions, transcript accumulation was not regulated appropriately in response to auxin or dark exposure.

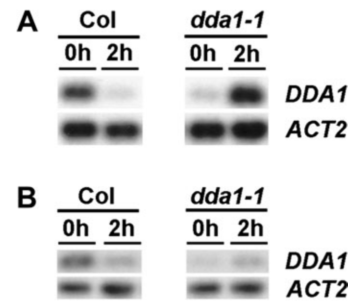

Fig. 6. Transcript levels are aberrantly regulated by auxin and dark exposure in *dda1-1* mutants. (A) RT-PCR analysis of *DDA1* transcript levels in 7-day-old Col and *dda1-1* seedlings following 0 h or 2 h exposure to 20 μM IAA. (B) RT-PCR analysis of *DDA1* transcript levels in 7-day-old Col and *dda1-1* seedlings transferred to the dark. RT-PCR products were detected by blotting and probing with gene-specific probes, following either 15 cycles (*DDA1*) or 10 cycles (*ACT2*) of amplification.

Since the T-DNA insertion in *dda1-1* is in the sole intron, it was speculated that the differential transcript accumulation compared with Col might be due to effects on splicing efficiency. If this were the case, then different regions of the transcript might differ in abundance in mutant plants. To test this possibility, *dda1-1* cDNA was amplified using primers spanning the first exon of *DDA1*, which is upstream of the insertion site. These primers produced an RT-PCR product similar in abundance to that obtained with primers spanning the entire coding region (data not shown), indicating that the increase in transcript levels is not likely to be due to changes in splicing efficiency. The nature of the altered *DDA1* regulation in *dda1-1* is not clear.

dda1-1 *is a hypermorphic allele in the presence of auxin and in the dark*

If *dda1-1* plants exhibited increased *DDA1* activity in the presence of auxin, as would be predicted for a hypermorphic allele, then it is expected to behave in a semi-dominant manner. To test this hypothesis, *dda1-1*/+ heterozygotes were analysed for auxin responses. *dda1-1*/+ seedlings exhibited moderate auxin resistance, showing an intermediate level of growth inhibition between that seen in *dda1-1* homozygotes and wild-type seedlings. This result is consistent with the conclusion that *dda1-1* is a hypermorphic allele in the presence of exogenous auxin (Fig. 3B).

To investigate further the role of *DDA1* in plant development, transgenic plants were generated expressing a dexamethasone (DEX)-inducible form of DDA1, a translational fusion to the hormone-binding domain of the rat GR, under control of the ubiquitously expressed cauliflower mosaic virus *35S* promoter. In the absence of DEX, the response of *35S*:*DDA1-GR* seedlings to auxin was not significantly different from that of the wild type (Fig. 3B). When grown in the presence of DEX, however, *DDA1-GR* seedlings showed reduced auxin sensitivity compared with

wild-type plants grown on DEX (Fig. 3B). It is noted that wild-type plants grown on DEX also exhibited a diminished response to auxin. However, DEX-grown *DDA1-GR* seedlings showed a mild but significant reduction in auxin sensitivity compared with wild-type seedlings grown on DEX. *DDA1-GR* plants on DEX showed ~15% root growth inhibition due to auxin, while wild-type plants on DEX showed ~30% inhibition. Reduced sensitivity to auxin in plants that had increased levels of *DDA1* transcript, in auxin-treated *dda1-1* mutants and *DDA1-GR* plants, indicates that *DDA1* acts as a negative regulator of the auxin signalling pathway.

As *dda1-1* mutants had higher transcript levels following dark exposure, in contrast to wild-type plants, which had reduced transcript accumulation (Fig. 6B), this allele also appears to be hypermorphic under dark-grown conditions. *dda1-1*/+ heterozygotes and *DDA1-GR* plants were therefore examined for aberrant hypocotyl elongation in the dark. Dark-grown *dda1-1*/+ seedlings produced hypocotyls that were intermediate in length between Col wild type and *dda1-1* homozygotes, consistent with *dda1-1* being a semi-dominant allele (Fig. 5A). *DDA1-GR* plants grown on DEX showed a reduction in hypocotyl length of ~33% compared with the wild type on DEX, while *DDA1-GR* grown on medium without DEX exhibited normal hypocotyl elongation (Fig. 5A). These data support the hypothesis that *dda1-1* behaves as a hypermorphic allele in the dark and suggest that *DDA1* is involved in suppressing hypocotyl elongation during photomorphogenesis.

Overexpression of DDA1 fused to a transcriptional repression domain reveals differences in DDA1 function in the auxin and photomorphogenesis pathways

Several LBD proteins have been shown to bind DNA, and the closely related LOB protein has transcriptional activation activity (Husbands *et al.*, 2007). DDA1 is therefore likely to function as a transcriptional regulator. To examine the role of this protein further in processes related to photomorphogenesis and auxin signalling, transgenic plants were generated expressing a fusion of DDA1 to an EAR domain, which has strong transcriptional repression activity (Ohta *et al.*, 2001; Hiratsu *et al.*, 2003). This fusion protein is expected to function as a strong transcriptional repressor, which should provide insights into the function of DDA1. Given that it was not possible to obtain plants with significantly elevated levels of *DDA1* when using a constitutive promoter (data not shown), transgenic plants with inducible *DDA1-EAR* expression were generated using the two-component *alc* system (Deveaux *et al.*, 2003). Transgenic plants expressing *DDA1-EAR* under the control of the *AlcA* promoter (*AlcA:DDA1-EAR*) were crossed to plants expressing the AlcR transcription factor under control of the *35S* promoter (*35S:AlcR*). AlcR is active only in the presence of ethanol (Lockington, 1987), allowing *DDA1-EAR* expression to be induced by ethanol vapour. F_1 plants, designated *35S>>DDA1-EAR*, were examined for ethanol-dependent phenotypes.

In the absence of ethanol, *35S>>DDA1-EAR* transgenic plants were phenotypically normal, indistinguishable from wild-type plants grown in either the presence or absence of ethanol (data not shown). The phenotypes of *dda1-1* and *DDA1-GR* plants, both of which have increased DDA1 activity in the presence of exogenous auxin, indicated that *DDA1* is a negative regulator of auxin signalling. In order to investigate the nature of this negative regulation, *35S>>DDA1-EAR* plants were examined for auxin-response phenotypes. Root growth inhibition assays were conducted on *35S>>DDA1-EAR* and wild-type plants that were either induced with ethanol vapour or grown in control conditions without exposure to ethanol. Relative to uninduced plants, ethanol-induced *35S>>DDA1-EAR* plants exhibited reduced growth inhibition in response to auxin exposure (Fig. 7A), while the response of wild-type plants to auxin was unaffected by ethanol treatment (data not shown). Following treatment with ethanol vapour, auxin treatment of *35S>>DDA1-EAR* roots resulted in ~14% growth inhibition, while roots of uninduced control plants showed ~28% growth inhibition (Fig. 7A). The reduced auxin sensitivity of *35S>>DDA1-EAR* plants is similar to that observed in *DDA1-GR* plants, suggesting that the addition of a repressor domain to DDA1 did not

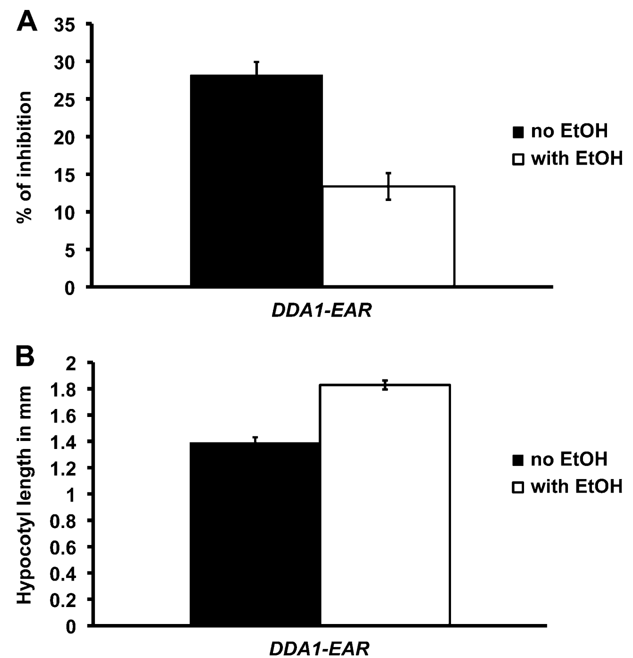

Fig. 7. Phenotypes observed in *DDA1-EAR* seedlings. (A) Reduction in root growth resulting from 2,4-D exposure. Seedlings were grown as described in Fig. 3. On the third day, ethanol induction was initiated and maintained for 4 d. The percentage growth inhibition was calculated from growth on 2,4-D relative to growth on unsupplemented medium. A minimum of 10 seedlings was assayed for each condition. Error bars represent the standard error. *t*-test P <0.001 (no ethanol×with ethanol). (B) Hypocotyl length of 7-day-old seedlings grown under a 16 h light/8 h dark photoperiod. A minimum of 12 seedlings was assayed for each condition. Error bars represent the standard error. *t*-test P <0.000001 (no ethanol×with ethanol).

alter its function. These data are consistent with DDA1 functioning as a transcriptional repressor to suppress some aspects of the auxin response.

Based on the phenotypes of *dda1-1* and *DDA1-GR* plants, which have increased levels of DDA1 activity, *DDA1* appears to contribute to the repression of hypocotyl elongation during photomorphogenesis. To gain further insights into this aspect of *DDA1* function, light-grown *35S>>DDA1-EAR* plants were examined for ethanol-dependent changes in hypocotyl length. Following treatment with ethanol vapour, the hypocotyls of *35S>>DDA1-EAR* plants grown in standard growth conditions were slightly longer than those of uninduced control plants, while exposure to ethanol vapour did not affect hypocotyl length in wild-type plants (data not shown). Hypocotyls of induced *35S>>DDA1-EAR* plants were ~30% longer than those of uninduced controls (Fig. 7B). No difference in hypocotyl length was observed between induced and control dark-grown *35S>>DDA1-EAR* plants (data not shown). The phenotype of induced *35S>>DDA1-EAR* plants resembled that of *hy5* mutants, which also have longer hypocotyls in the light (Oyama *et al.*, 1997), although the *hy5* phenotype is more dramatic. The observation that *35S>>DDA1-EAR* plants exhibited a longer hypocotyl in the light while the gain-of-function *DDA1-GR* and *dda1-1* plants exhibited a shorter hypocotyl in the dark suggests that the activity of the DDA1-EAR protein is different from that of the native DDA1 protein, consistent with the fusion protein functioning as a dominant negative. It is worth noting that when grown under a long-day photoperiod, in which the transcript levels of *DDA1* were reduced (Fig. 2B), *dda1-1* plants had slightly longer hypocotyls than Col plants (Fig. 4A). These data are consistent with a model in which *DDA1* functions as a transcriptional activator to repress hypocotyl elongation in the light. Collectively, the results suggest that DDA1 acts as a transcriptional repressor during auxin response while it acts as a transcriptional activator in the photomorphogenesis pathway.

Discussion

Nature of the dda1-1 *allele*

Although the T-DNA insertion in *dda1-1* produced a hypomorphic allele under standard growth conditions, the *dda1-1* mutant behaves as a gain-of-function allele in the presence of exogenous IAA or in the dark. The reason for this discrepancy is not yet clear. The T-DNA insertion in *dda1-1* does not appear to alter splicing efficiency, as RT-PCR using primers annealing to the first exon, which is upstream of the insertion in *dda1-1*, also revealed elevated transcript levels. It is possible that the T-DNA insertion, which is in the sole *DDA1* intron, disrupts a *cis*-acting element required for the transcriptional down-regulation of *DDA1* in response to auxin or growth in the dark. However, the intron sequences are not essential for this regulation, as a *DDA1* promoter:*GUS* construct lacking the intron conferred regulation by auxin and dark. Another possibility is that transcript accumulation is due to reduced post-transcriptional degradation of the *DDA1* transcript. As the transcript produced by the *dda1-1* mutant is predicted to be identical to the wild-type *DDA1* transcript, this explanation seems implausible.

dda1-1 *mutants do not present severe phenotypes*

dda1-1 mutant plants exhibited a diminished response to both auxin and dark growth conditions. Although consistent, the phenotypes were quite subtle compared with those of other auxin and light signalling mutants (Lincoln *et al.*, 1990; Wei *et al.*, 1994; Leyser *et al.*, 1996). Because both gain-of-function *DDA1* and *35S>>DDA1-EAR* phenotypes were fairly subtle, it is likely that *DDA1* would not have been identified in conventional mutagenesis screens. The subtle nature of the phenotypes may result from the fact that *DDA1* functions in both the auxin and light perception pathways, perhaps contributing quantitatively to both responses.

In recent years, a large body of data relating to the auxin and light signal transduction pathways has been amassed, leading to a dramatic increase in our understanding of these important responses. Several mutant screens led to the identification of major players in both pathways. Most of those screens identified components that act very early in the respective pathway (Wei and Deng, 1996, 1999; Holm and Deng, 1999; Hardtke and Deng, 2000; Dharmasiri and Estelle, 2002, 2004; Liscum and Reed, 2002; Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005). To gain a complete understanding of the auxin and dark responses, it will be crucial also to identify and characterize late-acting genes. *DDA1* appears to be one such gene, participating in both light and auxin pathway responses.

LBD *genes involved in auxin-related processes*

DDA1 is one of a number of *LBD* genes that play a role in plant responses to auxin. The expression of several *Arabidopsis LBD* genes has been shown to be regulated by auxin (Nemhauser *et al.*, 2004; Paponov *et al.*, 2008). Although biological functions for most of the auxin-regulated *LBD* genes have not been reported, the rice gene *Crl1/Arl1*, which is a direct target of OsARF1 (Inukai *et al.*, 2005; Liu *et al.*, 2005), is required for crown root formation. The *Arabidopsis* genes *LBD16*, *LBD18*, and *LBD29*, which are closely related to *Crl1/Arl1*, also function in lateral root formation and are regulated by ARF7 and ARF19 (Okushima *et al.*, 2007; Lee *et al.*, 2009), indicating that function within this *LBD* subfamily is conserved across monocots and dicots. The *Arabidopsis LBD* gene *JLO* is also involved in auxin responses. *JLO* activity negatively regulates the expression of members of the *PIN* family of auxin efflux factors (Borghi *et al.*, 2007), although it remains to be shown if this regulation is direct.

Cross-talk between the auxin and light pathways

Several pieces of evidence support the idea that there is communication between the auxin and light signal

transduction pathways. HY5, a bZIP transcription factor involved in the light response pathway, promotes the expression of the *Aux/IAA* genes *AUXIN RESISTANT2* and *SOLITARY ROOT*, which function as negative regulators of auxin signalling (Cluis *et al.*, 2004). HY5 also seems to promote the expression of *DDA1*, which has been shown to be a negative regulator of auxin responses. HY5 regulation of *DDA1* transcription is probably indirect, as HY5-binding sites were not found in the *DDA1* promoter (data not shown) and *DDA1* was not identified as a HY5 target in ChIP-chip experiments (Lee *et al.*, 2007). Other evidence of cross-talk between the light and auxin pathways comes from the observation that some gain-of-function Aux/IAA mutants are also constitutively photomorphogenic (Reed, 2001; Liscum and Reed, 2002). *DDA1* also functions in both pathways, contributing to negative regulation of auxin responses and to repression of hypocotyl elongation in the light. One mutant involved in both auxin and light responses is *axr3-1*, and it was shown that *DDA1* levels were stably maintained in this background even in the presence of auxin. The present data are consistent with the idea that *DDA1* is a negative regulator of the auxin signalling pathway and promotes hypocotyl elongation in the light.

DDA1 *is involved in auxin signalling and promotion of photomorphogenesis*

Taken together, the data shown here have led to a model that would explain the regulation of *DDA1* and its function. IAA negatively regulates Aux/IAA proteins such as AXR3 by inducing their proteolysis (Dharmasiri and Estelle, 2002, 2004). It was found that in the dominant *axr3-1* mutant, the level of *DDA1* transcripts was stabilized in the presence of IAA, which normally causes a decrease in *DDA1* transcript accumulation. Therefore, the IAA-induced reduction of *DDA1* transcription may act through the degradation of AXR3 or related Aux/IAA proteins. In the presence of auxin, the levels of *DDA1* transcripts were increased in the *dda1-1* background. Hence, the reduced auxin responses in *dda1-1* are due to enhanced levels of *DDA1* transcripts, leading to the conclusion that *DDA1* is a negative regulator of auxin signalling. This is in agreement with the fact that AXR3, which is a positive regulator of *DDA1*, is also a negative regulator of this same pathway (Reed, 2001; Dharmasiri and Estelle, 2002, 2004; Liscum and Reed, 2002).

It is known that the photomorphogenesis-promoting transcription factor HY5 is targeted for degradation in the dark (von Arnim and Deng, 1994; von Arnim *et al.*, 1997; Ang *et al.*, 1998; Osterlund *et al.*, 2000). It has been shown here that HY5 positively regulates the expression of *DDA1* and, in the dark, when HY5 is absent, the transcript levels of *DDA1* are decreased. Based on these results, a model is proposed in which a mechanism for down-regulation of *DDA1* in the dark is through the degradation of its positive regulator, HY5. In the *dda1-1* mutant, the levels of *DDA1* transcripts are increased in the dark. Therefore, the aberrant dark responses in *dda1-1* are due to elevated levels of *DDA1*, leading to the conclusion that *DDA1* is involved in promotion of photomorphogenesis. The fact that HY5, a key player in the promotion of photomorphogenesis (Chattopadhyay *et al.*, 1998; Yadav *et al.*, 2002), is a positive regulator of *DDA1* corroborates this conclusion.

Based on the phenotypes observed in *35S>>DDA1-EAR* plants, *DDA1* appears to function as both a transcriptional activator and a transcriptional repressor depending on the pathway. Transcription factors in a number of different families have been reported to have both transcriptional activation and transcriptional repression activities depending on interactions with other factors or protein modifications (Hoecker *et al.*, 1995; Ammanamanchi *et al.*, 2003; Canon and Banerjee, 2003; Kesarwani *et al.*, 2007; Ikeda *et al.*, 2009). The ability of a transcription factor both to activate and to repress transcription depending on context contributes substantially to the overall complexity of the transcriptional response.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Dose–response curve comparing auxin sensitivity in *dda1-1*, Col, and *axr1-3*.

Figure S2. RT-PCR using different amounts of cDNA template to demonstrate that PCRs are quantitative.

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