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Seed germination of ethylene perception mutants of tomato and *Arabidopsis*

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Abstract

The involvement of ethylene in determining the time to radicle protrusion was investigated in ethylene-insensitive gain-of-function (GOF) receptor mutants in tomato and *Arabidopsis*, as well as in single and double loss-of-function (LOF) receptor mutants in *Arabidopsis*. Because ethylene evolution from seeds is coincident with radicle protrusion, and the ability to convert 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene is diagnostic for seed vigour, it was hypothesized that ethylene-insensitive mutants would require more time to complete germination compared to wild-type seeds. Mutant *Never Ripe* (*Nr*) tomato seeds from two genetic backgrounds refuted this hypothesis, while experiments with wild-type seeds, treated with the ethylene action inhibitors, 2,5-norbornadiene or silver thiosulphate, supported it. However, reciprocal crosses between wild-type and *Nr* demonstrated that ethylene insensitivity during seed development determined subsequent time to complete germination, rather than the ability of the embryo/endosperm to perceive ethylene in the mature seed during germination. Additionally, seed quality, determined by standard vigour tests, was reduced in *Nr* compared to wild-type seeds, establishing a disconnection between rapid completion of germination and seed vigour. In *Arabidopsis*, all ethylene-insensitive GOF, and five of six single LOF mutants, required more time to complete 50% radicle protrusion, while double LOF mutants required the same, or less, time to complete germination compared to wild-type seeds. These findings support a role for ethylene perception in determining the length of time *Arabidopsis* seeds remain in the lag phase prior to radicle protrusion.

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Introduction

Ethylene is a simple unsaturated hydrocarbon that plays significant roles in coordinating and regulating a wide variety of growth and developmental processes (Bleecker and Kende, 2000). It has been implicated in the coordinated control of seed development, dormancy and germination (Matilla, 2000). The majority, if not all, of the species evaluated have seeds that produce ethylene prior to, or concomitant with, radicle protrusion. However, except in isolated cases, ethylene does not appear to be required for completion of germination, but rather enhances the ability to complete germination under unfavourable conditions, as is the case for lettuce germinated at high temperature (Abeles, 1986). Supporting this contention is the observation that seeds with high vigour produce more ethylene than those of low vigour (Gorecki *et al*., 1991; Khan, 1994; Siriwitayawan, 2002).

The ethylene response is regulated at multiple levels, from hormone synthesis and perception to signal transduction and transcriptional regulation (Johnson and Ecker, 1998). Evidently, the ability to produce ethylene during germination is most closely associated with 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase synthesis and activity (De Rueda *et al*., 1995; Petruzzelli *et al*., 2000). It is less clear whether or not the ability to perceive ethylene is required for the initiation of germination, or is associated with germination performance, i.e. time required to complete germination. Most studies using inhibitors of ethylene production or action have not demonstrated a clear requirement for ethylene production for initiation of radicle protrusion (Matilla, 2000). Exceptions include marigold (*Tagetes erecta* L.) and chickpea (*Cicer arietinum* L.) seeds (Lalonde and Saini, 1992; Gallardo *et al*., 1994). The

completion of germination of the latter is dependent on ethylene synthesis in the embryo.

Ethylene perception in plants is coordinated by multiple hormone receptors, sharing common sequences with prokaryotic environmental sensor proteins known as two-component regulators (Lashbrook *et al*., 1998). In *Arabidopsis* (*Arabidopsis thaliana* L.), the receptor family is classified into two subfamilies, the Ethylene Response 1 (ETR1)- and ETR2-like subfamilies (Bleecker, 1999). The ETR1-like subfamily contains ETR1 and Ethylene Response Sensor 1 (ERS1), possessing three hydrophobic sub-domains at the N-terminus and a conserved histidine kinase domain. The ETR2-like sub-family [ETR2, Ethylene Insensitive 4 (EIN4) and ERS2] contains an additional hydrophobic extension at the N-terminus and a degenerate histidine kinase domain that lacks one or more elements essential for catalytic activity (Bleecker, 1999). In tomato (*Lycopersicon esculentum* L. Mill), homologues of the *Arabidopsis* ethylene receptors have been identified (Lashbrook *et al*., 1998). *LeETR3* maps to the chromosomal locus corresponding to *Never Ripe* (*Nr*) (Lashbrook *et al*., 1998), a tomato mutant identified by dysfunctional fruit ripening (Rick and Butler, 1956). *Nr* exhibited similarity to *Arabidopsis ERS1*, which is also missing the response regulator domain (Hua *et al*., 1995). In the ethylene signal transduction pathway, the CONSTITUTIVE TRIPLE RESPONSE 1 protein (CTR1) is downstream of the ethylene receptor genes and negatively regulates the ethylene response pathway by inhibiting EIN2 activity (Ecker, 1995).

Using the model published by Bleecker (1999), the premise that ethylene is involved in enhancing seed vigour (Khan, 1994), and the time required for radicle emergence as a measure of seed vigour (AOSA, 1983), it was hypothesized that seeds with reduced ability to perceive ethylene should take longer to complete germination. A corollary of this hypothesis is that loss-of-function (LOF) receptor mutants should increase the speed of seed germination (i.e. require less time to complete germination) as the number of non-functional receptors increases. Therefore, the objectives of the current study were to use dominant, gain-of-function (GOF) ethylene receptor mutants in tomato and *Arabidopsis*, and recessive, LOF receptor mutants in *Arabidopsis*, to study the association between ethylene perception and time to complete seed germination.

Materials and methods

Plant material and seed germination conditions

Wild-type and *Nr* tomato (*Lycopersicon esculentum* L. Mill. 'Rutgers' and 'Ailsa Craig') seeds were originally obtained from the C.M. Rick Tomato Genetics Resource Center (University of California, Davis, California, USA) and subsequently grown in field plots at the University of Kentucky Horticultural Research Farm. Mutant and wild-type plants were grown in the same plots, at the same time, and fertilization, watering and cultural practices were identical. Seeds, harvested from fruit on the same day and cleaned in the same manner, were subsequently dehydrated (8% moisture), placed in glass bottles and stored at 4°C.

Arabidopsis GOF ethylene receptor mutants, *etr1-1*, *etr1-3*, and *ein4-1* and wild ecotype Columbia were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, Ohio, USA) and subsequently grown in identical greenhouse conditions (25°C, under ambient light) at the same time. Seeds were harvested from mature siliques on the same day and stored at room temperature (20°C) for 3 months before use. *Arabidopsis* recessive LOF ethylene receptor mutants were obtained from Dr Elliot Meyerowitz, California Institute of Technology, Pasadena, California, USA. Wild-type Columbia, single mutants *etr1-6*, *-8*, *etr2-2*, *-3*, *ein4–4*, *-7*, and double mutants *etr1-6*/*etr2-3* and *etr1-7*/*etr2-3* were grown in a growth chamber at alternate cycles of 8 h at 20°C in darkness followed by 16 h at 25°C in light (45 μmol s^{-1} m^{-2}). Seeds from mature siliques were harvested on the same day, and used for germination tests before and after moist chilling treatment (seeds imbibed on 2 discs of moist blotter paper in Petri dishes for 3 d at 3°C).

Tomato and *Arabidopsis* seed germination tests were done in Petri dishes on moist blotters incubated at alternating temperatures of 25 and 30°C, with 8 h at 30°C in the dark (AOSA, 1993). Germination percentages were calculated as the total numbers of seeds completing germination at 60 and 120 h after imbibition for *Arabidopsis* and tomato, respectively. The completion of germination was defined as the radicle visibly protruding from the testa. The time required for seeds to attain 50% radicle protrusion (T_{50}) was determined from a linear regression of probit transformed germination percentage data collected every 8 or 4 h for tomato and *Arabidopsis*, respectively. Four replications of 50 seeds per treatment were statistically evaluated using the least significant difference (LSD) or Tukey's honestly significant difference tests (SAS Institute, 1988).

Saturated salt accelerated ageing and cold test

Tomato seeds (wild-type 'Ailsa Craig' and *Nr*) were placed on wire mesh screens over 40 ml of saturated potassium chloride (KCl) in 'accelerated aging boxes' (Hoffman Manufacturing Inc., Oregon, USA) at 45°C and approximately 78% relative humidity

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(McDonald, 1998). Seeds were treated for 3–8 d, followed by rapid forced-air drying to 10% moisture content [fresh weight basis (MCFW)]. Aged seeds were evaluated for germination within 24 h of drying.

For the saturated tray cold test (modified from TeKrony and Woltz, 1997), two replications of 50 seeds were planted on water-saturated soil (Lanton silty clay loam), obtained from the University of Kentucky Spindletop Research Farm. Before use, soil was sieved through a 20-mesh screen. Subsequently, seeds were planted on the soil surface and incubated at 10°C for 7 d in the dark, followed by 14 d at 25°C in the light before being evaluated for normal or abnormal seedlings.

Ethylene determination

Ethylene evolution was measured from four replications of 50 wild-type or *Nr* tomato seeds from 'Rutgers' or 'Ailsa Craig', treated with ACC (0, 1 or 5 mM) for 12, 24, 36, 48 and 60 h. Four replications of 50 *Arabidopsis* wild-type or *etr1-1* seeds were treated with ACC (0, 1 or 5 mM) for 20, 24, 28 and 32 h. Petri dishes of both tomato and *Arabidopsis* were placed into germinators under conditions described previously for each species.

Ethylene evolution was detected by moving tomato or *Arabidopsis* seeds from a Petri dish into dry 25-ml Erlenmeyer flasks or 2-ml vials for tomato or *Arabidopsis*, respectively. Flasks were sealed with serum stoppers and, after 2 h of incubation, a 1 ml gas sample was withdrawn for ethylene evaluation with a syringe. A gas chromatograph (Buck Scientific, East Norwalk, Connecticut, USA), with flame ionization detector (155°C) and alumina column (125°C), with a nitrogen flow rate of 1 ml min^{-1}, was used to determine ethylene evolution.

Endogenous ACC content

ACC content was measured in four replications of 50 wild-type or *Nr* tomato seeds from 'Rutgers' or 'Ailsa Craig', collected after 0, 12, 24, 36, 48 and 60 h imbibition on water under the germination conditions described previously. Seeds were frozen in liquid nitrogen and stored at –80°C until extracted. Seeds were placed in a mortar and pestle and ground in 2 ml of 80% ethanol. The slurry was transferred into a test tube and incubated at 70°C for 30 min. Following centrifugation, the supernatant was transferred to a new tube, the pellet re-extracted, centrifuged, and supernatants combined. Extracts were evaporated to dryness *in vacuo*, reconstituted in water, chloroform added (1:1 v/v), vortexed, centrifuged, and the aqueous layer recovered. Subsequently, the aqueous phase was assayed for ACC-derived ethylene production according to methods from McKeon *et al.* (1982) and Lizada and Yang (1979). Internal standards of ACC indicated an extraction efficiency for the assay of 88 ± 2%.

Ethylene action inhibitors and seed germination

Three replications of 25 wild-type and *Nr* tomato seeds from 'Rutgers' and 'Ailsa Craig' were imbibed in an atmosphere of 0, 1000 or 3000 p.p.m. 2,5-norbornadiene (NBD), a competitive inhibitor of ethylene at the receptor-binding site (Reid, 1995). Open Petri dishes containing seeds and water were placed on top of water-saturated paper towels inside airtight 0.5-litre Mason jars fitted with a serum stopper. The appropriate amount of NBD was injected into each jar, based on the volume of the jar with towels and Petri dishes added. Jars were vented daily and NBD replaced at its original concentration.

Four replicates of 50 tomato seeds were treated with silver thiosulphate (STS), an inhibitor of ethylene action that interferes with the ethylene receptor complex (Sisler and Yang, 1984), at concentrations of 0, 0.5, 1, 1.5 and 2.0 mM. Germination was tested in Petri dishes as described previously.

Reciprocal crosses

Tomato wild type and *Nr* from 'Rutgers' or 'Ailsa Craig' plants were grown under greenhouse conditions. Hand pollination of emasculated flowers provided reciprocal crosses between wild type and *Nr*. This yielded homozygous wild-type and *Nr* seeds, as well as seeds containing a heterozygous embryo (with a heterozygous endosperm containing one or two copies of *Nr*, if *Nr* was the male or female parent, respectively). These heterozygous tissues were enclosed within a testa homozygous for wild type or *Nr*, if *Nr* was the paternal or maternal parent, respectively. Mature fruits were harvested and seeds collected, dried, and stored at room temperature until germination was evaluated.

Reciprocal crosses were also made between *Arabidopsis* wild-type and *etr1-1* plants grown in a growth chamber at alternating cycles of 8 h at 20°C in darkness followed by 16 h at 25°C in light (45 µmol m^{-2} s^{-1}). Seeds from mature siliques were harvested and used in germination tests after moist chilling (3 d at 3°C).

Results

Tomato

Nr tomato seeds exhibited a reduction in time to complete germination compared to wild-type seeds in both 'Rutgers' and 'Ailsa Craig' genetic backgrounds

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(Table 1). Wild-type 'Rutgers' or 'Ailsa Craig' required approximately 12 or 24 additional hours, respectively, to attain 50% germination compared to their *Nr* counterparts. Treating seeds with 5 mM ACC reduced the time to 50% germination in wild-type seeds of both genotypes by approximately 5 h, but had no effect on the germination speed of *Nr* seeds (Table 1).

Endogenous ACC content was initially higher in dry seeds from *Nr* in both 'Rutgers' and 'Ailsa Craig', compared to wild-type seeds, and remained higher throughout imbibition and early radicle protrusion (Fig. 1). Correspondingly, ethylene evolution was greater in *Nr* seeds. Ethylene evolution after 48 h imbibition was 0.6 or 1.1 nl h^{-1} g dw^{-1} from wild-type, compared to 3.2 or 21.6 nl h^{-1} g dw^{-1} from *Nr* seeds of 'Rutgers' and 'Ailsa Craig' backgrounds, respectively. Similarly, *Nr* seeds treated with 5 mM ACC for 48 h had a two- or threefold increase in ethylene evolution (Fig. 1), relative to wild-type seeds on 5 mM ACC, for 'Rutgers' and 'Ailsa Craig', respectively. 'Ailsa Craig' seeds in both wild type and *Nr* began to evolve ethylene approximately 12 h sooner than 'Rutgers', and evolved large quantities of ethylene in the presence of 5 mM ACC.

The ethylene action inhibitor NBD (3000 µl l^{-1}) increased the time required to complete 50% germination in wild-type seeds of both 'Rutgers' and 'Ailsa Craig' (Table 2). The germination percentage of wild-type seeds of both genotypes declined by approximately 10–20% at 3000 µl l^{-1} NBD (Table 2). NBD at 1000 or 3000 µl l^{-1} significantly inhibited the speed, but not the percentage, of germination of *Nr* seeds (Table 2). Time to complete 50% germination of 'Rutgers' seeds in 3000 µl l^{-1} NBD was delayed by 31.0 h (WT) and 30.8 h (*Nr*) relative to air. In 3000 µl l^{-1} NBD, the time to 50% completion of germination of 'Ailsa Craig' seeds was delayed by 58.8 h (WT) and 58.1 h (*Nr*). Hence, the degree of delay to 50% germination induced by 3000 µl l^{-1} NBD was similar in WT and *Nr* mutant seeds within a cultivar. Silver thiosulphate (STS) increased the time needed to complete 50% germination in wild-type and *Nr* seeds of 'Rutgers', but only slowed germination in wild-type 'Ailsa Craig' (Table 3). Germination percentages were unaffected by STS treatment (data not shown).

Development in an *Nr* fruit environment had a significantly negative impact on seed quality (Table 4). Although the percentage of seeds that had completed germination after 3 d for *Nr* was considerably greater than wild type in both genetic backgrounds, there was an approximate 20% reduction in seeds capable of completing germination after accelerated ageing for both genotypes harbouring *Nr*, relative to their respective wild-type background (Table 4). Additionally, there was a 30% reduction in the completion of germination in the cold test for 'Rutgers' and a 15% reduction for 'Ailsa Craig'.

Time to complete 50% germination differed in reciprocal crosses between wild-type and ethylene-insensitive mutant seeds, depending on whether wild type or *Nr* plants were used as the female parent (Table 5). When wild-type plants were used as female parents, the time required to complete 50% germination was not different from seeds obtained from wild-type self-pollinated flowers. When *Nr* plants were used as female parents, seeds completed germination equally as fast as seeds from homozygous *Nr* plants for both 'Rutgers' and 'Ailsa Craig' (Table 5).

Arabidopsis

In contrast to tomato, *Arabidopsis* GOF ethylene receptor mutants (*etr1-1*, *etr1-3* and *ein4-4*) required 4–6 h longer to complete 50% germination compared to WT *Arabidopsis* (Table 6). Exogenously applied ACC reduced the time required to complete 50% germination in WT, *etr1-3* and *ein4-4*, but not *etr1-1*

Table 1. Average time to complete 50% radicle protrusion ± SE, and average germination percentage ± SE, of wild-type and *Never Ripe* (*Nr*) tomato seeds treated with 1-aminocyclopropane-1-carboxylic acid (ACC)

	ACC concentration (mM)			
	0		5	
Genotype	T_{50} [z]	Germination percentage	T_{50}	Germination percentage
'Rutgers'				
Wild type	79.8 ± 0.6 a, A [y]	90.0 ± 0.8 a	75.2 ± 0.7 a, A	89.0 ± 1.3 a
Nr	67.9 ± 0.9 b, A	91.0 ± 1.3 a	66.9 ± 0.6 b, A	90.0 ± 1.0 a
'Ailsa Craig'				
Wild type	77.6 ± 0.9 a, A	90.0 ± 0.8 a	72.0 ± 0.5 a, B	89.0 ± 0.7 a
Nr	52.8 ± 0.8 b, A	90.0 ± 0.8 a	50.3 ± 0.7 b, A	91.0 ± 0.5 a

[z] T_{50} is the time (h) required for 50% of the seeds to complete radicle protrusion.
[y] Means followed by the same lower-case letter within a column and cultivar, or upper-case letter within a row, for T_{50} were not significantly different by Tukey's test ($P < 0.05$).

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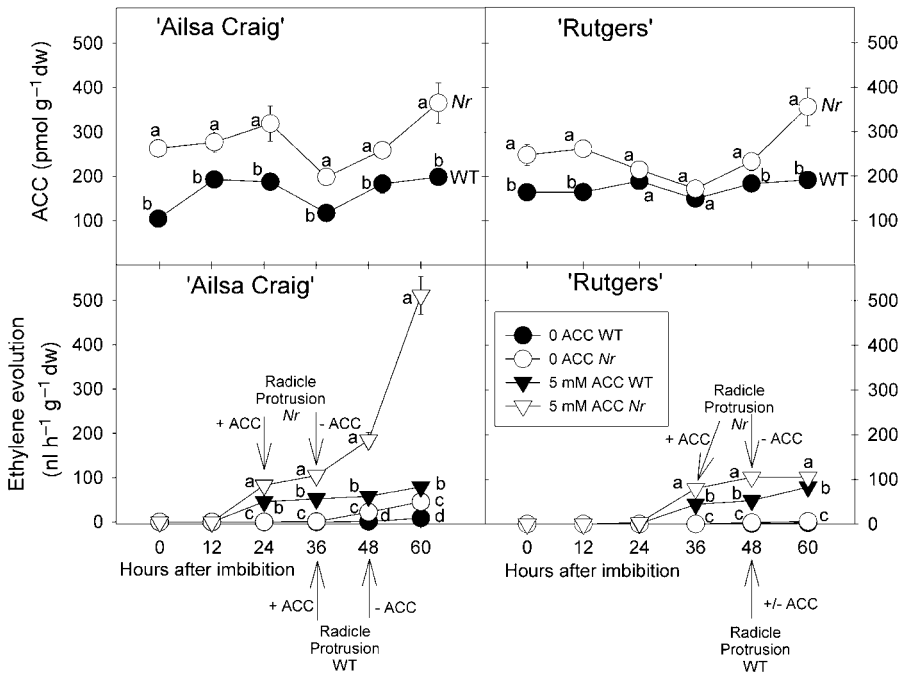

Figure 1. 1-Aminocyclopropane-1-carboxylic acid (ACC) content in, and ethylene evolution from, wild-type (WT) and *Never Ripe* (*Nr*) tomato seeds treated with or without 5 mM exogenous ACC. Means ± SE followed by the same letter at each time point were not significantly different by LSD test ($P \leq 0.05$). Radicle protrusion (arrows) commenced at 48 h for WT seeds on water regardless of cultivar, and at 48 and 36 h on ACC for 'Rutgers' and 'Ailsa Craig', respectively. Radicle protrusion for *Nr* commenced at 48 and 36 h for 'Rutgers' and 'Ailsa Craig' on water, respectively, and at 36 and 24 h on ACC. Open circles in graphs of ACC concentration are values for *Nr*; closed circles are values for wild type. Graphs depicting ethylene evolution from seeds use open symbols for *Nr* and closed symbols for wild type. Circles are evolved ethylene values from seeds imbibed on water; triangles are evolved ethylene values from seeds imbibed on 5 mM exogenous ACC.

Table 2. Average time to complete 50% radicle protrusion ± SE and average germination percentage ± SE of wild-type and *Never Ripe* (*Nr*) tomato seeds treated with 2,4-norbornadiene

	2,4-norbornadiene concentration (μl l^{-1})					
	0		1000		3000	
Genotype	T_{50}[z]	Germination percentage	T_{50}	Germination percentage	T_{50}	Germination percentage
'Rutgers'						
Wild type	84.6 ± 0.9 a, B[y]	94.0 ± 2 a, A	89.9 ± 1.4 a, B	83.0 ± 3 b, B	115.6 ± 3.0 b, A	74.0 ± 2 b, B
Nr	57.5 ± 1.9 b, C	92.0 ± 7 a, A	69.0 ± 1.2 b, B	97.0 ± 2 a, A	88.3 ± 2.8 c, A	92.0 ± 2 a, A
'Ailsa Craig'						
Wild type	81.1 ± 1.3 a, B	95.0 ± 1 a, A	78.1 ± 1.0 a, B	94.0 ± 2 a, A	139.9 ± 2.9 a, A	85.0 ± 3 a, B
Nr	56.4 ± 2.1 b, C	96.0 ± 2 a, A	78.1 ± 2.0 a, B	94.0 ± 2 a, A	114.5 ± 6.2 b, A	92.0 ± 4 a, A

[z] T_{50} is the time (h) required for 50% of the seeds to complete radicle protrusion.
[y] Means followed by the same lower-case letter within a column and cultivar, or upper-case letter within a row, for T_{50} or germination were not significantly different by Tukey's test ($P \leq 0.05$).

(Table 6). In WT and *etr1-3*, application of 0.1 mM ACC was more effective than 1.0 mM in reducing the time to complete 50% germination. Additionally, the germination percentage was increased to that of WT seeds by ACC application to seeds of *etr1-3* and *ein4-4* mutants, but did not affect the germination percentage in WT or *etr1-1* seeds.

Seeds from *Arabidopsis etr1-1*, imbibed on water, initially evolved more ethylene compared to wild-type seeds, but showed no differences after 32 h imbibition (Fig. 2). In contrast, there were no differences in ethylene production when seeds were imbibed on 1 or 5 mM ACC for 32 h, except for seeds treated with 1 mM ACC after 24 h (Fig. 2).

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Table 3. Average time (h) to complete 50% radicle protrusion ± SE of wild-type and *Never Ripe* (*Nr*) tomato seeds treated with silver thiosulphate

	Silver thiosulphate concentration (mM)				
Genotype	0	0.5	1.0	1.5	2.0
'Rutgers'					
Wild type	69.0 ± 2.3 c[z]	71.7 ± 0.9 ab	73.1 ± 0.8 bc	71.8 ± 1.1 bc	75.4 ± 0.9 a
Nr	59.6 ± 1.1 b	66.3 ± 1.0 a	57.7 ± 1.3 b	63.4 ± 2.5 ab	69.4 ± 1.4 a
'Ailsa Craig'					
Wild type	72.1 ± 1.8 b	74.7 ± 0.7 ab	72.7 ± 1.8 b	76.2 ± 1.1 a	77.2 ± 0.8 a
Nr	56.0 ± 1.5 a	59.8 ± 0.8 a	58.6 ± 0.5 a	60.2 ± 1.6 a	59.5 ± 0.5 a

[z] Means followed by the same lower-case letter within a row were not significantly different by Tukey's test ($P \leqslant 0.05$).

Table 4. Seed quality of wild-type and *Never Ripe* (*Nr*) tomato seeds as indicated by average time to radicle protrusion, standard germination and vigour tests. Averages (h) are ± SE

		Germination percentage			
Genotype	Moisture content (%)	3 d[z]	Standard	AA[y]	Cold test
'Rutgers'					
Wild type	8.0 ± 0.1 a[w]	41 ± 2 b	91 ± 1 a	78 ± 3 a	63 ± 4 a
Nr	7.7 ± 0.3 a	73 ± 2 a	96 ± 3 a	53 ± 3 b	32 ± 3 b
'Ailsa Craig'					
Wild type	8.0 ± 0.2 a	28 ± 2 b	87 ± 2 b	76 ± 3 a	55 ± 2 a
Nr	8.0 ± 0.2 a	92 ± 1 a	97 ± 1 a	57 ± 1 b	40 ± 2 b

[z] Radicle protrusion 3 d after imbibition.
[y] Germination percentage following accelerated ageing (AA).
[w] Mean separation within column and cultivar by LSD ($P \leqslant 0.05$).

Table 5. Average time (h) to complete 50% radicle protrusion ± SE and average germination percentage ± SE of seeds from reciprocal crosses between wild-type and *Never Ripe* (*Nr*) tomato

Genotype	T_{50}	Germination percentage
'Rutgers'		
Wild type (WT)	62.4 ± 1.6 a[z]	83 ± 3 c
Nr	56.8 ± 1.4 b	100 ± 2 a
WT × *Nr*[y]	63.5 ± 2.0 a	91 ± 4 b
Nr × WT	55.7 ± 3.0 b	98 ± 2 ab
'Ailsa Craig'		
Wild type (WT)	63.3 ± 0.7 a	89 ± 3 ab
Nr	54.9 ± 0.9 b	93 ± 2 a
WT × *Nr*	62.0 ± 0.8 a	81 ± 3 b
Nr × WT	54.9 ± 0.4 b	91 ± 3 a

[z] Means followed by the same lower-case letter within a column and cultivar were not significantly different by the LSD test ($P \leqslant 0.05$).
[y] The female parent is written first.

Without a moist chilling treatment, *Arabidopsis* LOF ethylene-receptor mutants imbibed on water exhibited differences in both speed of germination and germination percentages, depending on the specific gene(s) that was knocked out (Table 7). Compared to wild-type seed germination, *etr1-6*, *etr1-8*, *etr2–2*, *etr2-3* and *ein4-7* required more time to complete 50% germination, while *etr1-6*/*etr2-3* required less time. There were no differences in the speed or percentage germination detected for *ein4-4* and *etr1-7*/*etr2-3* relative to wild type (Table 7). The germination percentage for *etr1-6*, *etr1-8*, *etr2–2*, *etr2-3* and *ein4-7* seeds was less than that of *ein4-4*, *etr1-6*/*etr2-3*, *etr1-7*/*etr2-3* and WT seeds. However, when seeds received moist chilling for dormancy release, only *etr1-8* and *ein4-7* had lower germination percentages than WT seeds (Table 7). Moist chilling also reduced the time required to complete 50% germination between 15 and 40 h, compared to seeds without prior moist chilling. After moist chilling, only the double mutant *etr1-6*/*etr2-3* required less time to attain 50% germination compared to WT seeds, while *etr1-8*, *etr2-2*, *etr2-3*, *ein4-4* and *ein4-7* required more time to complete germination.

In *Arabidopsis*, reciprocal crosses between WT and *etr1-1* showed that when *etr1-1* was selfed or used as the female parent, seeds required more time to complete 50% germination (Table 8) and had a percentage germination significantly less than that of WT seeds or seeds produced from a cross with WT as the maternal parent (Table 8).

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Table 6. Average time to complete 50% radicle protrusion ± SE and average germination percentage ± SE of wild-type and ethylene-insensitive (gain-of-function) *Arabidopsis* seeds (Columbia) treated with 1-aminocyclopropane-1-carboxylic acid (ACC)

Genotype	ACC (mM)	T_{50} [z]	Germination percentage
Wild type	0	34.9 ± 0.3 b[y]	95 ± 1 a
	0.1	32.6 ± 0.5 c	95 ± 2 a
	1.0	34.8 ± 0.5 b	96 ± 1 a
etr1-1	0	38.7 ± 0.3 a	75 ± 4 c
	0.1	38.3 ± 0.7 a	76 ± 2 c
	1.0	38.4 ± 0.3 a	77 ± 2 c
etr1-3	0	41.5 ± 1.9 a	62 ± 3 d
	0.1	33.4 ± 1.5 c	94 ± 2 a
	1.0	35.6 ± 0.5 b	94 ± 2 a
ein4-4	0	41.0 ± 1.0 a	84 ± 4 b
	0.1	35.0 ± 0.4 b	93 ± 1 a
	1.0	35.6 ± 0.4 b	91 ± 1 a

[z] T_{50} is the time (h) required for 50% of the seeds to complete radicle protrusion.
[y] Means followed by the same lower-case letter within a column and ACC concentration were not significantly different by Tukey's test ($P \leq 0.05$).

Discussion

Application of ACC to tomato (Table 1) and *Arabidopsis* (Table 6) seeds reduced the time to radicle protrusion. In addition, previous work has shown that the time to complete germination was correlated with the ability to produce ethylene in high- and low-vigour tomato seeds (Siriwitayawan, 2002). These data suggest a physiological role for ethylene in determining the time interval between imbibition and radicle protrusion. Such a physiological role has been defined in tobacco seeds, where a gene for β-1,3-glucanase, an enzyme known to participate in endosperm rupture (Leubner-Metzger and Meins, 2000), is up-regulated by ethylene (Leubner-Metzger *et al*., 1998). However, a more compelling case for a physiologically significant role for ethylene could be derived if seeds impaired for ethylene perception exhibited slower germination compared to wild-type seeds. The present study using tomato and *Arabidopsis* ethylene-insensitive mutants and chemical inhibitors of ethylene perception generally supports a positive role for ethylene in determining the time required to initiate radicle protrusion. The evidence is more

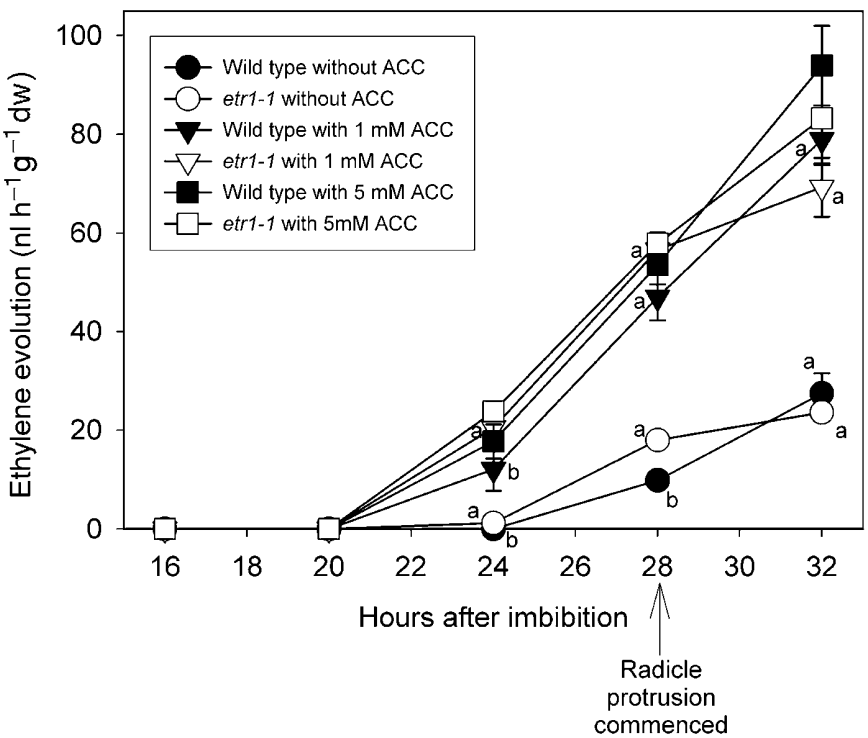

Figure 2. Ethylene evolution from WT and *etr1-1 Arabidopsis* seeds on water, or treated with 1 or 5 mM exogenous 1-aminocyclopropane-1-carboxylic acid (ACC). Within an ACC treatment, means ± SE, followed by the same letter at each time point, were not significantly different by an LSD test ($P \leqslant 0.05$). Ethylene evolution from wild-type and mutant seeds on 5 mM ACC did not differ significantly (letters not shown, for clarity). Radicle protrusion (arrow) commenced from wild-type Columbia and from *etr1-1* mutant seeds at 28 h, regardless of the imbibition media. Open symbols depict ethylene evolution from *etr1-1* seeds; closed symbols are wild-type seed values. Circles are evolved ethylene values from seeds imbibed on water; triangles and squares are evolved ethylene values from seeds imbibed on 1 or 5 mM exogenous ACC, respectively. Prior to 20 h imbibition, no ethylene was detected from the seeds, regardless of treatment.

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Table 7. Average time to complete 50% radicle protrusion ± SE and average germination percentage ± SE of wild type and single and double loss-of-function, ethylene-receptor mutant *Arabidopsis* seeds, with and without moist chilling

Genotype	Without moist chilling		With moist chilling	
	T_{50} [z]	Germination percentage	T_{50}	Germination percentage
Wild type	40.2 ± 0.6 e[y]	97 ± 1 a	21.1 ± 0.5 d	98 ± 1 ab
etr1-6	48.0 ± 0.9 d	86 ± 4 b	20.7 ± 0.4 d	96 ± 1 ab
etr 1-8	67.3 ± 1.9 b	64 ± 9 c	29.1 ± 0.4 b	83 ± 1 c
etr2-2	50.5 ± 0.6 d	85 ± 2 b	23.7 ± 1.6 c	90 ± 6 abc
etr2-3	56.1 ± 1.8 c	66 ± 3 c	28.3 ± 0.5 b	90 ± 2 bc
ein4-4	38.4 ± 0.7 e	98 ± 2 a	25.1 ± 0.8 c	91 ± 4 ab
ein4-7	74.0 ± 2.0 a	45 ± 3 d	38.2 ± 0.1 a	58 ± 1 d
etr1-6/etr2-3	30.0 ± 0.3 f	100 a	17.9 ± 0.6 e	98 ± 1 a
etr1-7/etr2-3	40.8 ± 0.5 e	95 ± 2 ab	21.0 ± 0.8 d	92 ± 3 ab

[z] T_{50} is the time (h) required for 50% of the seeds to complete radicle protrusion.
[y] Means followed by the same lower-case letters within a column were not significantly different by LSD test ($P \leqslant 0.05$).

Table 8. Average time (h) to complete 50% radicle protrusion ± SE and average germination percentage ± SE of seeds from reciprocal crosses between wild type and *etr1-1 Arabidopsis*

Genotype	T_{50}	Germination percentage
Wild type (WT)	33.6 ± 0.3 b[z]	87 ± 1 a
etr1-1	37.5 ± 0.5 a	80 ± 1 b
WT × *etr1-1*[y]	34.1 ± 0.4 b	85 ± 1 a
etr1-1 × WT	35.9 ± 0.7 a	72 ± 1 c

[z] Means followed by the same lower-case letter within a column were not significantly different by LSD test ($P \leqslant 0.05$).
[y] The female parent is written first.

compelling for *Arabidopsis* than tomato, suggesting that the mechanism controlling germination time is different in the two species.

The ethylene-insensitive tomato mutant *Nr* represents a GOF mutation in one of several tomato ethylene receptors (Lashbrook *et al*., 1998). Contrary to expectations based on our hypothesis, *Nr* seeds from two genetic backgrounds completed germination faster than wild-type seeds produced under identical cultural conditions (Table 1). The model for ethylene perception in *Arabidopsis* indicates that a GOF mutation in any ethylene receptor should continue to activate CTR1, the next identified protein in the signal transduction pathway, even in the presence of ethylene. The continuous activation of CTR1, even in the presence of ethylene, leads to repression of EIN2 and the silencing of the ethylene response, resulting in an ethylene-insensitive phenotype (Bleecker, 1999). Faster germination speed of *Nr* tomato seeds relative to wild-type seeds might be explained in one of two ways. First, because *Nr* is a co-dominant mutation that is leaky, it displays some limited ethylene perception (Lanahan *et al*., 1994). Therefore, ethylene may still be enhancing germination speed in the mutant. For germination speed of *Nr* mutant seeds to be faster than wild type, *Nr* mutant seeds must at least still be sensitive to ethylene, and more ethylene must be produced and/or the mutant, paradoxically, made hypersensitive to it. In the present study, 'Ailsa Craig' *Nr* seedlings exposed to exogenous ACC showed some characteristics of a triple response (data not presented). Furthermore, *Nr* in the 'Ailsa Craig' background showed faster completion of germination compared to *Nr* in 'Rutgers', whereas the germination speed of wild-type seeds from both cultivars was comparable, indicating that the same mutation does not behave in a similar fashion in different genetic backgrounds (Table 1). Similar results have been reported previously for developmental events unrelated to seed germination (Lanahan *et al*., 1994). This is particularly evident in the ethylene production from wild-type and mutant seeds of both cultivars, where 'Ailsa Craig' produced more ethylene (much more when imbibed on ACC) than 'Rutgers' (Fig. 1). Somewhat in contrast to *Arabidopsis*, from which Bleecker's (1999) model was derived, *Nr* gene expression was shown to be developmentally regulated in tomato (Lashbrook *et al*., 1998). Hence, at least for one receptor knockout mutant, the remaining tomato receptor genes encoding normal products were capable of compensatory expression (Tieman *et al*., 2000). *Nr* mRNA was present in germinating seeds, but was most abundant during fruit ripening. *LeETR2* mRNA was found in the greatest abundance during imbibition and seed germination, which is a more pronounced tissue specificity than is thought to occur in *Arabidopsis*. Speculating that greater transcript abundance leads to greater protein abundance, it is possible that the LeETR2 receptor, being more abundant than *Nr* in WT seeds and during seed germination, can compensate for and

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allow ethylene perception in *Nr* mutant seeds. This hypothetical compensatory activity of LeETR2, coupled with the greater than usual ethylene production from *Nr* mutant seeds, may result in a reduced time to completion of germination for *Nr* seeds.

The second manner by which faster germination speed of *Nr* seeds, relative to WT, might be explained is based on the seed developmental environment. Therefore, seeds from reciprocal crosses between WT and *Nr* plants were evaluated for the impact of the *Nr* genetic background on seed development. *Nr* plants used as the female parent produced seeds that completed germination at a speed similar to homozygous *Nr* seeds and faster than either WT seeds or seeds from crosses where WT plants were used as the female parent and *Nr* as the pollen source (Table 5). This suggests that the impact of ethylene perception during seed development (possibly related to reduced fruit ripening of the *Nr* mutant) influences seed germination speed upon imbibition. Certainly the vigour of *Nr* seeds was significantly reduced (Table 4), so it is possible that seed development in an ethylene-insensitive fruit environment may influence subsequent germination speed. The testa and endosperm cap are critical in determining germination characteristics of tomato (Groot and Karssen, 1987, 1992; Dahal and Bradford, 1990; Ni and Bradford, 1993; Hilhorst and Downie, 1996) and, due to the dominant nature of the *Nr* mutation, crosses with *Nr* as the female parent would express an ethylene-insensitive phenotype in the testa, endosperm and embryo while crosses with WT as the female would be ethylene insensitive in the endosperm and embryo only. Germination speed was normal for heterozygous plants with the WT as the female. Germination speed was equally rapid, relative to WT, for seeds from homozygous *Nr* plants and for heterozygous seeds from female *Nr* plants. There does not appear to be any dose effect of having two or three copies of *Nr* in the triploid endosperm (equally fast), and heterozygous embryos behave differently depending on the genotype of the plant they developed on. It is, therefore, tempting to ascribe more rapid completion of germination to an ethylene-insensitive testa developing in an ethylene-insensitive environment. Alternatively, the expression of the *Nr* phenotype during seed development could be subject to imprinting, the paternal contribution being silenced. Such epigenetic phenomena have been documented to affect various aspects of seed germination (Kollipara *et al*., 2002). Resolution of this quandary is ongoing, utilizing a backcross of *Nr* heterozygous plants as females with WT males and selfing of *Nr* heterozygous plants.

An alternative approach to ethylene-insensitive mutations was to use the ethylene antagonists NBD and STS to reduce ethylene perception in tomato seeds during germination. In accordance with a model in which ethylene perception stimulated germination speed, wild-type seeds of both cultivars and *Nr* seeds of 'Rutgers' treated with NBD and STS required more time to initiate radicle emergence than non-treated seeds (Tables 2 and 3). STS did not impact time to radicle emergence in 'Ailsa Craig' *Nr* seeds, while NBD tended to delay it. This could be further evidence that *Nr* seeds perceived ethylene during germination and that ethylene was significant in determining germination speed. Conversely, it could indicate that these two inhibitors had a negative, non-specific effect on germination speed. The germination percentage was not affected by NBD or STS, except for NBD with wild-type 'Rutgers' and the highest concentration of NBD with 'Ailsa Craig' wild-type seeds (Table 2 and data not shown).

In *Arabidopsis*, GOF mutations in any one receptor isoform should lead to dominant insensitivity to ethylene, by continuing to activate CTR1, even in the presence of ethylene (Bleecker, 1999). Accordingly, germination speed in *etr1-1*, *etr1-3* and *ein4* seeds was slower than that of WT seeds (Tables 6 and 8). Seeds from *etr1-1* showed no response to exogenous ACC, while *etr1-3* and *ein4* showed reduced time to radicle protrusion and increased germination percentage when germinated in the presence of ACC (Table 6). This corresponds to previous work investigating stem and root growth in ethylene-insensitive mutants exposed to exogenous ethylene, where *etr1-1* mutants did not respond to exogenous ethylene, while *etr1-3* showed only a reduction of ethylene responsiveness (Bleecker and Schaller, 1996). Poor germination in *etr1-1* has been reported previously (Bleecker *et al*., 1988), and one mechanism for this phenomenon may be dependent on abscisic acid (ABA). Mutant screens for seeds that either enhance or suppress ABA sensitivity during germination recovered mutants that were disturbed in their response to ethylene. Ethylene insensitivity led to a greater sensitivity to exogenous ABA and greater ABA biosynthesis (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000), while mutants displaying a constitutive triple response were able to complete germination on greater amounts of ABA than usual by a decrease in seed sensitivity to ABA (Beaudoin *et al*., 2000).

LOF receptor mutations in any single receptor did not result in an ethylene hyper/insensitive phenotype, but LOF for multiple receptors resulted in a more constitutive ethylene response phenotype (*ctr1*-like) (Hua and Meyerowitz, 1998). Therefore, germination speed in single LOF mutants should resemble that of wild-type seeds, while double mutants should show a decrease in time to radicle protrusion. However, without moist chilling, seeds from most single LOF receptor gene mutants (*etr1-6*,

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etr1-8, *etr2-2*, *etr2-3* and *ein4-7*) completed germination slower than WT seeds (Table 7). The relative increase in time for radicle protrusion was not equivalent across genotypes, with *etr1–8* and *ein4-7* germinating significantly slower than WT seeds and other single LOF mutants. Moist chilling increased germination percentages to above 90% in all LOF mutants except *etr1-8* and *ein4-7* (Table 7). The germination percentage of *ein4-7* remained low, suggesting that this mutant may require longer moist chilling to relieve dormancy. All single LOF mutants, as well as WT seeds, required substantially less time for radicle protrusion than did non-stratified seeds. This suggests that mutants were able to respond to dormancy release in a similar fashion to WT seeds. However, germination of four of the six single LOF mutants (*etr1-8*, *etr2-3*, *ein4-4* and *ein4-7*) still exhibited slower completion of germination after moist chilling compared to WT seeds. Slower completion of germination in the single LOF *etr1–8* mutant may be due to reduced capacity for cell elongation observed in hypocotyl and radicle tissue (Hua and Meyerowitz, 1998). However, single LOF *ein4* and *etr2* mutants did not show this defect in cell elongation (Hua and Meyerowitz, 1998), yet they still completed germination slower in the current study. Hua and Meyerowitz (1998) also noted later completion of germination in *etr2* single LOF mutants, but did not provide data for this aspect of development.

Double LOF mutants between *etr1* and *etr2* had greater than 95% germination (Table 7). One double LOF mutation in *Arabidopsis* (*etr1-6*/*etr2-3*) completed germination faster than the wild type, while a second double mutation (*etr1-7*/*etr2-3*) showed no difference from the wild-type control, although it was faster than all single mutants except unchilled *ein4-4* (Table 7). Hua and Meyerowitz (1998) indicated that these double mutants had similar phenotypes to single LOF receptor mutants. However, with regards to germination speed, *etr1* and *etr2* single LOF mutants had slower germination compared to the WT control, while the double mutants had faster or comparable germination to WT. The results with double mutants generally support a role for ethylene in determining germination speed.

Reciprocal crosses between WT and *etr1-1* *Arabidopsis* suggest that ethylene perception during seed development is important for determining germination speed. The cross with *etr1-1* as the maternal parent completed germination slower than WT and similar to the *etr1–1* homozygote (Table 8). Because *etr1-1* is a dominant mutation, the embryo should express an ethylene-insensitive phenotype with *etr1-1* as the maternal or paternal parent. The major parent-of-origin difference in these crosses related to seed development should be the ability to perceive ethylene in the developing fruit and the testa. In a related species, rapeseed (*Brassica napus* L.) embryos produced ethylene in a climacteric fashion early in the pre-desiccation stage (Ward *et al*., 1995). This was associated with ethylene-promoted chlorophyll loss in the embryo during this stage. It was suggested that the silique was involved in controlling ethylene concentration around the embryo (Rodriguez-Gacio and Matilla, 2001). The role of ethylene in seed development in *Arabidopsis* has not been investigated. However, using mutants that showed enhanced or suppressed response to ABA, it was demonstrated that endogenous ethylene promoted seed germination by decreasing sensitivity to endogenous ABA (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000). It is possible that this relationship also impacts ABA-promoted events during seed development. In addition, there is direct evidence that ethylene is important for normal seed development in tobacco. Both co- and antisense-suppression of ACC oxidase in tobacco resulted in an arrested ovule development that led to shorter than usual integuments and female sterility (De Martinis and Mariani, 1999). It is possible that ethylene-insensitive mutants in *Arabidopsis* have altered testa characteristics that could, in turn, impact subsequent seed germination.

Ethylene promotes its own biosynthesis through positive feedback in several tissue types, including tomato fruit (Blume and Grierson, 1997), etiolated pea seedlings (Peck *et al*., 1998) and germinating pea seeds (Petruzzelli *et al*., 2000). These studies demonstrated up-regulation of *ACC-OXIDASE* transcripts by ethylene, resulting in increased ethylene biosynthesis. In the current study using *Nr* tomato mutants, there was a significant increase in ethylene production during germination and an increased ability to convert ACC to ethylene, suggesting higher ACC oxidase activity (Fig. 1). This suggests that there is negative rather than positive feedback for ethylene-induced ethylene production in these ethylene-insensitive tomato mutants. However, as indicated earlier, these mutants still retain some ethylene perception. In *Arabidopsis*, *etr1-1* also produced more ethylene than wild type during early imbibition (24 and 28 h; Fig. 2). However, this difference was not detected after 32 h imbibition, and there was no significant increase in the ability to convert exogenous ACC to ethylene during germination (Fig. 2). Using exogenously applied ethylene or NBD, it was determined that ethylene promotes its own synthesis via increased ACC oxidase activity during pea germination (Petruzzelli *et al*., 2000). However, in tomato and *Arabidopsis*, no positive feedback for ethylene-regulated ethylene biosynthesis was observed. Rather, ethylene production was higher or not different in the ethylene-insensitive mutants

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compared to WT. The dramatic increase in ethylene production in *Nr* tomato suggests that ethylene may actually limit its own synthesis during germination.

In summary, results using ethylene-insensitive tomato mutants conflicted with the hypothesis that ethylene enhances the speed of seed germination, while inhibitors of ethylene action provided general support for a physiological role for ethylene in determining the time to radicle emergence. The deviation of the ethylene-insensitive mutant from the hypothesis was attributed to either the nature of the mutation and/or effects that ethylene insensitivity has on seed development. In *Arabidopsis*, the evidence for the involvement of ethylene in decreasing the length of the lag phase of germination was more compelling than in tomato. However, in *Arabidopsis*, both single GOF and LOF mutations in *ETR1* resulted in slower germination. None the less, double LOF mutants were equal to, or faster than WT, and double mutant seeds completed germination faster than seeds of single mutants from which the double mutant was comprised. It was determined that the impact of ethylene insensitivity on seed development must be considered when interpreting seed germination results in tomato and *Arabidopsis*.

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