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Hormonal interplay during adventitious root formation in flooded tomato plants

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SUMMARY

Soil flooding, which results in a decline in the availability of oxygen to submerged organs, negatively affects the growth and productivity of most crops. Although tomato (*Solanum lycopersicum*) is known for its sensitivity to waterlogging, its ability to produce adventitious roots (ARs) increases plant survival when the level of oxygen is decreased in the root zone. Ethylene entrapment by water may represent the first warning signal to the plant indicating waterlogging. We found that treatment with the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) and the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) resulted in a reduction of AR formation in waterlogged plants. We observed that ethylene, perceived by the Never Ripe receptor, stimulated auxin transport. In a process requiring the *Diageotropica* gene, auxin accumulation in the stem triggered additional ethylene synthesis, which further stimulated a flux of auxin towards to the flooded parts of the plant. Auxin accumulation in the base of the plant induces growth of pre-formed root initials. This response of tomato plants results in a new root system that is capable of replacing the original one when it has been damaged by submergence.

Keywords: tomato, flooding, submergence, adventitious root, ethylene, auxin.

INTRODUCTION

Flooding, which includes soil waterlogging and submergence, is one of the most frequent and extensive abiotic stresses that negatively influence terrestrial plant growth and productivity, affecting the composition of plant populations and in some cases resulting in plant death (Jackson and Colmer, 2005; Voesenek *et al.*, 2006). It has been calculated that approximately 16% of the fertile areas of the world are affected by soil waterlogging (Ahsan *et al.*, 2007). Climate change is likely to cause an increase in the occurrence of heavy rains leading to flooding of agricultural lands (Bailey-Serres and Voesenek, 2008). Moreover, resistance to flooding has not been included among the main objectives in improvement programs for most cultivated plants. An excess of water in the upper layers of soil may therefore have a dramatic effect on crop productivity (Bailey-Serres and Voesenek, 2008).

Reduction of gas exchange is the primary effect of soil waterlogging. The 10^4-fold decrease in the gas diffusion rate in water compared to air negatively affects oxygen supply, and causes accumulation of ethylene in submerged tissues (Jackson *et al.*, 2003; Visser and Voesenek, 2004). It is ethylene production and entrapment that trigger a number of plant responses to flooding and submergence (Jackson, 2002). These responses vary with plant species, and include epinasty, hyponastic growth, shoot elongation, aerenchyma formation and adventitious root development (Jackson, 2002; Visser and Voesenek, 2004; Voesenek *et al.*, 2006; Bailey-Serres and Voesenek, 2008). Through these adaptations, plants can escape the hypoxic environment or at least improve oxygen diffusion to the submerged organs.

Some species of crops, such as rice, are particularly tolerant to submergence, whereas others, such as tomato (*Solanum lycopersicon* L.), are highly intolerant (Ahsan *et al.*, 2007). Tomato is the second most important vegetable crop, from an economic perspective, after potato, and the fresh tomato trade has increased by 45% in the last 5 years. For several decades, its economic significance has made tomato the focus of extensive physiological, biochemical

and genetic studies, some of which have described its response to waterlogging stress (Else *et al.*, 2009). More recently, tomato has been the target of molecular research, proteomic analyses and also genome sequencing (Fei *et al.*, 2006; Ahsan *et al.*, 2007).

Two of typical tomato responses to flooding are epinasty and adventitious root formation. Epinasty results from accumulation of the ethylene precursor 1-aminocyclopropane-1-carbooxylic acid (ACC) in flooded roots, which is then transported through the transpiration stream to the aerial part of the plant, where the presence of oxygen allows it to be converted into ethylene, triggering petiole epinasty (Jackson, 2002). Adventitious roots are known to participate in tomato plant recovery from waterlogging (Kramer, 1951). McNamara and Mitchell (1991) proposed that auxin is required to stimulate the growth of root initials in hypocotyls before flooding treatment. However, it has also been suggested that submergence induces the development of root initials in hypocotyls (Jackson, 1985).

The development of ARs has also been observed in other species such as sunflower and sugar cane (Jackson, 1955). It has been demonstrated that ethylene participates in AR formation under flooding conditions, although its role may vary between species. For instance, in *Rumex palustris* Sm., ethylene increases auxin sensitivity and leads to the production of ARs (Visser *et al.*, 1996). In deepwater rice, on the other hand, ethylene facilitates the emergence of pre-formed ARs by causing death of the epidermal cells that cover the root tip (Mergemann and Sauter, 2000).

Nevertheless, the exact role of auxin in AR formation under waterlogging stress is still unclear for most plant species. In addition, ethylene may affect auxin transport, resulting in its accumulation at the stem base of flooded plants (Grichko and Glick, 2001).

Ethylene- and auxin-insensitive mutants are available in tomato as well as in Arabidopsis, making it easier to perform physiological and molecular experiments in order to better understand the mechanism by which these hormones regulate plant responses. Two such tomato mutants are *Never ripe* (*Nr*) and *diageotropica* (*dgt*). Plants carrying the *Nr* mutation are insensitive to ethylene due to a defective receptor protein, and consequently exhibit less AR production in cuttings in comparison to wild-type (WT) plants (Clark *et al.*, 1999). In the case of the *dgt* mutation, there is a lower sensitivity to auxin, and plants are characterized by several conspicuous phenotypes, including lack of lateral roots, reduced gravitropic response, hyponastic leaves and reduced apical dominance (Nebenfuhr *et al.*, 2000).

In this study, adventitious root formation in flooded tomato plants was studied in order to unravel the involvement of ethylene and auxin. The results demonstrate that ethylene and auxin signaling are strictly interconnected in the pathway leading to AR production.

RESULTS

Induction of adventitious roots in flooded tomato plants

The development of ARs in 4-week-old tomato plants at 24, 72 h and 7 days after the onset of flooding treatment is shown in Figure 1(a), in comparison with control plants. After 72 h of submergence, AR primordia were visible on the hypocotyl surface (Figure 1a), although only a few were long enough to emerge from the shoot epidermis. After 1 week, numerous ARs protruded from the lower part of the submerged stem (Figure S1a), which presented a slight hypertrophy (Figure 1a).

Microscopic studies revealed that root primordia had already formed 24 h after the start of the experiment (Figure 1b). Typically, primordia continued to grow and most reached the epidermis after 48 h of submergence (Figure 1b).

Alcohol dehydrogenase 2 (*LeADH2*) is known to be induced at low oxygen concentrations (Van Der Straeten *et al.*, 1991). *LeADH2* transcripts were induced by flooding in submerged organs (roots and hypocotyls) (Figure 1c), indicating that these tissues are hypoxic. One of the first events that takes place after the onset of flooding treatment is an increase in the ethylene level, resulting from entrapment or increased synthesis. We thus examined the expression of *LeACS3* and *LeACS7*, two genes encoding ACC synthase that are induced early in flooded tomato roots (Shiu *et al.*, 1998). *LeACS3* showed high induction in roots, and was transiently induced during the first 12 h of treatment in both the hypocotyl and epicotyl. This indicates that the genes responsible for the initial ethylene evolution were induced soon after the start of flooding (Figure 1c). *LeACS7* showed the highest induction after 4 h of submergence in both hypocotyls and epicotyls, which coincides with the early induction observed by Shiu *et al.* (1998). *LeACS3* and *LeACS7* expression levels were low for all tissues collected from control plants (Figure 1c). The expression level of the Never Ripe (NR) ethylene receptor was also studied. Interestingly, *LeNR* showed a bi-phasic increase in transcript levels in hypocotyls after 4 and 24 h of submergence.

Hypoxia is not sufficient to trigger adventitious root production

To determine whether the low oxygen level resulting from submergence was sufficient for AR formation under flooding conditions, 4-week-old tomato plants were placed inside a hypoxic chamber at 1% O_2. When removed from the chamber, plants showed signs of wilting (Figure 2a), but recovered in a few hours. No AR primordia were observed, even after 1 week of hypoxic treatment (Figure 2b). Ethylene production was measured, and the results indicated that, while submergence triggers ethylene synthesis, hypoxic treatment dramatically reduces ethylene production (Figure 2c).

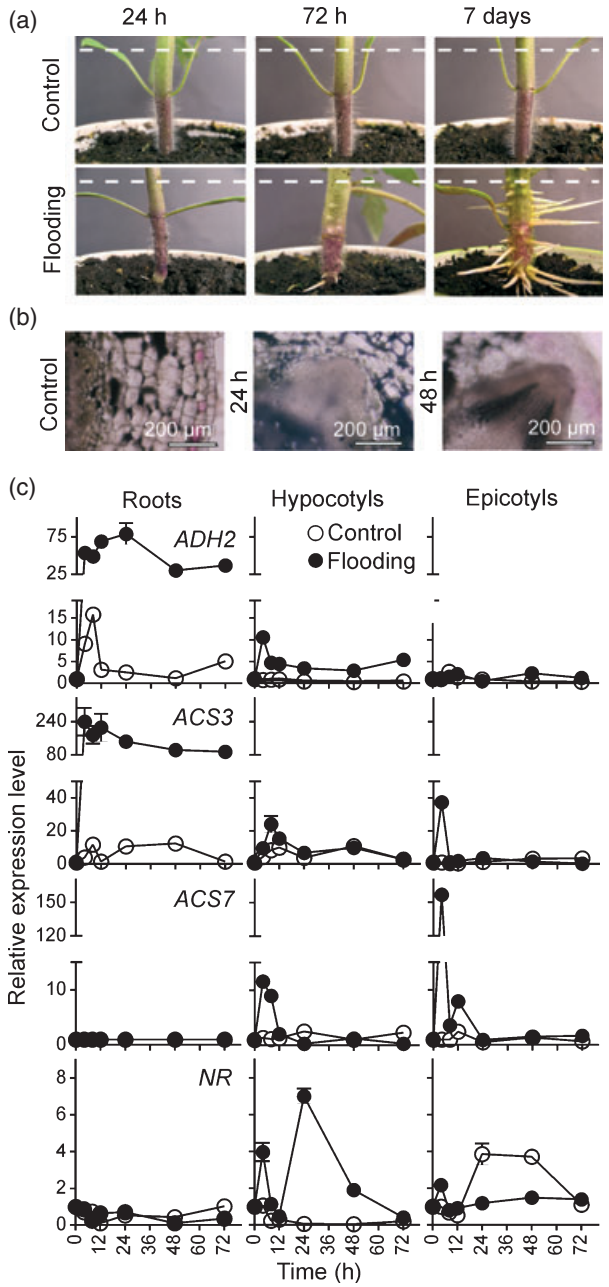

Figure 1. Adventitious root formation in 4-week-old wild-type (WT) tomato plants (cv. Ailsa Craig) under flooding conditions.
(a) Hypocotyl region showing AR primordia and AR emergence after 24, 72 h and 7 days of flooding treatment, respectively. The water level used for submergence treatments is indicated by a dotted line. Quantitative data for the root numbers are given in Figure S1(a).
(b) AR primordia before emergence: cross-sections of a hypocotyl from a 4-week-old tomato plant growing under normal conditions (control), a hypocotyl with AR primordium 24 h after the beginning of flooding treatment, and a hypocotyl showing an AR primordium elongating inside the stem tissue 48 h after the start of flooding treatment.
(c) Transcript levels of *ADH2*, *ACS3*, *ACS7* and *NR* in roots, hypocotyls and epicotyls. Transcript levels were normalized to *LeEF1A* and *ACT7* expression using Genorm, and are indicated as relative units, with the organ with the lowest value (epicotyls) assigned a value of 1. Data are means ± SD (n = 3).

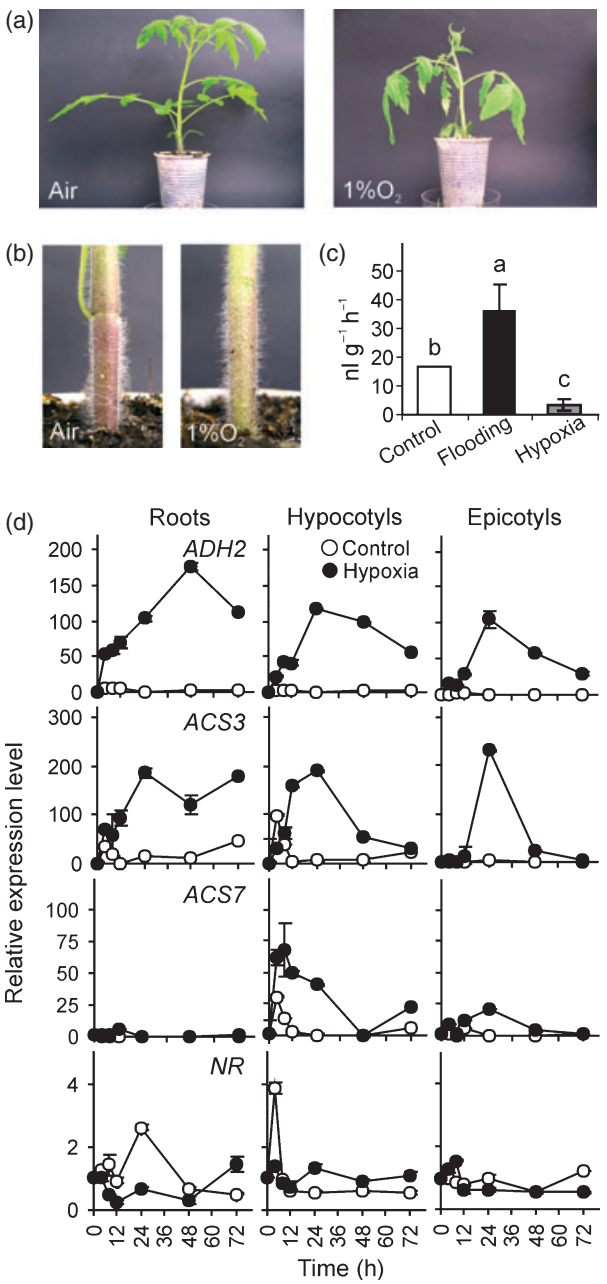

Figure 2. Adventitious root formation under 1% O_2 in WT (Ailsa Craig) tomato plants.
(a) Tomato plants in air and after 8 days of hypoxic treatment (1% O_2).
(b) Close-up of the hypocotyl region showing the absence of AR primordia in control and hypoxia-treated plants.
(c) Ethylene production in tomato plants under control, flooding and hypoxic conditions. For flooding treatment, 4-week-old tomato plants were submerged up to the first leaf insertion above the cotyledonary node for 24 h. Hypoxic treatment consisted of 1% O_2 supplied to a hypoxic chamber where plants were kept for 24 h. Data are means ± SD (n = 3). Different letters indicate significant differences between treatments at $P \leq 0.05$ (t test).
(d) *ADH2*, *ACS3*, *ACS7* and *NR* relative expression levels in roots, hypocotyls and epicotyls. Transcript levels were normalized to *LeEF1A* and *ACT7* expression using Genorm, and are indicated as relative units, with the organ with the lowest value (epicotyls) assigned a value of 1. Data are means ± SD (n = 3).

LeADH2 was induced by hypoxia in all tested tissues (Figure 2d), confirming that the whole plant was in contact with the oxygen-deficient atmosphere. *LeACS3* was also induced by hypoxia, and showed a similar expression level in all three tissues (Figure 2d). *LeACS7* showed higher expression levels in hypocotyls and epicotyls of hypoxic plants. However, there was no such increase in transcript abundance in roots collected from treated plants compared to the controls. With regard to *LeNR* induction, it was not possible to observe any difference in expression levels between the controls and treated plants, suggesting that ethylene production is required for modulating *LeNR*.

Ethylene biosynthesis and perception are required for adventitious root formation

As AR primordia were noticeable 48–72 h after flooding treatment, growth of the root initials must have occurred several hours prior to the emergence of these primordia. Ethylene is involved in several plant responses to flooding, including AR development (Visser and Voesenek, 2004). Ethylene production was measured 24 h after the start of the submergence treatment in leaves, epicotyls, hypocotyls and entire plants. A flooding-dependent increase in ethylene production by the entire plant was observed (Figure 3a). Ethylene production increased approximately twofold in all dissected tissues of flooded plants in comparison with controls (Figure 3a). In control plants, ethylene production in leaves was higher than that in hypocotyls and epicotyls (Figure 3a). The highest amount of ethylene was measured in leaves from flooded plants. Interestingly, submerged hypocotyls were able to increase ethylene release compared to those in air, almost doubling the amount produced (Figure 3a).

In order to assess the significance of ethylene production in the development of ARs, flooded and control plants were treated with aminoethoxyvinylglycine (AVG), an ethylene biosynthesis inhibitor (Visser *et al.*, 1996). After 72 h of submergence, AR primordia became visible in the stem base of flooded plants that had not been treated with AVG (Figures 3b and S1b). These primordia elongated and produced abundant ARs within 1 week (Figure 3b). Plant treatment with AVG drastically reduced AR production (Figures 3b and S1b).

Nr, an ethylene-insensitive mutant, showed reduced AR formation after flooding treatment compared with the parental WT (cv. Gimar), indicating that ethylene perception is required to elicit AR production (Figures 4a and S1c). Moreover, *Nr* roots were shorter than those produced by Gimar plants (Figure 4a). As AR formation takes place in submerged hypocotyls, gene expression was studied in these tissues. *LeADH2* expression in both genotypes showed a bi-phasic induction pattern following flooding treatment (Figure 4b). An increase in *LeACS3* and *LeACS7* transcript abundance was observed 8 h after the onset of

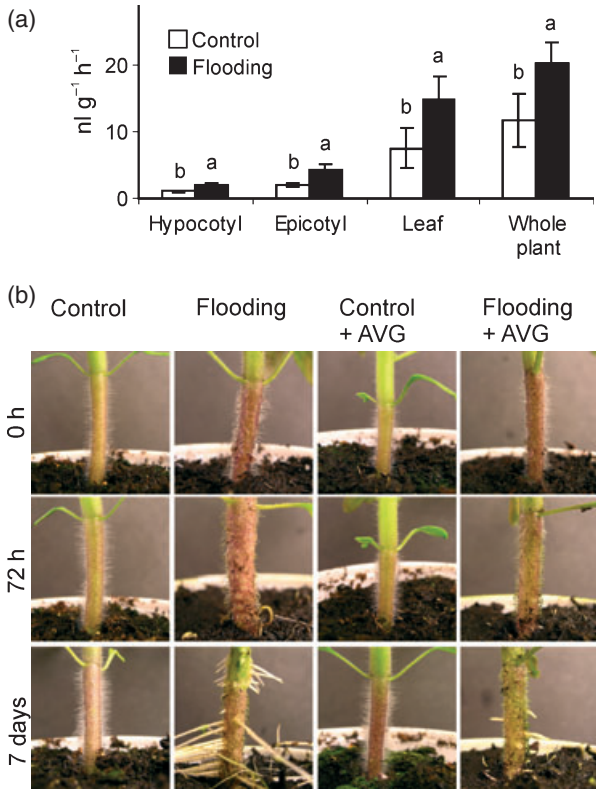

Figure 3. Ethylene is required for adventitious root formation in tomato.
(a) Ethylene production in flooded tomato plants. Four-week-old tomato plants were submerged up to the first leaf insertion above the cotyledonary node for 24 h, and ethylene production was quantified in excised hypocotyls, epicotyls, leaves and whole plants. Data are means ± SD ($n = 3$). Different letters indicate significant differences between treatments but within tissues at $P \leq 0.05$ (t test).
(b) Effect of AVG on AR formation in 4-week-old WT tomato plants (cv. Ailsa Craig). The aerial part of the plants was sprayed with 500 μM AVG 12 h before the start of flooding treatment, and then daily at the start of the light phase. Plants were flooded up to the first leaf insertion above the cotyledonary node. Quantitative data for root numbers are given in Figure S1(b).

flooding in the WT, but their expression levels in flooded *Nr* plants remained as low as in non-flooded plants (Figure 4b).

Interestingly, ethylene perception by NR is not required for flooding-enhanced ethylene synthesis (Figure 4c). Flooding increased auxin concentrations in both epicotyls and hypocotyls in the WT, but the auxin level in the *Nr* mutant did not increase due to flooding treatment (Figure 4d). These results highlight the existence of interplay between auxin and ethylene physiology in flooded tomato plants.

In order to ascertain whether ethylene perception was required only at the site of AR production, we produced grafted plants in which either the rootstock or scion was insensitive to ethylene (Figure 4e). Hypocotyls from grafted plants that carried the *Nr* mutation in both rootstock and scion produced fewer ARs than those in which at least one of the grafting parts was sensitive to ethylene, even when 10 days had passed since the start of the flooding treatment

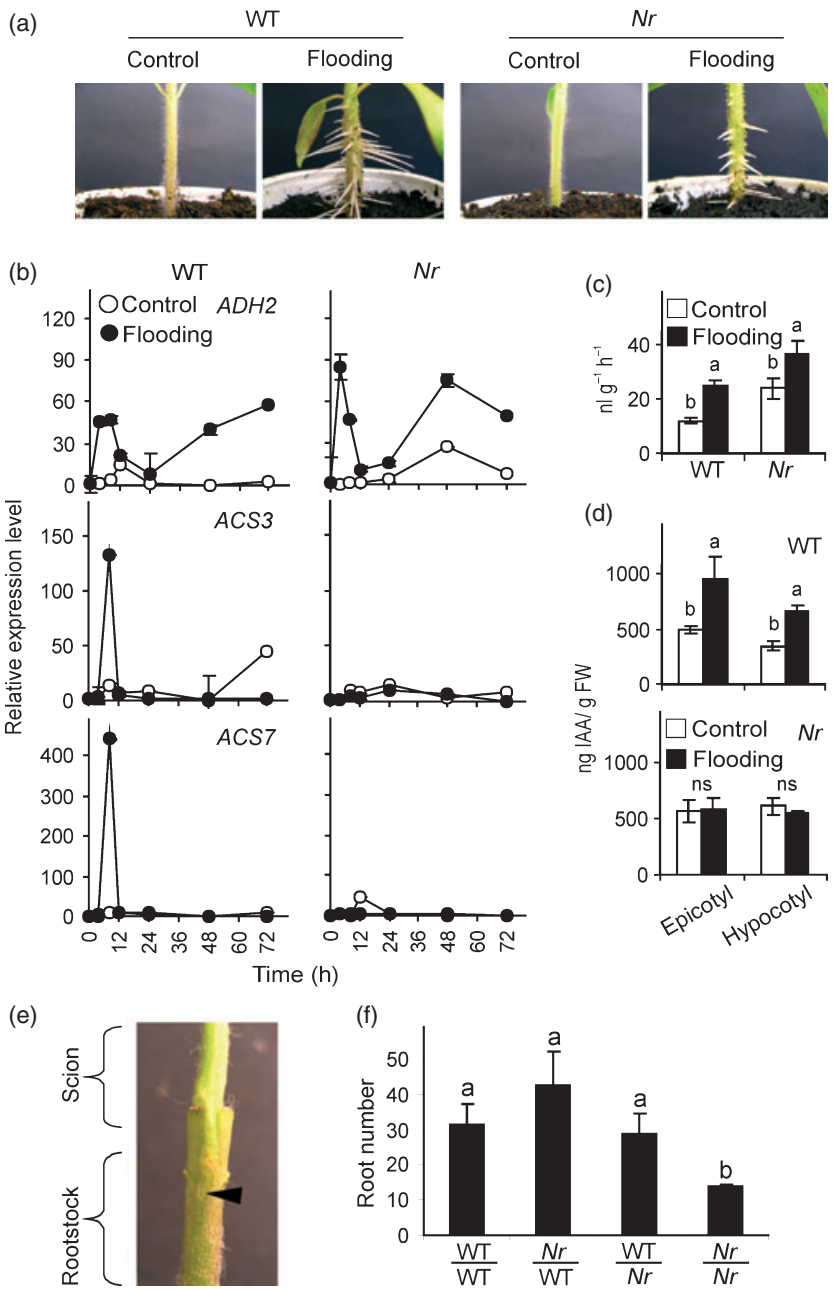

Figure 4. Adventitious root formation in ethylene-insensitive tomato mutants after 7 days of root and hypocotyl submergence.
(a) Hypocotyl region of the ethylene-insensitive tomato mutant *Nr* and the corresponding WT (Gimar) after 7 days of flooding treatment. Quantitative data for root numbers are given in Figure S1(c).
(b) *ADH2*, *ACS3* and *ACS7* relative expression levels in hypocotyls of WT and *Nr* plants under normal and flooding conditions. Transcript levels were normalized to *LeEF1A* and *ACT7* expression using Genorm and are indicated in relative units. Data are means ± SD (n = 3).
(c) Ethylene production in flooded WT and *Nr* tomato plants. Four-week-old tomato plants were submerged for 24 h up to the first leaf insertion above the cotyledonary node, and ethylene production was quantified. Data are means ± SD (n = 3). Different letters indicate significant differences between treatments at $P \leq 0.05$ (t test) within genotypes.
(d) IAA content in 4-week-old WT and *Nr* plants under control conditions and after 24 h of flooding treatment. The IAA content in epicotyl and hypocotyl tissues was determined. The water level covered only roots and hypocotyls, so that epicotyls and leaves were above the water surface. Values are means ± SD (n = 3). Different letters indicate significant differences between treatments at $P \leq 0.05$ (t test) within tissues.
(e) Close-up of the rootstock–scion junction area. The black arrow indicates the water level.
(f) AR formation in grafted tomato plants of *Nr* and the corresponding WT after 10 days of flooding up to the first node above the cotyledonary node. Means for root number and root length are shown for plants with the WT genotype in both scion and rootstock (WT/WT), in the rootstock (*Nr*/WT), in the scion (WT/*Nr*) or with the *Nr* genotype in both parts (*Nr*/*Nr*). Different letters indicate significant differences at $P \leq 0.05$ (t test).

(Figure 4f). All genotype combinations that were different from *Nr*/*Nr* produced as many roots as the WT/WT plants (Figure 4f), and showed a similar root length (data not shown). This indicates that ethylene perception in either the flooded or emerged stem sections is sufficient for AR production.

Auxin transport and perception greatly influence AR formation

A higher indole-3-acetic acid (IAA) content in flooded tissues from 4-week-old tomato plants was observed in both the Gimar (Figure 4d) and Ailsa Craig (Figure 5a) WT tomato cultivars. We used transgenic plants expressing an IAA-inducible promoter fused to the GUS reporter gene to visualize auxin localization. More intense GUS-dependent staining was observed in hypocotyls from flooded plants after 24 h of submergence compared to non-flooded plants (Figure 5b). A blue coloration, corresponding to the sites of auxin accumulation, was mainly observed in the vascular tissues and developing ARs (arrows in Figure 5b).

An increase in auxin content in the submerged organs suggested that increased basipetal auxin transport occurs in flooded plants. In order to evaluate the role of the transport of auxin to submerged organs in AR production, the auxin

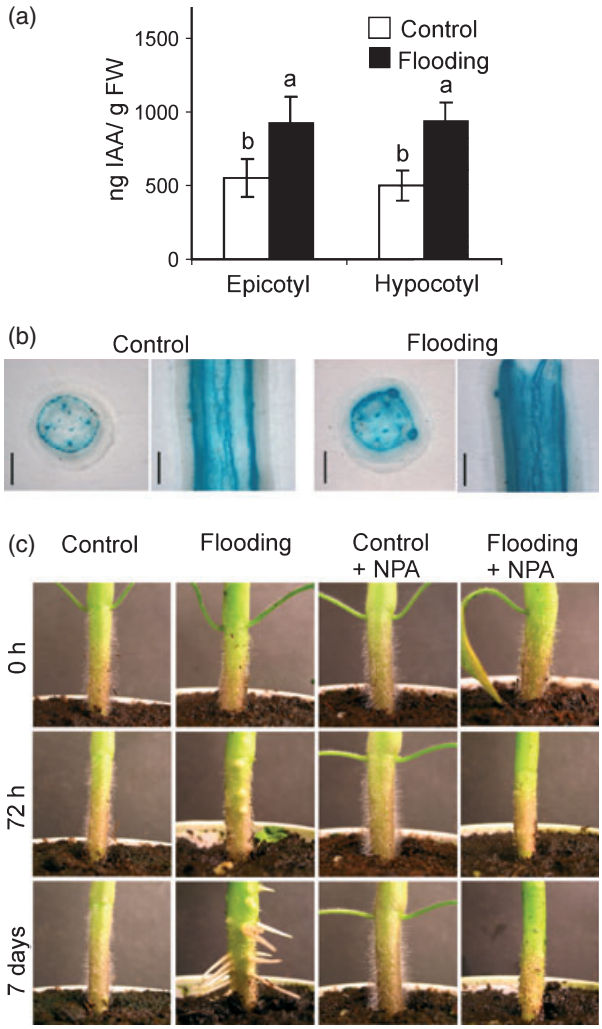

Figure 5. Auxin transport is critical for adventitious root formation.
(a) IAA content in 4-week-old WT (Ailsa Craig) plants under control conditions and after 24 h of flooding treatment. The water level covered roots and hypocotyls but epicotyls and leaves were above the water surface. Values are means ± SD (n = 3). Different letters indicate significant differences between treatments at $P \leq 0.05$ (t test) within tissues.
(b) GUS staining in hypocotyl cross-sections from treated and control plants 24 h after the start of root and hypocotyl flooding. Scale bar = 2 mm.
(c) Effect of NPA on AR production in 4-week-old WT tomato plants (Ailsa Craig). Leaves and stems were sprayed with 1 mM NPA 12 h before the start of flooding treatment, and then daily at the start of the light phase. The water level reached the insertion of the first leaf above the cotyledonary node. Quantitative data for root numbers are given in Figure S1(d).

transport inhibitor 1-naphthylphthalamic acid (NPA) was applied to flooded and control plants. Treatment with NPA clearly suppressed the proliferation of ARs in submerged hypocotyls. This effect continued over time and prevented *de novo* root formation, even 7 days after the start of flooding treatments (Figures 5c and S1d).

To verify whether auxin perception was required for AR initiation, experiments were performed using the auxin-insensitive mutant *dgt*. Under flooding conditions, AR formation was abundant in WT plants, but few ARs were observed in *dgt* plants (Figures 6a and S1e). In the latter, roots emerged only in the stem region close to the root neck instead of over the whole hypocotyl as in the WT plants. Interestingly, ARs of *dgt* plants grew upwards, confirming the reduced gravitropic response that characterizes the mutant (Ivanchenko *et al.*, 2006). Gene expression analysis showed higher *LeADH2* induction (Figure 6b) in hypocotyls from flooded plants compared to controls for *dgt* and WT. However, *dgt* had lower *LeACS3* and *LeACS7* expression levels than the WT for both treated and control plants (Figure 6b). Flooding-induced ethylene production was abolished in *dgt* (Figure 6c).

DISCUSSION

Almost six decades have passed since the first report that associated the ability of tomato plants to produce ARs with fast recovery following waterlogging stress (Kramer, 1951). Submergence-tolerant species develop larger adventitious root systems than intolerant species, and these newly formed roots often contain more aerenchyma (e.g. *Rumex* species, Laan *et al.*, 1989). The initiation and outgrowth of adventitious roots have been studied in deepwater rice and *Rumex* species, respectively (reviewed by Colmer and Voesenek, 2009). The accumulation of ethylene and auxin in submerged organs is known to play an important role in AR production (Visser *et al.*, 1996; Steffens and Sauter, 2009). Nevertheless, the hormonal interplay in *de novo* root formation in flooded tomato plants remained unclear.

Our results indicated that flooding-dependent AR production requires both enhanced auxin transport and accumulation, together with increased ethylene levels. Flooded tomato plants fail to produce ARs if treated with the auxin transport inhibitor NPA (Figure 5c). This confirms that auxin transport is required for AR production, in line with the evidence available in rice (Xu *et al.*, 2005) and tobacco (McDonald and Visser, 2003). Auxin perception is also a prerequisite for AR production, as indicated by the failure of the flooded *dgt* mutant to produce ARs (Figure 6a). The *dgt* mutant also fails to produce ARs in response to exogenous auxin (Oh *et al.*, 2006), indicating that AR production requires the same signaling pathway that operates following either flooding or exogenous auxin treatment.

Ethylene production is a typical response in waterlogged plants (Bailey-Serres and Voesenek, 2008). Although *dgt* petioles have been reported to be capable of producing ethylene after waterlogging (Bradford and Yang, 1980), ethylene production in waterlogged *dgt* plants was 37% less than in the WT (Jackson, 1979). In our experiments, ethylene production by flooded *dgt* plants did not differ from the controls, probably because whole plants (Figure 6c) were analyzed rather than petioles (Jackson, 1979; Bradford and Yang, 1980). The induction of *LeACS3* (Olson *et al.*,

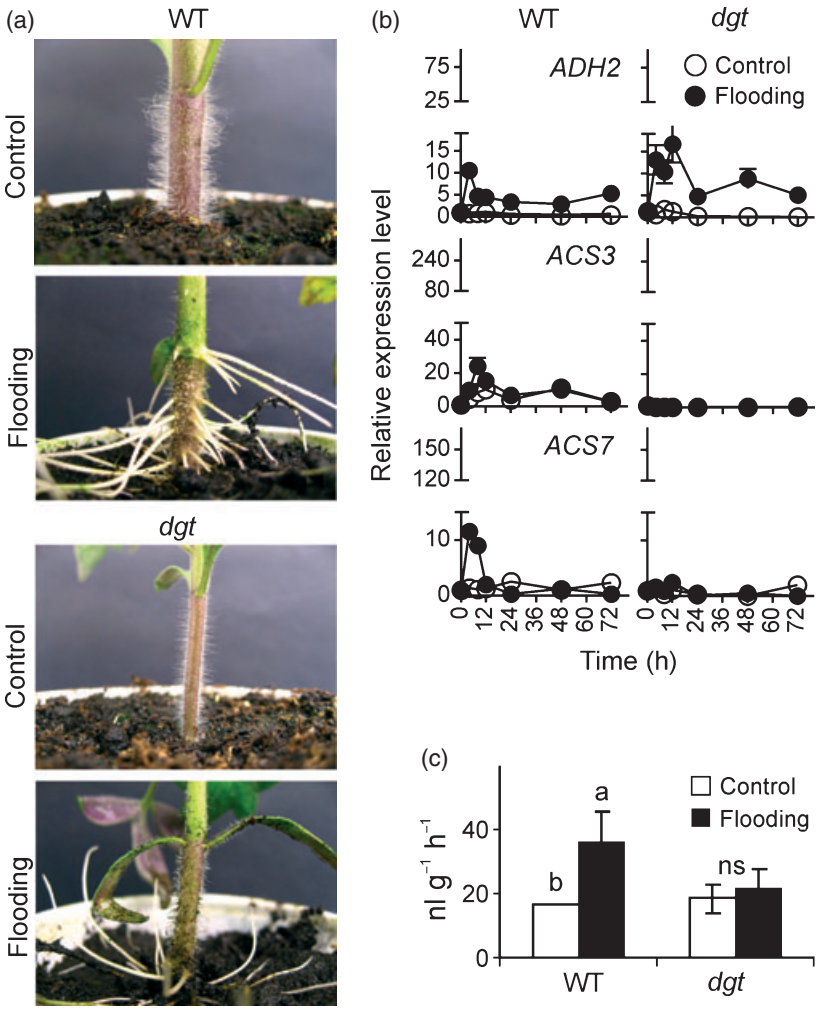

Figure 6. Adventitious root production in the auxin-insensitive mutant *diageotropica* (*dgt*) and the corresponding WT (Ailsa Craig).
(a) Hypocotyls of *dgt* and the corresponding WT plants under normal and flooding conditions, showing greater production of ARs in plants with normal auxin perception after 7 days of treatment. Flooding treatment involved root and hypocotyl submergence up to the first node above the cotyledonary node. Quantitative data for root numbers are given in Figure S1(e).
(b) *ADH2*, *ACS3* and *ACS7* relative expression levels in hypocotyls of WT (Ailsa Craig) and *dgt* plants under normal and flooding conditions. Transcript levels were normalized to *LeEF1A* expression and are indicated in relative units. Data are means ± SD (n = 3).
(c) Ethylene production in 4-week-old WT (Ailsa Craig) and *dgt* plants under control conditions and after 24 h of flooding treatment. The water level covered only roots and hypocotyls, so that epicotyls and leaves were above the water surface. Values are means ± SD (n = 3). Different letters indicate significant differences between treatments at $P \leq 0.05$ (t test) within genotypes.

1995; Shiu *et al.*, 1998) and *LeACS7* by flooding was absent in *dgt* plants (Figure 6b), consistent with the absence of an increase in ethylene evolution following submergence in *dgt* (Figure 6c). It also suggests that there is a link between auxin and ethylene physiology during flooding-induced AR formation. Both *LeACS3* (Coenen *et al.*, 2003) and *LeACS7* (Balbi and Lomax, 2003) are DGT-dependent auxin-induced genes.

Ethylene synthesis is required for AR production. The inhibition of ethylene synthesis by AVG (Figure 3b) or inhibition of ethylene perception (*Nr* mutant, Figure 4a) result in low levels of AR production. Moreover, experiments performed with WT and transgenic tobacco plants showing decreased ethylene sensitivity have indicated the positive effect of ethylene on AR formation under waterlogging (McDonald and Visser, 2003). It appears that the presence of a functioning ethylene receptor other than NR in *Nr* plants can in some way overcome the effect of the *Nr* mutation during root morphogenesis, allowing the plant to produce ARs when endogenous ethylene levels rise above a certain threshold. This may be the reason why AVG treatment, which completely prevents ethylene biosynthesis, resulted in a lower production of ARs than in the experiment with the *Nr* mutant (Figures 3b, 4a and S1b,c). Exogenous ACC treatment did not induce *LeACS3* and *LeACS7* (data not shown). *LeACS3* and *LeACS7* are induced by auxin (Balbi and Lomax, 2003; Coenen *et al.*, 2003), suggesting that both *ACS* genes are induced by flooding-dependent increased auxin levels (Figures 4d and 5a). *Nr* did not show increased IAA levels in flooded hypocotyls or epicotyls (Figure 4d), and, consistent with an auxin-dependent induction, *LeACS3* and *LeACS7* mRNA levels were unaffected by flooding in *Nr* (Figure 4b). Additionally, both *ACS* genes were induced by hypoxia, which is not associated with an increased ethylene level (Figure 2c,d). The fact that the IAA level in *Nr* plants did not increase when the plants were flooded (Figure 4d) indicates that ethylene perception is required for flooding-induced auxin accumulation. Ethylene enhances auxin synthesis and accumulation in Arabidopsis root tips (Stepanova *et al.*, 2005, 2007; Ruzicka *et al.*, 2007; Swarup *et al.*, 2007),

but negative regulation of IAA levels was demonstrated by Negi *et al.* (2010) in tomato roots. However, although ethylene exerted a negative effect on lateral root production, its effects were positive in terms of auxin-induced ARs (Negi *et al.*, 2010), suggesting that the cross-talk between ethylene and auxin affects lateral and AR production differently.

Our results suggest that, under flooding conditions, ethylene signaling is required for enhancing auxin content in tomato stems. Transgenic tobacco plants with a defective ethylene receptor produced fewer ARs than the WT, indicating the importance of ethylene signaling in this process (McDonald and Visser, 2003). Auxin plays a crucial role in the induction of AR formation (Torrey, 1976), and the increased IAA content in flooded stems is likely to be the trigger for AR production. In *A. thaliana*, a transient increase in the transcript levels of *IAA2* and *IAA3* (auxin-responsive genes) was observed in hypoxia-treated root cultures, and this appears to confirm the participation of IAA in flooding adaptations (Klok *et al.*, 2002). Aloni *et al.* (2006) suggested that ethylene released from the vascular bundle results in local accumulation of IAA, which is responsible for AR initiation in stems. Tomato seedlings (cv. Pearson) treated with ACC under aerobic conditions showed a reduction in auxin transport in hypocotyls and an enhancement of transport in roots, but no IAA accumulation was observed in these organs (Negi *et al.*, 2010). In waterlogged plants, the sites of IAA synthesis, the shoot apex and young leaves (Ljung *et al.*, 2005), also show higher flooding-induced ethylene production (Figure 3a). However, this is unlikely to inhibit IAA transport as increased ethylene production in the leaves of flooded tomato plants (Figure 3a) coincides with high IAA levels in flooded organs (Figure 5a). Experiments performed out with the ethylene-overproducing *epi* (*epinastic*) mutant revealed an increase in IAA transport in both hypocotyls and roots (Negi *et al.*, 2010), suggesting that, in agreement with our observations, ethylene may facilitate auxin transport. Moreover, NPA application to flooded WT tobacco plants resulted in a reduction in AR production, similar to the level observed in transgenic ethylene-insensitive plants, indicating that the lower AR formation exhibited by the latter may be related to a reduction in auxin transport towards the base of the plant (McDonald and Visser, 2003).

The increase in IAA levels observed in flooded tomato stems is not restricted to the submerged hypocotyl section (where ARs are produced), but is also observed in epicotyls (Figure 5a). Under flooding conditions, endogenous ethylene may be entrapped by the surrounding water, so physiologically active levels are reached within the submerged plant tissues (Voesenek *et al.*, 1993; Jackson, 2008). The build-up of ethylene is one of the first signals indicating to the plant a change in the gas composition of the root environment (Peeters *et al.*, 2002). Ethylene entrapment by water could explain the localization of ARs on the submerged stem. It is clear that the presence of water around the hypocotyl is required for AR production, and that hypoxia arising from submergence is not sufficient to trigger ARs (Figure 2b). Ethylene entrapment resulted in a higher local ethylene concentration in the submerged organs, triggering *NR* expression (Figure 1c). The expression of *NR* is up-regulated by flooding only in submerged hypocotyls, suggesting an attempt to mitigate ethylene signaling by increasing the presence of ethylene receptors, as these molecules are negative regulators of ethylene signaling (Bailey-Serres and Voesenek, 2008). Up-regulation of ethylene receptor genes has been observed in several plant species subjected to flooding, including *RpERS1* in *Rumex palustris* (Vriezen *et al.*, 1997), *OsERL1* in deepwater rice (Watanabe *et al.*, 2004) and *ETR2* in Arabidopsis (Klok *et al.*, 2002; Branco-Price *et al.*, 2005; Liu *et al.*, 2005; Loreti *et al.*, 2005).

Submergence-dependent ethylene entrapment may affect AR emergence by favoring cell-wall loosening through regulation of apoplastic pH or up-regulation of expansin genes, such as α-*expansin A1* (*RpEXPA1*) in *Rumex palustris* (Vreeburg *et al.*, 2005) and *LeEXP1* in tomato, which promote cell-wall disassembly during fruit maturation (Rose *et al.*, 2000; Zhaohui *et al.*, 2009). Cell-wall loosening would reduce the mechanical resistance of stem tissues and facilitate new root emergence. In Arabidopsis, ethylene and auxin interact to modify the expression of cell wall-related genes, and the action of ethylene is likely to be mediated by auxin (Stepanova *et al.*, 2007). In rice, ethylene induces programmed cell death in epidermal cells located in front of growing ARs. This occurs in order to weaken the epidermis and thus make it possible for the AR to emerge without being damaged (Mergemann and Sauter, 2000). The lack of cell-wall loosening in AVG-treated and *Nr* plants could be the reason why the ARs take longer to grow through fibrous stem tissues.

Interestingly, grafting experiments showed that ethylene perception in the non-flooded aerial parts of the plant (as in WT/*Nr* plants) is sufficient to trigger *de novo* root formation in the flooded stem (Figure 4f). When ethylene perception is normal in the flooded organs (*Nr*/WT plants), AR production does not differ from that is the WT combination (WT/WT). Only when the *Nr* mutation is present in both the rootstock and scion is a reduction in root number observed (*Nr*/*Nr* plants, Figure 4f), as for the non-grafted *Nr* plants shown in Figure 4(a). This indicates that AR production is only reduced when ethylene perception is hampered in both the aerial and flooded parts, as in *Nr* (Figure 4a) and *Nr*/*Nr* grafted plants (Figure 4f). Thus, although ethylene signaling takes place in both the aerial and flooded organs, perception of ethylene either in the aerial or submerged organs is sufficient for normal AR production (*Nr*/Wt and Wt/*Nr* plants). This may be the consequence of two *Nr*-related effects: a reduction in ethylene perception in non-flooded organs (epicotyl, apex, leaves), which may reduce auxin

transport (Figure 4d), and a decrease in ethylene-dependent cell-wall loosening at the stem base, where ARs are produced (Vreeburg *et al.*, 2005).

The experiments reported in this study indicate that there may be interplay between ethylene and auxin with respect to AR production in flooded tomato (Figure 7). Ethylene entrapment by water could be the first signal that warns the plant about the presence of waterlogging, stimulating auxin transport. This auxin accumulates in the stem and triggers additional ethylene synthesis (by inducing *LeACS3* and *LeACS7*) to further stimulate a flux of auxin towards the flooded parts of the plant. Auxin accumulation in the base of the plant induces growth of pre-formed root initials. In addition, ethylene entrapment in the submerged part of the stem may induce hypocotyl cell-wall loosening or programmed cell death, thus favoring AR emergence. Flooding-tolerant species form a large adventitious root system, which can replace the stress-damaged roots when in saturated soils (Jackson and Drew, 1984; Colmer and Voesenek, 2009). This response of tomato plants would be crucial since the formation of a new root system would allow the plant to recover after the damage of the original roots by submergence.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Tomato (*Solanum lycopersicon*) plants cv. Ailsa Craig were used as WT for all experiments, except for those involving ethylene and auxin perception mutants. The ethylene-insensitive mutant *Never ripe* (*Nr*) and the corresponding WT cv. Gimar were provided by Dr G.P. Soressi (Agrobiology and Agrochemistry Department, University of Tuscia, Viterbo, Italy). Seeds from transgenic plants containing the IAA-inducible promoter of *Agrobacterium tumefaciens* gene *5* (*p5*) fused to the GUS reporter in the Chico III background were also provided by Dr G.P. Soressi. The auxin-resistant *diageotropica* (*dgt*) in the Ailsa Craig background was provided by Dr M.G. Ivanchenko (Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR). Seeds were germinated on moist filter paper in Petri dishes, and, after 1 week, plantlets were transferred to 250 ml pots containing peat-based substrate (TG final retail soil, Hawita Flor, http://www.hawita-gruppe.de). Plants were grown at 26°C under a 15 h day/9 h night cycle and 250 μmol m^{-2} sec^{-1} of irradiance provided by high-pressure sodium lamps, and were watered with a nutrient solution (12.5 mM N; 1.30 mM P; 8 mM K; 4 mM Ca; 1.19 mM Mg; 9 mM Na; 1.59 mM S; 9.87 mM Cl; 19.5 mM Fe; 28.6 mM B; 3.6 mM Cu; 4.5 mM Zn; 10.9 mM Mn; 0.2 mM Mo) as required for 3 weeks before use in the experiments. The electrical conductivity of the nutrient solution was 2.84 mS cm^{-1}, and the pH was adjusted to 5.8. To prepare this solution, a 200-fold concentrated stock solution was produced by dissolving the following compounds in 10 L of water: 1.079 kg $Ca(NO_3)_2$, 1.317 kg KNO_3, 14 g chelated Fe with ethylenediamine-N, N′-bis(2-hydroxyphenylacetic acid) (EDDHA), 0.212 kg $MgSO_4$, 0.354 kg KH_2PO_4, 36 g K_2SO_4, 30 g Hidromix (Valagro, http://www.valagro.com) WG, 3 g H_3BO_3. Four-week-old plants were used in all experiments. Unless stated otherwise, each treatment was performed using 12 plants, and experiments were repeated three times.

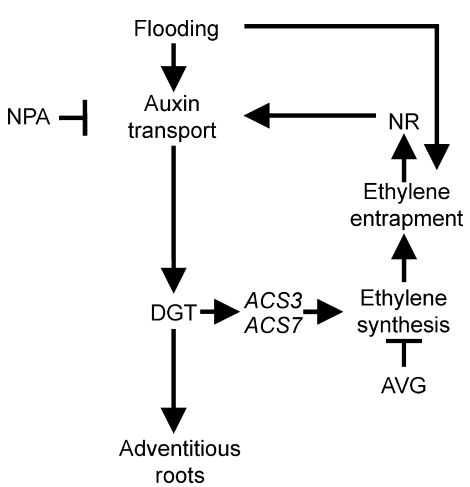

Figure 7. Ethylene and auxin involvement in flooding-induced AR formation in tomato.
Ethylene entrapment by water stimulates transport of auxin, which accumulates in the stem, triggers additional ethylene synthesis and induces growth of pre-formed root initials. Ethylene entrapment in submerged stems may induce hypocotyl cell-wall loosening or programmed cell death, favoring AR emergence. Arrows represent positive regulation and bars represent negative regulation.

Submergence and hypoxic conditions

Glass tanks (25 × 30 × 49 cm) were used for experiments involving plant submergence. Four-week-old plants still growing in the organic potting mix were placed inside the tanks and were flooded with tap water up to the first node above the cotyledons. For control treatments, plants were put inside the tanks and kept at field capacity. In all cases, plants were allowed to acclimatize for 2 days in the containers before the start of treatments. The light intensity inside the tanks where plants were kept was between 60 and 80 μmol m^{-2} sec^{-1} photosynthetically active radiation. Flooding treatments were always initiated at 9 am. Hypoxic treatments were performed using a transparent acrylic airtight chamber containing 1% oxygen.

Inhibitor treatments

Plants were treated with either the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) (32999, Fluka/Sigma-Aldrich, http://www.sigmaaldrich.com) or the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) (33371, Fluka/Sigma-Aldrich). The aerial part of the plants was sprayed with 500 μM AVG or 1 mM NPA 12 h before the start of flooding and at the beginning of the light phase for the first 3 days. Inhibitors were applied in aqueous solutions containing 0.1% Tween-20 to improve the adhesion of the chemical to the leaves. The same solution without addition of AVG and NPA was used to spray control plants simultaneously with treated ones.

Grafting experiment

Three-week-old plants of the ethylene-insensitive *Nr* mutant and the corresponding WT parent cv. Gimar were used. Plants of both genotypes were grafted in all four possible combinations using the cleft graft method. Stems were transversally sectioned approximately 2 cm above the cotyledonary node so that the wound did not affect the hypocotyl. Some plants carried *Nr* as the rootstock and WT (cv. Gimar) as the scion, and others had the opposite arrangement. Plants in which *Nr* and WT scions were grafted onto the same genotype were also produced. These two combinations provided a

control for the wounding effect. The graft area was covered with a piece of cotton, and the rootstock and the scion were held together using a clothes peg for 1 week. Ten plants were obtained per combination. Submergence treatments began 2 weeks after grafting, when plants were 5 weeks old. The water level completely covered the hypocotyls but did not reach the grafting point.

Sample harvest, RNA isolation and real-time PCR

Samples from roots, hypocotyls and epicotyls were collected at various times from the beginning of treatments, immediately frozen in liquid nitrogen, and kept at –80°C until use for RNA extraction. Total RNA from the various tissues was extracted as described previously (Perata *et al.*, 1997) with a minor modification (omission of aurintricarboxylic acid) to make the protocol compatible with subsequent PCR procedures. Electrophoresis using a 1% agarose gel was performed for all RNA samples to check RNA integrity, followed by spectrophotometric quantification. Contaminating DNA was removed using a TURBO DNA-free kit (Ambion, http://www.ambion.com/). RNA was then reverse-transcribed using a high-capacity cDNA archive kit (Applied Biosystems, http://www3.appliedbiosystems.com). Expression analyses of the genes *LeADH2* (*Lycopersicon esculentum ALCOHOL DEHYDROGENASE 2*), *LeACS3* (*Lycopersicon esculentum 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 3*), *LeACS7* (*Lycopersicon esculentum 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 7*), *LeNR* (*Lycopersicon esculentum Never Ripe ethylene receptor*), *LeEF1a* (*Lycopersicon esculentum ELONGATION FACTOR 1-α*) and *LeACT7* (*Lycopersicon esculentum ACTIN 7*) were performed by real-time PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems). Quantitative PCR was performed using 50 ng cDNA and *Power* SYBR® Green PCR master mix (Applied Biosystems), according to the manufacturer's instructions. Expression of *LeEF1A* and *LeACT7* was used as endogenous controls. Relative expression levels were calculated using Genorm (http://medgen.ugent.be/~jvdesomp/genorm/). The following specific primers were used: *LeADH2*, 5′-CTTCTGGGAAGCTAAGGGTCAA-3′ (forward), 5′-ATGGTCTCCTGGTGCAAGGT-3′ (reverse); *LeACS3*, 5′-GGTGCAACTTCCGCTAATGA-3′ (forward), 5′-TACAATCTCCGCCCCAGTTC3′ (reverse); *LeACS7*, 5′-CTTCCAGCTTTCAAAGATGC-3′ (forward), 5′-CGAGGCAAAACATGAGTGTC-3′ (reverse); *LeNR*, 5′-GCTGTTCGTGTACCGCTTTT-3′ (forward), 5′-TTCATCGGGAGAACCAGAAC-3′ (reverse); *LeACT7*, 5′-CGTACAACTGGTATTGTGTTGG-3′ (forward), 5′- CGGTGAGGATCTTCATCAGGT-3′ (reverse); *LeEF1a*, 5′-GCTGCTGTAACAAGATGGATGC-3′ (forward), 5′-GGGGATTTTGTCAGGGTTGTAA-3′ (reverse). Each sample was pooled from four plants, and experiments were repeated three times.

Ethylene quantification

Ethylene production was measured in various tissues harvested 24 h after the onset of flooding or hypoxic treatments, as well as in material collected from the control plants. Each treatment was applied to five plants, and experiments were repeated three times. Samples consisted of leaves (first young fully expanded leaf), epicotyls (pieces of stem approximately 2 cm long taken 1 cm above the water level) and hypocotyls (pieces of submerged stem below the cotyledonary node) from Ailsa Craig, *dgt*, Gimar and *Nr* plants. Once excised, tissues were immediately weighed, placed in 10 or 30 ml flasks for stem parts and leaves, respectively, and incubated for 1 h. In addition, whole plants were grown in a sterile substrate (expanded perlite 3, Agrilit, http://www.perlite.it/en) for 4 weeks and placed inside 2 L glass bottles for flooding treatment. After 24 h, the water was removed, bottles were sealed and gas samples were taken after 1 h incubation. Ethylene was therefore measured as 'de-submergence-induced ethylene'. This is a good indicator of ethylene production/ACS accumulation during flooding, and reflects the full potential of the ethylene biosynthetic machinery (Geisler-Lee *et al.*, 2010). All glass containers had plastic caps with holes in and rubber septa underneath through which 1 ml samples were collected from the head space using a hypodermic syringe. Ethylene concentrations were determined by gas chromatography (Hewlett-Packard 6890; Hewlett-Packard, http://www.hp.com) equipped with a dual flame ionization detector and a metal column (150 cm long, 0.4 cm internal diameter) packed with alumina (70–230 mesh). The column and detector temperatures were 70 and 350°C, respectively. N_2 was used as a carrier at a flow rate of 30 ml min^{-1}. Ethylene identification was performed by comparison with a pure standard. Quantification was achieved using the external standard technique. Ethylene production was calculated on the basis of the fresh weight (FW) of samples and expressed as nl g^{-1} h^{-1}.

Auxin quantification

Plant material (500 mg) harvested from flooded and control plants was collected. Samples consisted of epicotyls and hypocotyls (similar to those for ethylene quantification) from Ailsa Craig, Gimar and *Nr* plants. Each sample was the result of pooling material from five plants. Tissues were ground and extracted using 70% v/v aqueous acetone (5 ml). The homogenate was shaken for 3 h and then centrifuged at 2500 ***g*** for 10 min. The supernatant was collected and another 5 ml acetone was added to the pellet. Shaking and centrifugation were repeated three more times, one of which consisted of overnight shaking. Supernatants were then pooled, and acetone was evaporated using a rotating evaporator (Rotavapor R114; Büchi, http://www.buchi.com) and a water bath at 35°C (Waterbath B480, Büchi). The remaining aqueous solution was then acidified to pH 2.8 with HCl, shaken three times with equivalent volumes of ether for 30 sec, and the supernatant collected each time was pooled. Subsequently, samples were dried under N_2 and methylated using 200 μl diazomethane. Quantitative determination of the level of indole-3-acetic acid (IAA) was performed by competitive ELISA (Phytodetek® IAA test kit; Agdia, http://www.agdia.com). Experiments were repeated three times.

Microscopy and photography

Microscopic observations were performed using a Nikon TMS-F microscope or a Nikon SMZ-2T stereomicroscope (http://www.nikon.com). Photographs of hypocotyl sections under various treatments and at various times after the start of flooding were taken using a DS-U2 Nikon digital sight camera. NIS-Elements F2.20 imaging software (Nikon) was used to capture the images.

GUS assay

GUS activity was analyzed after staining hypocotyl sections for 40 min at 37°C. The staining solution comprised 1 mM X-glucuronide, 100 mM phosphate buffer pH 7, 10 mM EDTA, 0.01% Triton X-100 and 500 μM each of K_3FeCN_6 and K_4FeCN_6. Samples were de-stained using several changes of 95% ethanol at room temperature, and subsequently placed in 70% ethanol. Images were then obtained using the Nikon SMZ-2T stereomicroscope. Three-week-old transgenic plants and the corresponding WTs were submerged for 24 h before cross-sections were obtained.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Root numbers in hypocotyls of flooded and control tomato plants.

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