Arabidopsis RING E3 Ligase XBAT32 Regulates Lateral Root Production through Its Role in Ethylene Biosynthesis[1[W][OA]]

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XBAT32, a member of the RING domain-containing ankyrin repeat subfamily of E3 ligases, was previously identified as a positive regulator of lateral root development. Arabidopsis (*Arabidopsis thaliana*) plants harboring a mutation in *XBAT32* produce fewer lateral roots that wild-type plants. We found that *xbat32* mutants produce significantly more ethylene than wild-type plants and that inhibition of ethylene biosynthesis or perception significantly increased *xbat32* lateral root production. XBAT32 interacts with the ethylene biosynthesis enzymes AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE4 (ACS4) and ACS7 in yeast-two-hybrid assays. XBAT32 is capable of catalyzing the attachment of ubiquitin to both ACS4 and ACS7 in in vitro ubiquitination assays. These results suggest that XBAT32 negatively regulates ethylene biosynthesis by modulating the abundance of ACS proteins. Loss of *XBAT32* may promote the stabilization of ACSs and lead to increased ethylene synthesis and suppression of lateral root formation. XBAT32 may also contribute to the broader hormonal cross talk that influences lateral root development. While auxin treatments only partially rescue the lateral root defect of *xbat32*, they completely restore wild-type levels of *xbat32* lateral root production when coupled with ethylene inhibition. Abscisic acid, an antagonist of ethylene synthesis/signaling, was also found to stimulate rather than inhibit *xbat32* lateral root formation, and abscisic acid acts synergistically with auxin to promote *xbat32* lateral root production.

Ubiquitin ligases (E3) are central components of the ubiquitination pathway, an essential system that functions to covalently attach ubiquitin molecules to selected proteins. Posttranslational attachment of ubiquitin is accomplished via a conjugation cascade that begins with the ubiquitin-activating enzyme (E1), which activates and transfers ubiquitin to one of many ubiquitin-conjugating enzymes (E2). The substrate-recruiting E3 then facilitates the transfer of ubiquitin from the E2-ubiquitin intermediate to the substrate. One, few, or many ubiquitin moieties can be attached, and the types of ubiquitin-ubiquitin linkages utilized in polyubiquitin chain formation provide functional versatility (Weissman, 2001). The best-characterized outcome of ubiquitination is the degradation of proteins by the 26S proteasome. Proteins targeted for degradation are modified by the attachment of a Lys-48-linked polyubiquitin chain. A number of nonproteolytic functions, including endocytosis, protein activation, and sorting, have also been linked to ubiquitination. These processes utilize alternative ubiquitin linkages, such as Lys-63-linked polyubiquitin chain (Bonifacino and Weissman, 1998; Deng et al., 2000).

The selection of specific targets in the ubiquitination pathway is governed mainly by a very large and diverse family of E3 ligases. The Arabidopsis (*Arabidopsis thaliana*) genome, for example, is predicted to encode for over 1,300 E3 ligases, which can be classified into different subgroups based on the presence of the U-box, Really Interesting New Gene (RING), or Homologous to E6-AP C-Terminus E2-binding domain (Stone and Callis, 2007). A large complement of the Arabidopsis E3s belongs to the RING class, which is characterized by the presence of a Cys- and His-rich domain (Lovering et al., 1993). The Arabidopsis RING domain-containing E3s can further be classified into 30 different subgroups based on domain architecture (Stone et al., 2005). Members of several subgroups have been linked to important physiological and developmental processes, including XBAT32 (XB3 ortholog 2 in Arabidopsis), which belongs to the RING domain-containing ankyrin repeat subgroup (Nodzon et al., 2004; Stone et al., 2005). Members of this subgroup are referred to as XBAT due to their structural similarity to the rice (*Oryza sativa*) XB3 protein (Nodzon et al., 2004). XBAT32 has been characterized as a positive regulator of lateral root development (Nodzon et al., 2004). This E3 ligase, which is postu-

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lated to be involved in auxin transport, is required for the proper initiation of lateral roots.

Lateral roots are an essential part of the plant root system, which functions as a sensor for abiotic and biotic signals, facilitates the uptake of nutrients and moisture from the soil, and acts as a support for the aerial parts of the plant. The primary root is formed during embryogenesis and emerges from the seed during germination (Dittmer, 1937; Malamy and Benfey, 1997). As the seedling develops further, lateral roots emerge from the primary root to facilitate maximum nutrient absorption from the soil. Following germination, only certain pericycle cells in the primary root that are in contact with the xylem poles acquire the ability to undergo a series of asymmetric cell divisions. This process, referred to as the lateral root initiation stage, leads to the formation of lateral root primordium (Malamy and Benfey, 1997; Dubrovsky et al., 2000; De Smet et al., 2006a). A well-characterized series of cell divisions in the lateral root primordium then forms a meristem, which finally emerges from the primary root by cell elongation (Malamy and Benfey, 1997; Casimiro et al., 2003).

The developmental plasticity of the plant root system, which is affected by external signals and multiple internal hormonal cues, allows the root to adapt to the changing environment. Auxin, a key promoter of lateral root growth, regulates different stages of lateral root formation, including initiation (Casimiro et al., 2003; De Smet et al., 2006a), primordium development (Benková et al., 2003), and emergence (Laskowski et al., 2006). Functional auxin biosynthesis, transport, and signaling pathways are required for lateral root formation (Fukaki et al., 2007). Mutants that overproduce indole-3-acetic acid (IAA), the predominant naturally occurring form of auxin, have an increased number of lateral roots (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995). Altering acropetal and basipetal auxin transport by treatment with auxin transport inhibitors decreases lateral root production (Reed et al., 1998; Casimiro et al., 2001). Other hormones regulate lateral root development either directly or indirectly via their interactions with auxin (Nibau et al., 2008). There is evidence to support cross talk between auxin and abscisic acid (ABA), which is known to inhibit lateral root formation (De Smet et al., 2006b). The ABA signaling mutant *abscisic acid insensitive3* shows reduced lateral root formation in response to an auxin stimulus (Brady et al., 2003). Ethylene also inhibits lateral root production (Chilley et al., 2006; Fukaki et al., 2007; Negi et al., 2008). The regulatory effect of ethylene on lateral root production is auxin dependent (Stepanova et al., 2005; Ruzicka et al., 2007; Swarup et al., 2007). High levels of ethylene alter auxin transport, which in turn affects auxin uploading into root cells, to ultimately inhibit lateral root production (Gazzarrini and McCourt, 2001; Negi et al., 2008). Enhanced ethylene response mutants such as *polaris* display reduced IAA accumulation in roots and are deficient in lateral root development (Chilley et al., 2006). Other ethylene mutants, such as *ethylene overproducer1* (*eto1*) and the constitutive ethylene signaling mutant *constitutive triple response1* (*ctr1*), also display a reduced lateral root phenotype (Negi et al., 2008).

Regulated proteolysis also plays an important role in lateral root development. The auxin receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1) is an F-box protein that functions as the substrate-recruiting subunit for the SCF^{TIR1} E3 ligase complex (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). SCF^{TIR1} regulates the degradation of AUX/IAA transcriptional repressors that interact with and inhibit the activity of AUXIN RESPONSE FACTOR (ARF) transcription factors. The degradation of AUX/IAA proteins such as SOLITARY ROOT (SLR1)/IAA14 allows ARF7 and ARF19 to activate the transcription of genes required for lateral root initiation (Fukaki et al., 2002; Okushima et al., 2007). Mutations that stabilize SLR1/IAA14 prohibit the formation of lateral roots (Fukaki et al., 2005). Other E3 ligases with a role in lateral root development include the F-box proteins CEGENDUO (CEG), VIER F-BOX PROTEINE (VFB), ARABIDILLO-1 and ARABIDILLO-2, and the RING proteins SINAT5 and XBAT32 (Xie et al., 2002; Nodzon et al., 2004; Coates et al., 2006; Dong et al., 2006; Schwager et al., 2007). Plants that lack *VFB* and *ARABIDILLO* produce fewer lateral roots than wild-type plants, and an increase in the number of lateral roots is observed with loss of *CEG* (Coates et al., 2006; Dong et al., 2006; Schwager et al., 2007). SINAT5 regulates the abundance of the transcription factor NAC1, which promotes lateral root formation (Xie et al., 2000, 2002). Overexpression of *SINAT5* reduces NAC1 protein levels in roots and inhibits lateral root growth (Xie et al., 2002).

In this study, we analyzed three of the *XBAT* RING E3 ligase family members, *XBAT32*, *XBAT34*, and *XBAT35*. The reduced lateral root phenotype is unique to *XBAT32*. Further analysis of *xbat32* demonstrates that the mutant overproduces ethylene, and this contributes to its reduced lateral root phenotype. Ethylene antagonists silver nitrate and ABA stimulate lateral root production in *xbat32*, and both act synergistically with auxin to fully rescue the lateral root defect. XBAT32 interacts with AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE4 (ACS4) and ACS7 in a yeast two-hybrid assay, and XBAT32 was able to ubiquitinate both ACS4 and ACS7 in in vitro ubiquitination assays. Together, our results indicate that XBAT32 plays an essential role in ethylene biosynthesis as a negative regulator of ACS protein abundance. Loss of XBAT32 E3 activity may lead to an accumulation of ACS4/7 and increased ethylene production, which may account for the *xbat32* mutant phenotypes.

RESULTS

ABA Promotes Lateral Root Production in *xbat32*

Arabidopsis *XBAT* and *XBAT*-related genes from other plant species are involved in development, re-

production, ABA signaling, and biotic and abiotic stress tolerance (Nodzon et al., 2004; Huang et al., 2006; Stone et al., 2006; Wang et al., 2006; Xu et al., 2007; Yang et al., 2008). Loss of Arabidopsis *XBAT32*, for example, results in a delayed growth phenotype and reduced lateral root production due to a decrease in lateral root initiation (Nodzon et al., 2004). The difference between *xbat32* and the wild type was also evident in the larger flowers produced by *xbat32* plants (Supplemental Fig. S1). To determine whether other *XBAT* family members played similar roles in development, we examined the effects of *XBAT34* and *XBAT35* mutations on plant development. Phenotypic analysis of homozygous *XBAT34* and *XBAT35* T-DNA insertional lines shows that the *xbat32* phenotypes are unique to plants bearing the *XBAT32* mutation (Fig. 1A; Supplemental Fig. S1). Given the previous association detected between the *XBAT*-related genes and ABA signaling (Stone et al., 2006; Yang et al., 2008), *xbat32*, *xbat34*, and *xbat35* seedlings were treated with ABA and its inhibitory effect on root growth was analyzed. Three-day-old seedlings were transferred to growth medium (GM) with or without ABA, and root development was analyzed after 5 d. Interestingly, from this analysis, we discovered that ABA promoted rather than inhibited *xbat32* lateral root production (Fig. 1; Supplemental Fig. S2). In contrast, the response of *xbat34* and *xbat35* to ABA-mediated inhibition of lateral root production was similar to that of the wild type, because in those three genotypes ABA significantly reduced the number of lateral roots produced (Fig. 1A). Compared with the wild type, *xbat32* did not display an altered sensitivity to ABA-mediated inhibition of primary root growth, which suggests that other ABA-mediated responses remain intact (Supplemental Fig. S2).

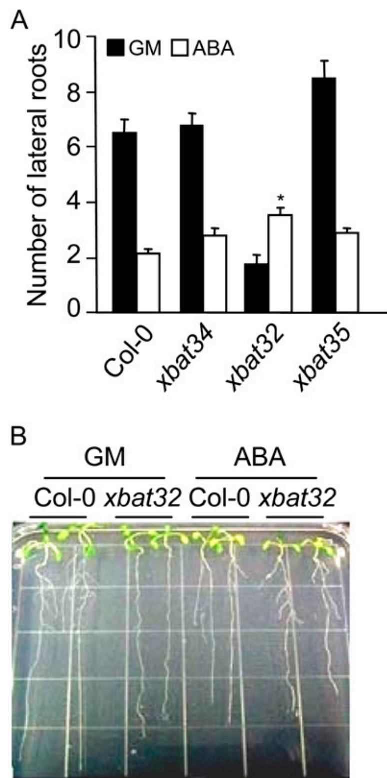

Figure 1. ABA treatment increases *xbat32* lateral root production. A and B, Three-day-old *xbat34*, *xbat32*, *xbat35*, and wild-type Arabidopsis (Col-0) seedlings were transferred to GM alone or GM with 5 μM ABA and grown vertically for 5 d, after which the number of visible lateral roots was quantified (A) and photographs were taken (B). Each bar represents the average number of lateral roots ± SE. Statistical analysis was performed using Student's *t* test, with significant differences relative to untreated *xbat32* and ABA-treated Col-0 (* $P < 0.05$).

Inhibition of Ethylene Perception Drastically Increases *xbat32* Lateral Root Production

ABA stimulation of *xbat32* lateral root production could be due to ABA relieving the inhibitory effects of ethylene on root growth. During lateral root development, high levels of ethylene inhibit lateral root formation (Ivanchenko et al., 2008; Negi et al., 2008). If ABA is indeed alleviating the inhibitory effects of ethylene on *xbat32* lateral root development, then blocking ethylene synthesis or perception should increase *xbat32* lateral root numbers. To determine if this is the case, 3-d-old *xbat32* seedlings were treated for 5 d with increasing concentrations of silver nitrate ($AgNO_3$), an antagonist of the ethylene receptor (Beyer, 1976). Blocking ethylene perception greatly increased the number of lateral roots produced by *xbat32* (Fig. 2, A and B; Supplemental Fig. S3). An increase in lateral root production was also observed for wild-type seedlings. However, the effect of $AgNO_3$ on *xbat32* lateral root production was considerably greater than that observed for the wild type (Fig. 2A). The fact that *xbat32* mutants are more sensitive than wild-type plants to the effects of the ethylene receptor antagonist suggests hyperactivity of ethylene production or response in the mutant. Overall, these results indicate that ethylene signaling is a target for the *xbat32* mutation.

Inhibition of Ethylene Perception or Signaling Enhances Auxin's Ability to Rescue *xbat32* Lateral Root Production

Recent studies have suggested an interaction between ethylene and auxin in regulating lateral root development (Ivanchenko et al., 2008; Negi et al., 2008). Auxin can suppress the inhibitory effects of ethylene on lateral root formation, and auxin mutants are insensitive to ethylene-induced inhibition of lateral root initiation (Ivanchenko et al., 2008; Negi et al., 2008). Treatment of *xbat32* seedlings with IAA and the synthetic auxin analogs naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxy acetic acid (2,4-D) only partially rescued the reduced lateral root phenotype (Fig. 2C; Nodzon et al., 2004). Therefore, we

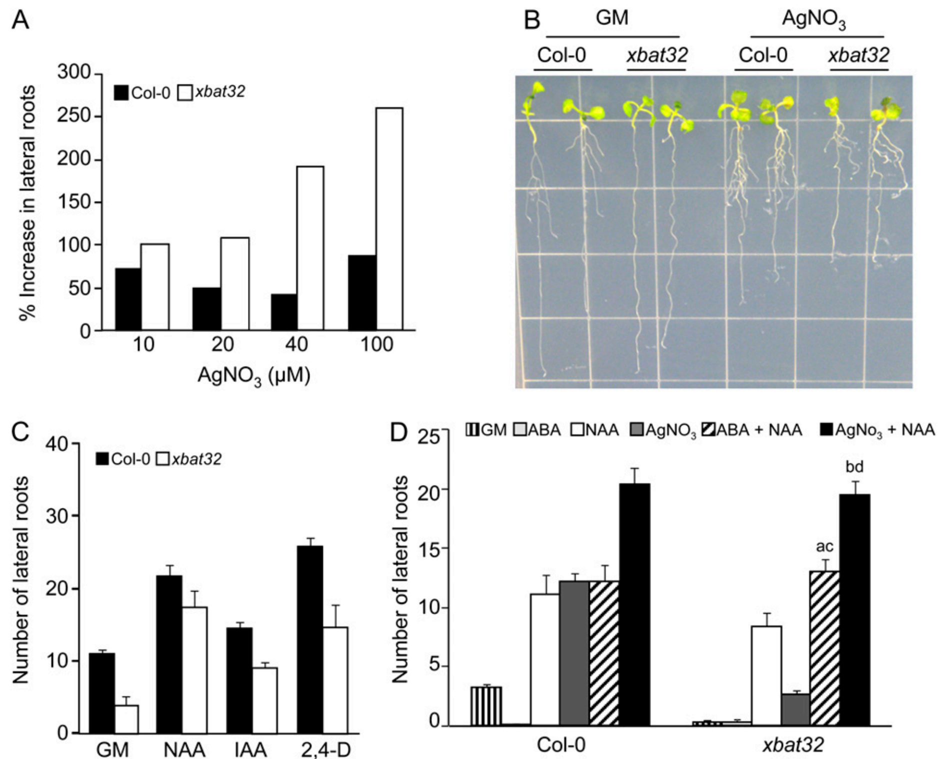

Figure 2. Response of *xbat32* to inhibition of ethylene signaling and/or increase in auxin levels. A and B, Three-day-old Col-0 and *xbat32* seedlings were treated with increasing concentrations of $AgNO_3$ for 5 d, after which the number of visible lateral roots was scored and seedlings were photographed. A shows percentage increase in lateral root numbers on $AgNO_3$ compared with the control (without $AgNO_3$). C, Comparison of lateral root growth of *xbat32* and Col-0 seedlings grown on GM and GM supplemented with 2 μM NAA, 2 μM IAA, or 2 μM 2,4-D. The average number of lateral roots ± SE is shown. D, Col-0 and *xbat32* seedlings were grown for 3 d on GM and then transferred to GM supplemented with 2 μM NAA, 0.5 μM ABA, or 100 μM $AgNO_3$ alone or in combination, 0.5 μM ABA plus 2 μM NAA or 100 μM $AgNO_3$ plus 2 μM NAA. Lateral root growth was assessed 5 d after transfer. Each bar represents the average number of lateral roots ± SE. Significant differences ($P < 0.05$) between *xbat32* on NAA alone and *xbat32* on NAA with ABA (a) or NAA with $AgNO_3$ (b) is indicated. No significant difference ($P > 0.05$) was found between Col-0 and *xbat32* treated with NAA and ABA (c) or NAA and $AgNO_3$ (d). Statistical analysis was performed using Student's *t* test.

analyzed the combined effect of blocking ethylene perception and/or signaling and increasing auxin levels. Three-day-old *xbat32* and wild-type seedlings were treated with NAA or $AgNO_3$ alone or NAA plus $AgNO_3$ for 5 d, after which lateral root production was quantified. The combined effects of blocking ethylene perception and increasing auxin levels brought about a greater rescue of the lateral root defect of *xbat32* compared with auxin or $AgNO_3$ alone (Fig. 2D). Since ABA promotes *xbat32* lateral root formation, we also examined the combined effect of ABA and auxin on lateral root production. As observed with $AgNO_3$, ABA acted synergistically with auxin to fully rescue *xbat32* lateral root production compared with auxin or ABA treatment alone (Fig. 2D). The fact that $AgNO_3$ and ABA treatment enhances the actions of auxin on *xbat32* lateral root production suggests that increased ethylene synthesis and/or signaling contributes to the *xbat32* lateral root phenotype.

Increased Ethylene Signaling Inhibits Lateral Root Production on Preexisting *xbat32* Primary Roots

Applications of exogenous ethylene have been shown to inhibit lateral root initiation only on roots that have formed in the presence of a treatment with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC; Ivanchenko et al., 2008). The ethylene-induced inhibitory effect is not observed on roots formed prior to ACC treatment; in fact, in this instance, ethylene is observed to moderately promote the emergence of lateral roots. To determine if *xbat32* response to ethylene was similar to that of the wild type, we observed the effects of ACC treatment on roots formed prior to the ACC treatment (designated as proximal) and roots formed during the treatment (designated as distal; Fig. 3A). For wild-type seedlings, ACC inhibited lateral root formation on the distal portion of primary roots. A slight increase in lateral root numbers was observed for ACC-treated

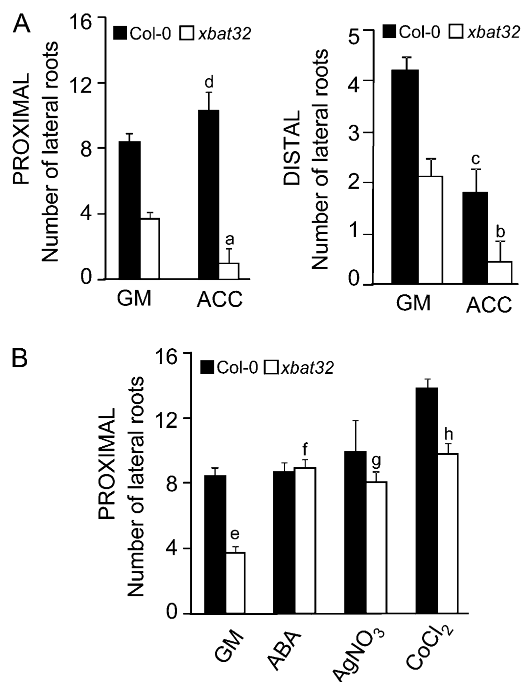

Figure 3. Effects of modulating ethylene signaling on lateral root production on preexisting and new primary root growth. A, Five-day-old Col-0 and *xbat32* seedlings were grown on GM or GM supplemented with 50 μM ACC for 5 d, after which lateral roots produced were quantified. Lateral roots produced on preexisting primary roots at the time of transfer (proximal) and those produced on primary roots that developed during treatment (distal) were quantified separately. Differences between ACC-treated *xbat32* (a and b) or Col-0 (c) and untreated are statistically significant ($P < 0.05$). ACC-treated Col-0 (d) is not significantly different from untreated Col-0 ($P > 0.05$). B, Col-0 and *xbat32* seedlings were grown on GM for 5 d and then transferred to GM containing 5 μM ABA, 100 μM $AgNO_3$, or 100 μM $CoCl_2$ for another 5 d. The number of lateral roots that developed on preexisting primary roots at the time of transfer (proximal) was quantified. Each bar represents the average number of lateral roots ± SE. Significant difference ($P < 0.05$) between Col-0 and *xbat32* on GM (e) is indicated. No significant difference ($P > 0.05$) between *xbat32* treated with ABA (f), $AgNO_3$ (g), or $CoCl_2$ (h) and untreated Col-0 was found. Statistical analysis was performed using Student's *t* test.

proximal primary roots; however, the change was not significantly different from untreated roots (Fig. 3A). For *xbat32* seedlings, both proximal and distal root portions showed reduced lateral root numbers in response to ACC (Fig. 3A). These results suggest that the *xbat32* mutation enhanced ethylene synthesis or signaling, which led to increased sensitivity to ethylene and inhibition of lateral root formation in the proximal portion of the primary root. Therefore, treatment with ACC only serves to enhance the effects of the *xbat32* mutation. To further explore the role of ethylene in the *xbat32* mutant phenotype, the effect of the ethylene antagonists ABA and $AgNO_3$ on the production of lateral roots on the proximal portion of primary roots was analyzed. Five-day-old seedlings were treated with $AgNO_3$ or ABA for 5 d, after which the number of lateral roots produced on the proximal portion of the primary root was quantified (Fig. 3B). Application of either ABA or $AgNO_3$ restored *xbat32* lateral root numbers to wild-type levels. The fact that treatment with ABA and $AgNO_3$ had similar effects on lateral root formation indicates that the *xbat32* mutation alters ethylene biosynthesis. To determine if increased ethylene synthesis is involved in producing the *xbat32* phenotype, seedlings were treated as described above with cobalt chloride ($CoCl_2$), which blocks the ACC oxidase activity required for the conversion of ACC to ethylene. Exposure to $CoCl_2$ increased *xbat32* lateral root numbers on proximal roots to wild-type levels (Fig. 3B). An increase in wild-type lateral root numbers was also observed. These results further support the hypothesis that ethylene overproduction contributes to the *xbat32* lateral root defect.

XBAT32 Mutant Seedlings Overproduce Ethylene

The results obtained from growth assays using ethylene inhibitors are consistent with *XBAT32*'s involvement in regulating ethylene biosynthesis. Based on these observations, we predicted that the *xbat32* mutation would enhance ethylene production. To test this hypothesis, we observed the *xbat32* dark-grown phenotype, studied the response of *xbat32* etiolated seedlings to ethylene, and measured ethylene production. Dark-grown wild-type seedlings exposed to ethylene and ethylene-overproducing mutants display a characteristic triple response that includes shorter hypocotyls, shorter roots, and exaggerated apical hooks (Guzman and Ecker, 1990). Dark-grown *xbat32* seedlings had shorter hypocotyls than wild-type seedlings when grown in air (Fig. 4, A and B). The *xbat32* dark-grown phenotype is consistent with ethylene overproduction; however, this phenotype could result from alterations in ethylene signaling. To demonstrate that ethylene synthesis and not signaling is affected by the mutation, we examined the response of *xbat32* etiolated seedlings to ACC, $CoCl_2$, and aminoethoxyvinylglycine (AVG), an inhibitor of ACS activity that is required for the conversion of *S*-adenosyl-L-methionine (SAM) to the ethylene precursor ACC. Similar to the wild type, *xbat32* hypocotyl growth was inhibited upon exposure to ACC (Fig. 4, A and B). Treatment with the ethylene biosynthesis inhibitors AVG and $CoCl_2$ restored *xbat32* hypocotyl length to wild-type levels (Fig. 4, A and B). The difference between wild-type and *xbat32* hypocotyl length became progressively smaller as the concentration of cobalt was increased (Fig. 4C). Consistent with the results from the growth assays, *xbat32* seedlings were repeatedly found to produce significantly more ethylene than the wild type (Fig. 4D). These findings indicate that *xbat32*'s phenotype is due to an increase in ethylene synthesis.

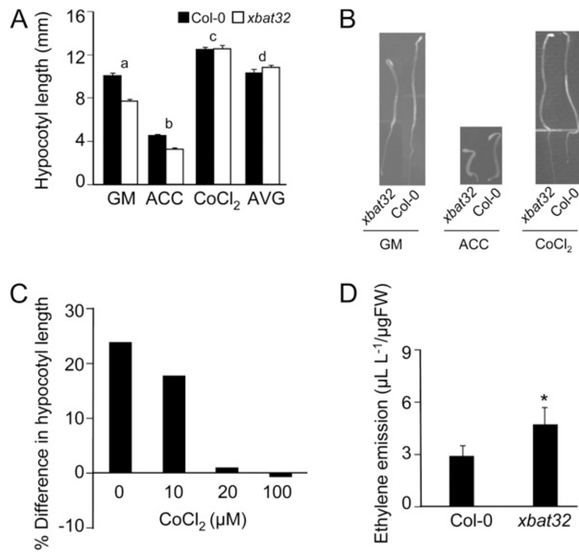

Figure 4. Response of etiolated *xbat32* seedlings to ethylene, cobalt, and AVG and measurement of ethylene emission. A, Hypocotyl lengths of Col-0 and *xbat32* etiolated seedlings grown on GM or GM supplemented with 50 μM ACC, 100 μM $CoCl_2$, or 2 μM AVG. Each bar represents the average number of lateral roots ± SE. Significant differences ($P < 0.05$) between *xbat32* and Col-0 on GM (a; $P = 0.000$) and with ACC treatment (b; $P = 0.000$) are indicated. No significant difference ($P > 0.05$) was found between $CoCl_2$-treated (c) and AVG-treated (d) Col-0 and *xbat32*. Statistical analysis was performed using Student's *t* test. B, Representative 3-d-old etiolated Col-0 and *xbat32* seedlings grown on GM or GM supplemented with 50 μM ACC or 100 μM $CoCl_2$. C, Hypocotyl elongation of etiolated *xbat32* seedlings in response to increasing concentrations of $CoCl_2$. Percentage difference in hypocotyl length between Col-0 and *xbat32* is shown. D, Ethylene emission from 10-d-old Col-0 and *xbat32* seedlings. Col-0 and *xbat32* seedlings were grown in sealed vials, and ethylene emitted was quantified by gas chromatography. Each bar represents the average measurement ± SE from two separate trials each with duplicate vials. Each vial was measured in triplicate. Difference between *xbat32* and Col-0 is statistically significant (* $P < 0.05$) using Student's *t* test. FW, Fresh weight.

XBAT32 Interacts with and Ubiquitinates ACS4 and ACS7

The increased production of ethylene observed for *xbat32* is most likely due to an increase in the activity or levels of one or more of the ethylene biosynthesis enzymes. The rate-limiting conversion of SAM to ACC by ACS is regulated by ubiquitin-dependent proteasomal degradation (Vogel et al., 1998; Chae et al., 2003; Wang et al., 2004; Yoshida et al., 2005; Christians et al., 2009). XBAT32, a functional RING-type E3 ligase capable of E2-dependent protein ubiquitination (Nodzon et al., 2004), may regulate the abundance of one or more of the ACS enzymes. The ACSs are grouped into three classes, type 1 (ACS1, ACS2, and ACS6), type 2 (ACS4, ACS5, ACS8, and ACS9), and type 3 (ACS7 and ACS11; Yoshida et al., 2005). We first used a yeast two-hybrid screen to determine if XBAT32 can interact with representative type 1 (ACS2 and ACS6), type 2 (ACS4), or type 3 (ACS7 and ACS11) ACSs. To prevent ubiquitination and subsequent degradation of any interacting proteins, the RING domain of XBAT32 was made nonfunctional by mutating two essential zinc coordinating residues to Ala (XBAT32-AA). XBAT32 was found to interact with ACS4 and ACS7 (Fig. 5A). XBAT32-AA repeatedly induced higher β-galactosidase activity with ACS4 and ACS7 relative to controls and other ACSs tested. This interaction was also observed with XBAT32 containing a functional RING domain; however, evidence for this interaction (blue color) took longer to appear (data not shown). This observation can be explained by the existence of a conserved ubiquitination pathway in yeast; ACS4 and ACS7 protein levels may have taken longer to accumulate and turn on expression of the reporter gene if XBAT32 ubiquitinated and targeted some of the ACS4 and ACS7 for degradation by the

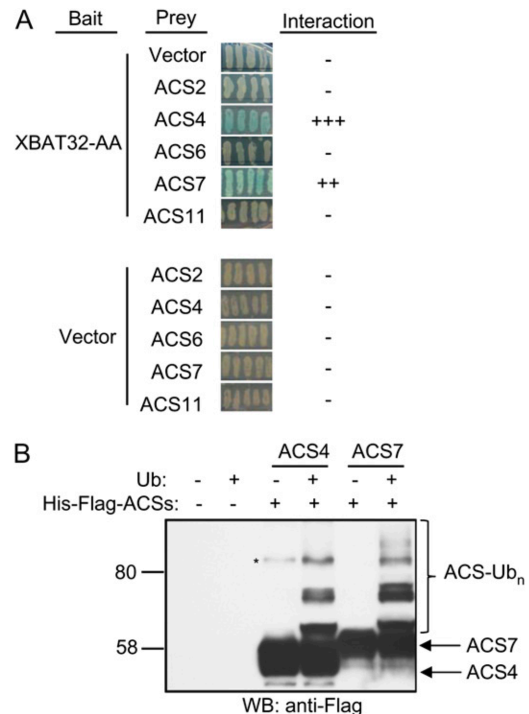

Figure 5. XBAT32 interacts with and ubiquitinates ACS4 and ACS7 in vitro. A, Full-length XBAT32 with a mutated RING domain (XBAT32-AA) fused to a LexA DNA binding domain (bait) in pNLexAattR was tested by yeast two-hybrid screening for interactions with various ACSs fused to a Gal4 activation domain (prey) in pJZ4attR. Empty pNLex-AattR and pJZ4attR vectors were used as controls. Interaction between XBAT32-AA and ACS is indicated by β-galactosidase activity (blue color). −, No interaction; ++, moderate interaction; +++, strong interaction. B, His-GST-XBAT32 (E3) promotes the ubiquitination of Flag-His-ACS4 and -ACS7 in in vitro ubiquitination assays containing recombinant yeast E1, His-AtUBC8 (E2), and ubiquitin (Ub). Omission of ubiquitin from the assay resulted in a loss of ACS ubiquitination. ACS and ubiquitinated ACSs were visualized by western-blot analysis using Flag antibodies. The asterisk indicates a nonspecific protein detected by Flag antibodies. WB, Western blot.

yeast 26S proteasome. Recombinant His-glutathione *S*-transferase (GST)-tagged XBAT32 was capable of attaching ubiquitin to both His-Flag-tagged ACS4 and ACS7 in in vitro ubiquitination assays (Fig. 5B). This is evident by the higher M_r forms of His-Flag-ACS4 and -ACS7 detected by Flag antibodies following assays carried out in the presence of ubiquitin. The higher M_r forms of His-Flag-ACS4 or -ACS7 were not observed when ubiquitin was omitted from the assay (Fig. 5B). These results suggest that XBAT32 negatively regulates ethylene biosynthesis via ubiquitin-dependent degradation of ACS enzymes, in particular ACS4 and ACS7.

DISCUSSION

Ethylene is known to play a major role in regulating root development (Hussain and Roberts, 2002). Older studies established that in Arabidopsis high levels of ethylene inhibit primary root growth (Le et al., 2001; Swarup et al., 2007), and recent experiments have indicated that high levels of ethylene also negatively regulate lateral root formation (Ivanchenko et al., 2008; Negi et al., 2008). Previous studies have shown that the *xbat32* mutation causes a number of aberrant phenotypes, including a striking and pronounced reduction in the number of lateral roots (Nodzon et al., 2004). Here, we show that XBAT32, a RING domain-containing ankyrin repeat E3 ligase, is a negative regulator of ethylene biosynthesis and that through this activity XBAT32 modulates lateral root development. Homozygous *xbat32* plants display an ethylene overproduction phenotype when grown in the dark and produce significantly more ethylene than the wild type. The dark-grown ethylene overproduction phenotype and reduced lateral root numbers were rescued by treating *xbat32* seedlings with inhibitors of ethylene biosynthesis and perception. XBAT32 interacts with the ethylene biosynthesis enzymes ACS4 and ACS7, which convert SAM to the ethylene precursor ACC. Furthermore, XBAT32 is capable of catalyzing the attachment of ubiquitin to ACS4 and ACS7 in vitro. Altogether, these data suggest that XBAT32 regulates ethylene biosynthesis by modulating the stability of one or more ACS enzymes.

Exogenous auxin treatment partially rescued the lateral root phenotype in *xbat32* (Nodzon et al., 2004; this study). Based on our findings, a feasible explanation for only a partial rescue for *xbat32* could be the presence of high levels of ethylene in the mutant plants. Several studies point to an interaction between ethylene and auxin during lateral root production (Stepanova et al., 2007; Swarup et al., 2007; Ivanchenko et al., 2008; Negi et al., 2008). High ethylene levels promote both acropetal and basipetal auxin transport (Negi et al., 2008), which affects auxin uploading into root cells of the elongation zone, thus resulting in reduced lateral root production (Negi et al., 2008). In enhanced ethylene signaling mutants, such as the *polaris* mutant, which displays reduced lateral root production, auxin transport in roots is also defective (Chilley et al., 2006). The overproduction of ethylene in *xbat32* could be the factor responsible for preventing a complete rescue of the lateral root defect by auxin. Since high ethylene enhances auxin transport (Negi et al., 2008), which alters its availability to root cells, simply adding exogenous auxin would not be sufficient to fully rescue the lateral root defect. Blocking ethylene signaling would restore proper auxin transport, allowing the exogenously supplied auxin to be made more available for lateral root production. ABA, another developmentally essential plant hormone known to inhibit Arabidopsis lateral root development (De Smet et al., 2006b), was found to increase the number of lateral roots in *xbat32* seedlings. In contrast to Arabidopsis, ABA stimulates lateral root development in legumes. ABA has been shown to rescue the meristem defect and promote lateral root formation in the *Medicago truncatula lateral root organ defective* mutant (Liang et al., 2007). A possible explanation for stimulatory effects of ABA on *xbat32* lateral root production may be that ABA inhibits ethylene synthesis and/or signaling. ABA and ethylene are antagonistic during many stages of plant development. For example, ABA inhibits early seedling establishment, whereas ethylene has the opposite effect. Interactions between ABA and ethylene may occur during hormone synthesis and/or between signaling pathways (Beaudoin et al., 2000; Ghassemian et al., 2000; Spollen et al., 2000; LeNoble et al., 2004; Cheng et al., 2009). Several lines of evidence support the hypothesis that ABA negatively regulates ethylene synthesis and vice versa (Spollen et al., 2000; LeNoble et al., 2004; Cheng et al., 2009). Inhibition of ABA synthesis chemically via fluridone treatment or genetically using ABA-deficient mutants such as *abscisic acid deficient2* increases ethylene

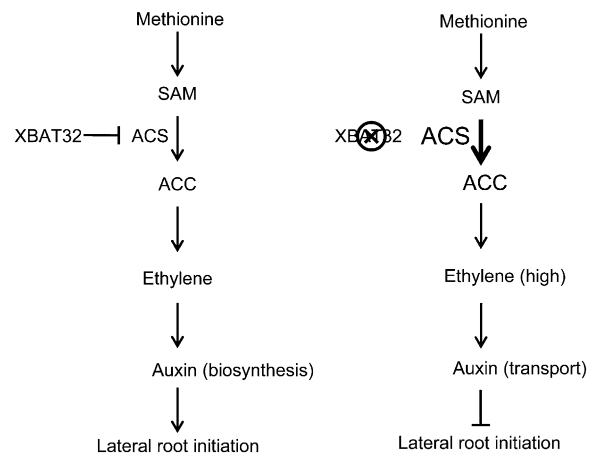

Figure 6. Model for XBAT32's role during ethylene/auxin regulation of lateral root development. XBAT32 negatively regulates ethylene biosynthesis, possibly by modulating the stability of specific ACS through ubiquitin-dependent proteasomal degradation. Loss of XBAT32 leads to stabilization of these ACSs and increased ethylene synthesis, which results in inhibition of lateral root initiation. Ethylene's role in regulating lateral root development may occur through modulation of auxin biosynthesis and transport.

production, and application of ABA inhibits ethylene production (Spollen et al., 2000; LeNoble et al., 2004; Cheng et al., 2009). Exogenous ABA, therefore, may reduce *xbat32* ethylene levels, thus alleviating the negative effects of ethylene on auxin transport, allowing for lateral root initiation and primordia development.

The role of *XBAT32* as a negative regulator of ethylene biosynthesis is further strengthened by the display of an ethylene-associated phenotype of a shortened hypocotyl in *xbat32* etiolated seedlings. Wild-type Arabidopsis seedlings exposed to ethylene and ethylene-overproducing mutants such as *eto1* and *ctr1-1* that activate the ethylene response pathway (Kieber et al., 1993) display a dark-grown constitutive triple response phenotype, which is characterized by a shortened hypocotyl as well as an exaggerated apical hook and a short seedling root (Guzman and Ecker, 1990). Gas chromatography results indicated that *xbat32* produced significantly more ethylene than the wild type. *XBAT32* mutant seedlings do not seem to have an exaggerated apical hook or shorter roots; however, the shorter hypocotyls compared with wild-type seedlings indicate that the level of ethylene overproduction in *xbat32* does alter some aspects of growth. Blocking ethylene synthesis via cobalt or AVG application rescues the shortened hypocotyl phenotype and restores it to wild-type levels, as for ethylene-overproducing mutants like *eto1* (Guzman and Ecker, 1990) and the triple mutant *eto1 eol1 eol2* (Christians et al., 2009). Similar to *xbat32*, the ethylene overproducer *eto1* also shows reduced lateral root formation, whereas ethylene-insensitive mutants such as *ein2* and *ert1* produce more lateral roots than the wild type (Negi et al., 2008). The fact that XBAT32 can interact with and ubiquitinate ACS4 and ACS7 provides further evidence for a role for XBAT32 in ethylene biosynthesis. The abundance of the type 2 ACSs, ACS5 and ACS9, is regulated by ETO1 and ETO1-like (EOL1) broad complex/tramtrack/bric-a-brac proteins, which are substrate-recruiting components of cullin-based RING E3 ligases (Vogel et al., 1998; Chae et al., 2003; Wang et al., 2004; Yoshida et al., 2005; Christians et al., 2009). Similarly, XBAT32 may negatively regulate the abundance of ACSs to modulate ethylene production. Our results suggest a model where XBAT32 regulates lateral root production by maintaining low levels of specific ACSs and reducing ethylene synthesis (Fig. 6). The low levels of ethylene may support lateral root formation by up-regulating auxin biosynthesis, which promotes lateral root primordia initiation (Swarup et al., 2007; Ivanchenko et al., 2008). Loss of XBAT32 would result in a stabilization of ACSs and increased ethylene synthesis (Fig. 6). Increased ethylene levels may enhance auxin transport, which negatively affects auxin's ability to promote lateral root formation. The role of XBAT32 in regulating ethylene synthesis may also be responsible for the delayed growth phenotype of *xbat32* plants. Similar to ethylene overproducers or constitutively active ethylene signaling mutants (Ogawara et al., 2003; Christians et al., 2009), *xbat32* plants are shorter in stature than wild-type plants. However, unlike these ethylene mutants, the short stature phenotype of *xbat32* is only observed early in development (Nodzon et al., 2004; this study). XBAT32 may be required to regulate ACS abundance and ethylene synthesis during early seedling and plant growth and development. Thus, the *XBAT32* mutation may only affect early plant development. Other E3 ligases may regulate ACS abundance at later stages of development, allowing *xbat32* plants to achieve wild-type levels of growth later in development.

Collectively, our results indicate that an overproduction of ethylene in *xbat32* seedlings contributes to the lateral root defect. The mechanism by which high ethylene inhibits lateral roots in *xbat32* is not yet clear; however, this study provides evidence to support the recent line of studies that suggest an interaction between ethylene and auxin in lateral root development, and it also emphasizes the essential role of E3 ligases in regulating hormonal levels during root development.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds from Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) or mutant plant lines surface sterilized with 30% (v/v) bleach and 0.1% (v/v) Triton X-100 were grown on solid GM (pH 5.7) containing 0.8% agar with half-strength Murashige and Skoog medium (Sigma-Aldrich) supplemented with 1% Suc. Sterilized seeds were stratified for 2 to 3 d in the dark at 4°C and then transferred to room temperature under continuous light. Seven-day-old seedlings were transferred to soil and grown under photoperiodic cycles of 16 h of light and 8 h of dark at 22°C.

Identification of T-DNA Insertional Plants

All T-DNA insertional lines were obtained from the Salk Institute Genomic Analysis Laboratory via the Arabidopsis Biological Resource Center (ABRC; Alonso et al., 2003). *xbat34* (SAIL_395_E02), *xbat32* (Salk_015002), and *xbat35* (Salk_104813) are T-DNA insertion lines in At4g14365, At5g57740, and At3g23280, respectively. T-DNA homozygous plants were confirmed by PCR analysis of genomic DNA using the Extract N′ Amp kit (Sigma-Aldrich) as per the manufacturer's instructions. Genotyping was done using gene-specific primers for the wild-type allele in combination with T-DNA-specific primers for the mutant allele.

Growth Assays

For root growth assays, surface-sterilized and stratified seeds were germinated on GM and grown vertically for 3 or 5 d before transfer to GM with or without the indicated concentrations of ABA, ACC, AVG, $AgNO_3$, $CoCl_2$, NAA, or 2,4-D. All chemicals used were obtained from Sigma-Aldrich. Seedlings were then grown vertically for 5 d, after which time the number of lateral roots and/or primary root length was quantified. The number of lateral roots was scored with a compound light microscope. For hypocotyl growth assays, surface-sterilized and stratified seeds were germinated on GM with or without the indicated quantities of ACC, $CoCl_2$, or AVG and grown for 3 d in the dark at room temperature. Seedlings were then photographed, and measurements of root and hypocotyl length were performed using ImageJ Image Processing and Analysis Software (Abramoff et al., 2004). All assays were repeated a minimum of four times with two replicates for each trial and at least 10 seedlings per replicate.

Ethylene Production Measurements

Surface-sterilized and stratified seeds were germinated and grown on GM for 3 d before transfer to vials containing liquid GM. Each vial contained 50

seedlings. After 3 d, vials were sealed and seedlings were grown for an additional 3 d. The accumulated ethylene in each vial was measured by gas chromatography (3900 gas chromatograph; Varian) on the 4th d after sealing. An injection volume of 1 mL was used. An ethylene standard (μL L^{-1}; Praxair) was used as a positive control. Assays were carried out at two separate times with two replicates per sample. Measurements were done in triplicate for each vial. Results from each vial were represented as means of the three readings. Ethylene concentration was calculated per milligram of seedling weight.

Yeast Two-Hybrid Analysis

XBAT32, *ACS2*, *ACS4*, *ACS6*, *ACS7*, and *ACS11* cDNAs were obtained from the ABRC. To create *XBAT32-AA*, Phusion site-directed mutagenesis (New England Biolabs) was used to mutate RING domain metal ligand residues Cys-336 and His-338 to Ala. cDNAs were introduced into the Gateway entry vector pDONR201 (Invitrogen). *XBAT32* and *XBAT32-AA* were then introduced into the bait Gateway destination vector pNLexAattR containing the LexA DNA binding domain as well as a nuclear localization signal. *ACS2*, *ACS4*, *ACS6*, *ACS7*, and *ACS11* cDNAs were introduced into the prey Gateway destination vector pJZ4attR, which contains the activation domain and a Gal-inducible promoter. Sequence-verified yeast two-hybrid Gateway destination vectors were a gift from the Finley laboratory (Wayne State University; Serebriiskii et al., 2001). The reporter plasmid pSH18-34 was first transformed into yeast stain EGY48 using the TRAFO Protocol (Agatep et al., 1998), followed sequentially by the bait and prey vectors. Transformed yeast were selected and maintained on dropout medium (−His/uracil/Trp; BioShop Canada). To detect an interaction, yeast colonies containing all three plasmids were streaked onto medium containing Gal/raffinose and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (BioShop Canada). Binding of activation domain fusion proteins to binding domain fusion proteins was detected by the presence of a blue color produced by the metabolism of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside by β-galactosidase, the protein product of the reporter gene.

In Vitro Ubiquitination Assays

XBAT32, *ACS4*, and *ACS7* cDNAs were cloned via Gateway into the pDEST565 or modified pDEST527 (a FLAG tag was inserted into the vector to create a Flag-His tag) vectors to obtain His-GST- or Flag-His-tagged fusion proteins, respectively. pDEST565 and pDEST527 were obtained from Addgene, plasmids 11520 and 11518, respectively (plasmids donated by Dominic Esposito, National Cancer Institute). Fusion proteins were expressed in *Escherichia coli* strain Rosetta (DE3) and purified using nickel-charged resin (Bio-Rad) according to the manufacturer's protocols. Ubiquitination assays were performed as described previously (Stone et al., 2006). Briefly, reactions (30 μL) containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.05 mM $ZnCl_2$, 1 mM ATP (Sigma-Aldrich), 0.2 mM dithiothreitol, 10 mM phosphocreatine, 0.1 unit of creatine kinase (Sigma-Aldrich), 50 ng of yeast E1 (BostonBiochem), 250 ng of purified 6× His-AtUBC8 (E2), 2 μg of ubiquitin (BostonBiochem), 500 ng of His-GST-XBAT32, and 500 ng of Flag-His-ACSs were incubated at 30°C for 2 h. Reactions were stopped by adding sample buffer (125 mM Tris-HCl, pH 6.8, 20% [v/v] glycerin, 4% [w/v] SDS, and 10% [v/v] β-mercaptoethanol) and analyzed by SDS-PAGE followed by protein gel blotting using anti-Flag antibodies.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: At4g14365, DQ086842; At5g57740, NM_125157.3; At3g23280, DQ086844.

Supplemental Data

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

Supplemental Figure S1. Phenotypic analysis of *xbat* mutants.

Supplemental Figure S2. Comparison of wild-type Col-0 and *xbat32* response to ABA.

Supplemental Figure S3. Inhibition of ethylene signaling in *xbat32*.

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