Ligand-Induced Alterations in the Phosphorylation State of Ethylene Receptors in Tomato Fruit

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Perception of the plant hormone ethylene is essential to initiate and advance ripening of climacteric fruits. Since ethylene receptors negatively regulate signaling, the suppression is canceled upon ethylene binding, permitting responses including fruit ripening. Although receptors have autophosphorylation activity, the mechanism whereby signal transduction occurs has not been fully determined. Here we demonstrate that LeETR4, a critical receptor for tomato (Solanum lycopersicum) fruit ripening, is multiply phosphorylated in vivo and the phosphorylation level is dependent on ripening stage and ethylene action. Treatment of preclimacteric fruits with ethylene resulted in accumulation of LeETR4 with reduced phosphorylation whereas treatments of ripening fruits with ethylene antagonists, 1-methylcyclopropene and 2,5-norbornadiene, induced accumulation of the phosphorylated isotypes. A similar phosphorylation pattern was also observed for Never ripe, another ripening-related receptor. Alteration in the phosphorylation state of receptors is likely to be an initial response upon ethylene binding since treatments with ethylene and 1-methylcyclopropene rapidly induced the LeETR4 phosphorylation state rather than protein abundance. The LeETR4 phosphorylation state closely paralleled ripening progress, suggesting that the phosphorylation state of receptors is implicated in ethylene signal output in tomato fruits. We provide insights into the nature of receptor on and off states.

The gaseous plant hormone ethylene regulates multiple developmental processes including germination, seedling growth, organ senescence and abscission, and fruit ripening (Abeles et al., 1992). Ripening of climacteric fruits in particular is profoundly controlled by ethylene action, and the mechanism of this control has been intensively investigated in a number of species, most notably tomato (Solanum lycopersicum), an important model for the study of fleshy fruit development. Climacteric fruits such as tomato are characterized by an increase in respiration and a concomitant increase in ethylene biosynthesis that is required for triggering ripening processes including pigment accumulation, cell wall degradation, and volatile evolution. While ethylene biosynthesis is required to initiate and advance fruit ripening, sensing the hormone is necessarily the first step in controlling ripening.

Ethylene signaling begins with perception of the hormone by multiple receptors. Genetic analysis in tomato and Arabidopsis (Arabidopsis thaliana) has shown that receptors act as negative regulators of the signaling pathway (Hua and Meyerowitz, 1998; Tieman et al., 2000). Receptors actively suppress ethylene responses in the absence of the hormone, and upon ethylene binding suppression is canceled, permitting the response to occur. In tomato, there are seven ethylene receptors, LeETR1-2, -4 to -7, and Never ripe (NR; Wilkinson et al., 1995; Zhou et al., 1996; Lashbrook et al., 1998; Tieman and Klee, 1999), five of which have been shown to bind ethylene with high affinity; LeETR6 and LeETR7 were not tested (O’Malley et al., 2005). NR, LeETR4, and LeETR6 transcripts are induced at the onset of fruit ripening and these three genes are the most highly expressed during ripening (Kevany et al., 2007). Reduced expression of either LeETR4 or LeETR6 by antisense transgenes resulted in phenotypes consistent with constitutive ethylene responses including earlier fruit ripening (Tieman et al., 2000; Kevany et al., 2007). On the other hand, presence of the dominant Nr mutation, which has a single amino acid change in the ethylene-binding region of NR, resulted in ethylene insensitivity and non-ripening fruit (Lanahan et al., 1994; Wilkinson et al., 1995). An ethyl-methanesulfonate-induced tomato LeETR1 mutant having a single amino acid change similar to Nr also exhibited a delayed ripening phenotype although the effects were more modest than Nr (Okabe et al., 2011).

Ethylene insensitivity found in dominant mutants (e.g. Nr) can be chemically mimicked by treatment with ethylene antagonists. 1-Methylcyclopropene (1-MCP), one of the most potent of the cyclic olefin ethylene antagonists, has higher affinity for receptors than does ethylene and prevents ethylene signaling (Sisler and Serek, 1997; Hall et al., 2000). 1-MCP treatment has been shown to effectively delay ripening and senescence.
Phosphorylation of Tomato Ethylene Receptors

in a wide range of horticultural crops, most notably climacteric fruits. The response intensity and recovery of climacteric fruits to 1-MCP depend on the developmental stage at the time of treatment (Huber, 2008). Application to preclimacteric fruits can result in strong and irreversible effects, with fruits exhibiting loss of capacity to fully ripen. In contrast, fruits treated after ripening initiation retain full ripening capacity yet can quickly lose responsiveness as treatment is applied at progressively advanced stages of ripening (Huber, 2008). The processes contributing to loss of responsiveness to and recovery from 1-MCP are not well understood. Elucidation of the molecular events following 1-MCP binding to the receptor will aid in our understanding of the events underlying the efficacy of cyclic olefin ethylene antagonists.

Ethylene receptors are ancestrally related to bacterial two-component His kinases (Bleecker, 1999) and act as disulfide-linked dimers (Schaller et al., 1995) primarily located on endoplasmic reticulum membranes (Chen et al., 2002, 2007; Ma et al., 2006). Each receptor contains an N-terminal transmembrane domain where ethylene binds, followed by a GAF domain, and a kinase domain. A subset of the receptors additionally possess a receiver-like domain at the C terminus. Receptors can be classified into two subfamilies based on structural features. The subfamily 1 members are the most homologous to His kinases whereas the subfamily 2 members have diverged and lost most of the amino acids associated with His kinase activity (Gamble et al., 1998; Moussatche and Klee, 2004). Instead, subfamily 2 members have acquired autophosphorylation activities on Ser/Thr residues (Xie et al., 2003; Moussatche and Klee, 2004; Zhang et al., 2004; Wuriyaphan et al., 2009). It was demonstrated that His kinase activity of subfamily 1 Arabidopsis ETR1 is suppressed by ethylene in vitro (Voet-van-Vormizeele and Groth, 2008). Recent genetic analysis concluded that His kinase activity of ETR1 is not essential but modulates ethylene signaling (Hall et al., 2012). Ser/Thr kinase activity of NTHK1, a subfamily 2 member in tobacco (Nicotiana tabacum), influences responses to ethylene and salt when expressed in Arabidopsis (Chen et al., 2009). It is thus likely that ethylene binding to the receptors alters kinase activity in vivo, modulating signal output.

Although receptor autophosphorylation has been known for a number of years, its actual function in signal transduction remains unknown. For example, it is not known whether phosphorylated receptors suppress or permit signal transduction or whether phosphorylation state is related to receptor turnover. In this report, we examined the phosphorylation state of receptors during tomato fruit development to link phosphorylation state with ethylene signaling. Analyses with phosphate affinity SDS-PAGE (Phos-tag PAGE) revealed that the phosphorylation levels of LeETR4 and NR changed before and after ripening initiation. Further, the phosphorylation levels were altered following treatments with ethylene and antagonists. These results provide insights into the nature of receptor on and off states.

RESULTS

Phosphorylation State of LeETR4 during Fruit Development

We initially analyzed protein levels of receptors in tomato fruit during development and ripening. Both LeETR4 and NR proteins increased at the onset of ripening and persisted at high levels until fruits were fully ripe (Supplemental Fig. S1). Since receptors act as negative regulators of ethylene responses, this increase in receptor abundance cannot explain ethylene-dependent ripening, i.e. more receptor would be expected to reduce ethylene responses. Accordingly, a receptor inactivation process must occur upon ethylene binding. To elucidate the initial response to ethylene binding, we examined the receptor phosphorylation state. Due to the availability of highly specific antibodies with strong affinity for the antigen, we focused on LeETR4.

To analyze the phosphorylation state of LeETR4, microsomal proteins from pericarp tissues were subjected to Phos-tag PAGE and detected with anti-LeETR4 antibody. In the gel containing Phos-tag, which has strong affinity for phosphate, migration of phosphorylated proteins is reduced compared with that of the nonphosphorylated counterparts (Kinoshita et al., 2006). Although a single band corresponding to LeETR4 was detected in SDS-PAGE at all developmental stages, multiple up-shifted bands were detected at different positions in Phos-tag PAGE (Fig. 1A). Signals were observed at relative mobility (R) 0.2 and 0.5 to 0.6 in immature (IM) fruits, and at R 0.7 with minor bands at R 0.9 and 1.0 in mature green (MG) fruits. From breaker through red ripe stages, doublet bands were detected at R 0.9 and 1.0. Analysis of an antisense line in which LeETR4 expression is greatly reduced validated the identity of the immunoreactive bands (Fig. 1A). Treatment of microsomal proteins with alkaline phosphatase gave a single band at the lowest position (R 1.0; Fig. 1B), indicating that LeETR4 proteins detected at R 1.0 correspond to the nonphosphorylated isotype whereas the up-shifted LeETR4 proteins (R 0.2–0.9) are phosphorylated isotypes. Although the phosphorylation position can also influence the protein mobility shift, the distance of the shift is generally dependent on the number of phosphorylation sites (Kinoshita-Kikuta et al., 2007). The Phos-tag PAGE result therefore suggests that LeETR4 is highly and multiply phosphorylated at the IM stage, with successively less phosphorylation at the MG stage and ripening stages. Here, we refer to an isotype with the greatest mobility shift (R 0.2) as highly phosphorylated, isotypes with medium mobility shifts (R 0.4–0.7) as intermediately phosphorylated, and an isotype with slight mobility shift (R 0.9) as minimally phosphorylated. In MG fruits, the phosphorylation state was mainly intermediate but varied among experiments,
probably because of difficulty in visually staging MG
fruit (Fig. 1, A and B).

Alterations in the Phosphorylation State of LeETR4 in
Response to Ethylene and Antagonists

Since the minimally phosphorylated and nonphos-
phorylated LeETR4 isotypes appeared concomitant with
ripening initiation, when autocatalytic ethylene produc-
tion was initiated, we speculated that ethylene binding
to LeETR4 alters the phosphorylation state. The effect
of ethylene treatment on the phosphorylation state of
LeETR4 was examined in IM and MG fruits (Fig. 2).
Although the phosphorylation state in IM fruits was
unaltered in air, continuous treatment with 50 μL L⁻¹
ethylene reduced phosphorylation within 4 h. This
level of ethylene is within the physiological range ob-
served in ripening fruits (Burg and Burg, 1962). A
similar response was also observed in MG fruits. The
levels of minimally phosphorylated and nonphos-
phorylated isotypes gradually increased in response
to 50 μL L⁻¹ ethylene treatment. SDS-PAGE indicated
that ethylene treatment had negligible effect on the
total amount of LeETR4 protein in both IM and MG
fruits. These results indicate that ethylene binding to
LeETR4 reduces the phosphorylation level in precli-
macteric fruits.

Figure 1. LeETR4 phosphorylation state during fruit development and
ripening. A, Analysis of LeETR4 phosphorylation state in fruits of wild-
type and LeETR4 antisense plants. Microsomal proteins prepared from
pericarp tissues at different stages were subjected to SDS-PAGE and
Phos-tag SDS-PAGE, followed by LeETR4 detection by immunoblot-
ting. A band labeled with an asterisk is a nonspecifically detected
protein. A scale bar beside Phos-tag SDS-PAGE indicates the relative
distance of protein mobility (R_f) to nonphosphorylated LeETR4. The
upper edge of the gel was defined as R_f 0. Bip protein was visualized as
an endoplasmic reticulum-localized loading control. B, Characteri-
zation of up-shifted LeETR4 by dephosphorylation. Microsomal pro-
teins were incubated with or without calf intestinal alkaline phosphatase
(CIP). Abbreviations depicting fruit developmental stages are as follows:
BR, Breaker; TR, turning; PK, pink; RR, red ripe.

Figure 2. Alteration of LeETR4 phosphorylation state by ethylene
treatment in preclimacteric fruits. IM or MG fruits were treated with or
without 50 μL L⁻¹ ethylene for indicated times up to 16 h. The
phosphorylation state of LeETR4 was detected as described in Figure 1.
A band labeled with an asterisk is a nonspecifically detected protein.
We next analyzed the effect of treatment with the ethylene antagonist 1-MCP on the phosphorylation state. The binding affinity of 1-MCP for the receptor is much stronger than that of ethylene (Sisler and Serek, 1997). Accordingly, if ethylene binding is responsible for the observed change in phosphorylation state, 1-MCP binding should antagonize the effect of ethylene on phosphorylation state. The effect of continuous 12-h treatment with 2 μL L⁻¹ 1-MCP on the phosphorylation state was examined in pink stage fruits, where LeETR4 is present mainly in the minimally phosphorylated and nonphosphorylated states (Fig. 3A). SDS-PAGE confirmed that the total amount of LeETR4 remained unchanged throughout the 12-h exposure to 1-MCP. However, the levels of minimally phosphorylated and nonphosphorylated isotypes started to decline after 4 h of 1-MCP treatment accompanied by the appearance of the highly and intermediately phosphorylated isotypes. At 8 and 12 h of 1-MCP treatment, a high proportion of LeETR4 was highly or intermediately phosphorylated, comparable to levels observed in IM fruits. Comparison with the electrophoresis pattern to an LeETR4 antisense line confirmed that the up-shifted proteins are LeETR4 isotypes (Fig. 3B). Treatments of microsomal proteins with alkaline phosphatase shifted the signals to the position of the nonphosphorylated isotype (Fig. 3B), indicating that the 1-MCP-mediated up shifts were due to LeETR4 phosphorylation.

We also tested the effect of 2,5-norbornadiene (NBD), a reversible cyclic-olefin ethylene antagonist (Sisler and Yang, 1984), on the phosphorylation state. Application of 100 μL L⁻¹ NBD to pink stage fruits for 16 h resulted in accumulation of phosphorylated isotypes (Fig. 4A). However, the phosphorylation level gradually reduced over the 60 h after NBD removal. A second NBD treatment after 60 h in air again induced accumulation of phosphorylated isotypes even though fruits at the time of the treatment were fully red ripe. This reversible phosphorylation pattern observed in response to intermittent NBD treatment is consistent with the reversible binding character of NBD. A competition experiment with different concentrations of ethylene and 50 μL L⁻¹ NBD confirmed that phosphorylation was induced by NBD binding to LeETR4 (Fig. 4B).

**Correlation of the Phosphorylation State of LeETR4 and Fruit Ripening Progress**

We monitored alterations in the phosphorylation state of LeETR4 for extended periods following 1-MCP treatment to associate the phosphorylation state with ripening progress. Fruits were treated at MG or turning stages with 2 μL L⁻¹ 1-MCP or air (control) for 12 h and then transferred to air for up to 9 d. When MG fruits were treated, control fruits exhibited ripening initiation (external red color) at 2 to 4 d and fully ripened at 9 d. However, 1-MCP-treated fruits showed no visible color change even at 9 d (Supplemental Fig. S2). The amount of LeETR4 protein gradually increased as the control fruits ripened, following transcriptional up-regulation (Fig. 5A). On the other hand, LeETR4 protein level remained constant in 1-MCP-treated fruits due to transcriptional suppression. Phos-tag SDS-PAGE showed dynamic alterations in LeETR4 phosphorylation state. Although LeETR4 in MG fruits was mainly in the intermediately phosphorylated states before treatments, the ratio of minimally phosphorylated and nonphosphorylated isotypes gradually increased concomitant with ripening in control fruits.
In contrast, 1-MCP treatment switched LeETR4 to the highly phosphorylated state, which was maintained until 9 d. Almost all LeETR4 proteins detected in 1-MCP-treated fruits at 9 d should be derived from 0-d fruits since LeETR4 transcription was completely suppressed, suggesting that 1-MCP-bound LeETR4 in MG fruits was neither dephosphorylated nor degraded through the storage period.

1-MCP efficacy was attenuated when turning fruits were treated; ripening progress was clearly suppressed until 4 d, but gradually recovered after 6 d (Supplemental Fig. S2). In control fruits, transcript and protein levels of LeETR4 remained high through the storage period (Fig. 5B). LeETR4 transcript level was substantially reduced following 1-MCP treatment, but gradually recovered from 6 d. Although the LeETR4 protein level was constant in 1-MCP-treated fruits, the phosphorylation state was significantly altered (Fig. 5B). The highly phosphorylated isotype induced by 1-MCP treatment was maintained until 2 d but disappeared at 4 d. At 4 d, the minimally phosphorylated and nonphosphorylated isoforms started to accumulate. Taken together, the phosphorylation state closely paralleled ripening progress, suggesting that the phosphorylation modulates ethylene signaling.

**Phosphorylation State of NR in Fruits**

The phosphorylation state of NR, a subfamily 1 receptor, was also examined in fruits. Although a single band of NR was detected in SDS-PAGE, two major bands were observed following Phos-tag PAGE (Fig. 6A). When microsomal proteins from MG fruits were treated with alkaline phosphatase, the signal intensity of the lower band increased while the upper band disappeared (Fig. 6A), indicating that the upper band corresponds to a phosphorylated isotype of NR. It was therefore evident that the proportion of nonphosphorylated NR increased upon initiation of ripening. We were unable to determine the phosphorylation state in IM fruits because the protein was not present at a detectable level. To elucidate the effect of ethylene binding on NR, MG and pink stage fruits were treated with ethylene and 1-MCP, respectively. Continuous 16-h treatment of MG fruits with 50 μL L⁻¹ ethylene resulted in a large increase in the nonphosphorylated isotype (Fig. 6B). On the other hand, continuous 8-h treatment of pink stage fruits with 2 μL L⁻¹ 1-MCP induced accumulation of multiple up-shifted bands while the amount of nonphosphorylated isotype was decreased (Fig. 6C). Treatment with alkaline phosphatase and analyses of a cosuppressed line of NR confirmed that the up-shifted bands were due to NR phosphorylation (Fig. 6C).

**DISCUSSION**

Here we demonstrated an ethylene-dependent alteration in the phosphorylation state of LeETR4, which is one of the critical receptors regulating tomato fruit ripening. Based on the observations that LeETR4 is highly or intermediately phosphorylated at the preclimacteric stages but minimally phosphorylated or nonphosphorylated after ripening initiation (Fig. 1), the isotypes with minimal and no phosphorylation presumably correspond to the off state of the receptor. This idea is supported by the results in which ethylene treatment of preclimacteric fruits resulted in accumulation of LeETR4 with reduced phosphorylation (Fig. 2). Previous studies indicated that ethylene exposure of IM fruits significantly hastens the onset of maturity (Burg and Burg, 1962; Kevany et al., 2007). This acceleration could be explained by an ethylene-induced
Figure 5. Transition of LeETR4 phosphorylation state in MG and turning fruit after 1-MCP treatment. MG (A) and turning (B) fruits were treated with 2 μL L⁻¹ 1-MCP for 12 h and then kept in air until 9 d. The phosphorylation state of LeETR4 was detected as described in Figure 1. A band labeled with an asterisk is a nonspecifically detected protein. In SDS-PAGE of section B, the sample of MG fruit (0 d) was included for comparison of protein abundance. LeETR4 transcript level was quantified with real-time PCR. Levels of the transcripts are given as the copy number of mRNA per ng of total RNA. Real-time PCR was performed in triplicate, and values shown are means ± se (n = 3).
alteration in the receptor phosphorylation state resembling that of ripening fruits. Although previous studies have indicated that 1-MCP and NBD bind to receptors (Sisler and Yang, 1984; Sisler and Serek, 1997), preventing ethylene binding and locking the receptor on state, a subsequent molecular event has not been elucidated. Here we showed that either 1-MCP or NBD binding to LeETR4 increased its phosphorylation level regardless of ripening stage (Figs. 3–5), reinforcing a model of phosphorylation-based regulation of ethylene receptors. Since an ethylene-dependent alteration in the phosphorylation state was also observed in NR, this response would be conserved among ripening-related receptors including LeETR6.

Although 2 μL L⁻¹ 1-MCP treatments of both MG and turning fruits resulted in significant ripening inhibition, turning fruits recovered from ripening inhibition more rapidly than did MG fruits (Supplemental Fig. S2). This result is consistent with previous observations in which the efficacy of 1-MCP is attenuated when applied after ripening initiation in climacteric fruits (Huber, 2008). Ripening recovery in 1-MCP-treated turning fruits coincided with disappearance of the highly phosphorylated isotype and appearance of the minimally phosphorylated and nonphosphorylated isotypes.

Figure 6. NR phosphorylation state and the effects of ethylene and 1-MCP treatments. A, Analysis of NR phosphorylation state in fruits. After SDS-PAGE or Phos-tag PAGE, NR was detected by immunoblotting. Abbreviations depicting fruit developmental stages are the same as in Figure 1. For dephosphorylation analysis, microsomal proteins from MG fruits were incubated with or without calf intestinal alkaline phosphatase (CIP). B, The effect of ethylene treatment. MG fruits were treated with or without 50 μL L⁻¹ ethylene for 16 h. C, The effect of 1-MCP treatment. Pink stage fruits of wild-type and NR cosuppressed (Cosup.) plants were treated with or without 2 μL L⁻¹ 1-MCP for 8 h and microsomal proteins were analyzed as described in A. Thirty micrograms of proteins was used for all the samples.

Figure 7. Proposed model of phosphorylation-based regulation of LeETR4 in tomato fruit. In preclimacteric fruits, LeETR4 is multiply phosphorylated, which suppresses ethylene signaling (left). Different isotypes with both quantitative and qualitative differences in phosphorylation are likely to exist. During ripening, ethylene binding reduces phosphorylation, which inactivates the receptor (I). 1-MCP binding reactivates the receptor even in the presence of ethylene (II). Dissociation or metabolism of 1-MCP allows ethylene binding (III).
This alteration in the phosphorylation state implies substitution of 1-MCP by endogenous ethylene on the LeETR4, possibly because of slow dissociation from the LeETR4-binding site (Serek and Sisler, 2001; Jayant et al., 2004) and/or metabolism of 1-MCP (Huber et al., 2010). In contrast, 1-MCP-induced phosphorylation persisted for 9 d in MG fruits that produce little ethylene (Fig. 5A), suggesting that 1-MCP dissociation is accelerated by endogenous ethylene. There is also significantly higher LeETR4 transcript synthesis in 1-MCP-treated turning fruits relative to MG fruits, which would likely result in a higher rate of new receptor synthesis.

While we cannot exclude the possibility that LeETR4 and NR are phosphorylated by other receptor family members or a CTR (for CONSTITUTIVE TRIPLE RESPONSE1), we have shown that the phosphorylation state is altered in vivo; ethylene binding attenuates that phosphorylation while 1-MCP maintains a high level of phosphorylation. Biochemical analysis has previously shown that in vitro autophosphorylation activity of Escherichia coli-produced Arabidopsis ETR1 is turned off by ethylene, but is maintained by treatment with 1-MCP (Voet-van-Vormizeele and Groth, 2008). Thus, the phosphorylation of LeETR4 and NR is most likely associated with autophosphorylation. It should be noted that LeETR4 and NR are somewhat different from ETR1 with respect to kinase activity. While ETR1 has a single phosphorylation site on a His residue (Gamble et al., 1998), our results indicated that LeETR4 and NR have multiple phosphorylation sites. Further, their phosphorylation presumably occurs on Ser and/or Thr residues since LeETR4 is a subfamily 2 member lacking the conserved His kinase domains and NR is the closest homolog of Arabidopsis ERS1 that exhibited Ser kinase activity in natural ionic conditions (Moussatche and Klee, 2004). Since ethylene receptors diverged structurally and functionally from canonical two-component regulators, it is not surprising that some receptors acquired different phosphorylation activities.

The shifts to reduced LeETR4 phosphorylation were observed in ethylene-treated preclimacteric fruits (Fig. 2) and in turning fruits at the timing of ripening recovery from 1-MCP (Fig. 5B). To accomplish these shifts, an additional mechanism should be required as well as turning kinase activity off. Previous studies proposed a ligand-induced receptor degradation; upon ethylene binding, receptors are degraded by the 26S proteasome-dependent pathway (Chen et al., 2007; Kevany et al., 2007). Additionally, LeETR4 transcription is up-regulated by ethylene (Kevany et al., 2007). Thus, ethylene-induced degradation and de novo synthesis of receptors might contribute to the shift in phosphorylation state. Alternatively, receptor dephosphorylation can be proposed. The nonphosphorylated LeETR4 isotype appeared in 1-MCP-treated turning fruits at 4 d, when LeETR4 transcription is still significantly suppressed, suggesting that the nonphosphorylated isotype is derived from dephosphorylation of phosphorylated isotypes upon replacement of 1-MCP by ethylene. An enhanced ethylene response phenotype was reported in the Arabidopsis mutant (rcn1/ee1) of RCN1 (for ROOTS CURL IN NAPHTHYLPHTHALIC ACID1) that is a component of Ser/Thr protein phosphatase PP2A (Larsen and Chang, 2001; Larsen and Cancel, 2003). PP2A could have a role in receptor dephosphorylation.

Receptor phosphorylation may regulate interactions with other signaling elements. ETR1 has been shown to physically interact with several components of ethylene signaling (Clark et al., 1998; Bisson et al., 2009; Dong et al., 2010) and form high-molecular-mass protein complexes with other receptors (Chen et al., 2010). Specifically, CTR1 associates with the kinase domain of ETR1 (Clark et al., 1998; Gao et al., 2003), where autophosphorylation occurs (Gamble et al., 1998). Further, auto-phosphorylation of ETR1 reduced its affinity for ETHYLENE INSENSITIVE2 (EIN2; Bisson and Groth, 2010). Since proteins homologous to CTR1 (Leclercq et al., 2002; Adams-Phillips et al., 2004) and EIN2 (Wang et al., 2007) have been identified in tomato, receptor phosphorylation is likely to modulate the interactions with those proteins.

Previous studies have shown that receptor concentration can alter ethylene sensitivity (Hua and Meyerowitz, 1998; Tieman et al., 2000). However, our results indicate that the total amount of LeETR4 protein was not greatly affected by ethylene or 1-MCP treatment in fruits (Figs. 2 and 3). Since the abundance of receptor proteins alone cannot account for altered ethylene sensitivity in 1-MCP-treated fruits, the shift in phosphorylation level must determine receptor functionality. In conclusion, receptors likely exist in several different phosphorylation states in vivo. The possibilities for quantitative (number of phosphates) and qualitative (position of phosphorylation) differences exist. The multiply phosphorylated isotypes are likely to actively suppress downstream ethylene responses while less-phosphorylated isotypes would be inactive (Fig. 7). Future work will be needed to characterize the precise impact of different phosphorylation levels on the activity of the receptor.

MATERIALS AND METHODS

Plant Materials and Treatments

Tomato (Solanum lycopersicum) plants were grown in a greenhouse or at the field at Live Oak, Florida. Unless otherwise noted, fruits of cv Flora-Dade were used for experiments. Antisense lines of LeETR4 and NR (Tieman et al., 2000), and a cosuppressed line of NR were grown in the same conditions as wild-type plants. Fruits were harvested at distinct development stages. Ripening stages were determined according to the definition by Grierson and Kader (1986). Treatments were performed by incubating fruits in sealed 20-L containers at 20°C. Ethylene was injected to final concentrations of 5 to 500 μL L⁻¹. A total of 2 μL L⁻¹ 1-MCP atmosphere was generated by adding 0.055 g 1-MCP powder (SmartFresh quality system, 0.14% active ingredient, AgroFresh, Inc.) to a flask containing 25 mL of distilled water in a container. For long-term experiments, MG fruits and turning fruits were treated with 2 μL L⁻¹ 1-MCP for 12 h as described above and then kept in air at 20°C until 9 d. For NBD treatment, a beaker containing a certain amount of NBD was put in a container to evaporate. Pericarp tissues
were frozen in liquid nitrogen and then stored at −80°C until use. At least three fruits were used in each experimental unit.

Preparation of Microsomal Proteins
Pericarp tissue was homogenized at 4°C using a polytron (IKA Labor-technik) in 2 volumes of homogenization buffer (100 mM Tris-HCl [pH 8.2], 300 mM NaCl, 20 mM EDTA, 5 mM dithiothreitol [DTT], and 20 mM [v/v] glycerol with complete protease inhibitor cocktail [Roche Diagnostics]), and centrifuged at 8000g for 15 min at 4°C. The supernatant was strained through Miracloth (Millipore), and centrifuged at 100,000g for 30 min at 4°C. The pellet was resuspended in homogenization buffer containing 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), and 0.1% SDS (w/v). The suspension was incubated on ice for 1 h with agitation and centrifuged at 100,000g for 5 min at 4°C. Proteins in the supernatant were recovered by a combination of methanol and chloroform as described previously (Wessel and Flügge, 1984). A protein concentration of EDTA (1 mM) was determined with a DC protein assay kit (Bio-Rad) using bovine serum albumin as a standard. Samples were then incubated in the presence of 100 mM DTT for 1 h at 37°C.

For phosphate treatment, microsomal proteins were prepared from 6 g (fresh weight) pericarp tissue using homogenization buffer containing a low concentration of EDTA (1 mM). After centrifugation at 100,000g for 30 min, the pellet was washed twice with 20 mM Tris-HCl (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride to remove EDTA. Microsomal proteins were solubilized in SDS sample buffer (125 mM Tris-HCl [pH 6.8], 2.5% [w/v] SDS, 25% [v/v] glycerol, and 0.002% [w/v] bromphenol blue). Protein concentrations were determined with a DC protein assay kit (Bio-Rad) using Bio-Rad protein assay standards. Three fruits were used in each experimental unit.

PAGE and Immunoblot Analysis
Microsomal proteins were separated on both conventional SDS-PAGE (Laemmli, 1970) and Phos-tag SDS-PAGE (Kinoshita et al., 2006). Unless otherwise noted, 40, 30, and 20 μg of proteins were used for analyses of IM, MG, and ripening stages, respectively. A 30% acrylamide solution (Bio-Rad) was used with complete protease inhibitor cocktail (Roche Diagnostics), and centrifuged at 100,000g for 30 min at 4°C. The pellet was washed twice with 20 mM Tris-HCl (pH 7.5) containing 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), and 0.1% SDS (w/v) with Complete Protease Inhibitor Cocktail as described above. A resultant supernatant was split into two halves. Samples were incubated for 30 min at 30°C in the presence or absence of 50 units of calf intestinal alkaline phosphatase (New England BioLabs).

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

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Quantitative Reverse Transcription PCR
Total RNA was prepared from fruit tissues using plant RNA purification reagent (Invitrogen). Possible genomic DNA contamination was removed by DNase treatment (Qiagen) and total RNA was purified using GeneJet plant RNA purification (Fermentas). Real-time PCR was performed using StepOnePlus real-time PCR system using Power SYBR Green RNA-to-Ct 1-step kit (Applied Biosystems). The following set of primers was used to amplify DNA fragments of LeETR4 (sense, 5′-CCATGGCAACTATGACGGCCG-3′; antisense, 5′-CCATGGCAATTGATGGCCG-3′). A standard curve was generated using the pENTR plasmid (Invitrogen) carrying LeETR4 fragment.
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