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Genetic dissection of the role of ethylene in regulating auxin-dependent lateral and adventitious root formation in tomato

Sangeeta Negi[1,†], Poornima Sukumar[1,†], Xing Liu[2], Jerry D. Cohen[2] and Gloria K. Muday[1,*]

[1]*Department of Biology, Wake Forest University, Room 226 Winston Hall, Box 7325, Winston-Salem, NC 27109, USA, and*
[2]*Department of Horticultural Science and Microbial and Plant Genomics Institute, University of Minnesota, Saint Paul, MN 55108, USA*

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*For correspondence (fax +336 758 6008; e-mail muday@wfu.edu).
†These two authors contributed equally to this work.

SUMMARY

In this study we investigated the role of ethylene in the formation of lateral and adventitious roots in tomato (*Solanum lycopersicum*) using mutants isolated for altered ethylene signaling and fruit ripening. Mutations that block ethylene responses and delay ripening – *Nr* (*Never ripe*), *gr* (*green ripe*), *nor* (*non ripening*), and *rin* (*ripening inhibitor*) – have enhanced lateral root formation. In contrast, the *epi* (*epinastic*) mutant, which has elevated ethylene and constitutive ethylene signaling in some tissues, or treatment with the ethylene precursor 1-aminocyclopropane carboxylic acid (ACC), reduces lateral root formation. Treatment with ACC inhibits the initiation and elongation of lateral roots, except in the *Nr* genotype. Root basipetal and acropetal indole-3-acetic acid (IAA) transport increase with ACC treatments or in the *epi* mutant, while in the *Nr* mutant there is less auxin transport than in the wild type and transport is insensitive to ACC. In contrast, the process of adventitious root formation shows the opposite response to ethylene, with ACC treatment and the *epi* mutation increasing adventitious root formation and the *Nr* mutation reducing the number of adventitious roots. In hypocotyls, ACC treatment negatively regulated IAA transport while the *Nr* mutant showed increased IAA transport in hypocotyls. Ethylene significantly reduces free IAA content in roots, but only subtly changes free IAA content in tomato hypocotyls. These results indicate a negative role for ethylene in lateral root formation and a positive role in adventitious root formation with modulation of auxin transport as a central point of ethylene–auxin crosstalk.

Keywords: ethylene, lateral roots, auxin transport, *Solanum lycopersicum*, tomato, adventitious roots.

INTRODUCTION

The development of lateral and adventitious roots is a highly plastic process which is sensitive to nutrients, moisture, and other environmental parameters, with plant hormones acting as one important signaling mechanism (Malamy and Ryan, 2001; Li *et al.*, 2009). Primary roots form in the embryo and emerge from seeds during germination. As roots mature, quiescent cells within their pericycle layer begin dividing and form lateral root primordia via a precise series of divisions, which are best characterized in Arabidopsis (Malamy and Benfey, 1997). Ultimately, the lateral root elongates and undergoes further reiterative branching. Additionally, when shoot tissues of many plant species contact the soil, they can undergo an intriguing, but poorly characterized, process by which shoot tissues differentiate to form adventitious roots. Plant propagation relies heavily on the ability of shoot cuttings to effectively generate adventitious roots, yet there is dramatic variation between species in their propensity to form adventitious roots (De Klerk *et al.*, 1999) and little molecular information on this important developmental process.

Auxin positively regulates both lateral and adventitious root formation in most plant species. Elevated endogenous or exogenous concentrations of auxin increase the formation of adventitious and lateral roots (Torrey, 1976; Sitbon *et al.*, 1992; Boerjan *et al.*, 1995), while reductions in auxin signaling or transport, due to either mutations or inhibitors, reduce both their initiation and elongation (Reed *et al.*, 1998; Casimiro *et al.*, 2001; Laskowski *et al.*, 2008). The role of

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auxin in lateral root development has been extensively studied in Arabidopsis (Malamy, 2009), but only a few papers have examined the mechanism for auxin's induction of adventitious root formation in this model species (Ludwig-Muller *et al.*, 2005; Sorin *et al.*, 2005, 2006; Li *et al.*, 2009). A limited number of studies have examined root development in tomato (*Solanum lycopersicum*) (Muday and Haworth, 1994; Clark *et al.*, 1999; Tyburski and Tretyn, 2004; Ivanchenko *et al.*, 2006). In tomato, auxin increases lateral (Muday and Haworth, 1994; Muday *et al.*, 1995; Ivanchenko *et al.*, 2006) and adventitious root growth (Clark *et al.*, 1999; Tyburski and Tretyn, 2004), while auxin-transport inhibitors reduce the formation of both root types (Muday and Haworth, 1994; Tyburski and Tretyn, 2004). In the auxin-insensitive *diageotropic* (*dgt*) mutant, lateral root development is completely inhibited (Muday *et al.*, 1995; Ivanchenko *et al.*, 2006). Together, these reports suggest that auxin and auxin transport may modulate root development in similar ways in both tomato and Arabidopsis.

Recent studies in Arabidopsis have also identified a role for the gaseous plant hormone ethylene in lateral root formation, utilizing the diversity of mutants with altered ethylene signaling or synthesis (Ivanchenko *et al.*, 2008; Negi *et al.*, 2008). The *ctr1* mutant, with enhanced ethylene signaling (Kieber *et al.*, 1993; Huang *et al.*, 2003), and the *eto1* mutant, with enhanced ethylene synthesis (Guzman and Ecker, 1990; Kieber *et al.*, 1993), exhibited significant reductions in lateral root numbers compared with wild type. Additionally, treatment with the ethylene precursor 1-aminocyclopropane carboxylic acid (ACC) reduced lateral root formation (Negi *et al.*, 2008). In contrast the ethylene-insensitive mutants *etr1*, which has a dominant negative receptor mutation (Hua *et al.*, 1998; Sakai *et al.*, 1998), and *ein2*, which has a defect in an ethylene signaling protein (Kendrick and Chang, 2008), showed enhanced lateral root formation which was insensitive to ACC treatment (Negi *et al.*, 2008). These effects of ethylene are on the earliest stages of lateral root initiation (Ivanchenko *et al.*, 2008) and alter auxin transport, suggesting that crosstalk with auxin is a critical component of the activity of ethylene in lateral root development (Negi *et al.*, 2008).

In tomato, genetic approaches have identified a number of mutants which have defects in ethylene signaling and fruit ripening (Klee, 2004; Barry and Giovannoni, 2007; Kendrick and Chang, 2008). The *Never-ripe* (*Nr*) gene was cloned using a candidate gene approach, as *NR* exhibits sequence similarity to the Arabidopsis *ETR1* gene (Wilkinson *et al.*, 1995; Yen *et al.*, 1995) and is part of the *LeETR1-6* gene family (Klee, 2004; Barry and Giovannoni, 2007; Kendrick and Chang, 2008). Another ethylene signaling mutant with a ripening phenotype is *green ripe (gr)*, a dominant gain-of-function mutant, which exerts its effect by ectopic expression of the *GR* gene, an ortholog of the Arabidopsis *RTE1* gene (Barry and Giovannoni, 2006; Resnick *et al.*, 2006; Kendrick and Chang, 2008). Additional tomato fruit ripening mutants include *ripening-inhibitor* (*rin*) and *non-ripening* (*nor*) (Barry and Giovannoni, 2006). The identity of the *NOR* gene has not yet been published, but it has been suggested to function upstream of the ethylene signaling pathway (Lincoln and Fischer, 1988; Yokotani *et al.*, 2004). The *RIN* locus encodes a MADS-box transcription factor (Vrebalov *et al.*, 2002) that regulates the expression of genes including *LeACS2*, which encodes ACC synthase (Ito *et al.*, 2008), suggesting that the *rin* mutation alters ethylene synthesis but may also affect other aspects of ripening. The *epi* (*epinastic*) mutant was isolated on the basis of severe leaf epinasty; it exhibits an enhanced triple response in the absence of ethylene and has elevated ethylene levels in some tissues (Fujino *et al.*, 1988; Barry *et al.*, 2001). Although the molecular defect in the *epi* mutant has not been reported it does not share a map position with the tomato *CTR1* ortholog (Barry *et al.*, 2001). *epi* does not demonstrate a global constitutive ethylene response, but shows altered phenotypes in a subset of ethylene-responsive tissues (Barry *et al.*, 2001).

The role of ethylene in the formation of adventitious roots has been examined in a variety of plant species, but the results have been contradictory (Geneve and Heuser, 1983; Robbins *et al.*, 1983). In tomato there have been reports of a positive effect of ethylene on adventitious root formation (Hitchcock and Zimmerman, 1940; Roy *et al.*, 1972; Phatak *et al.*, 1981) and of negative effects (Coleman *et al.*, 1980). These contradictory findings may be due to variation in the different tissues, growth conditions, and methods of quantifying adventitious root formation.

In this study we explored the role of ethylene in lateral and adventitious root formation in tomato. Few studies have employed a genetic approach to examine the role of these tomato ethylene signaling mutants in processes beyond fruit ripening and the triple response. Exceptions include evidence that the *Green ripe* mutant showed reduced ethylene sensitivity in root elongation (Barry and Giovannoni, 2006), while cuttings of mature stems of the *Nr* mutant grown in soil have altered adventitious root formation (Clark *et al.*, 1999; Kim *et al.*, 2008). The gene expression patterns of tomato ethylene receptors at distinct developmental stages have been reported (Lashbrook *et al.*, 1998; Tieman and Klee, 1999; Ciardi *et al.*, 2001), suggesting that ethylene signaling occurs in a diversity of tissues. We utilized a series of mutants with altered ethylene signaling and fruit ripening combined with treatments with the ethylene precursor, ACC, to raise ethylene levels. Lateral root formation is negatively regulated by ethylene, while adventitious root formation is positively regulated. We also examined the effect of ethylene on free indole-3-acetic acid (IAA) levels in these plants and examined the effect of ethylene on auxin transport in the primary root and hypocotyl. These results provide insight into the mechanistic basis of ethylene-regulated root formation and the crosstalk between auxin and ethylene in

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the control of adventitious and lateral root formation. This study broadens the current understanding of the genetic controls of ethylene signaling that regulate root architecture and extends our understanding of this process beyond Arabidopsis into a second agriculturally important species.

RESULTS

Ethylene-insensitive mutants exhibit enhanced lateral root formation

We examined mutants defective in ethylene signaling and fruit ripening to ask if ethylene modulates root formation in tomato. Root elongation and lateral root formation in the *Never ripe* mutant (*Nr*) in two different backgrounds was compared with the parental wild-type lines Pearson (P) and Ailsa Craig (AC). Seedlings were grown along the surface of agar media for 8 days after sowing (Figure 1a–c). Both *Nr* mutants formed more lateral roots than the appropriate wild type with statistically significant 1.4-fold enhanced numbers ($P < 0.005$). We quantified the root biomass of seedlings grown in soil for 15 days and *Nr* showed 2.6-fold enhanced root biomass compared with Pearson ($P < 0.0005$) (Figure 1e,f).

The root phenotype of other fruit-ripening mutants, *gr*, *nor*, and *rin*, are also shown in Figure 1(a). All three mutants exhibit a 1.4-fold statistically significant increase in number

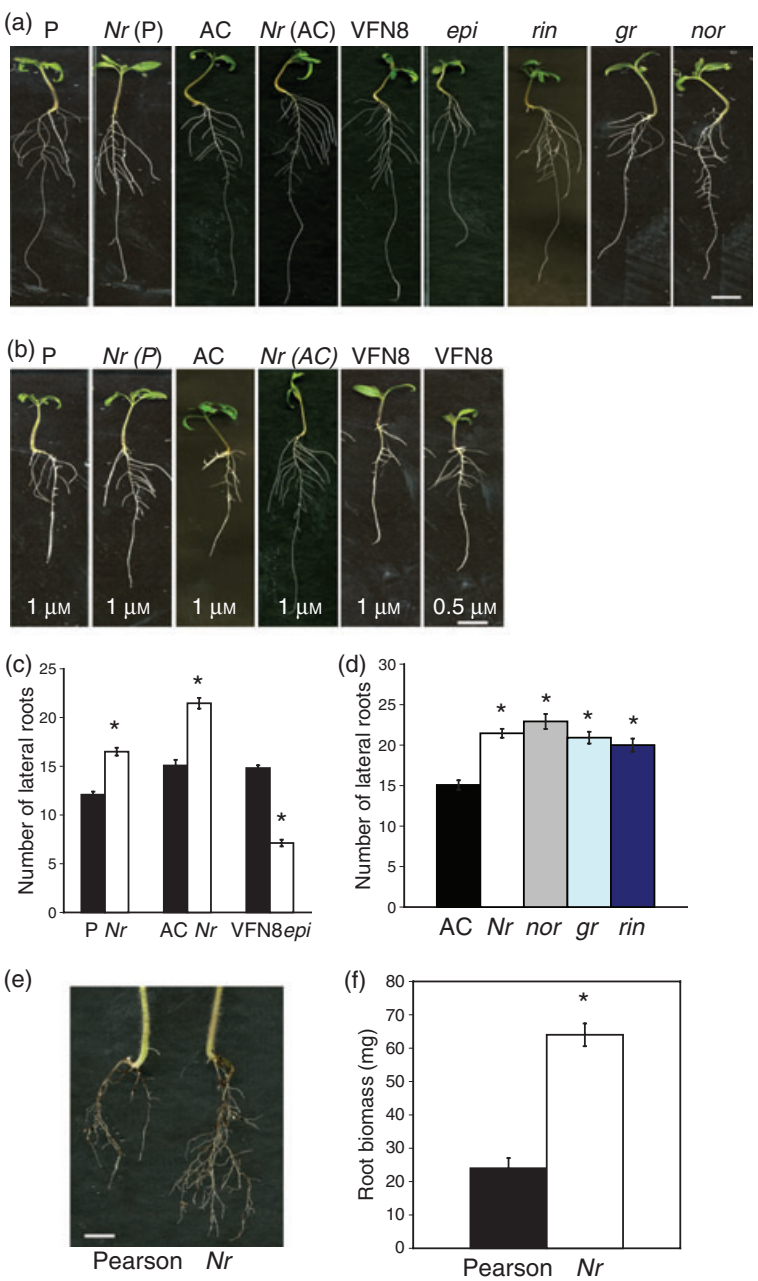

Figure 1. Lateral root formation in tomato (*Solanum lycopersicum*) is influenced by mutations that alter ethylene signaling and synthesis.
Roots were grown for 8 days on nutrient agar.
(a) Root phenotypes of seedlings on control or (b) 0.5 or 1 μM 1-aminocyclopropane carboxylic acid (ACC), as indicated.
(c) The average number of lateral roots and SE from 15 seedlings.
(d) The average number of emerged lateral roots in each genotype and SE of 15 seedlings.
(e) Seedlings grown for 15 days in soil. Size bar = 1 cm.
(f) The average and SE of biomass for 10 seedlings.
The asterisk (*) indicates a statistically significant differences between genotypes, determined by Student's *t*-test, with $P < 0.005$.

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of lateral roots (Figure 1d; $P < 0.005$). This enhanced root branching of these mutants is not tied to increases in hypocotyl or leaf growth, as is evident in Figure 1(a). The altered lateral root developmental patterns of *Nr*, *gr*, *rin*, and *nor* all suggest that these gene products function in roots or that they control the long distance transmission of signals, such as ethylene, to the roots. The known activities of *Nr*, *gr*, and *rin* in regulating ethylene signaling or synthesis, suggest a negative role of ethylene in lateral root formation.

Elevated ethylene levels inhibit lateral root formation

We examined root formation in the *epi* (epinastic) mutant, which has enhanced ethylene synthesis and signaling (Fujino *et al.*, 1988) and some ethylene-independent phenotypes (Barry *et al.*, 2001), and in wild-type and *Nr* seedlings treated with ACC, a precursor of ethylene (Figure 1a–c). The *epi* mutant showed a statistically significant twofold reduction in lateral root formation compared with its wild-type parental line VFN8 (Figure 1a,c). Treatment of wild-type roots with ACC phenocopies the *epi* mutant, resulting in reduced primary root elongation and a reduced number of emerged lateral roots (Figure 1b). This inhibitory effect of ACC on lateral root formation was lost in the *Nr* mutant, although root elongation is still reduced in *Nr* at higher doses of ACC (Figure 1b).

We quantified the effect of ACC on the number of lateral roots in the wild type and *Nr* (Figure 2a). Pearson demonstrated a dose-dependent decrease in lateral root formation with a twofold reduction in the number of roots at 10 μM ACC and this effect was lost in the *Nr* mutant. At the highest dose of ACC, there is a 2.5-fold difference in lateral roots between Pearson and *Nr*. In contrast, *Nr* was not resistant to the effect of ACC on primary root elongation (Figure 2b). We also examined the effect of ethylene gas on wild-type and *Nr* seedlings and found that at doses between 0.5 and 10 μl L^{-1} there was an inhibition of lateral root formation in Pearson and AC, but no inhibition in *Nr* (data not shown).

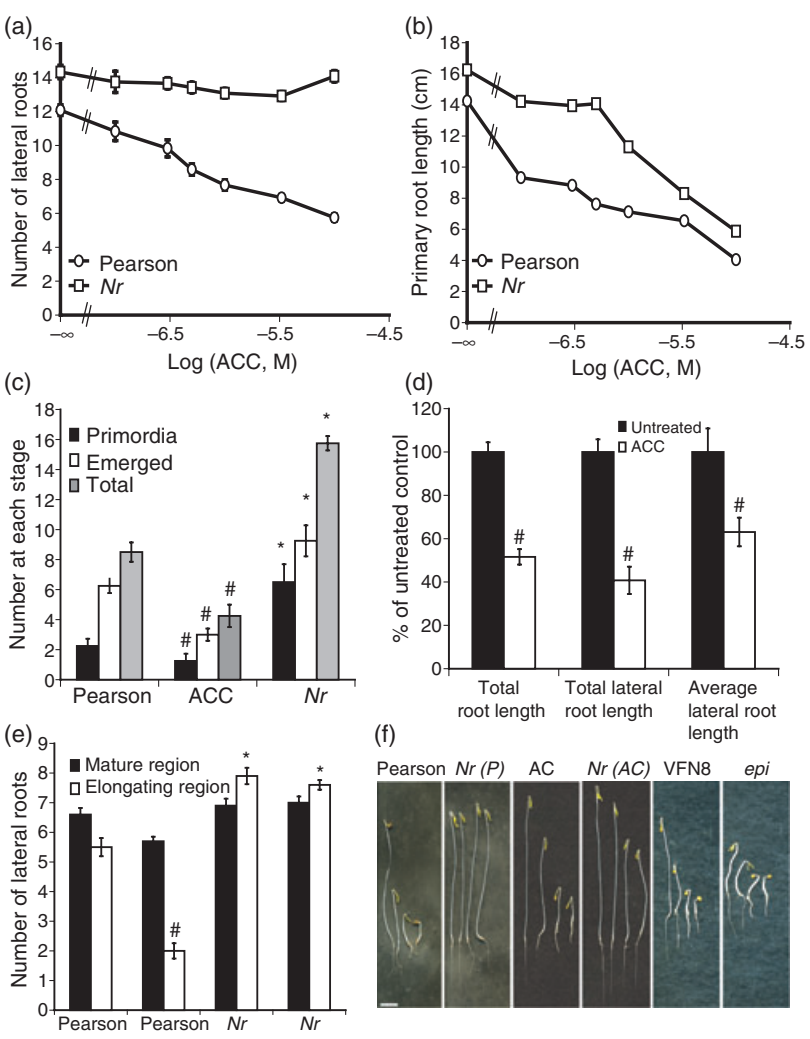

Figure 2. 1-Aminocyclopropane carboxylic acid (ACC) reduces root initiation in Pearson, but not in the *Nr* mutant.
(a) The effects of ACC on the number of lateral roots were determined, with the average and SE of 15 seedlings from three separate trials.
(b) The effect of ACC concentration on the elongation of the primary root, with the average and SE of 15 seedlings from three separate trials.
(c) The average number and SE of lateral root primordia, emerged lateral roots, and combined totals determined for 6-day-old cleared roots 1 day after transfer to control medium or medium containing 1 μM ACC. ($n = 10$ seedlings, from three trials).
(d) Lateral root elongation in Pearson seedlings treated with 1 μM ACC was quantified in several ways. The lengths of all lateral and primary root were summed (total root length), the lengths of only lateral roots were summed (total lateral root length), and the average of the root length reported for 7-day-old roots is shown. The average and SE for five seedlings are shown.
(e) The number of lateral roots that formed on primary root in the mature region elongating region is shown for seedlings 7 days after transfer to control or ACC-containing media. The average and SE of 10 seedlings, from three separate trials are reported.
(f) The triple response of two *Nr* alleles and the *epi* mutant are compared with the appropriate wild type grown in the dark on control medium or medium containing ACC for 4 days after radicle emergence. The seedlings were treated with 0, 1, 5, and 10 μM ACC from left to right.
The asterisk (*) indicates statistically significant differences between similarly treated Pearson and *Nr* as determined by a Student's *t*-test, with $P < 0.005$. The hash (#) indicates statistically significant differences between untreated and ACC treated seedlings within genotypes as determined by a Student's *t*-test, with $P < 0.05$.

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The *Nr* mutant in the AC background was slightly sensitive to the effect of ACC on lateral root formation. At 10 μM ACC only 80% of the untreated number of roots were formed (21 versus 26 lateral roots, for ACC-treated and control roots, respectively; $P < 0.0005$). This small effect of ACC in *Nr* in the AC background is consistent with its description in the literature as *Nr* exhibiting a weaker phenotype in the AC background (Lanahan *et al.*, 1994). We therefore compared the triple response in the *Nr* mutant in both backgrounds to define this difference (Figure 2f). At the highest dose *Nr* (Pearson) shows no apparent growth inhibition, while *Nr* (AC) shows partial responses. Together, these results are consistent with ethylene negatively regulating lateral root formation in tomato.

Ethylene inhibits lateral root initiation and elongation

We asked whether ethylene exerts its negative role at the stages of lateral root initiation and/or elongation. Wild-type and *Nr* roots treated with and without ACC were cleared to allow visualization and quantification of the early stages of root formation (Figure 2c). With ACC treatment there was a significant 1.4-fold reduction in the number of initiated and elongated lateral roots ($P < 0.05$). Cleared *Nr* mutant roots had significantly more primordia and emerged lateral roots than the wild type, with 2.6- and 1.4-fold increases, respectively. These results indicate that the most profound effects of ACC treatment are at the early stages of lateral root initiation.

To investigate the magnitude of the effect of ethylene on lateral root elongation, we calculated the total lateral root length in ACC-treated seedlings by summing the length of all the lateral roots in each treatment (Figure 2d). This parameter has been called a 'tot value' and has been used previously to quantify overall lateral root formation (Macgregor *et al.*, 2008). The average lengths of lateral roots in ACC-treated and control seedlings are shown in Figure 2(d). Treatment with ACC significantly reduced both the number of lateral roots and the elongation of lateral roots in total and on average for each lateral root. The effect on lateral roots is more profound than on primary roots.

In Arabidopsis, ACC affects lateral root formation in a position-specific manner with decreases in lateral root formation evident only on the primary root formed after transfer to ACC-containing media (Ivanchenko *et al.*, 2008; Negi *et al.*, 2008). We asked if the ACC effect was also position specific in tomato by examining the effect of ACC on the mature region (formed before transfer to ACC) and in the elongating region (formed after transfer to ACC-containing medium). In Pearson, the number of lateral roots was significantly reduced in both regions, but with a greater threefold reduction in the elongating region. In *Nr*, the ACC effect was lost and there were significantly more lateral roots in the elongating region (Figure 2e). These results are consistent with similar developmental sensitivity to ACC in Arabidopsis and tomato.

Ethylene positively regulates adventitious root formation

Although the negative effect of ACC treatment on lateral root formation in tomato parallels the effect seen previously in Arabidopsis (Ivanchenko *et al.*, 2008; Negi *et al.*, 2008), the inhibitory effect of ethylene contrasts with previous reports on adventitious root formation from hypocotyl tissues in tomato (Clark *et al.*, 1999; Kim *et al.*, 2008), which was examined in mature plants grown in soil. We therefore asked whether adventitious roots in tomato showed a similar ethylene response to the lateral roots examined in young seedlings grown under similar conditions. After germination, seeds were grown in low light (5–10 μmol m^{-2} sec^{-1}) for 3 days to elongate the hypocotyl and then transferred to high light (100 μmol m^{-2} sec^{-1}) for 7 days to observe adventitious root formation (Figure 3a,b). The *Nr* mutant showed a statistically significant 40% reduction in the number of adventitious roots ($P < 0.005$), consistent with two previous reports that examined older tissues grown under very different conditions (Clark *et al.*, 1999; Kim *et al.*, 2008). The *epi* mutant showed a statistically significant 1.8-fold increase in adventitious root formation ($P < 0.005$). Pearson had greater numbers of adventitious roots than *Nr* at all ACC doses (Figure 3c; $P < 0.005$ at 1 and 10 μM). Treatment of the wild type with ACC enhanced adventitious root formation in a dose-dependent manner, with significant 1.4- and 1.8-fold increases at 1 and 10 μM ACC, respectively, while *Nr* was insensitive to this effect. This suggests an opposite role for ethylene in the regulation of the formation of lateral and adventitious roots.

Ethylene positively regulates auxin transport in tomato roots

We asked whether ethylene might alter root formation through the modulation of auxin transport. Acropetal IAA transport was measured in Pearson and *Nr* tomato roots in the presence and absence of ACC (Figure 4a). Acropetal transport was quantified by the level of tritiated IAA moving from the site of application at the root–shoot junction to the root tip and is significantly reduced in *Nr* ($P < 0.05$). In contrast, ACC treatment resulted in a twofold enhancement in the number of acropetal IAA transport in Pearson, but not in *Nr*. The *epi* mutant exhibited a 2.5-fold increase in acropetal auxin transport relative to its wild type (Figure 4b). Treatment with ACC also significantly enhanced the acropetal transport of auxin in wild-type seedlings by twofold (Figure 4a; $P < 0.005$). Similarly, ACC treatment and the *epi* mutation significantly increase basipetal auxin transport by 2- and 3.5-fold, respectively (Figure 4b,c; $P < 0.0005$). The *Nr* mutant showed less basipetal IAA transport than Pearson and the ACC effect was lost in *Nr* (Figure 4b). These results indicate that eth-

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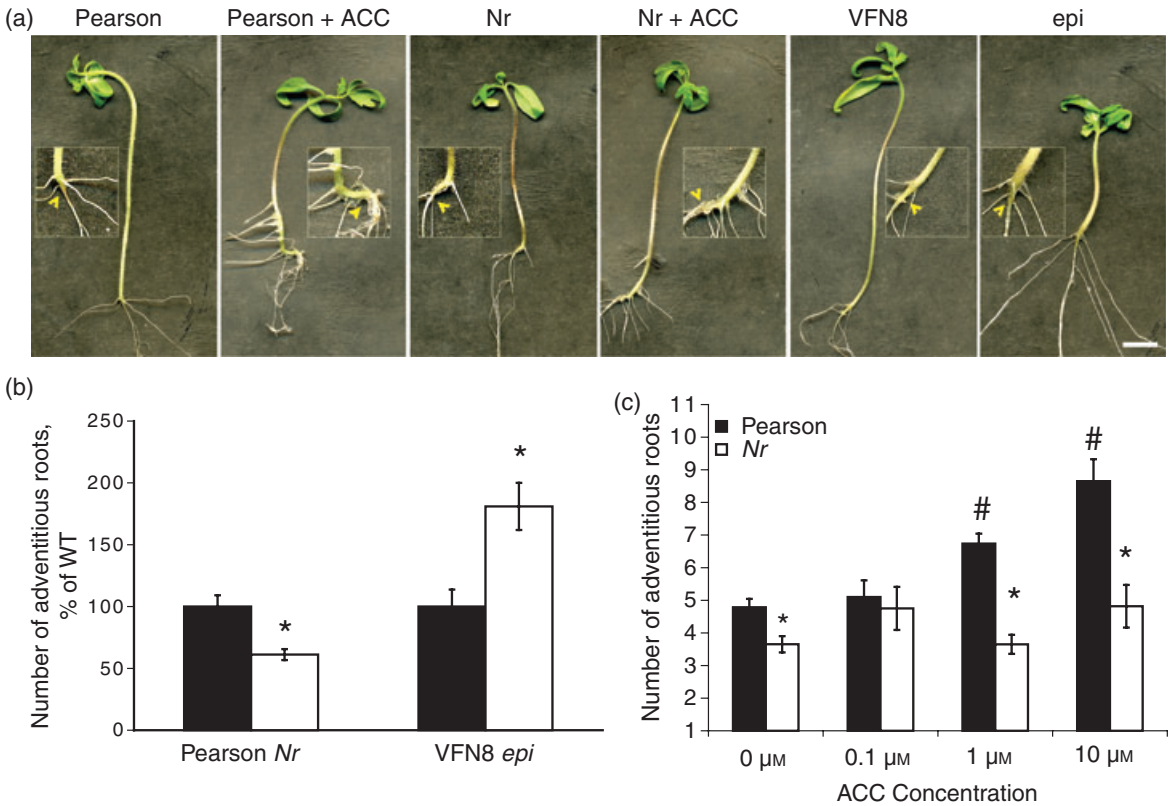

Figure 3. Ethylene enhances adventitious root formation in tomato (*Solanum lycopersicum*) hypocotyls.
The number of adventitious roots formed 7 days after transfer of seedlings to control plates or plates with 1-aminocyclopropane carboxylic acid (ACC). The average and SE for adventitious roots is shown.
(a) Adventitious root formation in control and 10 μM ACC-treated Pearson and *Nr*. The insert to this figure shows a magnified image of the root shoot junction to allow differentiation of adventitious roots arising from the hypocotyl, from lateral roots emerging from the root. Size bar = 1 cm.
(b) The number of adventitious root was quantified in the wild type (WT) and mutants grown on control media: n = 8–11, from two separate trials for Pearson and *Nr* and n = 22–25, from three separate trials for VFN8 and *epi*.
(c) The effects of a range of ACC concentrations on the number of adventitious roots with the average and SE of 19–28 seedlings, from four separate trials.
The asterisk (*) indicates statistically significant differences between genotypes determined by Student's *t*-test ($P < 0.005$). The hash (#) indicates statistically significant differences within genotype in response to ACC treatment determined by Student's *t*-test ($P < 0.005$).

ylene has a stimulatory effect on both acropetal and basipetal IAA transport in tomato roots.

Ethylene alters auxin transport in hypocotyls

We also examined the effect of ethylene on auxin transport in tomato hypocotyls. We applied 10 μl agar droplets containing [^{3}H]IAA at the shoot apical end, after removal of the shoot apex. Five hours later, 5-mm sections were excised from each hypocotyl at a distance of 2–2.5 cm from the point of application, and the amount of tritiated IAA was quantified. *Nr* showed enhanced IAA transport in hypocotyls, with a significant increase compared with Pearson (Figure 5a; $P < 0.005$). In contrast, auxin transport was reduced in tomato hypocotyls treated with ACC, while treatment with silver nitrate ($AgNO_3$), which blocks ethylene signaling, increased auxin transport (Figure 5b). This suggests that ethylene negatively regulates auxin transport in hypocotyls in contrast to root tissues, where the capacity to transport auxin is increased. Surprisingly, the *epi* mutant also showed an increase in hypocotyl IAA transport with a 2.7-fold significant increase in transport (Figure 5b; ($P < 0.005$).

Ethylene alters the free IAA content in tomato roots and hypocotyls

We further investigated the effect of ethylene on free auxin content in roots. The free IAA levels in seedlings on control medium or medium containing 1 μM ACC for 48 h and root tissues were quantified (Figure 6a,b). Free IAA was extracted and measured using a gas chromatograph–mass spectrometer operated in the selected ion monitoring mode (GC-SIM-MS) by isotope dilution analysis, using [$^{13}C_6$]IAA as the internal standard (Barkawi *et al.*, 2008). In *Nr*, free IAA levels were higher than in Pearson, and consistent with these results ACC-treated Pearson roots showed a significantly lower concentration ($P < 0.05$) of free IAA than untreated

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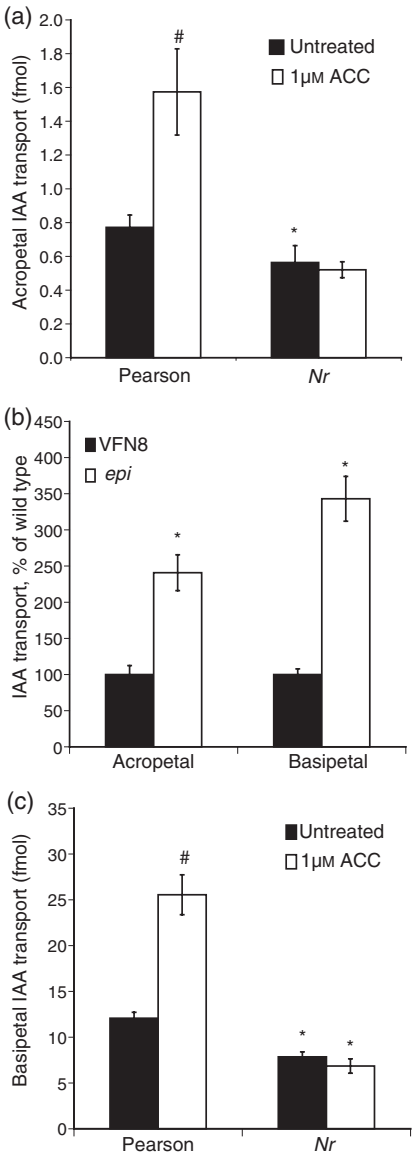

Figure 4. In tomato (*Solanum lycopersicum*) roots acropetal and basipetal indole-3-acetic acid (IAA) transport are positively regulated by ethylene.
The IAA transport was measured 2 days after seedlings were transferred to control medium or medium containing 1 μM 1-aminocyclopropane carboxylic acid (ACC). The average and SE of 15 seedlings from three separate experiments are reported in all panels.
(a) Acropetal IAA transport.
(b) Basipetal IAA transport.
(c) Acropetal and basipetal transport in VFN8 and *epi* as a percentage of wild type.
The hash (#) indicates statistically significant differences between untreated and ACC treated seedlings as determined by Student's *t*-test ($P < 0.05$). The asterisk (*) indicates a statistically significant difference between genotypes as determined by Student's *t*-test ($P < 0.05$).

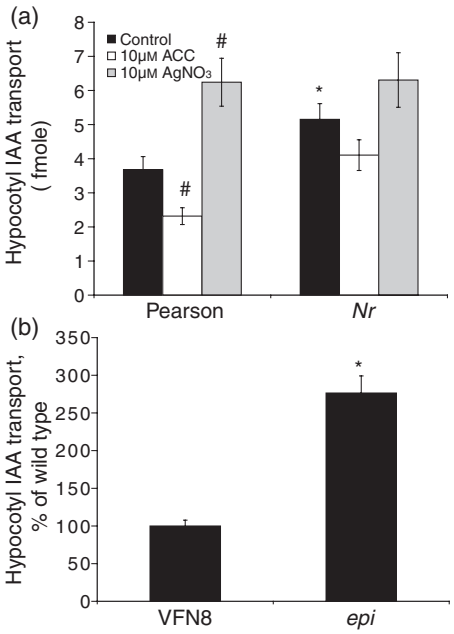

Figure 5. Ethylene alters basipetal auxin transport in tomato (*Solanum lycopersicum*) hypocotyls. The average ± SE is reported.
(a) Basipetal indole-3-acetic acid (IAA) transport in Pearson and *Nr* with and without 1-aminocyclopropane carboxylic acid (ACC) and $AgNO_3$ treatment is compared for 8–14 samples from three trials.
(b) Basipetal IAA transport in VFN8 and *epi* are compared in 7–18 samples from three trials.
The asterisk (*) indicates a statistically significant difference between untreated genotypes as determined by Student's *t*-test ($P < 0.005$). The hash (#) indicates a statistically significant difference in Pearson in response to ACC and $AgNO^3$ treatment as determined by Student's *t*-test ($P < 0.005$).

roots (Figure 6a,b). The effect of ACC is similar in all backgrounds (Pearson, AC, and VFN8) with a statistically significant 1.5-fold decrease in free IAA, as shown in Figure 6(a) ($P < 0.05$).

To test if ethylene regulates the accumulation of free IAA in hypocotyls, we measured free IAA levels in *Nr* and *epi*, as well as in the wild type treated with ACC. Low-light-grown seedlings were transferred to high-light conditions for 48 h and hypocotyl tissues were harvested and immediately frozen. Free IAA was extracted and quantified using GC-SIM-MS. *Nr* showed a slight, but not significant (20%), reduction in free IAA (Figure 7a). *epi* also showed a 40% reduction in free IAA levels compared with the wild type, but this reduction was also not significant. Additionally, we quantified free IAA levels in wild-type hypocotyls treated with 1 and 10 μM of ACC for 48 h (Figure 7b). Surprisingly, no changes in free IAA levels were observed in treated hypocotyls. This may be attributed to the lack of an effect of ACC on auxin accumulation. Alternatively, the treatment with ACC might not have been of sufficient duration to elicit a response or any response may have been transient, although similar treatments were found to alter IAA transport. Yet, the global effects of ACC treatment on free IAA in hypocotyls are minimal, suggesting that ethylene only subtly changes free IAA but has a more profound effect on IAA transport.

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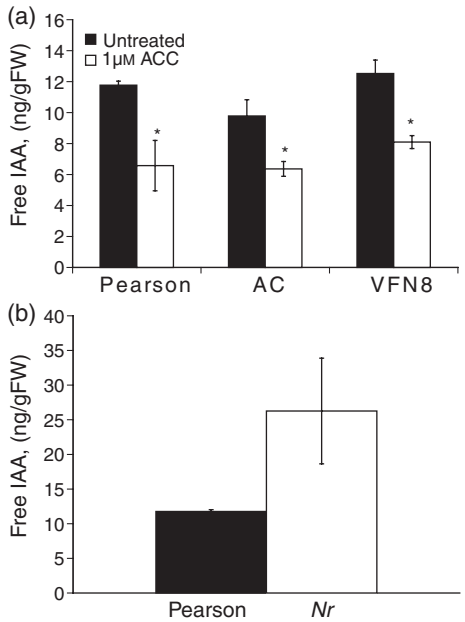

Figure 6. In tomato (*Solanum lycopersicum*) roots the free indole-3-acetic acid (IAA) content is reduced 2 days after treatment with 1 μM 1-aminocyclopropane carboxylic acid (ACC) treatment.
(a) Free IAA content in three different genotypes quantified after treatment with 1 μM ACC.
(b) Free IAA content in Pearson and the *Nr* mutant. The average and SE of three replicates are shown.
The asterisk (*) indicates a statistically significant difference relative to untreated wild-type as determined by Student's *t*-test ($P < 0.05$).

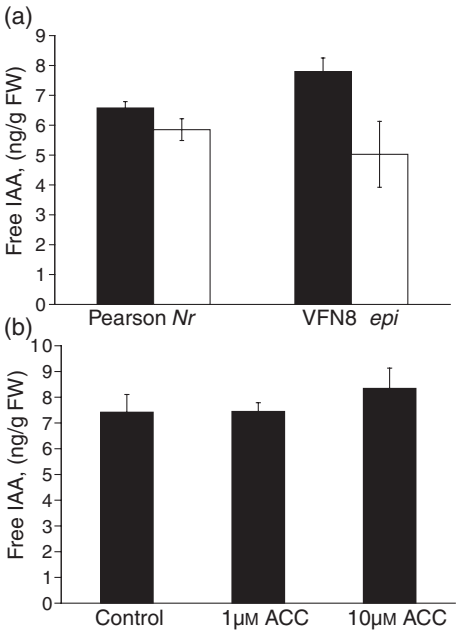

Figure 7. Effect of ethylene on the free indole-3-acetic acid (IAA) content in tomato (*Solanum lycopersicum*) hypocotyls.
(a) Free IAA in the ethylene mutants *Nr* and *epi* are compared with the respective wild types.
(b) Free IAA in tomato hypocotyls treated with 1-aminocyclopropane carboxylic acid (ACC) for 48 h. In both panels the average and SE of three replicates is reported.

Nr is insensitive to IAA-induced lateral root formation but exhibits a reduced response in adventitious root formation

We examined the role of auxin during lateral and adventitious root formation and asked whether there is crosstalk between auxin and ethylene signaling pathways. Pearson and *Nr* seedlings were grown on control medium for 1 day and then transferred to plates containing control medium or medium containing IAA. After 7 days the number of lateral roots was quantified. Pearson roots showed significant induction in lateral root formation ($P < 0.05$) with a 1.4-fold increase in lateral root number with 10 μM IAA; *Nr* remained insensitive to this response (Figure 8a). These results contrast with Arabidopsis, in which ethylene-insensitive mutants showed a similar induction to the wild type in lateral root number when treated with IAA (Negi *et al.*, 2008). These results suggest species-specific crosstalk between auxin and ethylene in the regulation of lateral root formation.

To examine the effect of IAA on adventitious root formation, low-light-grown Pearson and *Nr* seedlings were transferred to control agar or agar containing IAA, and seedlings were placed under high-light conditions with a yellow filter to prevent light-induced auxin degradation. Adventitious roots formed 7 days later and were quantified (Figure 8). The formation of adventitious roots in Pearson hypocotyls was enhanced by IAA in a dose-dependent manner, with a 1.7-fold induction at 10 μM IAA. These adventitious roots emerged from the lower half of the hypocotyls as well as at the root–shoot junction, similar to the pattern observed with ACC treatment. *Nr* showed similar induction to Pearson when treated with IAA. This suggests that both ethylene and auxin positively regulate adventitious root formation with auxin sensitivity not requiring ethylene signaling.

DISCUSSION

We examined the role of ethylene in the modulation of lateral root formation and adventitious root formation in tomato, utilizing an array of mutants with defects in ethylene signaling and fruit ripening, including *Never ripe* (*Nr*), *green ripe* (*gr*), *ripening inhibitor* (*rin*), *non ripening* (*nor*), and *epinastic* (*epi*). *Nr* in both Pearson and AC backgrounds formed significantly more lateral roots when grown on agar medium, as did *gr*, *rin*, and *nor*. Additionally, quantification of the root biomass of 15-day-old soil-grown seedlings of both Pearson and *Nr* showed that *Nr* has almost threefold more root biomass than Pearson. This observation suggests that enhanced root formation in *Nr* continues beyond the seedling stage and may be amplified when roots are grown in soil, which limits ethylene diffusion more than growth

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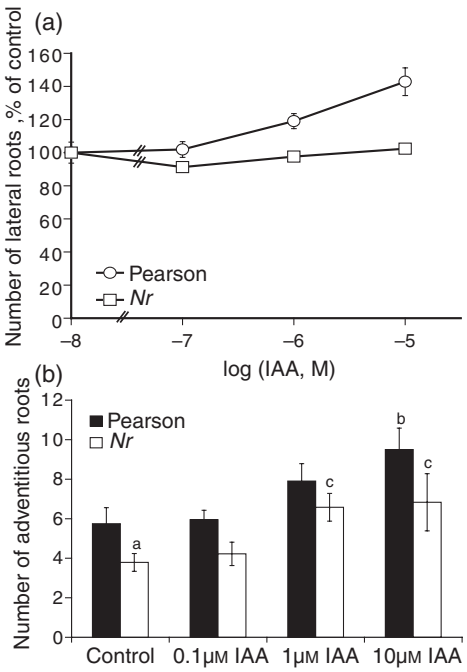

Figure 8. *Nr* has altered responses to auxin in both lateral and adventitious root formation.
(a) The effects of a range of indole-3-acetic acid (IAA) concentrations on the number of lateral roots. The average and SE of 15 seedlings were normalized to the untreated controls for each genotype (with Pearson having 13 and *Nr* having 16 adventitious roots).
(b) The effects of a range of IAA concentrations on the number of adventitious roots, with the average and SE of 6–21 seedlings being reported here. Significant differences were determined by Student's *t*-test, with significant differences between: a, untreated genotypes; b, with IAA treatment in Pearson; and c, with IAA treatment in *Nr* ($P < 0.05$).

along the surface of unsealed Petri dishes. Consistent with a negative role for ethylene in lateral root formation, treatment of the wild type with the ethylene precursor ACC reduces the number of initiated and elongated lateral roots; the *epi* mutant, which has been reported to have elevated ethylene levels (Fujino *et al.*, 1988; Barry *et al.*, 2001), also has reduced lateral root formation. The *Nr* mutant, however, was insensitive to the inhibition of lateral root formation by ACC. These experiments confirm the negative role of ethylene during lateral root formation in young tomato seedlings.

In contrast, adventitious root formation exhibited an opposite ethylene dependence. *Nr* had a reduced number of adventitious roots, while the ACC-treated wild type and the *epi* mutant had an enhanced number of adventitious roots. Treatment with ACC resulted in expansion of the zone of adventitious root formation, from proliferation only at the base of the hypocotyl, to a region extending 1–2 cm along the basal part of the hypocotyl. Examination of adventitious root formation on vegetative stem cuttings of Pearson and *Nr* found similar differences, with *Nr* forming reduced numbers compared with the wild type (Clark *et al.*, 1999). Moreover, *Nr* has been reported to have a reduced number of adventitious roots in intact plants grown under low phosphorus conditions, as compared with Pearson (Kim *et al.*, 2008). This positive effect of ethylene on adventitious roots has also been observed in other plant species, such as in intact plants of *Rumex palustris* under conditions of flooding or ethylene application (Visser *et al.*, 1996). These results suggest a differential role of ethylene in root formation with negative regulation during lateral root formation and positive regulation during adventitious root formation.

Ethylene–auxin crosstalk can occur at many different levels, including the modulation of auxin sensitivity, accumulation, and transport. We tested whether ethylene regulates auxin sensitivity during lateral and adventitious root formation in tomato by obtaining dose-response curves with exogenous IAA and quantifying differences in the *Nr* mutant. Auxin-induced promotion of lateral root formation is lost in *Nr*. Induction of lateral roots by IAA in the wild type was dependent upon the developmental stage at which treatment occurred, with more induction seen if longer primary roots were present at the time of transfer to a medium containing auxin, and no stimulation in numbers of lateral roots formed when seedlings are transferred to IAA immediately after germination (Muday and Haworth, 1994). In parallel, adventitious roots were induced by auxin treatment in both the wild type and *Nr*, but with *Nr* never reaching the wild-type number of adventitious roots even at 10 μM IAA, consistent with a previous report indicated that *Nr* is less responsive to the auxin indole-3-butyric acid (Clark *et al.*, 1999). Thus, even though auxin may affect lateral and adventitious root formation, the ethylene insensitivity alters the response to IAA differentially in hypocotyls and roots.

Additionally, we tested whether auxin transport is modulated via ethylene. Previous reports have found that ethylene inhibits polar auxin transport in shoot tissues (Morgan and Gausman, 1966; Suttle, 1988) and *Medicago* roots during nodulation (Prayitno *et al.*, 2006). Basipetal auxin transport was measured in ethylene-insensitive tomato mutants in stem tissues under conditions when adventitious root formation occurs. Treatment with ACC decreased the movement of IAA, consistent with previous reports. In contrast IAA transport was increased in *Nr* hypocotyls or wild-type hypocotyls treated with silver nitrate, an ethylene signaling antagonist, in comparison with the untreated wild type. Furthermore, *Nr* mutants were insensitive to both ACC and silver nitrate treatments. These two findings support a negative role for ethylene in the regulation of hypocotyl auxin transport.

Surprisingly, the *epi* mutant was found to have increased basipetal hypocotyl IAA transport similar to *Nr* and opposite to seedlings treated with ACC. As the *epi* mutation has yet to be cloned, the relationship between this mutation and ethylene signaling remains unclear. Studies that have examined ethylene synthesis have found that hypocotyls

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of *epi* had similar level of ethylene as the wild type even though the total ethylene content was higher in *epi* (Fujino *et al.*, 1988). Additionally, *epi* does not have the characteristics of an altered ethylene response mutant in all tissues (Barry *et al.*, 2001). Although vegetative growth is altered, fruit ripening and senescence in *epi* are similar to wild type. Therefore we still have no explanation for this contradiction between elevated ethylene and the *epi* phenotype.

In contrast to stem auxin transport, IAA transport was found to be decreased in *Nr* roots, while ACC treatment and the *epi* mutation increased root IAA transport. This suggests that ethylene has contrasting roles in roots and hypocotyls, with positive regulation by ethylene in root and negative modulation of auxin transport in stem tissue. We initially hypothesized that ACC might reduce root formation by negatively regulating IAA transport. This positive effect of ACC treatment on root auxin transport contrasted with this model, but confirms what we found in Arabidopsis (Negi *et al.*, 2008). These combined results in Arabidopsis and tomato lead us to speculate that enhanced long-distance polar IAA transport perhaps prevents localized accumulation of the auxin needed to drive lateral root formation.

We examined the effect of ACC treatment and mutants on free IAA levels in both roots and hypocotyls. Several reports have indicated that elevated ethylene levels enhance the accumulation and synthesis of IAA in Arabidopsis root tips (Ruzicka *et al.*, 2007; Stepanova *et al.*, 2007; Swarup *et al.*, 2007), so we predicted that free IAA might be elevated in the ACC-treated roots. In contrast to our expectation, significant reductions in free IAA levels were observed in root tissue in the presence of ACC, while in roots of the *Nr* mutant, there were increased levels of free IAA. This suggests that ethylene may negatively regulate free IAA levels in root tissue when the whole tissue is examined, rather than just the tip of Arabidopsis roots (Ruzicka *et al.*, 2007). Additionally, Swarup *et al.* (2007) found threefold increases in the rate of IAA synthesis in intact seedlings treated with 100 μM ACC by specifically measuring the rate of IAA synthesis using D_2O feeding studies. The doses used in that study were 100-fold higher than we used, and at doses that would completely block elongation. Therefore the lack of overall increase we see in free IAA levels, which is a combination of IAA synthesis, conjugation, and transport in whole root tissues, could be explained by differences in ACC dose, tissue segments, or growth conditions. We find no evidence for global changes in free IAA levels in stem tissue in *Nr* or *epi*, or in the wild type treated with ACC. As these experiments were performed with whole hypocotyls, there may be local changes in free IAA levels that were not detectable in this assay. Yet these findings are consistent with more profound effects of ethylene on auxin transport than free auxin levels in hypocotyls.

It is surprising that ACC treatment enhances long-distance polar transport within the root but decreases free IAA levels. If more auxin is being transported from the shoot into the root, then we would predict that there should be higher levels of free IAA. Our transport assays do not directly measure shoot to root movement of IAA, since we apply IAA below the root–shoot junction. To find out if there are differences in IAA transport from the shoot apex into the root, we applied [^{3}H]IAA at the top of hypocotyls and measured radioactivity along the hypocotyl of wild-type seedlings (data not shown). This assay found that the highest level of IAA was in the region directly above the root–shoot junction and that less IAA moved into the root. Therefore, there may be complex regulation of auxin flow as it crosses from the shoot into the root. Additionally, free levels may change as a result of changes in transport, but also synthesis and conjugation; therefore we cannot rule out ACC-dependent regulation of free IAA in roots that are transport independent.

An important general question is why it might be advantageous to a plant to enhance adventitious root formation and inhibit lateral root formation with rising ethylene levels. This might be a compensatory mechanism in plants where a lack of underground root growth is balanced by an increases in adventitious roots emerging from the stem of the plants. This has been seen under conditions of submergence in other plant species, wherein the development of lateral roots is diminished but at the same time there is an increase in the formation of adventitious roots (Visser *et al.*, 1996). Moreover, through the manipulation of auxin transport or synthesis, this balance between lateral and adventitious root formation has been shown to be regulated by ethylene (Visser *et al.*, 1996; Grichko and Glick, 2001).

In conclusion, we find that ethylene–auxin crosstalk drives root formation in tomato with tissue-specific mechanisms during lateral and adventitious root formation. The negative effect of ethylene on lateral root formation supports the previously reported effects on lateral root formation in Arabidopsis. Additionally, we expanded these studies and looked at the effect of ethylene on adventitious root formation; we found a positive influence of ethylene on adventitious root formation. This ethylene–auxin crosstalk includes negative regulation of free auxin accumulation, positive regulation of auxin transport in roots, and negative regulation of auxin transport in shoots. These differences in regulation give a better understanding of the complex pathways of ethylene–auxin crosstalk that regulate lateral and adventitious root development.

EXPERIMENTAL PROCEDURES

Chemicals

Triton X-100 was purchased from Fisher Scientific (http://www.fishersci.com/). Murashige and Skoog (MS) salts were purchased from Caisson Labs (http://www.caissonlabs.com/). The [5-^{3}H]IAA (specific activity, 23 Ci mmol^{-1}) was purchased from GE Healthcare Life Sciences (http://www6.gelifesciences.com).

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All other chemicals were acquired from Sigma (http://www.sigmaaldrich.com/).

Plant material and growth conditions

The *Nr* mutant in the Pearson background was provided by Harry Klee (University of Florida, Gainesville, FL) (Wilkinson *et al.*, 1995); *Nr*, *gr*, *rin*, *nor* (all in the AC background) and *epi* (in the VFN8 background) mutant seeds were provided by Jim Giovannoni (Boyce Thompson Institute, Ithaca, NY) (Barry *et al.*, 2001). All seeds were sterilized by incubation for 5 min in 95% ethanol, then for 30 min in freshly prepared 20% (v/v) bleach plus 0.01% (v/v) Triton X-100, and then washed with sterile water. The sterilized seeds were sown on sterilized blue filter papers and after emergence of the radicle they were transferred to control plates: 0.8% (w/v) Type M agar (A-4800; Sigma), MS nutrients (macro and micro salts, MSP0501; Caisson Labs) (Murashige and Skoog, 1962), vitamins (1 μg ml^{-1} thiamine, 1 μg ml^{-1} pyridoxine HCl, and 0.5 μg ml^{-1} nicotinic acid), 0.05% (w/v) 2-(*N*-morpholine)-ethanesulfonic acid (MES), with pH adjusted to 5.8. Seedlings were grown under 24-h fluorescent lights at 100 μmol m^{-2} sec^{-1} at 23°C or as noted. For experiments with IAA, seedlings were grown under yellow filters to prevent degradation of the IAA (Stasinopoulos and Hangarter, 1989). For quantification of root biomass, seedlings were grown in Metro-Mix 200 (Sun Gro Horticulture, http://www.sungro.com/) for 15 days under constant light at 40–50 μmol m^{-2} sec^{-1}.

Quantification of lateral and adventitious roots

Seeds were germinated, and after radicle emergence they were transferred to control agar plates or plated with agar containing the indicated amounts of ACC or IAA. The emerged lateral roots along the primary root that were longer than 1 mm were counted after seven additional days of growth using a dissecting scope.

For the quantification of adventitious roots, radicle-emerged seedlings were transferred to agar media in Petri dishes, and were placed vertically under 5–10 μmol m^{-2} sec^{-1}. After 3 days of growth they were transferred to 100 μmol m^{-2} sec^{-1}, either on control agar plates or plates with agar having different concentrations of IAA or ACC. A slab of control agar approximately 1 cm wide was placed across the hypocotyls, to keep the hypocotyls from growing away from the agar medium. Adventitious roots emerging from hypocotyl and root shoot junction were counted 7 days later.

Detection of lateral root initiation events

Roots from 5-day-old seedlings grown on either control medium or treatments were cut and fixed in ethanol:acetic acid [6:1(v/v) overnight]. Fixed roots were washed in 100% ethanol followed by washing in 70% ethanol, These roots were cleared in a mixture of chloral hydrate:glycerol:water [8:1:2(w/v)] overnight (Al-Hammadi *et al.*, 2003) and after clearing these roots were observed under a dissecting microscope for quantification of lateral root primordia.

Auxin transport assays

Seeds were germinated, and after radicle emergence were transferred to control or 1 μM ACC plates. After 48 h, a 100-nM [^{3}H]IAA agar cylinder was applied just below the aligned root–shoot junctions and the seedlings were incubated in the dark (in the inverted position to prevent [^{3}H]IAA from diffusing along the root) for 18 h. The apical 5 mm of each root tip was excised and the amount of radioactivity quantified. Individual segments from each plant and position were placed in 2.5 ml of scintillation liquid (Scintiverse® BD Cocktail; Fisher Scientific, http://www.fishersci.com) and radioactivity was measured for 2 min on a Beckman scintillation counter (model LS 6500; Beckman, http://www.beckman.com/). Measurement of radioactive basipetal auxin transport was performed using the method illustrated by Lewis and Muday (2009) using same-age seedlings as described above treated with a 100-nM [^{3}H]IAA agar cylinder applied adjacent to the root tip, the seedlings being incubated in the dark. After 5 h the apical 2 mm of root was excised and discarded and a 5-mm segment basal to that was quantified for radioactive IAA.

Quantification of basipetal hypocotyl transport

Seedlings were grown under low light (5–10 μmol m^{-2} sec^{-1}) for 3 days and were transferred to high-light conditions (100 μmol m^{-2} sec^{-1}) on control or treatment plates for 48 h. The transport was measured by the application of 10 mm wide agar droplets with 100 nM [^{3}H]IAA at the shoot apical end after removal of cotyledons. After 5 h of incubation in the dark, 5 mm sections at a distance of 2.5 cm from the shoot apical end were removed and radioactivity was determined using a scintillation counter.

Free IAA measurements

For root samples, seeds were germinated and after radicle emergence transferred to control or 1 μM ACC plates for 48 h. Roots were excised and frozen in liquid nitrogen. For hypocotyl tissues, samples were grown in low light for 3 days and transferred to high-light conditions onto media with and without 1 or 10 μM ACC for 48 h; hypocotyls were collected and frozen. For both types of sample 50–80 mg of frozen tissue was homogenized with a bead beater in 150 μl of homogenization buffer (35% of 0.2 M imidazole, 65% isopropanol, pH 7), containing 4 ng of [$^{13}C_6$]IAA as an internal standard. After 1 h on ice, samples were subjected to centrifugation at 10 000 ***g*** for 8 min. The homogenates were purified over two successive columns using an automated robotic system, methylated, dried, and redissolved in ethyl acetate (Barkawi *et al.*, 2008). The samples were then analyzed using GC-SIM-MS. The free IAA was quantified by isotope dilution analysis using [$^{13}C_6$]IAA as the internal standard (Barkawi *et al.*, 2008).

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REFERENCES

Al-Hammadi, A.S., Sreelakshmi, Y., Negi, S., Siddiqi, I. and Sharma, R. (2003) The polycotyledon mutant of tomato shows enhanced polar auxin transport. *Plant Physiol.* **133**, 113–125.

Barkawi, L.S., Tam, Y.Y., Tillman, J.A., Pederson, B., Calio, J., Al-Amier, H., Emerick, M., Normanly, J. and Cohen, J.D. (2008) A high-throughput method for the quantitative analysis of indole-3-acetic acid and other auxins from plant tissue. *Anal. Biochem.* **372**, 177–188.

Barry, C.S. and Giovannoni, J.J. (2006) Ripening in the tomato Green-ripe mutant is inhibited by ectopic expression of a protein that disrupts ethylene signaling. *Proc. Natl Acad. Sci. USA*, **103**, 7923–7928.

Barry, C.S. and Giovannoni, J.J. (2007) Ethylene and fruit ripening. *J. Plant Growth Regul.* **26**, 143–159.

Barry, C.S., Fox, E.A., Yen, H., Lee, S., Ying, T., Grierson, D. and Giovannoni, J.J. (2001) Analysis of the ethylene response in the epinastic mutant of tomato. *Plant Physiol.* **127**, 58–66.

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Boerjan, W., Cervera, M.-T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., Caboche, M., van Onckelen, H., Van Montagu, M. and Inze, D. (1995) *Superroot*, a recessive mutation in *Arabidopsis*, confers auxin overproduction. *Plant Cell*, **7**, 1405–1419.

Casimiro, I., Marchant, A., Bhalerao, R.P. *et al.* (2001) Auxin transport promotes *Arabidopsis* lateral root initiation. *Plant Cell*, **13**, 843–852.

Ciardi, J.A., Tieman, D.M., Jones, J.B. and Klee, H.J. (2001) Reduced expression of the tomato ethylene receptor gene LeETR4 enhances the hypersensitive response to *Xanthomonas campestris* pv. vesicatoria. *Mol. Plant Microbe Interact.* **14**, 487–495.

Clark, D.G., Gubrium, E.K., Barrett, J.E., Nell, T.A. and Klee, H.J. (1999) Root formation in ethylene-insensitive plants. *Plant Physiol.* **121**, 53–60.

Coleman, W., Huxter, T., Reid, D. and Thrope, T. (1980) Ethylene as an endogenous inhibitor of root regeneration in tomato leaf disc cultures in vitro. *Physiol. Plant.* **48**, 519–525.

De Klerk, G.-J., Van Der Krieken, W. and De Jong, J. (1999) The formation of adventitious roots: new concepts, new possibilities. *In Vitro Cell. Dev. Biol. Plants*, **35**, 189–199.

Fujino, D.W., Burger, D.W., Yang, S.F. and Bradford, K.J. (1988) Characterization of an ethylene overproducing mutant of tomato (*Lycopersicon esculentum* Mill. Cultivar VFN8). *Plant Physiol.* **88**, 774–779.

Geneve, R. and Heuser, C. (1983) The relationship between ethephon and auxin on adventitious root intiation in cuttings of *Vigna radiata* (L.) R. Wilcz. *J. Am. Soc. Hortic. Sci.* **108**, 330–333.

Grichko, V. and Glick, B. (2001) Ethylene and flooding stress in plants. *Plant Physiol. Biochem.* **39**, 1–9.

Guzman, P. and Ecker, J.R. (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *Plant Cell*, **2**, 513–523.

Hitchcock, A. and Zimmerman, P. (1940) Effects obtained with mixtures of root-inducing and other substances. *Contrib. Boyce Thompson Inst.* **11**, 155–159.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.G., Bleecker, A.B., Ecker, J.R. and Meyerowitz, E.M. (1998) EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. *Plant Cell*, **10**, 1321–1332.

Huang, Y., Li, H., Hutchison, C.E., Laskey, J. and Kieber, J.J. (2003) Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in Arabidopsis. *Plant J.* **33**, 221–233.

Ito, Y., Kitagawa, M., Ihashi, N., Yabe, K., Kimbara, J., Yasuda, J., Ito, H., Inakuma, T., Hiroi, S. and Kasumi, T. (2008) DNA-binding specificity, transcriptional activation potential, and the rin mutation effect for the tomato fruit-ripening regulator RIN. *Plant J.* **55**, 212–223.

Ivanchenko, M.G., Coffeen, W.C., Lomax, T.L. and Dubrovsky, J.G. (2006) Mutations in the *Diageotropica* (*dgt*) gene uncouple patterned cell division during lateral root initiation from proliferative cell division in the pericycle. *Plant J.* **46**, 436–447.

Ivanchenko, M.G., Muday, G.K. and Dubrovsky, J.G. (2008) Ethylene-auxin interactions regulate lateral root initiation and emergence in *Arabidopsis thaliana*. *Plant J.* **55**, 335–347.

Kendrick, M.D. and Chang, C. (2008) Ethylene signaling: new levels of complexity and regulation. *Curr. Opin. Plant Biol.* **11**, 479–485.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker, J.R. (1993) CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. *Cell*, **72**, 427–441.

Kim, H.J., Lynch, J.P. and Brown, K.M. (2008) Ethylene insensitivity impedes a subset of responses to phosphorus deficiency in tomato and petunia. *Plant Cell Environ.* **31**, 1744–1755.

Klee, H.J. (2004) Ethylene signal transduction. Moving beyond Arabidopsis. *Plant Physiol.* **135**, 660–667.

Lanahan, M.B., Yen, H.C., Giovannoni, J.J. and Klee, H.J. (1994) The *never ripe* mutation blocks ethylene perception in tomato. *Plant Cell*, **6**, 521–530.

Lashbrook, C.C., Tieman, D.M. and Klee, H.J. (1998) Differential regulation of the tomato ETR gene family throughout plant development. *Plant J.* **15**, 243–252.

Laskowski, M., Grieneisen, V.A., Hofhuis, H., Hove, C.A., Hogeweg, P., Maree, A.F. and Scheres, B. (2008) Root system architecture from coupling cell shape to auxin transport. *PLoS Biol.* **6**, e307.

Lewis, D.R. and Muday, G.K. (2009) Measurement of auxin transport in *Arabidopsis thaliana*. *Nat. Protoc.* **4**, 437–451.

Li, S.W., Xue, L.G., Xu, S.J., Feng, H.Y. and An, L.Z. (2009) Mediators, genes and signaling in adventitious rooting. *Bot. Rev.* **75**, 230–247.

Lincoln, J.E. and Fischer, R.L. (1988) Regulation of gene expression by ethylene in wild-type and rin Tomato (*Lycopersicon esculentum*) fruit. *Plant Physiol.* **88**, 370–374.

Ludwig-Muller, J., Vertocnik, A. and Town, C.D. (2005) Analysis of indole-3-butyric acid-induced adventitious root formation on Arabidopsis stem segments. *J. Exp. Bot.* **56**, 2095–2105.

Macgregor, D.R., Deak, K.I., Ingram, P.A. and Malamy, J.E. (2008) Root system architecture in Arabidopsis grown in culture is regulated by sucrose uptake in the aerial tissues. *Plant Cell*, **20**, 2643–2660.

Malamy, J. (2009) Lateral root formation. In *Root Development* (Beeckman, T., ed.), Vol. 37, pp. 83–126. Oxford, UK: Blackwell Publishing Limited.

Malamy, J. and Benfey, P. (1997) Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development*, **124**, 33–44.

Malamy, J.E. and Ryan, K.S. (2001) Environmental regulation of lateral root initiation in Arabidopsis. *Plant Physiol.* **127**, 899–909.

Morgan, P. and Gausman, H. (1966) Effects of ethylene on auxin transport. *Plant Physiol.* **41**, 45–52.

Muday, G.K. and Haworth, P. (1994) Tomato root growth, gravitropism, and lateral development: correlation with auxin transport. *Plant Physiol. Biochem.* **32**, 193–203.

Muday, G.K., Lomax, T.L. and Rayle, D.L. (1995) Characterization of the growth and auxin physiology of roots of the tomato mutant, *diageotropica*. *Planta*, **195**, 548–553.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–479.

Negi, S., Ivanchenko, M.G. and Muday, G.K. (2008) Ethylene regulates lateral root formation and auxin transport in *Arabidopsis thaliana*. *Plant J.* **55**, 175–187.

Phatak, S., Jaworski, C. and Liptay, A. (1981) Flowering and adventitious root growth of tomato cultivars as influenced by ethephon. *HortScience*, **16**, 181–182.

Prayitno, J., Rolfe, B.G. and Mathesius, U. (2006) The Ethylene-insensitive sickle mutant of *Medicago truncatula* shows altered auxin transport regulation during nodulation. *Plant Physiol.* **142**, 168–180.

Reed, R.C., Brady, S.R. and Muday, G.K. (1998) Inhibition of auxin movement from the shoot into the root inhibits lateral root development in arabidopsis. *Plant Physiol.* **118**, 1369–1378.

Resnick, J.S., Wen, C.K., Shockey, J.A. and Chang, C. (2006) REVERSION-TO-ETHYLENE SENSITIVITY1, a conserved gene that regulates ethylene receptor function in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **103**, 7917–7922.

Robbins, J., Kays, S. and Dirr, M. (1983) Enhanced rooting of wounded mung bean cuttings by wounding and ethephon. *J. Am. Soc. Hortic. Sci.* **108**, 325–329.

Roy, B., Basu, R. and Bose, T. (1972) Interaction of auxins with growth-retarding, -inhibiting and ethylene-producing chemicals in rooting of cuttings. *Plant Cell Physiol.* **13**, 1123–1127.

Ruzicka, K., Ljung, K., Vanneste, S., Podhorska, R., Beeckman, T., Friml, J. and Benkova, E. (2007) Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. *Plant Cell*, **19**, 2197–2212.

Sakai, H., Hua, J., Chen, Q.G., Chang, C., Medrano, L.J., Bleecker, A.B. and Meyerowitz, E.M. (1998) ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **95**, 5812–5817.

Sitbon, F., Hennion, S., Sundberg, B., Little, C.H.A., Olsson, O. and Sandberg, G. (1992) Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens* iaaM and iaaH genes display altered growth and indoleacetic acid metabolism. *Plant Physiol.* **99**, 1062–1069.

Sorin, C., Bussell, J.D., Camus, I. *et al.* (2005) Auxin and light control of adventitious rooting in Arabidopsis require ARGONAUTE1. *Plant Cell*, **17**, 1343–1359.

Sorin, C., Negroni, L., Balliau, T., Corti, H., Jacquemot, M.P., Davanture, M., Sandberg, G., Zivy, M. and Bellini, C. (2006) Proteomic analysis of different mutant genotypes of Arabidopsis led to the identification of 11 proteins correlating with adventitious root development. *Plant Physiol.* **140**, 349–364.

Stasinopoulos, T.C. and Hangarter, R.P. (1989) Preventing photochemistry in culture media by long-pass light filters alters growth of cultured tissues. *Plant Physiol.* **93**, 1365–1369.

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Stepanova, A.N., Yun, J., Likhacheva, A.V. and Alonso, J.M. (2007) Multilevel interactions between ethylene and auxin in Arabidopsis roots. *Plant Cell*, **19**, 2169–2185.

Suttle, J.C. (1988) Effect of ethylene treatment on polar IAA transport, net IAA uptake and specific binding of *N*-1-naphthylphthalamic acid in tissues and microsomes isolated from etiolated pea epicotyls. *Plant Physiol.* **88**, 795–799.

Swarup, R., Perry, P., Hagenbeek, D., Van Der Straeten, D., Beemster, G.T., Sandberg, G., Bhalerao, R., Ljung, K. and Bennett, M.J. (2007) Ethylene upregulates auxin biosynthesis in Arabidopsis seedlings to enhance inhibition of root cell elongation. *Plant Cell*, **19**, 2186–2196.

Tieman, D.M. and Klee, H.J. (1999) Differential expression of two novel members of the tomato ethylene-receptor family. *Plant Physiol.* **120**, 165–172.

Torrey, J.G. (1976) Root hormones and plant growth. *Annu. Rev. Plant Physiol.* **27**, 435–459.

Tyburski, J. and Tretyn, A. (2004) The role of light and polar auxin transport in root regenration from hypocotyls of tomato seedling cuttings. *Plant Growth Regul.* **42**, 39–48.

Visser, E., Cohen, J.D., Barendse, G., Blom, C. and Voesenek, L. (1996) An ethylene-mediated increase in sensitivity to Auxin induces adventitious root formation in flooded *Rumex palustris* Sm. *Plant Physiol.* **112**, 1687–1692.

Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W. and Giovannoni, J. (2002) A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (Rin) locus. *Science*, **296**, 343–346.

Wilkinson, J.Q., Lanahan, M.B., Yen, H.C., Giovannoni, J.J. and Klee, H.J. (1995) An ethylene-inducible component of signal transduction encoded by Never-ripe. *Science*, **270**, 1807–1809.

Yen, H.C., Lee, S., Tanksley, S.D., Lanahan, M.B., Klee, H.J. and Giovannoni, J.J. (1995) The tomato Never-ripe locus regulates ethylene-inducible gene expression and is linked to a homolog of the Arabidopsis ETR1 gene. *Plant Physiol.* **107**, 1343–1353.

Yokotani, N., Tamura, S., Nakano, R., Inaba, A., McGlasson, W.B. and Kubo, Y. (2004) Comparison of ethylene- and wound-induced responses in fruit of wild-type, rin and nor tomatoes. *Postharvest Biol. Technol.* **32**, 247–252.

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