New perspective in ethylene signaling

Zhefeng Lin and Don Grierson*
Division of Plant and Crop Sciences; School of Biosciences; University of Nottingham; Sutton Bonington Campus; Loughborough, UK

Key steps in understanding ethylene signaling have come from studying Arabidopsis mutants. The mechanisms of receptor signal output are still poorly understood and the discovery of new components has increased the apparent complexity. Not all receptors are equivalent and some appear to have unique functions. There are multiple CTR1-like proteins in tomato, which interact with more than one receptor. Focusing on mutants of the Arabidopsis triple response, which is primarily a growth response, may not uncover the complete range of components involved in developmental responses to ethylene and it is also possible there are real differences between species.

Ethylene regulates many aspects of the plant life cycle and understanding the control of ethylene synthesis and action are important objectives in plant biology. Most of the components responsible for ethylene perception and signaling have been discovered by molecular genetic studies in the model plant Arabidopsis, using alterations in the “triple response” to ethylene to identify mutants of the perception and signal transduction chain. The model that has emerged involves multiple ethylene receptors bound to a membrane that interact directly with a single downstream negative regulator, CTR1 (Constitutive triple response) (Fig. 1). In the absence of the gas ethylene responses are repressed and ethylene binding to the receptors releases the repression, leading to changes in gene expression in the nucleus. In the last few years several groups, including ourselves, have published results suggesting that the ethylene signaling model should be modified to take account of new information. For example: (1) The multiple ethylene receptors in Arabidopsis were proposed to be redundant, but there is increasing evidence suggesting that not all ethylene receptors are equivalent and that ETR1 may play a predominant and/or additional role in ethylene signaling. In tomato, different ethylene receptors have also been shown to have specific functions during plant development and ripening. (2) New receptor-interacting components have been described, and tomato has multiple CTR1-like proteins, which interact specifically with more than one ethylene receptor. In this article we discuss the implications of these findings for models of ethylene perception and signaling.

There are five ethylene receptors in Arabidopsis: ETR1, ERS1, ETR2, ERS2 and EIN4, and six in tomato: LeETR1-LeETR6. They share a similar domain structure with an N-terminal transmembrane domain, followed by a GAF domain, and a C-terminal signal output domain related to the bacterial two-component histidine kinases. Some, but not all receptors, have a receiver domain (Fig. 1). The downstream protein CTR1 (Fig. 1) resembles a Raf-like MAPKKK. The ethylene receptors are located in membranes and form both homo- and heterodimers. The basic functional unit for ETR1 is a disulfide-linked homodimer, which holds a single Cu(I) near the N-termini. Gao et al. showed that ETR1 can form heterodimers with ERS1, ETR2, ERS2 and EIN4 in vivo, and suggested that ethylene receptors exist in clusters, as found with histidine kinase-linked chemoreceptors of bacteria, and that such interaction may contribute to ethylene signal output. In the two-component system of bacteria...
the receiver protein functions to accept a phosphate transferred from the histidine kinase. Among the five ethylene receptors only ETR1 contains perfect histidine kinase and receiver domains. A MAPK cascade may form part of the ethylene signal cascade and dimerization of ETR1 with other ethylene receptors could play a role in a phosphorelay. Ethylene is perceived by a family of membrane-bound Type I or Type II receptors that dimerize and have direct protein-protein interactions with the CTR1 protein, which is a negative regulator. In the absence of ethylene, receptors associated with CTR1 suppress the downstream ethylene response. There are three other CTR1-like proteins in Arabidopsis and tomato, but evidence for involvement of these in ethylene signaling comes from tomato. When ethylene is present, it interacts with the Cu(II) associated with the membrane-located N-termini of the receptor dimers and initiates two processes: degradation of the receptors and possibly a phosphate relay cascading to downstream components. From receptor binding studies and the phenotypes of transgenic plants overexpressing SltPr1, it was proposed that this protein bound LeETR1 and NR and either interfered with binding of CTR-like proteins, or enhanced the degradation of the receptors. EIN2 is a membrane protein and functions downstream of CTR1. How CTR1 affects the membrane-associated EIN2 protein and how EIN2 transmits the ethylene signal into the nucleus remain to be determined, although it appears this may involve Ca2+ signaling. Inside the nucleus, EIN3 and ERF transcription factors are involved in the response to ethylene. MPK3 and MPK6 can influence the stability of EIN3 by phosphorylation. The level of EIN3 protein is also regulated by ubiquitination and proteasome degradation via EBF1 and EBF. XRN4 appears to govern the stability of EBF1/2 mRNA.

Figure 1. Schematic diagram of the ethylene perception and signaling network. Ethylene is perceived by a family of membrane-bound Type I or Type II receptors that dimerize and have direct protein-protein interactions with the CTR1 protein, which is a negative regulator. In the absence of ethylene, receptors associated with CTR1 suppress the downstream ethylene response. There are three other CTR1-like proteins in Arabidopsis and tomato, but evidence for involvement of these in ethylene signaling comes from tomato. When ethylene is present, it interacts with the Cu(II) associated with the membrane-located N-termini of the receptor dimers and initiates two processes: degradation of the receptors and possibly a phosphate relay cascades signaling to downstream components. From receptor binding studies and the phenotypes of transgenic plants overexpressing SltPr1, it was proposed that this protein bound LeETR1 and NR and either interfered with binding of CTR-like proteins, or enhanced the degradation of the receptors. EIN2 is a membrane protein and functions downstream of CTR1. How CTR1 affects the membrane-associated EIN2 protein and how EIN2 transmits the ethylene signal into the nucleus remain to be determined, although it appears this may involve Ca2+ signaling. Inside the nucleus, EIN3 and ERF transcription factors are involved in the response to ethylene. MPK3 and MPK6 can influence the stability of EIN3 by phosphorylation. The level of EIN3 protein is also regulated by ubiquitination and proteasome degradation via EBF1 and EBF. XRN4 appears to govern the stability of EBF1/2 mRNA.

Figure 1. Schematic diagram of the ethylene perception and signaling network. Ethylene is perceived by a family of membrane-bound Type I or Type II receptors that dimerize and have direct protein-protein interactions with the CTR1 protein, which is a negative regulator. In the absence of ethylene, receptors associated with CTR1 suppress the downstream ethylene response. There are three other CTR1-like proteins in Arabidopsis and tomato, but evidence for involvement of these in ethylene signaling comes from tomato. When ethylene is present, it interacts with the Cu(II) associated with the membrane-located N-termini of the receptor dimers and initiates two processes: degradation of the receptors and possibly a phosphate relay cascades signaling to downstream components. From receptor binding studies and the phenotypes of transgenic plants overexpressing SltPr1, it was proposed that this protein bound LeETR1 and NR and either interfered with binding of CTR-like proteins, or enhanced the degradation of the receptors. EIN2 is a membrane protein and functions downstream of CTR1. How CTR1 affects the membrane-associated EIN2 protein and how EIN2 transmits the ethylene signal into the nucleus remain to be determined, although it appears this may involve Ca2+ signaling. Inside the nucleus, EIN3 and ERF transcription factors are involved in the response to ethylene. MPK3 and MPK6 can influence the stability of EIN3 by phosphorylation. The level of EIN3 protein is also regulated by ubiquitination and proteasome degradation via EBF1 and EBF. XRN4 appears to govern the stability of EBF1/2 mRNA.

The C-terminal domains of the Arabidopsis ethylene receptor ETR1 and ERS1 have direct protein-protein interactions with the N-terminus of the serine/threonine protein kinase CTR1, which was found to co-localise with the receptors in the ER membrane. The C-terminal of CTR1 has high similarity to the mammalian Raf protein kinases, while its N-terminus functions as a regulatory domain with weak similarity to the Raf proteins. There are four CTR1-like genes in Arabidopsis (CTR1, EDR1, At1g18160 and At1g63660) (Fig. 2A) and all four proteins contain the conserved N- and C-terminal domains (Fig. 2B). A critical conserved G residue is also present in all four proteins (Fig. 2B highlighted with a star). The importance of this is shown by the ctr1-8 mutation, which alters this G to E, rendering CTR1 unable to bind ETR1, and resulting in a constitutive ethylene response in air, a phenotype resembling the ctr1 null mutation. Among the four Arabidopsis proteins only CTR1 has been implicated in ethylene signaling by mutant analysis and by its direct interactions with the receptors, although a link between ethylene and pathogen response has been suggested for EDR1. The functions of At1g18160 and At1g63660 remain to be determined. There are also four CTR1-like genes in tomato: LeCTR1, LeCTR2, LeCTR3 and LeCTR4. Phylogenetic tree analysis indicates that LeCTR1, 3 & 4 are in the same cluster with CTR1 (Fig. 2A), and in yeast 2-hybrid assays all three proteins interact with the tomato ethylene receptors LeETR1, 2 and NR (Table 1). Mutant complementation studies suggest that LeCTR3 most closely resembles CTR1 as it is able to complement the ctr1 mutation. LeCTR1 & 4 also partially complement ctr1, suggesting that several CTRs may mediate ethylene signaling in tomato.

LeCTR2 is more similar to EDR1 than to the other LeCTR (Fig. 2A). It has the same number, size and position of exons as EDR1 with homology in both the N-terminal and the C-terminal domains. Lin et al. showed it was able to interact with the subfamily I ethylene receptors LeETR1 (and LeETR2) but not with NR in yeast and in vitro. Overexpression of the LeCTR2 N-terminus in tomato resulted in enhanced hypersensitive response to the fungal pathogen Botrytis cinerea, and the transgenic plants also displayed abnormal development, but without any effect on the classical ripening response to ethylene. These observations raise important questions: (1) Do multiple CTRs interact with ethylene receptors in vivo, are the selective interactions of CTRs with the receptors
**Figure 2.** Analysis of CTR1-like sequences from Arabidopsis and tomato. (A) Phylogenetic tree produced in DNA Star software using the full-length protein sequences of CTRs, and the human B-raf was used as an outgroup. (B) Alignment of the N- and C-termini of Arabidopsis CTR1-like proteins. Consensus sequences are shown in red. The conserved amino acid residue Glycine (G) (circled and marked with *) in the CTR1 N-terminus, shown to be important for CTR1 function and discussed in the text, is conserved all CTR1-like proteins.
significant in ethylene signaling, and are there differences between species? (2) Do some CTR1-like proteins interact with other hormone receptors, such as cytokinin receptors? (3) Do different CTRs interact with each other? Dimerization is a common mechanism for regulating phosphorylation by protein kinases. The Raf protein can form “side by side” dimers through the kinase domains, thereby activating kinase activity even if one partner is catalytically “dead”.

Further investigation of the interacting partners of CTR-like proteins should shed more light on ethylene signaling.

Conformational changes in the ethylene receptors caused by ethylene binding probably hold the key to understanding ethylene signaling. The Arabidopsis RTE1 (REVERSION-TO-ETHYLENE SENSITIVITY)10 has been shown to regulate ETR1 functions specifically and to be co-localised with ETR1 in the ER.53 By double mutant analysis of the rte1 loss-of-function mutation and 11 etr1 ethylene-binding domain miss-sense mutations, Resnick et al.4 found that the ethylene binding domain of ETR1 is the target for RTE1 action and proposed that it regulates the conformational changes of the ETR1 receptor. A newly discovered protein SITPR1 binds the tomato NR and LeETR receptors in yeast and in vitro. A related protein AtTRP1 in Arabidopsis also interacts with the ERS1 ethylene receptor7 (Fig. 1). Overexpression of SITPR1 in tomato and AtTRP1 in Arabidopsis plants caused a variety of phenotypes suggesting altered hormone responses related to ethylene and auxin.18 It was postulated that SITPR1 might be involved in receptor degradation, or might interfere with the association of CTR1-like proteins with receptors (Fig. 1), and the mechanism of SITPR1/AtTRP1 action warrants further investigation. Protein degradation plays an important role in modulating ethylene synthesis and signal transduction. Ethylene-induced ethylene receptor degradation has been reported in both Arabidopsis and tomato. Chen et al.,36 showed that the Arabidopsis ETR2 receptor is degraded following treatment with ethylene at concentrations above 1 µl/litre in the wild type background and 0.2 µl/litre in the ctr1-2 background by the physical association of ethylene with the receptor through a proteasome-dependent pathway. Kevany et al.34 showed that the tomato ethylene receptors LeETR4 & 6 are subjected to degradation in the presence of ethylene and that the process depends on the 26S proteasome.

In order to understand ethylene signaling we need more information about ethylene-induced conformational changes in the receptors, the relationship to one or more CTR(s), the precise role of the possible phosphorelay cascade to downstream components, and how recruitment of receptor degrading machinery is regulated.

Table 1. Protein-protein interactions between tomato ethylene receptors and LeCTRs

| CTRs | LeETR1 | LeETR2 | LeETR3 (NR) | LeETR4 | LeETR5 | LeETR6 |
|------|--------|--------|-------------|--------|--------|--------|
| LeCTR1 | ++++   | ++++   | +++         | --     | --     | --     |
| LeCTR2 | ++++   | ++++   | --          | --     | --     | --     |
| LeCTR3 | ++++   | ++++   | ++++        | --     | --     | --     |
| LeCTR4 | ++++   | ++++   | +           | --     | --     | --     |

“+” indicates the extent of the interaction; “−” indicates no interaction. Data from6,17 and unpublished results of Z Lin.

References
1. Yoo S-D, Cho Y, Sheen J. Emerging connections in the ethylene signaling network. Trends in Plant Science 2009; 14:270-9.
2. Lin Z, Zheng S, Grierson D. Recent advances in ethylene research. J Exp Bot 2009; 60:3311-36.
3. Resnick JS, Wen CK, Shockey JA, Chang C. REVERSION-TO-ETHYLENE SENSITIVITY, a conserved gene that regulates ethylene receptor functions in Arabidopsis. Proc Natl Acad Sci USA 2006; 103:7917-22.
4. Resnick JS, Rivasola M, Chang C. Involvement of RTE1 in conformational changes promoting ETR1 ethylene receptor signaling in Arabidopsis. Plant J 2008; 56:423-31.
5. Lin Z, Arciga-Reyes L, Zhong S, Alexander L, Hackert R, Wilson I, et al. SITPR1, a tomato trarri- coperidide repeat protein, interacts with the ethylene receptors NR and LeETR1, modulating ethylene and auxin responses and development. J Exp Bot 2008; 59:4271-87.
6. Lin Z, Alexander L, Hackert R, Grierson D. LeCTR2, a CTR1-like protein kinase, interacts from tomato, plays a role in ethylene signaling, development and defence. Plant J 2008; 54:1083-93.
7. Lin Z, Ho CW, Grierson D. AtTRP1 encodes a novel TPR protein that interacts with the ethylene receptor ERS1 and modulates development in Arabidopsis. J Exp Bot 2009; 60:3607-714.
8. Xie F, Liu Q, Wen CK. Receptor signal output mediated by the ETR1 C terminus is primarily subfamily I receptor dependent. Plant Physiol 2006; 142:492-508.
9. Hall AE, Bleecker AB. Analysis of combinatorial loss-of-function mutants in Arabidopsis ethylene receptors reveals that the er1 erl double mutant has severe developmental defects that are EIN2 dependent. Plant Cell 2003; 15:2033-41.
10. Xu Q, Hall B, Gao Z, Schaller GEA. Strong constitutive ethylene-response phenotype conferred on Arabidopsis plants carrying null mutations in the ethylene receptors ETR1 and ERS1. BMC Plant Biol 2007; 7:3.
11. Cancel JD, Larsen PB. Loss-of-function mutations in the ethylene receptor ETR1 cause enhanced sensitivity and exaggerated response to ethylene in Arabidopsis. Plant Physiol 2002; 129:1957-67.
12. Binder BM, O’Malley RC, Wang W, Zaru TC, Bleecker AB. Ethylene stimulates mutations that are dependent on the ETR1 receptor. Plant Physiol 2006; 142:1069-700.
13. Tienman DM, Klee HJ. Differential expression of two novel members of the tomato ethylene-receptor family. Plant Physiol 1999; 120:165-72.
14. Whitelaw CA, Lysenko NN, Chen L, Zhou D, Mattoo AK, Tucker ML. Delayed absission and shorter Internodes correlate with a reduction in the ethylene receptor LeETR1 transcript in transgenic tomato. Plant Physiol 2002; 128:978-87.
15. Kevany BM, Tieman DM, Taylor MG, Dal Cin V, Klee HJ. Ethylene receptor degradation controls the timing of ripening in tomato fruit. Plant J 2007; 51:458-67.
16. Barry CS, Giovannoni JJ. Ripening in the tomato Green-ripe mutant is inhibited by ectopic expression of a protein that disrupts ethylene signaling. Proc Natl Acad Sci USA 2006; 103:7923-8.
17. Zhong S, Lin Z, Grierson D. Tomato ethylene receptor interaction: visualization of NEVER-RIPE interaction with multiple CTRs at the endoplasmic reticulum. J Exp Bot 2008; 59:965-72.
18. Schaller GE, Kieber JJ. Ethylene: The Arabidopsis Book. Rockville, MD: American Society of Plant Biologists 2002; (Online: http://www.aspb.org/publications/arabidopsis/).
19. Wilkinson JQ, Lanahan MB, Yen HC, Giovannoni JJ, Klee HJ. An ethylene-inducible component of signal transduction encoded by Neverripe. Science 1995; 270:1807-9.
20. Lashbrook CC, Tieman DM, Klee HJ. Differential regulation of the tomato ETR gene family through plant development. Plant J 1998; 15:243-52.
21. Kieber JJ, Rothenberg M, Roman G, Feldman KA, Ecker JR. CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. Cell 1993; 72:427-41.
22. Gao Z, Wen C, Binder BM, Chen Y, Chang J, Chiang Y, et al. Heteromeric interactions among ethylene receptors mediate signaling in Arabidopsis. J Biol Chem 2008; 283:23801-10.
23. Rodriguez FI, Esch JJ, Hall AE, Binder BM, Schaller GE, Bleecker AB. A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science 1999; 283:996-8.
24. Ouaked F, Reehow W, Lecourieux D, Hirt H. A MAPK pathway mediates ethylene signaling in plants. EMBO J 2003; 17:1282-8.
25. Moussatche P, Klee HJ. Histidine kinase activity is not necessary for ethylene receptor function in plants and suggest that ethylene signal transduction does not occur through a phosphorelay mechanism. J Biol Chem 2004; 279:48734-41.
26. Klee HJ. Ethylene signal transduction. Moving beyond Arabidopsis. Plant Physiol 2004; 135:660-7.
27. Clark KL, Larsen PB, Wang X, Chang C. Association of the Arabidopsis CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. Proc Natl Acad Sci USA 1998; 95:5401-6.
28. Gao Z, Chen YF, Randlett MD, Zhao XC, Findell JL, Kieber JJ, et al. Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of Arabidopsis through participation in ethylene receptor signaling complexes. J Biol Chem 2003; 278:34725-32.
29. Pelech S. Dimersation in protein kinase signaling. J Biol 2006; 5:12.
30. Huang Y, Li H, Hutchison CE, Laskey J, Kieber JJ. Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in Arabidopsis. Plant J 2003; 33:221-3.
31. Tang D, Christiansen KM, Innes RW. Regulation of plant disease resistance, stress responses, cell death and ethylene signaling in Arabidopsis by the EDR1 protein kinase. Plant Physiol 2005; 138:1018-26.
32. Adams-Phillips I, Barry C, Kannanz P, Leclercq J, Bouladif M, Giavazzi N. Evidence that CTR1-mediated ethylene signal transduction in tomato is encoded by a multigene family whose members display distinct regulatory features. Plant Mol Biol 2004; 54:387-404.
33. Rajakulendran T, Sahmi S, Lefrancois M, Sicheri F, Thirrien M. A dimerization-dependent mechanism drives RAF catalytic activation. Nature 2009; 461:542-6.
34. Bleecker AB, Esch JJ, Hall AE, Rodriguez FI, Binder BM. The ethylene-receptor family from Arabidopsis: structure and function. Phil Trans R Soc Lond B 1998; 353:1405-12.
35. Dong CH, Rivarola M, Resnick JS, Maggin BD, Chang C. Subcellular co-localization of RTE1 and ETR1 supports a regulatory role for RTE1 in ETR1 ethylene signaling. Proc Natl Acad Sci USA 2003; 95:5401-6.