Plant Signaling & Behavior 5:11, 1425-1429; November 2010; © 2010 Landes Bioscience

Further analysis of XBAT32, an Arabidopsis RING E3 ligase, involved in ethylene biosynthesis

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The Arabidopsis RING E3 ligase, XBAT32, was previously characterized as a regulator of lateral root initiation. However, how XBAT32 function to modulate lateral root initiation was unknown. In our recent paper, we demonstrated that XBAT32 is involved in ethylene biosynthesis and it is through this function that XBAT32 is able to regulate lateral root production. Here we discuss a few other findings, observed in the ethylene overproducing mutant, *xbat32*, that reflect the effect of elevated ethylene levels on plant growth and development. Ethylene signaling also regulates plant responses to adverse environmental conditions such as high salinity. Consistent with ethylene's role as a stress hormone, *xbat32* exhibited increased sensitivity to salt stress during seed germination and postgerminative growth. Thus, XBAT32 may also play a role in ethylene mediated response to abiotic stresses.

Key words: *Arabidopsis thaliana*, ethylene, lateral root, RING E3 ligase, salt stress, ubiquitination

Abbreviations: ACS, 1-aminocyclopropane-1-carboxylic acid synthase; ABA, abscisic acid; RING, really interesting new gene; UPS, ubiquitin-26S proteasome; XBAT32, XB3 ortholog 2 in *Arabidopsis thaliana*

Submitted: 08/09/10

Accepted: 08/10/10

Previously published online: www.landesbioscience.com/journals/psb/article/13294

DOI: 10.4161/psb.5.11.13294

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Addendum to: Prasad ME, Schofield A, Lyzenga W, Liu H, Stone SL. Arabidopsis RING E3 ligase XBAT32 regulates lateral root production through its role in ethylene biosynthesis. Plant Physiol 2010; 153:1587–96; PMID: 20511490; DOI: 10.1104/pp.110.156976.

Protein ubiquitination is an integral part of numerous cellular processes and is required for proper growth and development of all eukaryotic organisms. The ubiquitination pathway covalently attaches ubiquitin, a small regulatory peptide, to selected proteins. The fate of the ubiquitinated protein differs depending upon the number of attached ubiquitin molecules as well as polyubiquitin chain topology.[1,2] Most notable is degradation of proteins modified by the attachment of a lysine 48 linked polyubiquitin chain by the 26S proteasome, a multi-catalytic protease. Ubiquitination is a multi-step reaction involving the sequential action of three enzymes, E1 (ubiquitin activating enzyme) which initiates the conjugation cascade and E2 (ubiquitin conjugating enzyme) along with the substrate binding E3 (ubiquitin ligase), transfers ubiquitin from the E2-ubiquitin intermediate to the substrate.[2] Almost 6% of the *Arabidopsis thaliana* proteome is dedicated to the ubiquitin-26S proteasome system (UPS).[3] The majority of these proteins are ubiquitin ligases which, as the substrate recruiting component, govern specificity of the pathway. There are four main types of ubiquitin ligases found in plants, single subunit E3s containing either a U-box, Really Interesting New Gene (RING) or Homologous to E6-AP C-Terminus (HECT) E2-binding domain and multi-subunit cullin-RING ligases (CRL).[3] The CRLs are further divided into four subtypes each containing a cullin, an E2-binding RING protein and a substrate recognition subunit. The SCF type, for example, contains cullin 1 (CUL1), RING-box1 (RBX1) protein, S phase kinase-associated proteins 1 (SKP1) adaptor protein that allows for CUL1 interaction with the substrate recognition F-box protein (FBX).[3] The large number and diversity of the ubiquitin ligases illustrate the importance of this regulatory system to plant cellular biology.

The importance of the UPS to plant growth, development and survival under unfavorable conditions is due largely to its function in regulating the biosynthesis, perception and/or signaling pathways of major growth and stress hormones including abscisic acid (ABA), ethylene, jasmonic acid, gibberellin and auxin. The regulatory role of the UPS in hormone signaling is exemplified by its involvement in both

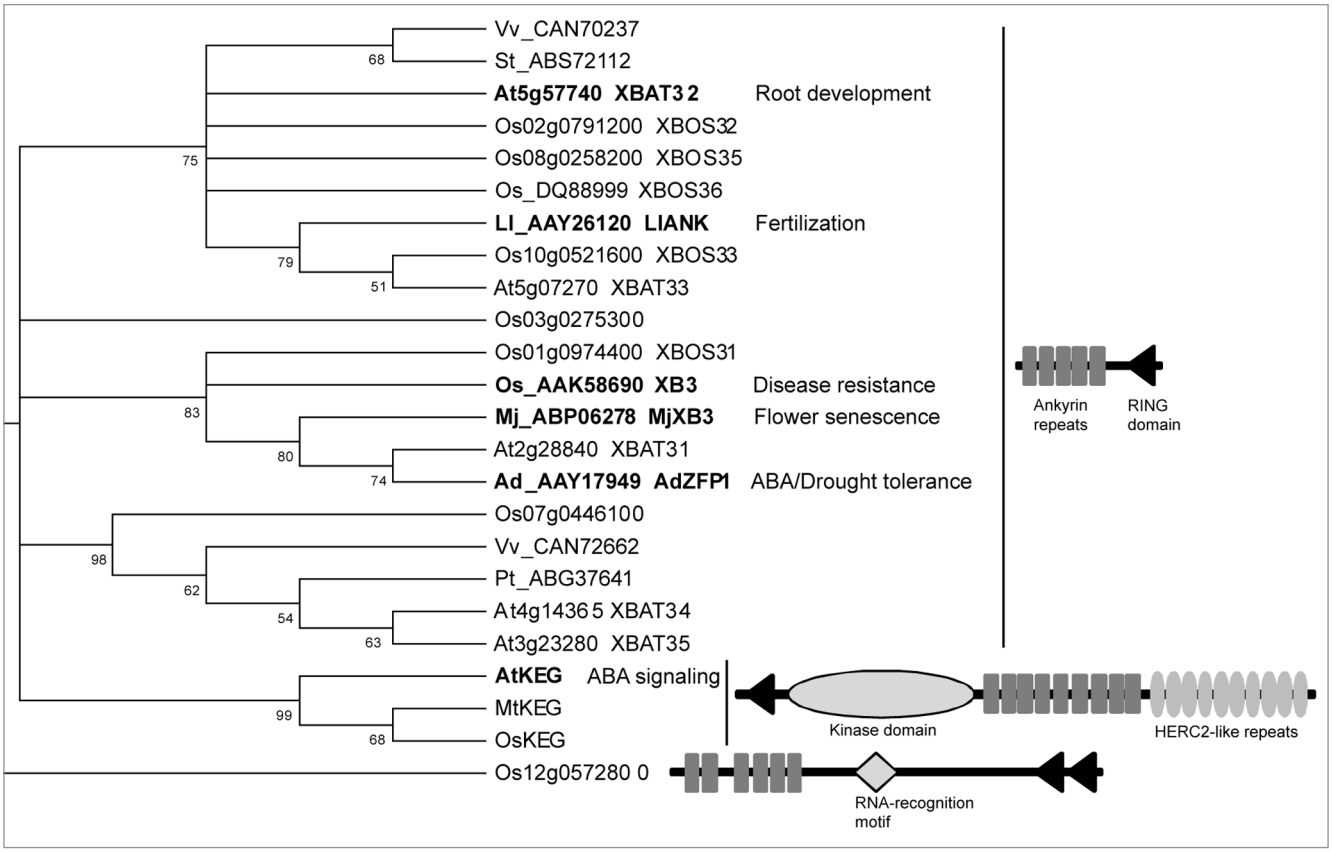

Figure 1. A protein similarity tree depicting the relationship between Arabidopsis XBAT and XBAT-related proteins from other plant species. Neighbor joining tree was generated using an alignment of the amino acid sequences of ankyrin repeats from each protein. Bootstrap values, based on 1,000 replicates, are indicated below each node. Representative schematic for each sub-group is shown to the right. Characterized proteins are shown in bold and functions are also listed. Species: Ad, *Artemisia desertorum Spreng*; At, *Arabidopsis thaliana;* Ll, *Lilium longiflorum (lily)*; Mt, *Medicago truncatula*; Mj, *Mirabilis jalapa*; Os, *Oryza sativa*; Pt, *Populus trichocarpa*; St, *Solanum tuberosum*; Vv, *Vitis vinifera.* The locus ID is shown for *Arabidopsis thaliana* and *Oryza sativa* and accession numbers are given for other species.

the biosynthesis and signal transduction pathway of ethylene. Within the ethylene signaling pathway, the abundance of ethylene receptor ETHYLENE RESPONSE2 (ETR2), integral membrane protein ETHYLENE INSENSITIVE2 (EIN2) and transcription factors EIN3 and EIN3-like1 (EIL1) are all regulated by the UPS.[4-8] Ethylene signaling blocks the degradation of EIN2 and EIN3 by SCF type CRL E3 ligases containing EIN2-TARGETING PROTEIN1/2 (ETP1/2) and EIN3-BINDING F-BOX1/2 (EBF1/2) substrate recruiting F-box proteins, respectively.[4-6] The UPS regulates ethylene production by modulating the stability of the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) enzymes which convert *S*-adenosylmethionine (SAM) to the ethylene precursor ACC. The ACSs are grouped into three classes: type-1 (ACS1, ACS2 and ACS6), type-2 (ASC4, ASC5, ACS8 and ASC9) and type-3 (ACS7 and ACS11).[9] Type-1 and -2 ACSs are short-lived in the absence of ethylene but are stable in the presence of the hormone.[9-11] E3 ligases responsible for the turnover of type-1 ACSs are unknown. The stability of the type-2 ACSs, particularly ACS5 and ACS9, is regulated mainly by ETHYLENE OVER PRODUCER 1 (ETO1) and ETO1-like (EOL1/2) broad complex/tram-track/bric-a-brac (BTB) proteins which are the substrate recruiting subunit of a CRL E3.[11] UPS regulation of type-3 ACS has not previously been established.

In our recent paper we showed that RING type E3 ligase, XBAT32 (for XB3 ortholog 2 in *Arabidopsis thaliana*), is involved in maintaining appropriate levels of ethylene.[12] Here we discuss other effects of ethylene overproduction on plant development exhibited by *xbat32* seedlings. XBAT32 is a member of the Arabidopsis RING ankyrin repeat subgroup of E3 ligases.[13] With the exception of one family member KEEP ON GOING (KEG), which also contains a kinase domain and a series of HERC2-like repeats, the XBAT proteins contain a series of amino terminal ankyrin repeats that facilitates protein-protein interactions followed by a RING-HC domain (**Fig. 1**). XBAT32 was found to interact with type-2 (ACS4) and type-3 (ACS7) but not with type-1 ACSs in yeast two hybrid assays.[12] XBAT32 can catalyze the attachment of ubiquitin to both ACS4 and ACS7 in in vitro ubiquitination assays.[12] Phenotypic, interaction and biochemical analyses suggest that XBAT32 modulate the stability of ACS4 and ACS7 via ubiquitination and degradation by the 26S proteasome. Our results also indicate that similar to the type-1 and -2 ACSs, type-3 ACSs, in particular ACS7, are also under proteolytic control.

Proteins with similar domain architecture to Arabidopsis XBAT32 have been identified in a number of other plant species including rice (*Oryza sativa*), lily (*Lilium longiflorum*) and the drought tolerant plant *Artemisia desertorum* (**Fig. 1**). Of the RING ankyrin repeat proteins studied so far XBAT32 is the only member found to be involved in ethylene synthesis/signaling. Other XBAT or XBAT-related proteins are involved in disease resistance, drought tolerance, flower senescence and fertilization[14-17] (**Fig. 1**). Loss of *XBAT32* produces a number of phenotypes that can be attributed to increase ethylene synthesis. Typically, dark-grown wild-type seedlings exposed to high levels of ethylene or ethylene overproducing mutants, such as *eto1-1*, demonstrate a constitutive triple response consisting of a shortened and thickened hypocotyl, exaggerated apical hook and shortened root.[18] Consistent with an ethylene overproducing phenotype, etiolated *xbat32* seedlings grown in air have shorter and thicker hypocotyls than wild-type seedlings. Similar to *eto1-1*, light grown *xbat32* seedlings exhibit up to a two-fold increase in ethylene production compared to wild-type.[12,18] It has been known for sometime that ethylene suppresses primary root growth by inhibiting cell elongation.[19-21] The effects of ethylene on root development are dependent upon the modulation of auxin synthesis and transport.[20,21] Recently, it was shown that increased ethylene synthesis or signaling inhibits lateral root formation.[22,23] *eto1-1* and the constitutive ethylene signaling mutant, *ctr1-1*, show reduced lateral root formation.[22] Similarly, *xbat32* seedlings exhibited a reduced lateral root phenotype.[12,24] Unlike *eto1-1* and *ctr1-1*, the *XBAT32* mutation did not affect primary root growth.[12] During root branching, elevated ethylene synthesis or signaling alter auxin transport leading to suboptimal levels of auxin for lateral root initiation.[22,23] This is consistent with the fact that complete rescue of the *xbat32* lateral root defect is achieved only when auxin treatment is coupled with the inhibition of ethylene synthesis/signaling. Auxin treatment alone only partially rescues *xbat32* lateral root production[12,24] (**Fig. 2**), suggesting that the added auxin is not fully available for initiation of lateral roots. The addition of low levels of abscisic acid (0.5 μM) during auxin treatment allowed *xbat32* to produce numbers of lateral root similar to wild-type[12] (**Fig. 2**). This concentration of ABA (0.5 μM) did not alter the number of lateral roots produced by auxin treated wild-type roots. Consistent with ABA's role as an inhibitor of root growth and development,[26] higher concentrations of ABA (1 to 5 μM) reduced auxin stimulated lateral root production for both wild-type and *xbat32* (**Fig. 2**). Since ABA is antagonistic to ethylene, treatment with low concentrations of ABA may have inhibited ethylene synthesis/signaling.[25] This may restore proper auxin transport and allows the exogenous auxin to promote *xbat32* lateral root initiation and growth. Further analysis is required to determine if auxin transport is altered in *xbat32* roots. Microarray analysis of roots from wild-type, ethylene insensitive mutant *ein2-5* and auxin transport mutant *aux1-7* etiolated seedlings identified several levels of interactions between ethylene and auxin.[27] The types of interaction found include auxin-dependent, -independent and -mediate ethylene responses or ethylene-dependent, -independent and -mediated auxin responses. From this analysis *XBAT32*'s expression was found to be regulated by ethylene, but not by auxin. Ethylene repression of *XBAT32* expression was altered in the *aux1-7* mutant background indicating that the regulation by ethylene is auxin-dependent. These results are somewhat contrary to those of Nodzon et al.[24] where using a promoter β-glucuronidase (GUS) reporter system, auxin was found to increase the expression of *XBAT32* in primary roots of light grown seedlings.[24] The discrepancies between the two studies could be explained by the differences in the experimental systems, for example light versus dark-grown roots. Overall, the results from the expression analyses indicate that XBAT32 may mediate tissue or cell specific cross-talk between ethylene and auxin to modulate lateral root initiation.

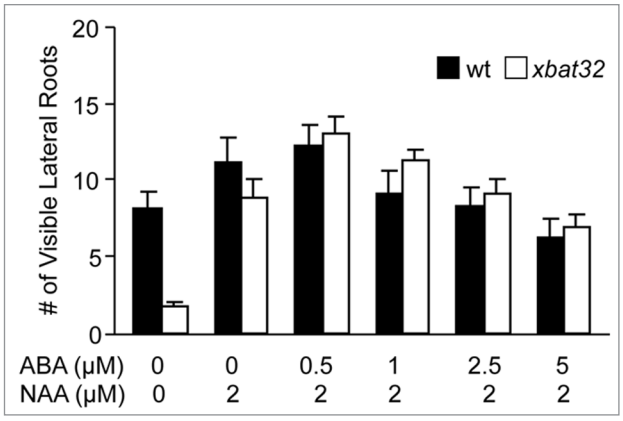

Figure 2. ABA coupled with auxin treatment can fully rescue the lateral root defect of *xbat32*. Three day old wild-type and *XBAT32* mutant seedlings were treated with increasing concentrations of abscisic acid (ABA) in the presence 2 μM naphthalenacetic acid (NAA), a synthetic auxin analog, for three days after which the number of visible lateral roots were quantified.

Several studies have illustrated the requirement for ethylene signaling in plant survival under salt stress.[28-33] Mutation in ethylene signaling components alters plant response to salt stress. For example, *ctr1-1* is tolerant of salt stress and *ein3-1* exhibit reduce salt tolerance during postgerminative development.[33] The ethylene overproducer, *eto1-1*, is salt-tolerant but only during seed germination and not during postgerminative growth.[34] Transgenic plants expressing a constitutively activated form of *MKK9*, a mitogen-activated protein kinase kinase that modulates ethylene synthesis, produce more ethylene than wild-type and exhibit enhanced sensitivity to salt stress.[35,36] Similarly, the *XBAT32* mutation renders plants sensitive to salt stress during germination and early seedling growth (**Fig. 3**). After 48 hours of growth on medium containing 100 mM sodium chloride (NaCl) less than 60% of *xbat32* seeds germinated compared to 80% of wild-type seeds (**Fig. 3A**). Only 20%

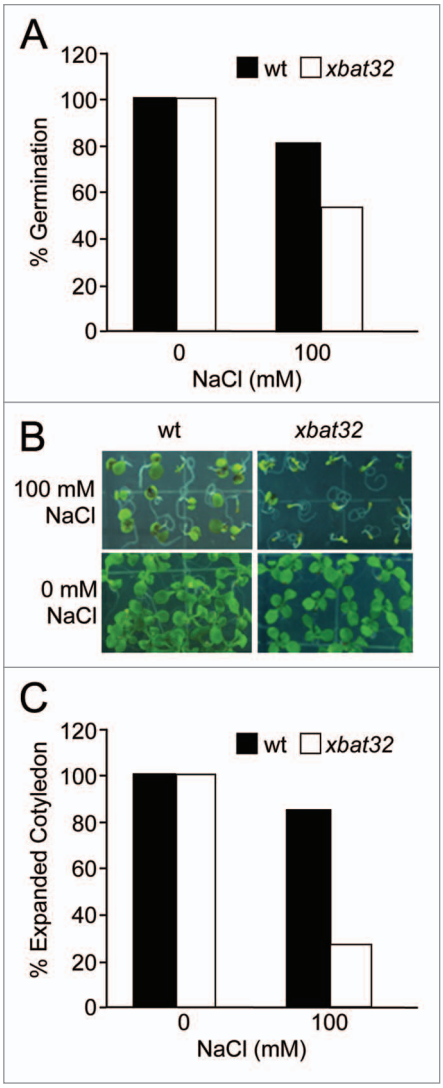

Figure 3. Response of *xbat32* and wild-type to salt stress. (A) Graph showing percentage (%) seed germination for wild-type (wt) and *xbat32* after 48 hours of growth on medium supplemented with 100 mM sodium chloride (NaCl). (B and C) Seven-day-old wild-type and *xbat32* seedlings grown on medium with or without 100 mM NaCl. Graph (C) shows percentage of seedlings with expanded cotyledons.

of *xbat32* seedlings on high salt medium exhibited proper cotyledon expansion compared to 80% of wild-type seedlings (**Fig. 3B and C**). Crosstalk between ethylene and ABA occurs during biosynthesis and signaling.[25] Therefore, it is likely that the altered sensitivity of *XBAT32* mutants to salt stress may in part be due to ethylene's interactions with ABA. However, disruption of *XBAT32* expression does not alter the plants response to ABA,[12] suggesting that *XBAT32* does not modulate plant stress responses through ABA.

Overall our studies provide evidence to support the role of XBAT32, a RING E3 ligase, in maintaining appropriate levels of ethylene during plant growth and development. XBAT32 may also be involved in modulating ethylene mediated responses to salt stress.

Acknowledgements

The authors would like to thank Dr. Hongxia Liu for comments on the manuscript. S.L.S. is supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and funds from a Human Frontier Science Program Organization (HFSPO) Career Development Award.

References

1. Weissman AM. Themes and variations on ubiquitylation. Nat Rev Mol Cell Biol 2001; 2:169-78.
2. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. Physiol Rev 2002; 82:373-428.
3. Vierstra RD. The ubiquitin-26S proteasome system at the nexus of plant biology. Nat Rev Mol Cell Biol 2009; 10:385-97.
4. Guo H, Ecker JR. Plant responses to ethylene gas are mediated by SCFEBF1/EBF2-dependent proteolysis of EIN3 transcription factor. Cell 2003; 115:667-77.
5. Potuschak T, Lechner E, Parmentier Y, Yanagisawa S, Grava S, Koncz C, et al. EIN3-dependent regulation of plant ethylene hormone signaling by two Arabidopsis F box proteins EBF1 and EBF2. Cell 2003; 115:679-89.
6. Gagne JM, Smalle J, Gingerich DJ, Walker JM, Yoo SD, Yanagisawa S, et al. Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proc Natl Acad Sci USA 2004; 101:6803-8.
7. Chen YF, Shakeel SN, Bowers J, Zhao XC, Etheridge N, Schaller GE. Ligand-induced degradation of the ethylene receptor ETR2 through a proteasome-dependent pathway in Arabidopsis. J Biol Chem 2007; 282:24752-8.
8. Qiao H, Chang KN, Yazaki J, Ecker JR. Interplay between ethylene, ETP1/ETP2 F-box proteins and degradation of EIN2 triggers ethylene responses in Arabidopsis. Genes Dev 2009; 23:512-21.
9. Yoshida H, Nagata M, Saito K, Wang KL, Ecker JR. Arabidopsis ETO1 specifically interacts with and negatively regulates type 2 1-aminocyclopropane-1-carboxylate synthases. BMC Plant Biol 2005; 5:14-27.
10. Kevin LCW, Yoshida H, Lurin C, Ecker JR. Regulation of ethylene gas biosynthesis by the Arabidopsis ETO1 protein. Nature 2004; 428:945-50.
11. Christians MJ, Gingerich DJ, Hansen M, Binder BM, Kieber JJ, Vierstra RD. The BTB ubiquitin ligases ETO1, EOL1 and EOL2 act collectively to regulate ethylene biosynthesis in Arabidopsis by controlling type-2 ACC synthase levels. Plant J 2009; 57:332-45.
12. Prasad ME, Schofield A, Lyzenga W, Liu H, Stone SL. Arabidopsis RING E3 ligase XBAT32 regulates lateral root production through its role in ethylene biosynthesis. Plant Physiol 2010; 153:1587-96.
13. Stone SL, Hauksdottir H, Troy A, Herschleb J, Kraft E, Callis J. Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis. Plant Physiol 2005; 137:13-30.
14. Yang X, Sun C, Hu Y, Lin Z. Molecular cloning and characterization of a gene encoding RING zinc finger ankyrin protein from drought-tolerant *Artemisia desertorum*. J Biosci 2008; 33:103-12.
15. Huang J, Chen F, Del Casino C, Autino A, Shen M, Yuan S, et al. An ankyrin repeat-containing protein, characterized as a ubiquitin ligase, is closely associated with membrane-enclosed organelles and required for pollen germination and pollen tube growth in lily. Plant Physiol 2006; 140:1374-83.
16. Wang YS, Pi LY, Chen X, Chakrabarty PK, Jiang J, De Leon AL, et al. Rice XA21 binding protein 3 is a ubiquitin ligase required for full Xa21-mediated disease resistance. Plant Cell 2006; 18:3635-46.
17. Xu X, Jiang CZ, Donnelly L, Reid MS. Functional analysis of a RING domain ankyrin repeat protein that is highly expressed during flower senescence. J Exp Bot 2007; 58:3623-30.
18. Guzman P, Ecker J. Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell 1990; 2:513-23.
19. Le J, Vandenbussche F, Van Der Straeten D, Verbelen JP. In the early response of Arabidopsis roots to ethylene, cell elongation is up and downregulated and uncoupled from differentiation. Plant Physiol 2001; 12:519-22.
20. Swarup R, Perry P, Hagenbeek D, Van Der Straeten D, Beemster GT, Sandberg G, et al. Ethylene upregulates auxin biosynthesis in Arabidopsis seedlings to enhance inhibition of root cell elongation. Plant Cell 2007; 19:2186-96.
21. Ruzicka K, Ljung K, Vanneste S, Podhorská R, Beeckman T, Friml J, et al. Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell 2007; 19:2197-212.
22. Negi S, Ivanchenko MG, Muday GK. Ethylene regulates lateral root formation and auxin transport in *Arabidopsis thaliana*. Plant J 2008; 55:175-87.
23. Ivanchenko MG, Muday GK, Dubrovsky JG. Ethylene-auxin interactions regulate lateral root initiation and emergence in *Arabidopsis thaliana*. Plant J 2008; 55:335-47.
24. Nodzon LA, Xu WH, Wang Y, Pi LY, Chakrabarty PK, Song WY. The ubiquitin ligase XBAT32 regulates lateral root development in Arabidopsis. Plant J 2004; 40:996-1006.
25. Cheng WH, Chiang MH, Hwang SG, Lin PC. Antagonism between abscisic acid and ethylene in Arabidopsis acts in parallel with the reciprocal regulation of their metabolism and signaling pathways. Plant Mol Biol 2009; 71:61-80.
26. De Smet I, Zhang H, Inzé D, Beeckman T. A novel role for abscisic acid emerges from underground. Trends Plant Sci 2006; 11:434-9.
27. Stepanova AN, Yun J, Likhacheva AV, Alonso JM. Multilevel interactions between ethylene and auxin in Arabidopsis roots. Plant Cell 2007; 19:2169-85.
28. Cao YR, Chen SY, Zhang JS. Ethylene signaling regulates salt stress response: An overview. Plant Signal Behav 2008; 3:761-3.
29. Wang Y, Wang T, Li K, Li X. Genetic analysis of involvement of ETR1 in plant response to salt and osmotic stress. Plant Growth Reg 2008; 54:261-9.
30. Zhou HL, Cao WH, Cao YR, Liu J, Hao YJ, Zhang JS, et al. Roles of ethylene receptor NTHK1 domains in plant growth, stress response and protein phosphorylation. FEBS Lett 2006; 580:1239-50.
31. Cao WH, Liu J, He XJ, Mu RL, Zhou HL, Chen SY, et al. Modulation of ethylene responses affects plant salt-stress responses. Plant Physiol 2007; 143:707-19.

32. Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR. EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science 1999; 284:2148-52.
33. Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, et al. Integration of plant responses to environmentally activated phytohormonal signals. Science 2006; 311:91-4.
34. Divi UK, Rahman T, Krishna P. Brassinosteroid-mediated stress tolerance in Arabidopsis shows interactions with abscisic acid, ethylene and salicylic acid pathways. BMC Plant Biol 2010; 10:151-65.
35. Xu J, Li Y, Wang Y, Liu H, Lei L, Yang H, et al. Activation of MAPK kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in Arabidopsis. J Biol Chem 2008; 283:26996-7006.
36. An F, Zhao Q, Ji Y, Li W, Jiang Z, Yu X, et al. Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 Binding F-Box 1 and 2 that requires EIN2 in Arabidopsis. Plant Cell 2010; 22:2384-401.