Control of ethylene-mediated processes in tomato at the level of receptors

Harry J. Klee

University of Florida, Horticultural Sciences, PO Box 110690, 1143 Fifield Hall, Gainesville, FL 32611 USA

Abstract

The plant hormone ethylene controls many aspects of development and response to the environment. In tomato, ethylene is an essential component of flower senescence, organ abscission, adventitious root initiation, and fruit ripening. Responses to ethylene are also critical for aspects of biotic and abiotic stress responses. Clearly, much of the control of these events occurs at the level of hormone synthesis. However, it is becoming apparent that levels of the ethylene receptors are also highly regulated. The tomato ethylene receptors are encoded by a family of six genes. Levels of expression of these genes are spatially and temporally controlled throughout development. Further, a subset of the receptor genes respond to external stimuli. Genetic and biochemical evidence supports a model in which the ethylene receptors act as negative regulators of downstream responses; in the absence of ethylene, receptors actively suppress expression of ethylene responsive genes. Consistent with this model, a reduction in the overall level of receptor increases ethylene responsiveness of a tissue while higher expression of receptor decreases ethylene sensitivity. Evidence to support this model will be presented.

Key words: Arabidopsis, fruit ripening, Never-ripe, phytohormone, transcription.

Introduction

Phytohormones have vital roles in assimilating many aspects of plant growth and development. Regulation of hormonal signals can occur via fluxes in the level of the hormone or in its perception (Bradford and Trewavas, 1994). Sensitivity to hormones can be regulated both spatially and temporally. For example, adjacent cells can respond differentially to a hormone, as occurs during organ abscission, or sensitivity to a hormone may change over time, as occurs during fruit ripening. The focus of this research has been to define how differential sensitivity to the phytohormone ethylene is regulated during development of the tomato plant. In the context of this article, the term ‘sensitivity’ refers to the response of a tissue or organ to the hormone. A change in sensitivity indicates that the concentration of hormone that initiates a response is altered.

Ethylene is a small, readily diffusible phytohormone with an important role in integrating developmental signals and responses to biotic and abiotic external stimuli. Ethylene is a critical component of such diverse developmental processes as seed germination, fruit ripening, abscission, and senescence (Abeles et al., 1992). It is also widely viewed as a stress hormone. Adverse biotic or abiotic cues usually stimulate ethylene synthesis. This ethylene, in turn, slows down plant growth until the stress is removed. At the level of gene expression, ethylene has been shown to induce transcription of a wide range of genes involved in wound signalling (O’Donnell et al., 1996), pathogen defence (Boller, 1991; Ecker and Davis, 1987) and fruit ripening (Giovannoni, 2001). How ethylene, and more specifically its receptor family, mediates such a diverse range of dissimilar functions is not well established.

Ethylene synthesis is highly regulated, being induced in response to certain developmental and stress cues. Genes encoding the two committed steps to ethylene synthesis, ACC synthase and ACC oxidase, are highly transcriptionally regulated (Barry et al., 1996; Rottmann et al., 1991).
In addition to tight developmental control over ethylene synthesis, there is regulation of its perception by many tissues. For example, experiments conducted with tomato (Yang, 1987) indicate that immature fruits do not ripen in response to exogenous ethylene. While the fruits do perceive ethylene, evident by the activation of a subset of ethylene-inducible genes, they do not initiate the developmental sequence that leads to ripening. While much is known about the regulation of ethylene synthesis in tomato, less is known about its perception and subsequent signal transduction. A tomato mutant has been identified that is altered in its ability to perceive ethylene. Never ripe (Nr) is a semi-dominant mutant in one of the ethylene receptor genes characterized by the inability of its fruit to undergo ripening (Wilkinson et al., 1995). Analysis of Nr indicated a number of pleiotropic effects indicative of ethylene insensitivity throughout the plant (Lanahan et al., 1994). The mutant is greatly impaired in floral abscission and there are significant delays in leaf and flower petal senescence. Several researchers have used Nr as a tool to assess the role of ethylene in a range of developmental (Aloni et al., 1998; Clark et al., 1999; Hansen and Grossmann, 2000; Llop-Tous et al., 2000), gene expression (Nakatsuka et al., 1998; Rose et al., 1997) and stress (Ciardi et al., 2000; Lund et al., 1998; O’Donnell et al., 2001) processes.

Ethylene signal transduction in Arabidopsis

There has been significant progress in elucidating the ethylene signal transduction pathway. A rapid, high-throughput screen utilizing the effect of ethylene on dark grown seedlings, the ‘triple response’, has permitted isolation of many Arabidopsis mutants altered in ethylene synthesis and response (for reviews see Bleecker and Kende, 2000; Stepanova and Ecker, 2000). Many genes involved in signal transduction including CTR1 (Kieber et al., 1993), EIN2 (Alonso et al., 1999), EIN3 (Chao et al., 1997), ERF1 (Solano et al., 1998), and ETR1 (Chang et al., 1993), have been cloned. Of particular interest were the dominant mutant alleles of ETR1 (Bleecker et al., 1988; Guzman and Ecker, 1990). The cloning of ETR1 permitted tomato homologues to be identified, including NR.

ETR1 is homologous to the prokaryotic family of signal transducers known as two-component regulators. In bacteria, the two components, the sensor and the response regulator, modulate responses to a wide range of developmental and environmental stimuli (reviewed in Stock et al., 2000). Multiple mutant alleles of ETR1 have been identified and all of these confer dominant ethylene insensitivity (Guzman and Ecker, 1990). Both genetic and biochemical data confirm that ETR1 encodes an ethylene receptor. ETR1 functions as a dimer and exhibits copper-mediated high affinity ethylene binding (Schaller and Bleecker, 1995; Schaller et al., 1995). The ETR1 family in Arabidopsis consists of five proteins (Chang et al., 1993; Hua et al., 1995, 1997, 1998; Sakai et al., 1998). The ETR1, ETR2 and EIN4 genes were initially identified by mutations, while the ERS1 and ERS2 genes were identified by their homology to ETR and subsequently shown to confer dominant ethylene insensitivity in transgenic plants. Ethylene receptor proteins can be structurally separated into three domains.

1) The sensor domain (amino acids 1–313) contains three hydrophobic, putative transmembrane stretches. Ethylene binding occurs within this amino terminal hydrophobic region and all of the known mutations of ETR1 are located within the hydrophobic regions. Ethylene binding is abolished in several of the ethylene-insensitive mutant proteins (such as etr1-1). The amino terminal domain also contains the amino acids necessary for dimerization and copper binding. Although homodimerization has been demonstrated (Schaller et al., 1995), proof of heterodimer formation is lacking. The first domain also contains a sub-domain referred to as GAF, whose function has yet to be established.

2) The kinase domain (amino acids 314–581) has extensive sequence homology to histidine kinases (HK). There are five sub-domains that define the catalytic core of histidine kinases (H, N, G1, F, G2). While ETR1 and ERS1 contain all of these sub-domains, the other three receptors lack one or more of them. Notably, ETR2 and ERS2 lack the histidine that is autophosphorylated. This histidine is not essential for the dominant ethylene insensitivity conferred by etr1-1 (Chang and Meyerowitz, 1995). ETR1 has been shown to have in vitro HK activity (Gamble et al., 1998). To date, this is the only ethylene receptor with demonstrated HK activity. In this paper, ETR1 and ERS1 will be referred to as Type 1 receptors. The structurally divergent ETR2, EIN4 and ERS2 will be designated as Type 2. In theory, the HK would act to transmit the signal to downstream components of the ethylene pathway. However, an obvious question is how do Type 2 receptors act since they clearly cannot have HK activity.

3) The receiver (or response regulator) domain (amino acids 582–738). This region has sequence identity to the output portion of bacterial two-component systems and contains an aspartate that is active in phosphorelay in bacterial proteins. In prokaryotes, there are two classes of first-component proteins, those that contain a receiver domain within the same protein as the sensor/HK and those that do not. Even where the proteins do contain the receiver domain contiguous to the HK, there is always a separate protein that is the ultimate receiver in signal transduction. In the yeast SLN1 and some bacterial two-component systems, the receiver domain is integral to transfer of the phosphate to the response regulator (see below). As in bacteria, some members of the plant ethylene receptor
family are missing the receiver domain; ERS1 and ERS2 lack it while the other three contain it. That some of these proteins maintain the receiver domain with a high degree of conservation while others completely lack it, suggests an important but undetermined function for this domain.

All of the etr1 mutants display a semi-dominant ethylene-insensitive phenotype. Single gene knock-outs, by contrast, have no obvious phenotype. Experiments using combinations of receptor knock-outs indicate that the receptors act as negative regulators of ethylene responses (Hua and Meyerowitz, 1998). Single and double loss-of-function mutants likely do not show an obvious ethylene-related phenotype due to functional redundancy. By contrast to the single and double knock-outs, triple mutants exhibit constitutive ethylene hypersensitivity. The mutants exhibit ethylene responses at levels below that which induces the response in wild-type plants. A quadruple mutant is more severe yet and does not reach maturity. The model derived from these experiments predicts a default state in which the receptor is active and ethylene acts to inactivate it. The most logical explanation is that a kinase acts to suppress ethylene-inducible gene expression and a receptor incapable of binding ethylene cannot be inactivated. The prediction of this model, shown schematically in Fig. 1, is that less ethylene to inactivate the remaining receptors. However, precisely what activity is being regulated is not clear since there are receptors such as ERS2 that cannot have HK activity.

The tomato ethylene receptor family

The wealth of background physiological and molecular research in tomato makes it an outstanding system in which to study the ethylene response. The well-defined roles of ethylene in mediating tomato fruit ripening, petal wilt and flower abscission illustrate the advantages of tomato. At the molecular and biochemical level, developmental regulation of an ethylene receptor was first demonstrated when it was shown that Nr expression significantly increased during fruit ripening (Wilkinson et al., 1995). Six tomato ethylene receptors, LeETR1-6, have been isolated and characterized (Lashbrook et al., 1998; Tieman and Klee, 1999; Wilkinson et al., 1995; Zhou et al., 1996a, b). For historical reasons, LeETR3 will continue to be referred to as NR. The predicted structures of the LeETR receptor family are very similar to those of the Arabidopsis receptor family. The proteins are quite divergent, exhibiting less than 50% identity in primary sequence at the extremes. Two members have a potential extra amino terminal membrane spanning domain. This domain may be a signalling sequence or may serve to direct the amino terminus to the cytoplasm. Only one receptor, NR, lacks the receiver domain. At least one and
possibly three (LeETR4–6) do not have the complete set of conserved HK domains, thus resembling the Type 2 Arabidopsis receptors. Despite the extensive structural differences between them, it has been shown experimentally that all except LeETR6, which was cloned much later than the others and has not yet been tested, are ethylene receptors, as defined by their ability to bind ethylene (F Rodriguez, A Bleecker, H Klee, unpublished results). In addition, NR can function in vivo to compensate for the loss of LeETR4, despite them being less than 50% identical (Tieman et al., 2000).

**Table 1. Expression patterns of the tomato ethylene receptor genes LeETR1–LeETR5**

Expression levels are indicated as follows: %mRNA 0.00002–0.00009 (*); 0.0001–0.0009 (**); 0.001–0.009 (***); 0.01–0.05 (****).

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<tr>
<th>Tissue/Jacquardia Xanthomonas</th>
<th>LeETR1</th>
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<td>Virulent Xanthomonas</td>
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**Expression of the tomato receptors**

The patterns of expression of the tomato receptor genes have been characterized. Each gene has a distinct pattern of expression throughout development and in response to external stimuli (Lashbrook et al., 1998; Tieman and Klee, 1999). The patterns of gene expression for LeETR1–5 are summarized in Table 1. LeETR1 and LeETR2 are expressed at constant levels in all tissues throughout development with LeETR1 expressed at about 5-fold higher than LeETR2. There was no sign of any altered expression of these two genes in response to any external stimulus such as ethylene or pathogen infection. By contrast, expression patterns of the other four genes are highly regulated. For example, during fruit development, ovaries express high levels of NR mRNA at anthesis. The level then drops ~10-fold until the onset of ripening when it rises ~20-fold. The ripening-associated rise is an example of developmentally-dependent ethylene inducibility, i.e. the gene is ethylene-inducible during ripening but not in immature fruit (Wilkinson et al., 1995). The LeETR4, LeETR5 and LeETR6 genes are expressed abundantly in reproductive tissues (flowers and fruits) and less so in vegetative tissues (D Tieman, H Klee, unpublished results) (Tieman and Klee, 1999). The levels of LeETR4 and LeETR5 also increase significantly as fruits mature and ripen. The significance of the higher expression of these receptors in reproductive tissues is not known at present. NR and LeETR4, but not the other genes, are induced by pathogen infection. This pathogen inducibility of LeETR4 is associated with increased ethylene synthesis in the case of infection with an avirulent pathogen. This induction is an important component of the defence response, functioning to reduce ethylene sensitivity of the infected tissue, thus limiting tissue damage (Ciardi et al., 2001) (described below).

**Is expression related to function?**

When considering the importance of receptor expression in terms of regulating overall ethylene response, there are a few points that must be kept in mind. First, the receptors apparently work as negative regulators. A receptor in the absence of ethylene actively suppresses the expression of ethylene-inducible gene expression. This model predicts that there should be an inverse correlation between receptor levels and ethylene sensitivity of a tissue. It should take more ethylene to inactivate high levels of receptors in order to relieve the repression and vice versa. This model explains why the multiple receptor knockouts in Arabidopsis exhibit constitutive ethylene responsiveness, despite unaltered levels of the hormone in the plant. The basal levels of ethylene produced by the plant are sufficient to inactivate the full complement of receptors. The second factor to consider in ethylene responses is that the receptors have a very long half-life for ethylene dissociation. The measured half-life of dissociation for yeast-expressed ETR1 was approximately 12 h (Schaller and Bleecker, 1995). This measured dissociation rate is most likely an underestimate since it does not account for protein turnover. Indeed, dissociation could be substantially longer than 12 h. In practical terms this means that once a receptor has bound ethylene, it will be unable to repress ethylene-inducible genes for a long time, if ever. Thus, the only way that a tissue can turn off an ethylene response once it has been initiated is via synthesis of new receptor. In terms of terminal, irreversible types of responses such as abscission, flower senescence or fruit ripening, there is no need to reverse the process. However, it must be remembered that ethylene serves a critical role in mediating a wide range of environmental responses. Many of these types of responses, such as water stress or wounding, disappear after some period of time. A plant must be capable of shutting down the ethylene response
appropriately. This requires synthesis of new receptors. It should be noted that turnover has not been measured in planta. There is also the possibility that ethylene dissociation kinetics could be different in vivo. An accurate measure of binding kinetics and protein turnover in plants is certainly warranted.

Within this context, receptor gene regulation in tomato is considered. Transgenic plants were used to test the model developed in Arabidopsis. It is important to note that most of the data collected is at the level of RNA accumulation. However, the levels of NR protein have been checked with antibodies in both over- and under-expressing lines and there is a good correlation between RNA and protein levels. A wild-type form of NR was over-expressed using transgenic plants containing the cDNA fused to the constitutive 35S promoter (Ciardi et al., 2000). These plants are, indeed less sensitive to ethylene. Thus, more receptor leads to reduced ethylene response. Conversely, antisense reduction in expression of individual receptors does not have a major effect on ethylene sensitivity. These results are entirely consistent with the Arabidopsis data and indicate that there is a degree of redundancy built into the system. There is, however, one very notable exception; antisense-reduced LeETR4 plants are severely affected, exhibiting a constitutive ethylene response. This is the only receptor that, when substantially reduced, causes a severe ethylene hypersensitive phenotype. The effects include epinasty, loss of flowers and substantially earlier fruit ripening. These effects occur without any increase in ethylene synthesis. Thus, plants have become more sensitive to ethylene, exhibiting a significant reduction in the dose of ethylene required to initiate a biological response. This phenotype was initially surprising since LeETR4, although abundant in reproductives tissues, is not the only highly expressed receptor. A closer examination of receptor gene expression in transgenic plants revealed what has been termed functional compensation (Tieman et al., 2000). In particular, when NR expression is reduced by antisense, expression of LeETR4 increased proportionately. In some manner, the plant compensates for reduced expression of NR by increasing expression of LeETR4. Thus, the overall receptor content of NR antisense lines is not substantially affected, whereas the receptor content in LeETR4 antisense lines is substantially reduced. To date, this compensatory mechanism has not been observed in Arabidopsis. Nonetheless, the tomato data are consistent with the model of reduced expression increasing ethylene sensitivity.

So far, significant alterations in the expression of multiple receptors have been seen in tomato, either during development or in response to external stimuli. However, in every case examined, the regulation is always positive. Expression of a receptor gene is increased. There is yet to be seen any occurrence of reduced expression correlating to increased ethylene responsiveness. Thus, it appears that tomato may use receptor expression to reduce ethylene responsiveness or, more likely, to restore the ability to respond to ethylene. How does tomato initiate an ethylene response? Apparently, it relies upon the finely tuned system of ethylene synthesis. When an ethylene response is initiated, there is increased ethylene synthesis frequently followed by increased receptor synthesis. While it may seem counterproductive to reduce hormone sensitivity shortly after synthesis, it appears to follow closely a pattern exhibited by many phytohormones. Typically, when a plant synthesizes or is exposed to a sudden increase in a hormone, it responds by increasing mechanisms to inactivate the response. Usually that response involves the synthesis of enzymes that inactivate the hormone directly. For example, auxin application to Arabidopsis results in substantial increases in auxin-conjugating enzymes (Ostin et al., 1998). In the case of ethylene, there are no known inactivating enzymes. Indeed, because of its rapid diffusion, there is probably no need for inactivation. Since only recently has any other hormone receptor gene been cloned, it is not known whether the levels of other hormone receptors respond to the hormone increase in a similar way. In summary, then, it is entirely normal for a plant to act to reduce a hormone response shortly after it is initiated. Increased ethylene receptor synthesis probably serves this purpose.

The patterns of expression of the six cloned receptor genes cannot be reconciled with the substantial alteration in ethylene sensitivity that occurs at the mature green phase of fruit development. The increased ethylene sensitivity of ripening fruits could only be explained by a significant decrease in receptor content. However, the sum total of expression of all receptors is actually higher in ripening fruits than in immature green fruits. It is far more likely that the ripening response is mediated by some combination of transcription factors, i.e. a subset of ethylene-inducible genes require the ethylene-associated transcriptional machinery plus an as yet unidentified developmentally-regulated factor. The identity and mode of regulation of such a hypothetical factor remains undetermined.

Finally, it has been observed that the increased expression of the LeETR4 gene in response to pathogen infection appears to have a role in disease response. Normally, the LeETR4 gene is induced during the hypersensitive response triggered by infection with Xanthomonas campestris pv. vesicatoria (Ciardi et al., 2001). In antisense lines with greatly reduced LeETR4 expression, there is an accelerated hypersensitive response that occurs upon infection with the pathogen. Increases in ethylene synthesis and pathogenesis-related gene expression are greater and more rapid in the infected antisense line, indicating an enhanced defence response. However, this enhanced response comes at a price to the plant since damage to the plant surrounding the infection site can be
much greater. If the receptor level does not increase in response to infection, the tissue is overly sensitive to ethylene. Thus, it appears that the normal pattern of pathogen and ethylene-induced increase in receptor has the effect of dampening the subsequent ethylene response to limit overall damage to the plant.

**Acknowledgements**

The author wishes to thank the US Department of Agriculture and the National Science Foundation for continuing support of the research described in this paper.

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