J. Plant Biol. (2009) 52:65–72
DOI 10.1007/s12374-008-9005-7

ORIGINAL RESEARCH

Identification and Functional Analysis of Phosphoproteins Regulated by Auxin in *Arabidopsis* Roots

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Received: 2 October 2008 / Revised: 23 November 2008 / Accepted: 26 November 2008 / Published online: 16 January 2009

Abstract The phytohormone auxin modulates diverse aspects of plant growth and development. Protein phosphorylation is believed to play a key role in regulating auxin-mediated responses. To determine the phosphoproteins affected by auxin in *Arabidopsis*, we used phospho-specific antibodies to analyze their profiles on two-dimensional gels, then identified them by mass spectrometry. We found two phosphoproteins, enolase and the beta subunit of succinyl-CoA synthetase (SCS-beta), and noted that their phosphorylation was increased by auxin. To investigate their importance in auxin-mediated processes, we characterized *Arabidopsis* knockout mutants of the two genes. A homozygous null mutation in the gene for SCS-beta conferred embryo lethality. The enolase knockout mutants showed defects in root development similar to those of auxin-related mutants such as *alf3* and *xbat32*. Therefore, we suggest that enolase is involved in auxin-regulated processes.

Keywords *Arabidopsis* · Auxin · Enolase · Phosphorylation · Succinyl-CoA synthetase

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Abbreviations

eno1	Enolase knockout mutant
IAA	Indole-3-acetic acid
SCS-beta	Succinyl-CoA synthetase beta subunit

The plant hormone auxin regulates many aspects of plant growth and development, including cell elongation, embryogenesis, and lateral root formation [3]. In *Arabidopsis*, embryogenesis is interrupted by mutations of the genes involved in auxin synthesis, transport, or perception [7, 10, 13]. Furthermore, homozygous knockout null mutants of the auxin-binding protein 1 (ABP1) gene are embryo lethal [6]. Mutational analyses in that species also have shown the importance of auxin in promoting lateral root formation; mutants with elevated levels of endogenous auxin have more lateral roots, whereas those defective in auxin transport or sensitivity have fewer such roots [14].

Protein phosphorylation plays an important regulatory role in both auxin transport and auxin signaling [9]. Auxin-stimulated mitogen-activated protein kinase (MAPK) activity has been reported in *Arabidopsis* roots [20]. MAPK activity is also required for adventitious root formation induced by indole-3-acetic acid (IAA), the principal natural auxin [24]. In addition, MAPK pathway kinases are involved in suppressing the early expression of auxin-responsive genes [15]. Besides MAPK pathway kinases, a serine–threonine protein kinase, PINOID, is critical to the regulation of auxin signaling in *Arabidopsis* [8]. Protein phosphatases have also been implicated in auxin-regulated processes. A mutation in protein phosphatase 2A regulatory subunit A in *Arabidopsis* results in reduced protein phosphatase 2A activity and alterations in auxin transport and lateral root growth [26]. Mutation in the dual-specificity phosphatase IBR5 gene leads to attenuated responses to auxin [21]. In agreement with this involvement

of protein phosphorylation in regulating auxin transport, the auxin efflux carrier protein PIN1 is phosphorylated in a PINOID-dependent manner in vivo [19]. Furthermore, the effect of PINOID kinase on PIN1 phosphorylation is antagonistically regulated by protein phosphatase 2A; the phosphorylation status of PIN1 dictates its localization at the apical or basal plasma membrane [19].

Here, we report the identification of several auxin-regulated phosphoproteins in *Arabidopsis* roots. We investigated the roles of two of them—enolase and succinyl-CoA synthetase beta subunit—in root development and embryogenesis.

Materials and Methods

Plant Materials and Protein Extraction

Arabidopsis thaliana (Columbia ecotype) seeds were germinated on half-strength Murashige and Skoog (MS) agar plates supplemented with 1% sucrose. The seedlings were then placed vertically for 3 weeks on MS plates in a growth cabinet at 22°C and under a 16-h photoperiod (70 to 90 μmol m^{-2} s^{-1}). For auxin treatment, the seedling plates were gently submerged for 30 min in either 5 μM IAA (Sigma) or a mock solution (control). Their roots were then excised and immediately frozen in liquid nitrogen. Samples were ground to a fine powder in liquid nitrogen, and proteins were extracted on ice for 10 min with an extraction buffer [50 mM Tris–HCl (pH 7.5) and 5 mM EDTA] containing protease and phosphatase inhibitor cocktails (Sigma). Insoluble material was removed by centrifugation at 16,000×*g* for 10 min at 4°C. Protein concentrations in the supernatant solutions were determined by Bradford assays [4].

Two-Dimensional Native/SDS Gel Electrophoresis

Native/SDS gel electrophoresis was performed as previously described [27], with modifications. Briefly, the first-dimension native gel electrophoresis was run at 4°C on 5% to 18% acrylamide gradient gels. Lanes containing proteins were then excised and soaked for 45 min at room temperature (RT) in equilibration buffer [62.5 mM Tris–HCl (pH 6.8), 3% SDS, 10% glycerol, and 10 mM dithiothreitol]. Afterward, the excised strips were placed horizontally on SDS-denaturing gels (7% to 15% acrylamide), and the proteins were resolved vertically by SDS-PAGE. The gels were then either stained with Coomassie blue or silver or used for immunoblotting.

Immunoblot Analysis of Phosphorylated Proteins

Proteins within the two-dimensional gels were transferred to Immobilon P membranes (Millipore) using an electrophoretic blotting system (C.B.S. Scientific) at 30 V overnight in a cold room. The membranes were blocked for 2 h at RT with 1% (*w*/*v*) bovine serum albumin in TBST [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% (*v*/*v*) Tween-20]. These were then incubated with rabbit anti-phosphoserine antibody (Zymed Laboratories) at 1 μg ml^{-1} in TBST for 2 h at RT. After washing with TBST, the membranes were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Pierce) at a 1:10,000 dilution for 1 h at RT. The antibody complexes were detected by enhanced chemiluminescence (Pierce), followed by exposure to X-ray film according to the manufacturer's instructions.

Dephosphorylation of Proteins with Alkaline Phosphatase

To ensure that the immunoreactive signals were specific for phosphoproteins, membranes with transferred proteins were incubated with calf intestinal alkaline phosphatase (New England Biolabs) according to Pezet et al. [25]. The alkaline phosphatase-treated membranes were then probed with anti-phosphoserine antibody as described above.

Protein Digestion and Tandem Mass Spectrometry Analysis

Protein spots detected on the immunoblots were cut out from the corresponding gel and digested with sequencing-grade trypsin (Promega) per Soskic et al. [30]. The resultant peptides were separated on a microcapillary C18 column (100 μm inner diam., 10 cm long) coupled inline with an LCQ-Deca XP ion-trap mass spectrometer (ThermoFinnigan). Eluting peptides from the column were ionized by electrospray ionization and analyzed by mass spectrometry, which was configured to optimize the duty cycle length with the data acquire, alternating between a single full MS scan followed by three MS/MS scans on the three most intense precursor masses. The resultant tandem mass spectra were searched against the *Arabidopsis* non-redundant protein database, which was downloaded from the National Center for Biotechnology Information using the SEQUEST computer algorithm (ThermoFinnigan) as described previously [17].

Characterization of T-DNA Insertion Mutants

T-DNA insertion lines in the enolase gene (At2g36530) and succinyl-CoA synthetase beta subunit gene (SCS-beta; At2g20420) were obtained from the SALK collection [1] at the Arabidopsis Biological Resource Center. T-DNA insertions were verified through PCR genotyping, with T-DNA left border (LB) and gene-specific primers. The *SCS-beta* mutant line (SALK_090643) was examined

for T-DNA insertion using the T-DNA LB primer LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) and the *SCS-beta*-specific primer S1 (5′-ATGAGGGGATTGGTGAA CAAG-3′). The *enolase* mutant line (SALK_021737) was examined for T-DNA insertion with the LBa1 primer and the *enolase*-specific primer E1 (5′-AGATCCTCCATCCCT CAGCTC-3′). Homozygotes of each line were identified by PCR from F2 generation plants, using a pair of primers flanking the T-DNA (gene-specific primer S1 or E1 and another gene-specific primer: S2, 5′-AGCAGCTCATC ATTTGCGTAG-3′ for *SCS-beta* or E2, 5′-ACAGATCTTC GACAGTCGTGG for *enolase*).

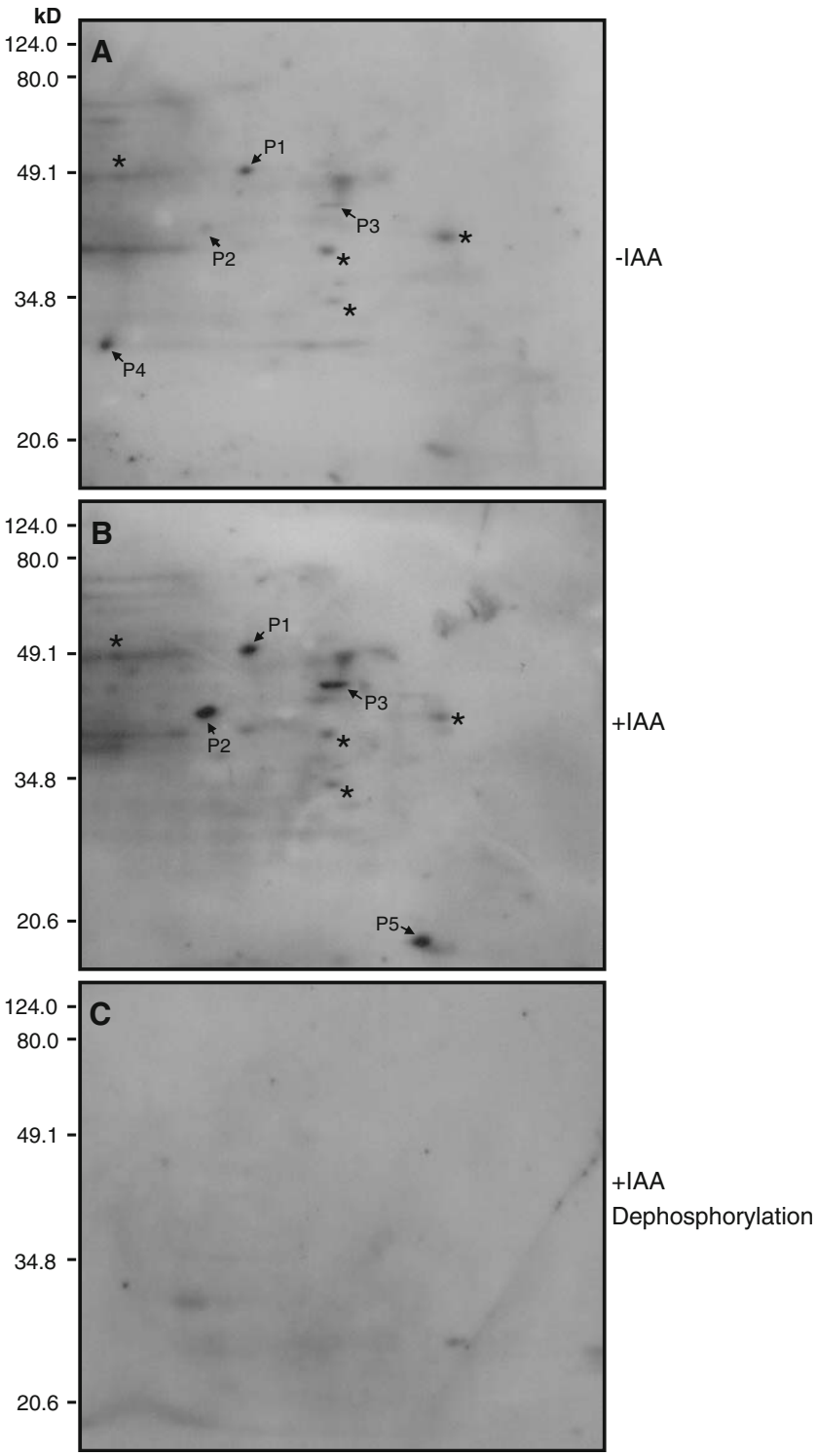

Fig. 1 Phosphoprotein patterns of *Arabidopsis* root proteins in response to auxin. Three-week-old seedlings were treated with 5 μM of IAA for 30 min. Crude proteins (300 μg) extracted from roots (control and treated) were separated by two-dimensional gel electrophoresis with non-denaturing PAGE in first dimension and SDS-PAGE in second dimension. Proteins were then transferred from gels to membranes and probed with anti-phosphoserine antibody. Phosphoproteins showing distinct differences between control (**a**) and IAA treatment (**b**) are marked with *P*. Unchanged spots are marked with *stars* and serve as references. To ensure that antibody was specific for phosphorylated proteins, membrane with transferred proteins from IAA-treated roots was incubated with calf intestinal alkaline phosphatase (to remove phosphates), then probed with anti-phosphoserine antibody (**c**). Positions of molecular mass standards (in kilodalton) are at the *left*. Results were reproducible in three independent experiments

RT-PCR

Total RNA was isolated from roots of 3-week-old *Arabidopsis* seedlings, using Trizol reagent (Invitrogen). cDNA was synthesized with the SuperScript First-Strand Synthesis System and an oligo(dT) primer (Invitrogen). PCR amplification was performed as follows: 5 min at 94°C; 34 cycles of 30 s at 94°C, 30 s at 58°C, 2 min at 72°C, and finally, 10 min at 72°C. The following primer pair was used for specific amplification of enolase cDNA: 5′-ATCCAGCCTTCTCGATAGCAG-3′ and 5′-ACA GATCTTCGACAGTCGTGG-3′. Constitutively expressed *Actin-2* served as an internal control for RT-PCR, using specific primers (5′-ATGGCTGAGGCTGATGATATT CAAC-3′ and 5′-TCTCAGCACCAATCGTGATGACTTG-3′). The amplified DNA was visualized on a 1% agarose gel stained with ethidium bromide.

Auxin Response Assays

Wild-type (Columbia ecotype) and *eno1* seeds were planted on half-strength MS agar media containing 1% sucrose. Plates were placed in a growth cabinet at 22°C and under a 16-h photoperiod (70 to 90 μmol m^{-2} s^{-1}). Three days after germination, the seedlings were transferred to half-strength MS agar media containing 1% sucrose with or without 1 μM IAA. Roots were measured 9 days after this transfer, and the lateral roots from each primary root were counted after 11 days. Measurement of IAA content in seedlings was conducted according to Lin et al. [18] using an enzyme-linked immunosorbent assay (ELISA) kit (Agdia, Elkhart, IN, USA).

Results and Discussion

Identification of Auxin-Regulated Phosphoproteins in *Arabidopsis* Roots

Auxin signaling plays a principal role in mediating root growth. To detect the phosphoproteins regulated by auxin, we treated *Arabidopsis* seedlings with indole-3-acetic acid, the major type of natural auxin. Root proteins from IAA-treated seedlings and controls were separated by two-dimensional native/SDS gel electrophoresis, transferred to membranes and probed with an anti-phosphoserine antibody. Five phosphoproteins (P1 through 5) displayed distinct changes in their response to auxin (Fig. 1). Spots P1, P2, P3, and P5 showed IAA-increased phosphorylation, whereas P4 exhibited a decrease. When the proteins on these membrane blots were first treated with alkaline phosphatase (to remove the phosphate groups), then probed with the phosphoserine-specific antibody, immunoreactivity was almost completely abolished (Fig. 1c). These results indicate that the phosphoserine antibody could specifically detect phosphoproteins in *Arabidopsis* by immunoblotting analysis.

To identify the auxin-regulated phosphoproteins, P1 through P5 were digested with trypsin and analyzed by tandem mass spectrometry. P1, P2, and P5 were identified

Table 1 Phosphoproteins identified by tandem mass spectrometry (MS/MS)

Spot	NCBI accession no.	Protein	Gene accession no.	Molecular mass	Unique peptide sequences identified by MS/MS
P1	NP_181192	Enolase	At2g36530	47,719	AVGNVNNIIGPALIGK AGAVVSGIPLYK IVLPVPAFNVINGGSHAGNK MGVEVYHHLK ISGDALKDLYK AIAEKSCNALLLK AGWGVMTSHR LAKYNQLLR
P2	NP_179632	Succinyl-CoA synthetase beta subunit	At2g20420	45,345	YGVNVPK GVAASSLEEVK AIQDVFPNESELVVK SQILAGGR MLGQVLVTK SAGPLIIACK LYELFR
P5	NP_194098	Major latex protein, putative	At4g23670	17,517	SWDYTYDGK GLDGHVMEHLK ITMIWEK

respectively as enolase, SCS-beta, and a putative major latex protein (Table 1). This demonstrated that the phosphorylation of all three was stimulated by IAA. In contrast, P3 and P4 could not be identified due to their low abundance.

Enolase is an enzyme of the glycolytic pathway. Apart from its role in glycolysis, it is found in the nucleus in several cell types and has transcriptional regulatory functions [12, 16, 31]. SCS catalyzes the formation of succinate and coenzyme-A in the citric acid cycle. SCS consists of two different subunits—alpha and beta [28]. The putative major latex protein shows significant similarity to a group of low-molecular-weight polypeptides that have been isolated from the latex of opium poppy [22]. Their functions are not known. Although enolase and SCS-beta were earlier identified as phosphoproteins in *Arabidopsis* [29], these major latex proteins were not reported previously as phosphoproteins.

Homozygous Mutations in the Gene for Succinyl-CoA Synthetase Beta Subunit Lead to Embryo Lethality

To explore further the role of SCS-beta in the auxin response, we took a reverse genetics approach to find mutants in the SCS-beta gene. One insertion line (SALK_090643) was identified from the Salk T-DNA collection, and the positions of the T-DNA insertions were confirmed by PCR analysis. The *scs-beta* mutant allele carried T-DNA in the second exon of the SCS-beta gene (Fig. 2a). Plants heterozygous for those insertions were isolated by PCR screening and showed no obvious phenotype. Moreover, no homozygous mutants were found from an extensive screening of the progeny of the heterozygous plants (Fig. 2b). These results suggest that homozygous null alleles might be lethal. Because immature *Arabidopsis* seeds within a single silique develop at approximately the same rate and because normal seeds are green, it is possible to score segregating individuals with aberrant development [11]. Therefore, we examined by microscope the immature seeds from siliques of heterozygous plants and found that approximately 25% of them

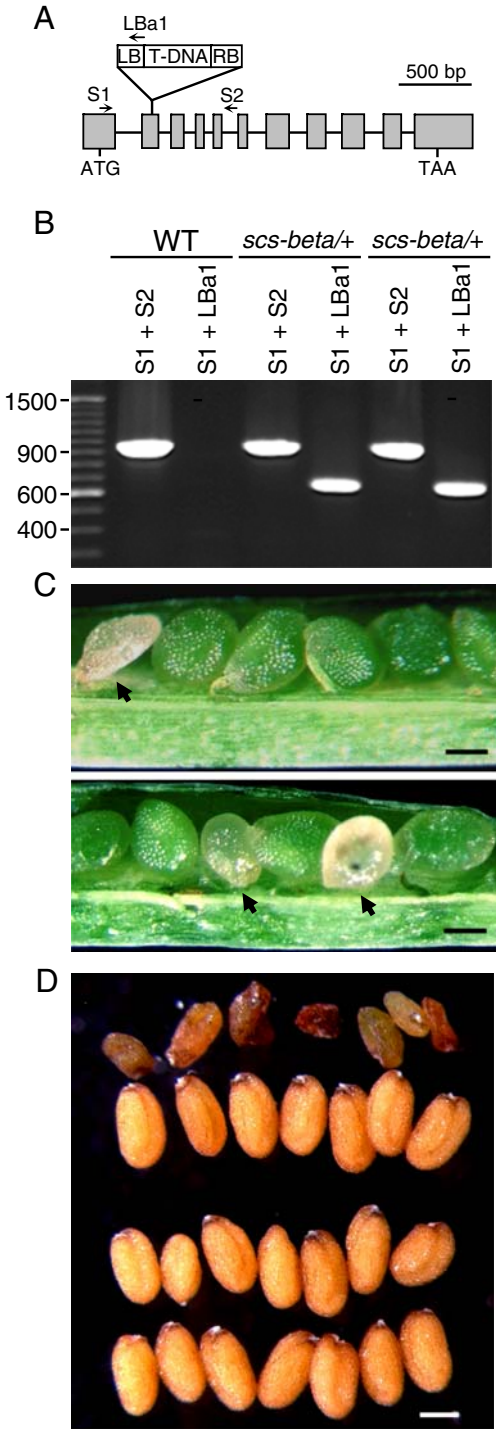

Fig. 2 Homozygous *Arabidopsis scs-beta* mutants are embryo lethal. **a** Schematic structure of SCS-beta gene and inserted T-DNA. Exons and introns are represented by *filled boxes* and *lines*, respectively. T-DNA insert is not drawn to scale. *LB* and *RB* are left and right borders of T-DNA, respectively. Primers used to determine T-DNA insertion in *SCS-beta* are displayed. *S1* and *S2* are *SCS-beta*-specific primers while LBa1 is T-DNA LB primer. **b** PCR screening for *scs-beta* T-DNA insertion mutants. Genomic DNA was extracted and subjected to two sets of PCR reactions (S1 + S2 and S1 + LBa1). For heterozygous lines (one in chromosome pair having T-DNA insertion, *scs-beta/+*), a 951-bp product should have been produced in S1 + S2 reaction, whereas homozygous lines (*scs-beta/scs-beta*) should not have any product in that reaction due to insertion of large piece of T-DNA (>8,000 bp) in both chromosomes. Both hetero- and homozygous lines should have yielded a 684-bp product (SCS-beta-T-DNA) in S1 + LBa1 reaction. Wild type (*WT*, no insertion) should have produced 951-bp fragment in S1 + S2 reaction but nothing in S1 + LBa1 reaction. No homozygous *scs-beta* mutants were identified, despite extensive screening. **c** Immature seeds in siliques of heterozygous lines (*scs-beta/+*). Normal, immature seeds appear *green*; abnormal seeds are *white* (*arrows*). Scale bar = 0.2 mm. **d** Normal and wrinkled seeds from *scs-beta/+* plant segregating in 3:1 ratio. Scale bar = 0.3 mm

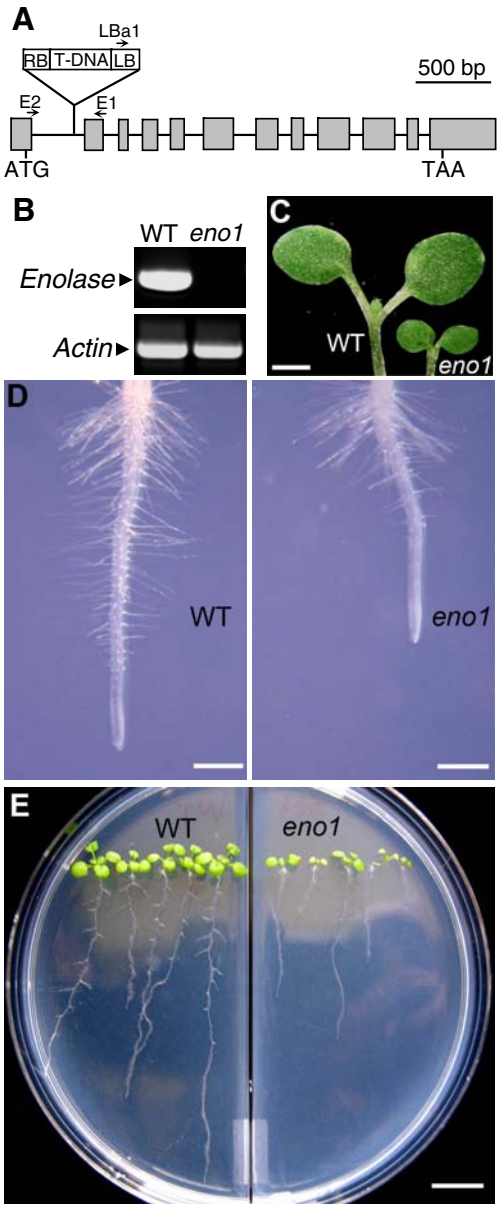

Fig. 3 Phenotypes of *Arabidopsis* enolase knockout mutant (*eno1*). **a** Schematic structure of enolase gene and inserted T-DNA. Exons and introns are represented by *filled boxes* and *lines*, respectively. T-DNA insert is not drawn to scale. *LB* and *RB* are left and right border of T-DNA, respectively. Primers (E1, E2, and LBa1) used to determine T-DNA insertion in *eno1* are displayed. **b** RT-PCR analysis of enolase expression in roots from *eno1* knockout mutants and wild-type (*WT*) plants. Mutant contains no detectable enolase transcript. Actin served as internal control for RT-PCR. **c** Cotyledons of WT and *eno1* seedlings grown for 5 days. Mutant has smaller cotyledons than WT. Scale bar = 0.6 mm. **d** Root phenotypes of 4-day-old seedlings. Primary roots of *eno1* mutant are shorter than from WT. Scale bar = 0.6 mm. **e** WT (*left*) and *eno1* (*right*) seedlings were grown for 12 days on MS medium in segmented dishes with two sections. WT roots are highly branched, whereas *eno1* seedlings have few, if any, lateral roots. Scale bar = 1 cm

were abnormal (Fig. 2c). They aborted and became shrunken when the siliques matured (Fig. 2d). Normal and wrinkled seeds from mature siliques of those heterozygous plants segregated in a 3:1 ratio, as is expected for a recessive mutation in an essential nuclear gene (normal/wrinkled = 369:117, χ^2=0.22). These results indicate that homozygous mutations in the SCS-beta gene lead to embryo lethality.

Mutations in *Arabidopsis* genes that are involved in auxin synthesis, transport, or perception affect embryogenesis [7, 10, 13]. Furthermore, a homozygous knockout null mutation of the ABP1 gene confers embryo lethality [6]. This lethality caused here by homozygous mutations (Fig. 2) implies that SCS-beta is important for embryogenesis. However, such a phenomenon makes difficult any

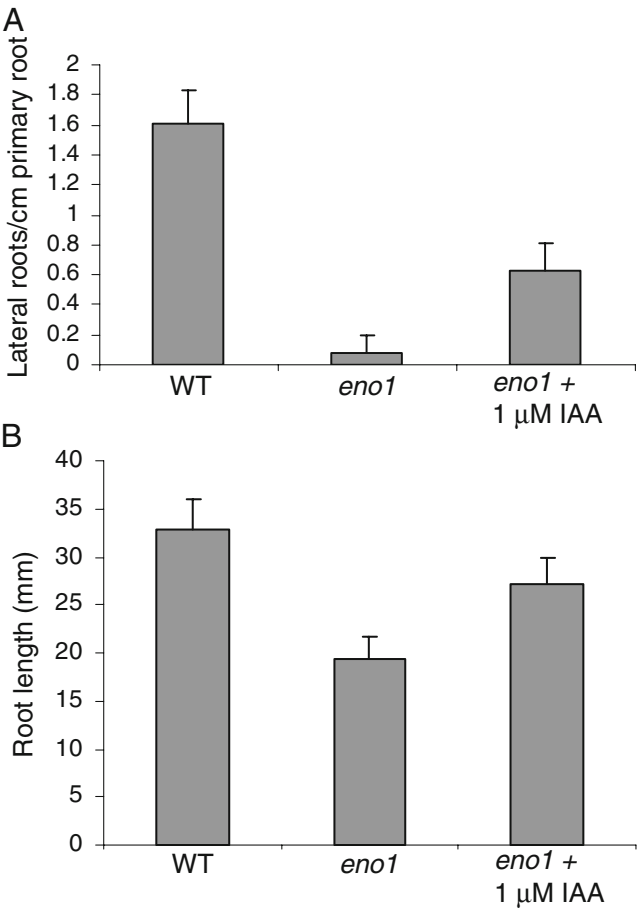

Fig. 4 **a** Auxin can partially rescue lateral root defects. WT plants were grown vertically on MS medium for 11 days. For mutant, 3-day-old *eno1* seedlings were transferred from MS medium to same media type supplemented with 0 or 1 μM IAA, then allowed to grow vertically for another 9 days. Each data point is average number of lateral roots counted from 50 primary roots. Error bars represent standard errors of means. **b** Effect of exogenous auxin on primary root growth. WT and *eno1* mutants were grown under same conditions as described above. Primary roots were measured after 11 days. Error bars represent standard errors of means; n=50

genetic analysis of a post-embryonic role for SCS-beta in auxin-mediated responses.

The Enolase Knockout Mutant Exhibits Root Defects

To determine the role of enolase in the auxin response, we isolated a T-DNA insertion knockout mutant for that gene from the Salk T-DNA collection (Fig. 3a). Its expression in the homozygous mutants was determined by RT-PCR, and no amplification product of the transcript was detected (Fig. 3b). We designated this mutant as *eno1*. When grown on MS media, *eno1* mutants had smaller cotyledons (Fig. 3c) and shorter primary roots (Fig. 3d) than the wild-type plants. They also had few, if any, lateral roots (Fig. 3e). Similar results were obtained in another knockout mutant (SALK_077784) of the enolase gene (data not shown). These *eno1* phenotypes resemble those of other auxin-related mutants, e.g., *xbat32*, *alf3*, and *sir1* [5, 23, 32]. The *xbat32* (XB3 ortholog 2 in *Arabidopsis*) mutant shows defects in lateral root formation and primary root growth, and the *XBAT32* gene is thought to be involved in auxin transport [23]. In the *alf3* (aberrant lateral root formation 3) mutant, growth of both lateral and primary roots is arrested [2, 5], but such defects can be rescued by exogenous application of IAA [2, 5]. The *sir1* (sirtinol resistant 1) mutant is hypersensitive to exogenous auxin, and SIR1 is thought to be an upstream component in auxin signaling [32]. Therefore, the phenotypic similarities among *eno1, xbat32*, *alf3*, and *sir1* suggest that enolase plays a role in auxin-regulated root development.

Exogenous Auxin Can Partially Restore the Root Deficiency in eno1 Mutant Plants

To investigate whether auxin can rescue lateral-root defects in mutants, we transferred 3-day-old *eno1* seedlings from their MS media to the same media now supplemented with 1 μM of IAA. Those on media containing IAA had significantly more lateral roots than did seedlings on IAA-free media (Fig. 4a). However, those mutant seedlings treated with IAA had fewer lateral roots than the wild-type plants. Although various IAA concentrations (0.1, 0.5, 1 and 5 μM) were tried, none was capable of fully restoring the level of *eno1* lateral root development to that of the wild type. These results indicate that auxin can partially restore the deficiency of lateral roots caused by mutation in the enolase gene. Likewise, exogenous application of IAA partially restored the defect in primary root growth in *eno1* mutants (Fig. 4b). Defect mutations in the genes involved in auxin biosynthesis, transport, or signaling can affect root development [14]. Our ELISA assay revealed that the seedlings of *eno1* had the same IAA content as those of wild-type plants. Thus, the root phenotype of eno1 did not appear to be due to an alteration in IAA synthesis. This observation that auxin can only partially restore the eno1 phenotype is similar to that found with the *xbat32* mutant [23]. There, a loss-of-function mutation in the ubiquitin ligase gene *XBAT32* causes root-growth defects that include shorter primary roots and a lack of lateral roots, but that root phenotype is partially rescued by auxin. Nodzon et al. [23] have proposed that XBAT32 functions in auxin transport. Although we make a similar speculation, we cannot rule out the possibility that enolase is involved in auxin signaling.

Conclusion

We identified enolase, SCS-beta, and a putative major latex protein as auxin-regulated phosphoproteins in *Arabidopsis* roots. This was accomplished through a combination of two-dimensional gel electrophoresis, phospho-specific antibody immunoblotting, and mass spectrometry. RT-PCR analysis also indicated that auxin did not affect the expression of these genes (data not shown). Taken together, these results suggest that all three are regulated by auxin at the protein level. The enolase knockout mutant exhibited defects in root development, but exogenous IAA partially restored that deficiency. Homozygous mutations in the SCS-beta gene conferred embryo lethality. We were unable to isolate a T-DNA insertion mutant for the putative major latex protein. However, its function might be determined later via RNA interference technology.

Acknowledgments We thank Drs. Din Ma, Zhaohua Peng, and Kenneth Willeford for critical reading of the manuscript and Dr. Tibor Pechan for help with mass spectrometry. This research was partially supported by start-up funds from Mississippi State University and by the US National Science Foundation (MCB0505458). Zenglan Wang's visit to Mississippi State University was partially supported by the National Basic Research Program of China (No. 2006CB100104). This article was approved for publication as No. J-11393 of the Mississippi Agricultural and Forestry Experiment Station.

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