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The *Never Ripe* Mutation Blocks Ethylene Perception in Tomato

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Seedlings of tomato fruit ripening mutants were screened for their ability to respond to ethylene. Ethylene induced the triple response in etiolated hypocotyls of all tomato ripening mutants tested except for one, *Never ripe* (*Nr*). Our results indicated that the lack of ripening in this mutant is caused by ethylene insensitivity. Segregation analysis indicated that *Nr*-associated ethylene insensitivity is a single codominant trait and is pleiotropic, blocking senescence and abscission of flowers and the epinastic response of petioles. In normal tomato flowers, petal abscission and senescence occur 4 to 5 days after the flower opens and precede fruit expansion. If fertilization does not occur, pedicel abscission occurs 5 to 8 days after petal senescence. If unfertilized, *Nr* flowers remained attached to the plant indefinitely, and petals remained viable and turgid more than four times longer than their normal counterparts. Fruit development in *Nr* plants was not preceded by petal senescence; petals and anthers remained attached until they were physically displaced by the expanding ovary. Analysis of engineered 1-aminocyclopropane-1-carboxylate (ACC) synthase-overexpressing plants indicated that they are phenotypic opposites of *Nr* plants. Constitutive expression of ACC synthase in tomato plants resulted in high rates of ethylene production by many tissues of the plant and induced petiole epinasty and premature senescence and abscission of flowers, usually before anthesis. There were no obvious effects on senescence in leaves of ACC synthase overexpressers, suggesting that although ethylene may be important, it is not sufficient to cause tomato leaf senescence; other signals are clearly involved.

INTRODUCTION

Ethylene plays a regulatory role in integrating the developmental effects of internal signals and external stimuli in plants (Abeles et al., 1992). In recent years, ethylene's role in regulating climacteric fruit ripening and the triple response of etiolated seedlings has been the subject of intense investigation. In climacteric fruits, ethylene promotes ripening by coordinately inducing the expression of a large number of genes that encode enzymes responsible for different aspects of the ripening process (reviewed in Speirs and Brady, 1991; Giovannoni, 1993; Theologis, 1993). Ethylene synthesis is regulated at the level of 1-aminocyclopropane-1-carboxylate (ACC) synthase gene transcription (Rottmann et al., 1991; Liang et al., 1992; O'Neill et al., 1993). It has been suggested that distinct ACC synthase genes regulate ethylene synthesis in response to different developmental or environmental stimuli by controlling the availability of the precursor ACC (Rottmann et al., 1991; Liang et al., 1992; Kieber and Ecker, 1993; Theologis, 1993). Ethylene is generated from ACC by ACC oxidase, an enzyme that is constitutively present at low levels in many plant tissues. Often the levels of this enzyme are increased by ethylene as well (Hamilton et al., 1991; Picton et al., 1993).

The effect of ethylene on dark-grown seedlings, the so-called "triple response," is an ethylene response that can be visually scored and that has been useful for identifying mutants altered in their ethylene synthesis, perception, and response (for a review, see Kieber and Ecker, 1993). Multiple mutations affecting ethylene perception have been identified in Arabidopsis (Guzmán and Ecker, 1990; Kieber and Ecker, 1993). *Ethylene response 1* (*Etr1*) and *Ethylene insensitive 1* (*Ein1*) are both dominant alleles of a gene that regulates ethylene perception in Arabidopsis (Bleecker et al., 1988; Guzmán and Ecker, 1990). Recessive alleles at other loci have been discovered (such as *ein2*, *ein3*, and *constitutive triple response 1* [*ctr1*]); they affect ethylene perception or response, indicating that a series of events transduce primary ethylene perception in target cells (Guzmán and Ecker, 1990; Kieber et al., 1993).

Pleiotropic effects that these mutations may have on aspects of Arabidopsis growth and development, other than the seedling triple response, are difficult to detect. For example, fruit ripening and flower abscission and senescence cannot be easily studied in Arabidopsis. In contrast, a great deal is known about the physiological effects of ethylene in tomato (Abeles et al., 1992). For example, the epinastic response of tomato petioles to ethylene results from differential expansion of the cells on the upper and lower halves of the petiole. This response in tomatoes is rapid (begins in 1 to 3 hr) and serves as an excellent assay for ethylene action. In tomato flowers, pedicel abscission and regulation of petal senescence have also

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proven to be useful systems for studying ethylene effects (Tucker et al., 1984; Roberts et al., 1993).

Many mutations that affect fruit ripening without obvious pleiotropic effects upon the growth and development of the rest of the plant have been described previously (Tigchelaar et al., 1978; Grierson et al., 1987). Among these are *ripening inhibitor* (*rin*; Robinson and Tomes, 1993), *non-ripening* (*nor*; Tigchelaar et al., 1978), *alcobaca* (*alc*; Kopeliovitch et al., 1980), and *Never ripe* (*Nr*; Rick and Butler, 1956). Fruit from these mutants fail to undergo many of the processes associated with normal fruit ripening. Exogenous ethylene does not significantly affect the ripening phenotype of these mutants, suggesting that they may be ethylene insensitive or defective in steps early in the regulatory pathway of the ripening process (Giovannoni, 1993). *Nr* was previously described as a dominant mutation that results in an incomplete and delayed ripening phenotype; after many months, fruit matures to an orange color externally and softens only marginally (Rick and Butler, 1956; Hobson, 1967). Ripening-associated gene expression has been reported to be ~10 to 30% of normal levels in *Nr* fruit (DellaPenna et al., 1989; Knapp et al., 1989). Modest effects of the *Nr* gene on the rate of flower abscission have also been reported (Tucker et al., 1984). Despite a great deal of work to characterize these ripening mutants, the lesions responsible for the altered ripening phenotypes have not been determined.

Here, we demonstrate that the *Nr* mutant has lost the capacity to respond to either endogenously generated or exogenously applied ethylene in all tissues examined. The *Nr* gene is partially dominant and has pleiotropic effects on plant development. Other ripening mutants examined seem to have lost the ability to develop ripening competence, because they retain ethylene responsiveness.

Arabidopsis mutants that overproduce ethylene (such as *eto1*) or display a constitutive ethylene-response phenotype (such as *ctr1*) have been described previously (Guzmán and Ecker, 1990; Kieber et al., 1993; Kieber and Ecker, 1993). Here, we also document the effects of constitutive ethylene overproduction on tomato plant development. Similar to the ethylene-insensitive phenotype, ethylene overproduction in tomato has effects beyond those previously observed in Arabidopsis. Ethylene overproduction in tomato has clear effects on rates of senescence and abscission in flowers as well as on epinasty in petioles, and it mimics the effects of the *epinastic* (*epi*) mutation in tomato (Fujino et al., 1988, 1989; Ursin and Bradford, 1989).

RESULTS

Nr Seedlings Are Ethylene Insensitive in the Triple Response Assay

In an attempt to identify tomato mutants impaired in ethylene perception or response, previously described mutants with altered fruit ripening phenotypes were selected and tested for ethylene sensitivity. Treatment of the seedlings with ethylene was accomplished by including ACC in the agar media on which the seeds were germinated. This ACC is converted to ethylene by the seedling and will induce a triple response in competent seedlings. Figure 1 shows seedlings of the normal cultivars Ailsa Craig, Pearson, and their corresponding near isogenic lines for the *Nr* mutation 12 days after germination in the presence or absence of 20 μM ACC. In the absence of ACC, seedling hypocotyls and roots were similar among all lines tested. In the presence of ACC, ethylene sensitivity in the normal seedlings (both Pearson and Ailsa Craig) is characterized by short, thick hypocotyls with exaggerated hooks, while the near isogenic *Nr* seedlings lacked a triple response. Figures 1E and 1F show that seedlings homozygous for the *rin* and *nor* mutations (nearly isogenic with cultivars Ailsa Craig and MH1, respectively) are ethylene sensitive. The ripening mutant *alc* also responded normally to ethylene (data not shown). Seedlings heterozygous for the *Nr* mutation (nearly isogenic with Ailsa Craig) displayed an attenuated triple response in the presence of ACC (Figures 1A to 1D). Germination in the presence of 10 ppm of ethylene of all genotypes described resulted in seedling phenotypes identical to those shown in Figure 1 (data not shown).

Nr-Associated Ethylene Insensitivity Is Regulated at a Single Locus

Although a mutation resulting in impaired ripening may seem consistent with the observation of ethylene insensitivity, it was nevertheless necessary to test for the possibility that *Nr*-associated ethylene insensitivity might be due to a genetic lesion at an alternative locus. Consequently, experiments were performed to ascertain (1) the effect of the ethylene-insensitivity allele on seedling phenotype in a segregating population and (2) the genetic linkage relationship between the fruit and seedling phenotypes associated with *Nr*. The first experiment was designed to determine whether (1) *Nr*-associated ethylene insensitivity resides at a single genetic locus or (2) the apparent intermediate ethylene insensitivity observed in the *Nr*/*nr* heterozygote can be utilized in conjunction with the extreme phenotypes as accurate indicators of genotype at the *Nr* locus.

F_2 seed was obtained from a cross between *Lycopersicon esculentum* (cv Ailsa Craig; *Nr*/*Nr*) and *L. cheesmannii* (*nr*/*nr*) followed by selfing of the F_1 progeny. This seed stock had been employed previously for generation of an F_2 restriction fragment length polymorphism mapping population to approximate the position of the *Nr* locus on the tomato genetic map (Tanksley et al., 1992).

F_2 seeds (119) were germinated on water agar supplemented with 20 μM ACC. Following 12 days in the dark, seedlings were scored as ethylene "sensitive" (30), "intermediate" (57), or "insensitive" (32) (Figure 1H). The resulting 1:2:1 ratio is consistent with the hypothesis that *Nr*-associated

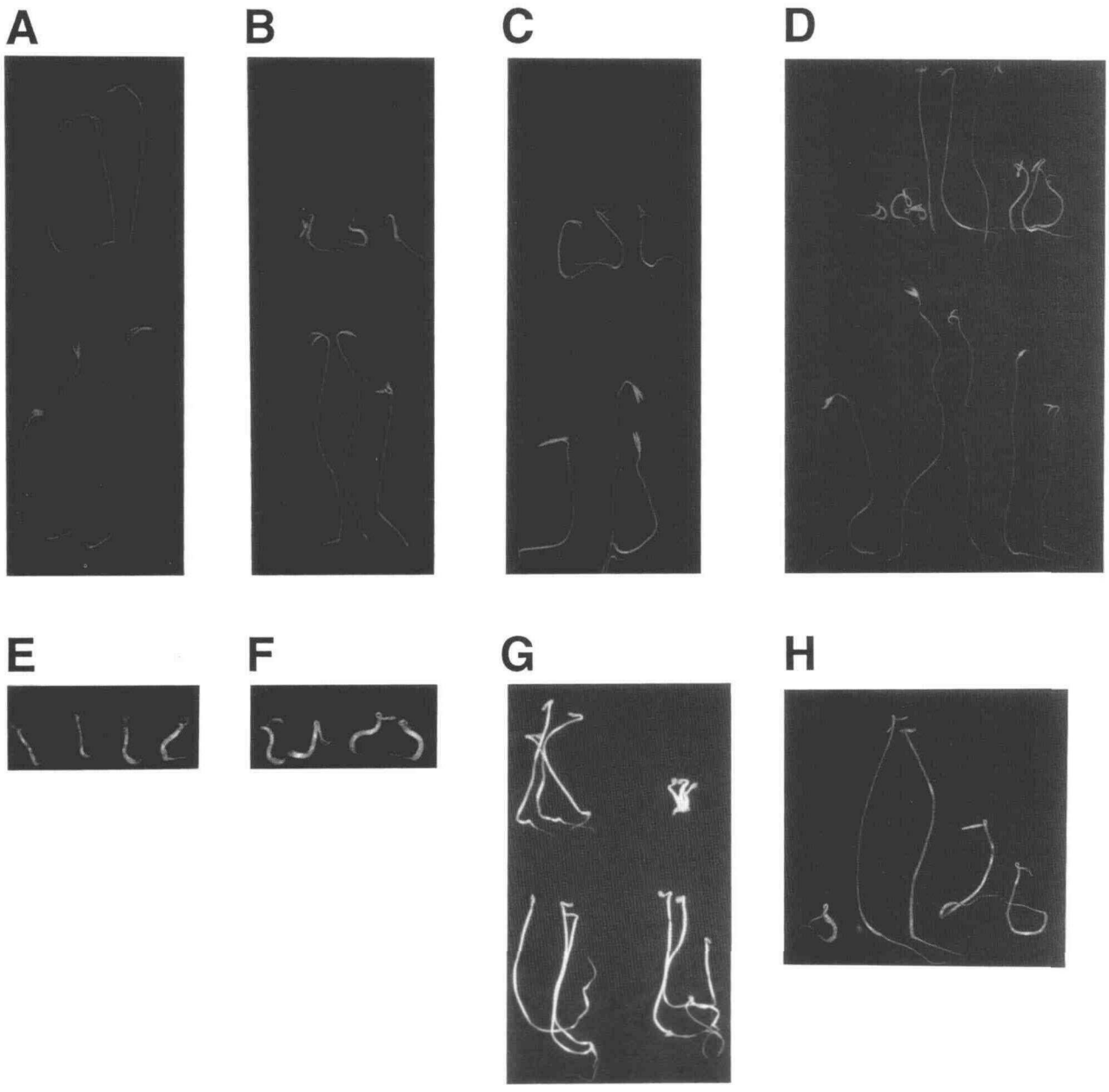


Figure 1. The *Nr* Gene Inhibits the Triple Response of Tomato Hypocotyls and Is Codominant.

(A) to **(D)** Seeds of *Nr* (Ailsa Craig *Nr/Nr*), normal (Ailsa Craig *nr/nr*), F_1 heterozygotes (*Nr/nr*) or progeny of a segregating F_2 population (Ailsa Craig *Nr/Nr* × Ailsa Craig *nr/nr*, F_2), respectively, were germinated on water agar with (top row) or without (bottom row) 20 μM ACC in the dark for 12 days and then photographed. Seedlings in the upper row of **(D)** were grouped according to their lengths to show the three phenotypic classes.
(E) and **(F)** Seeds of the *nor* (MHI *nor/nor*) and *rin* (Ailsa Craig *rin/rin*) mutants, respectively, were germinated on 20 μM ACC for 12 days.
(G) Effects of the *Nr* gene in the Pearson background are shown. *Nr* seedlings (Pearson *Nr/Nr*, left side) and normal seedlings (Pearson *nr/nr*, right side) were germinated in the presence (top) and absence (bottom) of 20 μM ACC.
(H) Phenotypes of seedlings from the segregating F_2 progeny derived from the cross Ailsa Craig *Nr/Nr* × *L. cheesmannii nr/nr* are shown. Seedlings were germinated in the presence of 20 μM ACC.

ethylene insensitivity is regulated at a single locus (χ^2 = 0.28, $P > 0.75$). Ethylene "sensitive" individuals were characterized by short, thick hypocotyls with tight hooks; "insensitive" individuals possessed long, thin, straighter hypocotyls without hooks; those scored as "intermediate" were of intermediate height and thickness and had partial hooks. It is important to note that we had more confidence in our ability to distinguish sensitive from intermediate and insensitive than intermediate from insensitive individuals. Consequently, we suspect that a small number of seedlings classified as insensitive may in fact be intermediate (heterozygous) and vice versa.

Nr-Associated Ethylene Insensitivity and Fruit Ripening Inhibition Are Regulated by the Same or Tightly Linked Loci

F_2 seed resulting from a cross between Ailsa Craig (*Nr*/*Nr*) × Ailsa Craig (*nr*/*nr*) was grown to maturity and scored for a fruit ripening phenotype prior to isolation of F_3 seed. Ripening inhibition segregated as a single dominant trait in the 19 individuals scored (3:1 ratio; 15 mutants:4 normal; χ^2 = 0.16, $P > 0.5$). Germination of F_3 seed from the same 19 individuals indicated 1:2:1 segregation of *Nr*-associated ethylene insensitivity in the F_2 population (5 insensitive:10 intermediate:4 sensitive; χ^2 = 0.16, $P > 0.9$). In addition, the 15 individuals with impaired fruit ripening (*Nr*/*Nr* or *Nr*/*nr*) were the same ones that yielded F_3 seed scored as insensitive (all ethylene insensitive) or intermediate (segregating for ethylene insensitivity). The four F_2 individuals bearing normally ripening fruit (*nr*/*nr*) were the same four that yielded only ethylene sensitive F_3 seed. The five individuals producing only ethylene insensitive F_3 seed demonstrated greater ripening inhibition than those classified as intermediate; this is similar to the difference between *Nr*/*Nr* and *Nr*/*nr* fruit described below. These results suggested that *Nr*-associated fruit ripening inhibition and ethylene insensitivity result from a lesion at the same or a tightly linked loci (0 recombinants in a population of 19 diploid individuals corresponds to a genetic distance of 0 ± 5.6 centimorgans).

The Epinastic Response of Petioles to Ethylene Is Absent in the *Nr* Mutant

Epinasty, one of the many effects that ethylene exerts on tomato development, is the induction of downward bending or curling of the petiole. Single leaves of young tomato plants can be treated with ethylene by infiltration with a solution of 20 μM ACC. The ACC is converted into ethylene by ACC oxidase, which, as in seedlings, is present constitutively in leaves. After 16 to 18 hr, the petioles of infiltrated leaves display an epinastic response. Figure 2A shows representative examples of the response of normal leaves to the ethylene produced after ACC infiltration (UC82B, left). When plants expressing ACC deaminase (line 5673; Klee et al., 1991), which degrades ACC, were infiltrated with ACC, there was little ethylene production and no epinastic response (Figure 2A, right).

Figure 2B shows the effects of ethylene overproduction on plant development. In tomato line 7776, an ACC synthase coding region under the transcriptional control of the cauliflower mosaic virus (CaMV) 35S promoter conferred high levels of constitutive ethylene synthesis. The developmental consequences of this ethylene overproduction are readily apparent. The stems and petioles are woodier than wild-type controls, and the leaves are thicker and leathery. Petioles display a pronounced swelling of their proximal portion and a severe constitutive epinastic response. Flowers become chlorotic and abscise well before flower opening. The plants resemble the previously described mutant *epi* (Fujino et al., 1988, 1989).

Following ACC infiltration, petioles of Pearson plants displayed a significant epinastic response (Figure 2C), whereas *Nr* plants never displayed this response (Figure 2D). The *Nr* leaves were able to convert ACC into ethylene. In a separate experiment, leaves that had been infiltrated with ACC (or uninfiltrated controls) were removed 0.5 hr after infiltration and placed in sealed tubes. After 1 hr, gas from the tube was collected and ethylene content was measured by gas chromatography. The rates of ethylene production by the leaves were calculated. As Figure 3 shows, the rate of ethylene production in single leaflets of uninfiltrated leaves was low. The transgenic 5673 plants, which express ACC deaminase, also produced very little ethylene following ACC infiltration (<0.2 nL/g/hr). Infiltration of either normal (UC82B or Pearson) or *Nr* leaves, however, resulted in high rates of ethylene production. Thus, *Nr* is not impaired in ACC oxidase function. In addition, we found that following infiltration with a bacterial pathogen (*Pseudomonas syringae* pv *tomato* DC3000; Whalen et al., 1991), leaves of both Pearson and *Nr* plants synthesized large amounts of ethylene de novo, indicating that *Nr* is not impaired in any step of ethylene biosynthesis.

Ethylene biosynthesis rates were also determined for plant line 7776, which expresses ACC synthase constitutively. Leaf and stem tissues of normal UC82B plants synthesized ~0.2 to 2 nL/g/hr. The 7776 plants synthesized 50 to 80 nL/g/hr of ethylene.

Regulation of Senescence and Abscission of Flowers by Ethylene

Because ethylene has been shown to participate in regulating flower abscission and senescence in many species, we sought to determine the effects of the *Nr* mutation on the development of flowers. Flowers of both Pearson and *Nr* plants were tagged on the day the flower petals opened (day 0) and inspected daily thereafter. Most Pearson flowers began to senesce (wilted) 4 days after opening (number observed >20, all were wilted by day 5). Figure 4E shows a 6-day-old Pearson flower that has wilted. Conversely, *Nr* flower petals did not wilt or senesce even after 13 days ($n > 20$). Figure 4G shows an 11-day-old *Nr* flower. All flowers observed remained turgid for

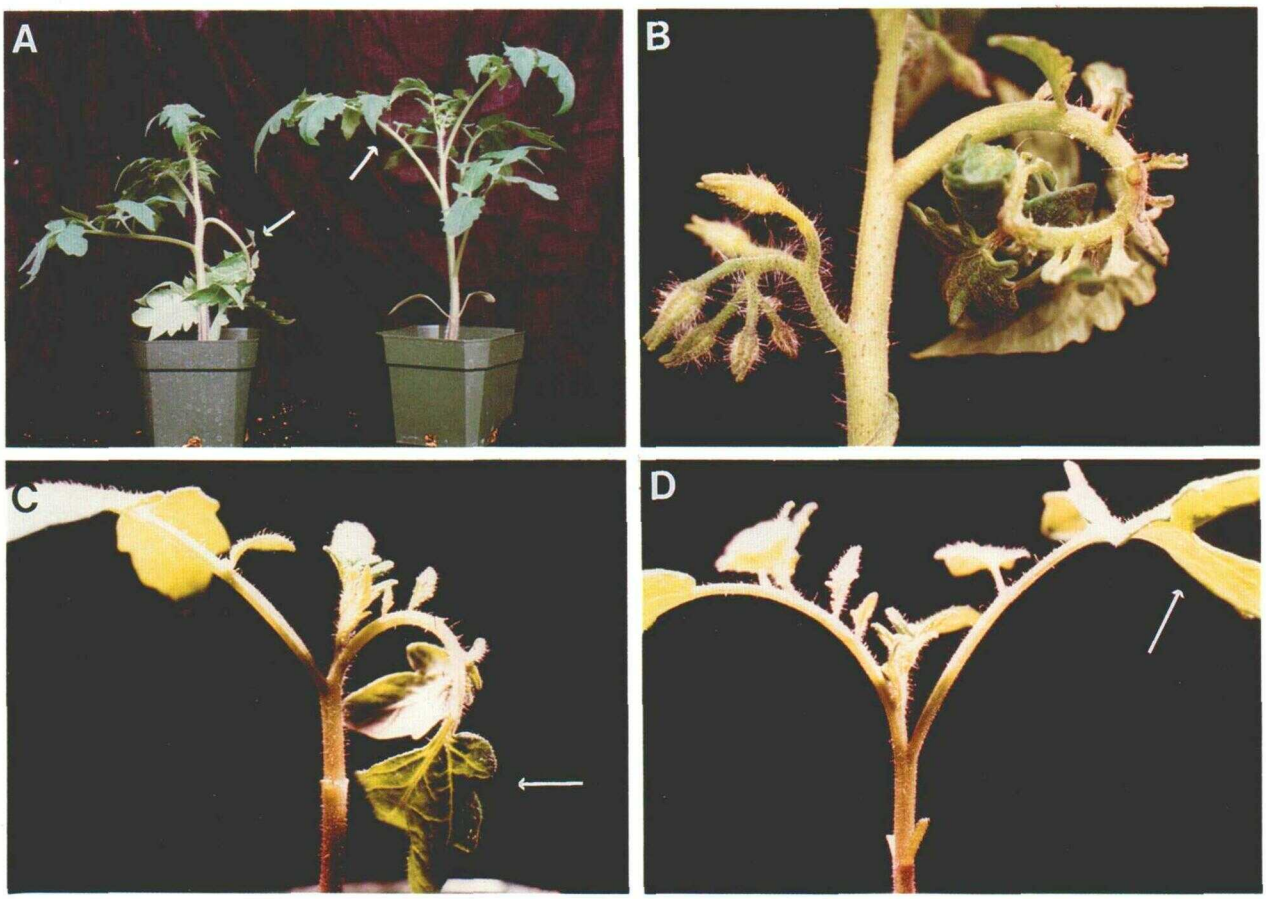


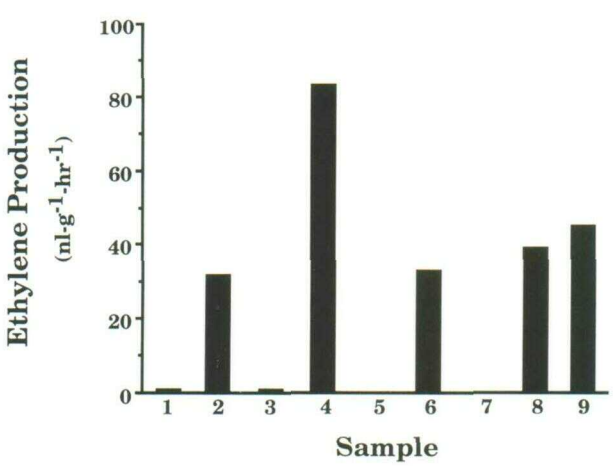
Figure 2. The Epinastic Response to Ethylene Is Inhibited in *Nr* Petioles.

(A) Leaflets of UC82B (left) and line 5673, which expresses ACC deaminase (right), that were infiltrated with 20 μL of 20 mM ACC and photographed 16 hr later (arrows indicate infiltrated leaves).

(B) UC82B plant that expresses the ACC synthase gene constitutively (line 7776).

(C) Pearson *nr/nr* plant that was infiltrated with 20 μL of 20 mM ACC (in leaf indicated by arrow) and photographed 16 hr later.

(D) Pearson *Nr/Nr* plant that was infiltrated with 20 μL of 20 mM ACC (in leaf indicated by arrow) and photographed 16 hr later.



at least 16 days or longer, provided that fertilization and subsequent fruit expansion had not occurred. Unfertilized flowers then slowly became pale, and progressive browning and shriveling eventually enveloped the entire flower (Figure 4I). This type of petal death is both temporally and developmen-

Figure 3. Ethylene Production Is Not Impaired in *Nr* Plants.

Rates of ethylene production (nanoliters per gram per hour) by a leaf of UC82B, column 1; ACC-infiltrated UC82B, column 2; ACC-infiltrated line 5673, column 3; ACC-overproducing line 7776, column 4; Pearson *nr/nr*, column 5; ACC-infiltrated Pearson *nr/nr*, column 6; Pearson *Nr/Nr*, column 7; ACC-infiltrated Pearson *Nr/Nr*, column 8; Pearson *Nr/Nr* leaf infiltrated with *Pseudomonas syringae* pv *tomato* DC3000, column 9.

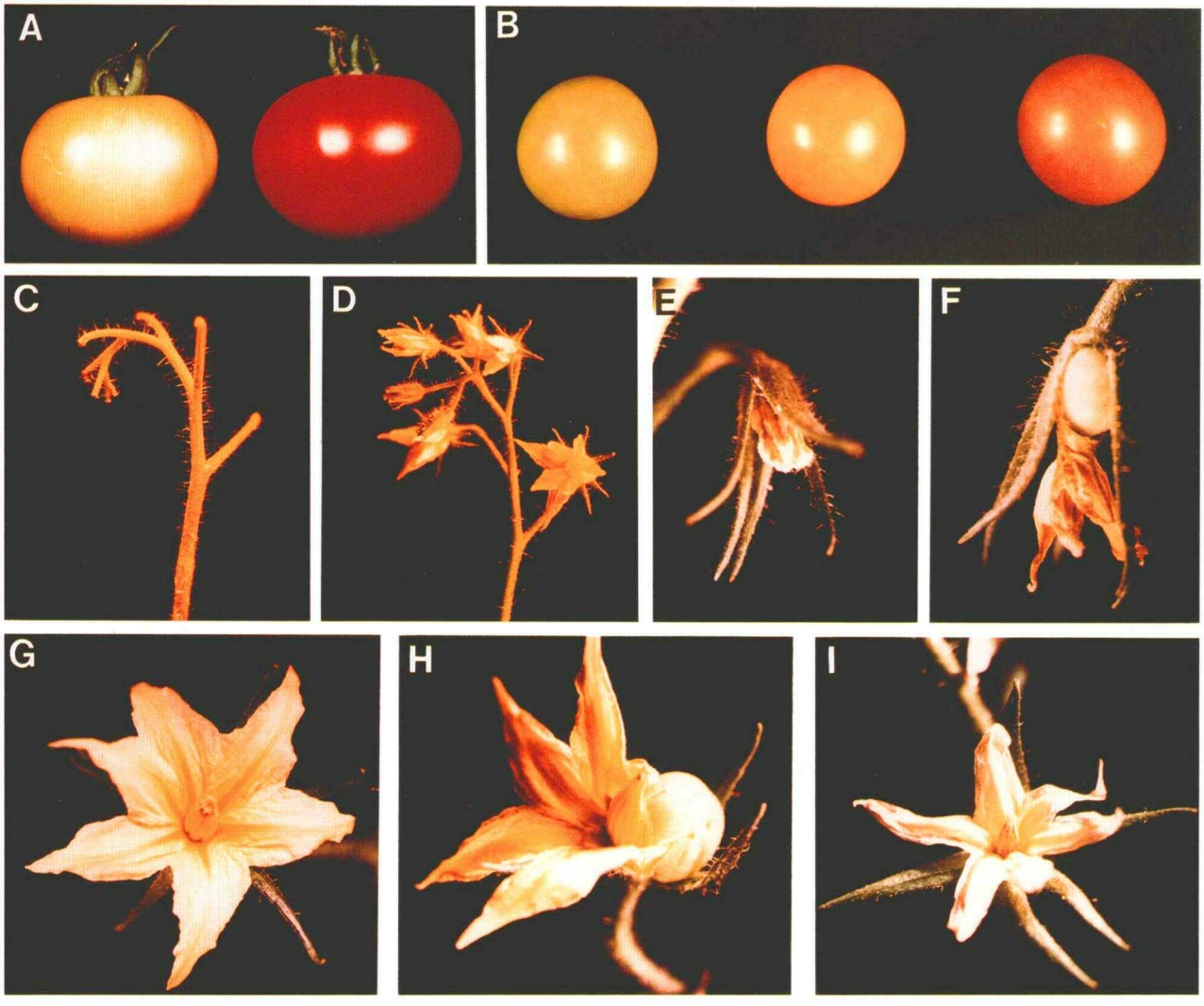


Figure 4. The Effects of Ethylene on Abscission and Senescence in Tomato.

(A) Tomato fruit from Pearson *Nr*/*Nr* (left) and Pearson *nr*/*nr* (right) plants ~3 and 2 weeks, respectively, after breaker stage.
(B) Tomato fruit from Ailsa Craig *Nr*/*Nr* (left), Ailsa Craig *Nr*/*nr* (middle), and Ailsa Craig *nr*/*nr* (right) plants 7 days after breaker stage.
(C) Tomato inflorescence from a Pearson *nr*/*nr* plant after 3 days of exposure to 50 μL/L ethylene.
(D) Tomato inflorescence from a Pearson *Nr*/*Nr* plant after 3 days of exposure to 50 μL/L ethylene.
(E) A 6-day-old senescent Pearson *nr*/*nr* flower.
(F) A 9-day-old senescent Pearson *nr*/*nr* flower with a small developing fruit.
(G) An 11-day-old Pearson *Nr*/*Nr* flower showing no signs of senescence.
(H) A 13-day-old Pearson *Nr*/*Nr* flower (a sepal was removed for clarity) with a developing fruit showing limited signs of senescence.
(I) A Pearson *Nr*/*Nr* flower (>15 days old) showing the type of petal senescence characteristic of unfertilized flowers of the mutant.

tally distinct from that which occurs in the wild-type control flowers.

If a Pearson flower was not fertilized, it typically abscised at the pedicel abscission zone 5 to 8 days after the flower began to senesce. *Nr* flowers rarely abscised, even after extended periods. Of 80 Pearson flowers in this study that had not set fruit, only 10 (12.5%) had not abscised after 20 days, whereas only two of the 53 *Nr* flowers more than 20 days old had abscised (<4%).

In a separate experiment, young inflorescences (oldest flower <4 days old) from *Nr* and Pearson plants were removed, and their cut stems were placed in distilled water. The cut flowers were then placed in a large desiccation jar, ethylene was added to the atmosphere inside the jar (50 μL/L), and it was left on the benchtop for 2 days. The percentage of abscised flowers was then determined; 77% (23 of 30) of the Pearson flowers had abscised, whereas only 11% (3 of 28) of the *Nr* flowers had abscised. Following 3 days of ethylene treatment, there was

93% (28 of 30) abscission of the Pearson flowers and 28% (8 of 28) abscission of the *Nr* flowers. Representative examples of flowers following 3 days of ethylene treatment are shown in Figures 4C and 4D (Pearson and *Nr*, respectively).

If fertilization took place and fruit was set in Pearson plants, fruit expansion was invariably preceded by complete flower senescence. Figure 4F shows a 9-day-old Pearson flower with a small developing fruit. In *Nr* plants, however, the onset of fruit expansion was not preceded by flower senescence. Figure 4H is of a 13-day-old *Nr* flower that shows limited signs of senescence, although a developing fruit had disrupted much of the vasculature that attached the flower petals to the plant. Flower petals and stamens of *Nr* plants appeared to die only when their vascular connections were substantially disrupted by the expanding fruit.

A significant effect of ethylene overproduction in line 7776 was premature senescence and abscission of the flowers (Figure 2B). The distal portion of the pedicels became chlorotic, and abscission occurred before the flowers opened, causing a marked decrease in fruit set in these plants. This observation is analogous to the abscission that occurred when flowers of normal plants (even young unopened flowers) were treated with exogenous ethylene (see above). In the transgenic lines producing the highest levels of ethylene, maturation of flowers and successful fruit set rarely occurred.

Ethylene insensitivity can reduce the rate of leaf senescence and delay flower petal abscission in Arabidopsis (Bleecker et al., 1988; A.B. Bleecker, personal communication), and a recent report indicates that inhibition of ACC oxidase synthesis by antisense expression can delay leaf senescence in tomato (Picton et al., 1993). Leaf senescence can be induced in older leaves by a number of environmental conditions, including nutrient deprivation, roots becoming pot bound, shading, or water stress (Abeles et al., 1992). With the growth conditions we used to grow our tomato plants, there was a small but noticeable delay in the rate of senescence of the lower leaves of *Nr* compared to Pearson plants (data not shown). Effects of stress on leaf senescence in *Nr* and Pearson plants were not determined.

Interestingly, the leaves of ethylene-overproducing plants did not show obviously increased rates of senescence relative to controls. Rather, the lowest leaves after becoming buried beneath a canopy of younger leaves often remained green for up to 6 months. The lack of senescence of leaves may be correlated with a lack of fruit set due to flower abortion. Thus, there is not a clear correlation between ethylene production and initiation of leaf senescence.

DISCUSSION

We have presented evidence indicating that the *Nr* mutant of tomato is insensitive to ethylene. Several lines of evidence support this conclusion: (1) as previously described, *Nr* fruit does not ripen fully, even in the presence of exogenous ethylene, (2) seedlings of *Nr* plants do not display the triple response to ethylene, (3) the epinastic response of petioles to ethylene is absent in the mutant, (4) pedicel abscission zones do not respond to exogenous ethylene, and (5) flower petal wilting (abscission and senescence) is inhibited in the mutant. In addition, we have shown that overproduction of ethylene results in a constitutive ethylene response phenotype. It is intriguing that inhibition of ethylene action, as in *Nr* plants, has relatively minor effects on plant morphology and development. Saturation of the response by constitutive ethylene synthesis causes no gross effects on plant morphology and development but does cause premature abscission of flowers. These lines should be useful in determining the role that ethylene plays in response to other stimuli, such as stresses, pathogens, and gravity. It has been shown that both 2,4-D and ethephon induce leaf epinasty in the Pearson cultivar, but only 2,4-D will cause epinasty in *Nr* plants. In addition, *Nr* plants were used to study regulation of gibberellin down regulated (GAD) gene expression. In Pearson, the plant growth regulators gibberellin, 2,4-D, and ethephon suppress the accumulation of GAD mRNAs. In *Nr* plants, however, GA and 2,4-D could suppress the accumulation of these transcripts, but ethephon had little or no effect on accumulation of the transcripts. Both of these results are consistent with ethylene insensitivity in *Nr* plants and help to clarify the role of ethylene in mediating GA and 2,4-D regulated processes (S. Jacobsen, personal communication).

In the cultivar Ailsa Craig, heterozygous *Nr* fruit ripen to a greater extent than the homozygous *Nr* fruit (Figure 4B). It was also observed that homozygous *Nr* fruit ripen considerably more so than fruit homozygous for *Nr* in the Pearson background (J.J. Giovannoni, data not shown). This suggests that the *Nr* gene is more effective in blocking ripening in Pearson. The partial dominance of the *Nr* gene in the hypocotyls (and probably fruit) of Ailsa Craig indicates incomplete expressivity in this genetic background. Routine delays in abscission had been observed for detached *Nr* tomato flowers previously, but in those experiments 100% abscission of *Nr* flowers occurred after 14 hr compared to 100% abscission at 6 hr for the control flowers (Tucker et al., 1984; no data on intact flower senescence was presented). Because the previous experiments used the *Nr* gene in the cultivar Ailsa Craig, the genetic background appears to have a significant effect on the sensitivity of the pedicel abscission zone to ethylene. The *Nr* gene in the Ailsa Craig background delayed flower abscission but did not inhibit abscission as dramatically as it did in the Pearson background. Complete abscission was reached in the Ailsa Craig background after 14 hr, whereas our results in the Pearson background showed that abscission of *Nr* pedicels was minimal (only 10%), even after 48 hr in 50 μL/L ethylene. Previous work has also shown polygalacturonase enzyme activity to be ~10% of the control in Ailsa Craig *Nr* (Tucker et al., 1980). We have found that polygalacturonase activity is undetectable in the Pearson *Nr* (data not shown). These results indicate that (1) the Ailsa Craig *Nr*/*Nr* mutant retains residual ethylene responsiveness and (2) the effects of the *Nr* gene are dependent upon the genetic background of the plant given that the same

Nr allele present in Pearson was introgressed into Ailsa Craig. Therefore, it is likely that the *Nr* fruit in the Ailsa Craig background ripen to a greater extent than those in the Pearson background, because they retain residual ethylene responsiveness. The aspects of ripening that do occur in the homozygous Pearson *Nr* fruit likely consist of the ethylene-independent aspects of tomato fruit ripening, but residual ethylene responsiveness in Pearson *Nr/Nr* cannot be ruled out.

The effects of the ethylene biosynthesis inhibitor L-α-(2-aminoethoxyvinyl)glycine, α-aminoisobutyric acid, and the ethylene antagonists norbornadiene and transcyclooctene on flowers have shown that abscission and petal senescence in many species are regulated by ethylene (Abeles et al., 1992). Our results confirmed these observations. Recently, carnation mutants affecting ethylene synthesis and sensitivity have been described (Brandt and Woodson, 1992). A 6- to 8-day increase in the life of the carnation flower was observed in the ethylene-insensitive line. Although the basis for the carnation phenotype is not known, their observations and ours confirm that ethylene insensitivity can significantly delay the onset of petal senescence.

The hypothesis that ethylene causes leaf senescence in tomato is complex. The recent report that plants expressing antisense mRNA corresponding to ACC oxidase are delayed in leaf senescence is intriguing (Picton et al., 1993). Regulation of tomato leaf senescence appears to be analogous to the ripening process that occurs in fruit. It is known that immature fruit will not respond to ethylene by ripening (Grierson and Kader, 1986). Furthermore, it is known that mature green *rin* and *nor* fruit will not ripen when exposed to exogenous ethylene. These fruit, however, do respond to ethylene (Herner and Sink, 1973; Lincoln and Fischer, 1988; Kieber and Ecker, 1993). Gene expression is affected and mRNAs are induced by ethylene in these mutants, but the fruit do not respond as normal mature green fruit do by ripening. Thus, competence for the ripening response to ethylene is acquired in the fruit in a developmentally regulated manner. The ability of a leaf to respond to ethylene by senescing may be analogous to the competence a fruit must develop to ripen. Thus, in *Nr* or ACC oxidase antisense lines, a lack of ethylene or response to ethylene can delay the onset of senescence. But, as is evidenced by the ethylene overproducing lines, ethylene alone is not sufficient to bring about senescence.

It is interesting to note that *Nr*-associated ethylene insensitivity of tomato seedlings is strikingly similar to that observed in Arabidopsis seedlings harboring the *Etr1* or *Ein1-1* mutations. Specifically, Arabidopsis seedlings germinated on media supplemented with ACC, and homozygous for the mutant *Etr1* allele, have phenotypes that are nearly identical to those described above for tomato *Nr* seedlings (Bleecker et al., 1988; Guzmán and Ecker, 1990), while less severe symptoms of ethylene exposure are observed in heterozygous seedlings (Chang et al., 1992). Consequently, tomato *Nr* and Arabidopsis *Etr1* mutations are likely to represent genetic lesions in similar or identical biochemical processes and possibly equivalent genes. Recent identification of the *Etr1* gene of Arabidopsis (Chang et al., 1993) will allow the isolation of tomato *Etr1* homologs from normal and *Nr* lines. In tomato, however, more profound physiological consequences are apparent because of ethylene's regulatory role in processes such as fruit ripening and flower senescence. Identification of *Nr* as an ethylene-insensitive mutant of tomato should greatly extend the ability to examine important aspects of ethylene-regulated plant development.

METHODS

Plant Materials

The *Never ripe* (*Nr*) mutation was originally identified in the Pearson cultivar and has been backcrossed to Pearson. Thus, the Pearson *Nr/Nr* and Pearson *nr/nr* are isogenic (Rick and Butler, 1956). The homozygous *Lycopersicon esculentum ripening inhibitor* (*rin*) mutant is a nearly isogenic line in the greenhouse-grown cultivar Ailsa Craig, whereas the homozygous *non-ripening* (*nor*) mutant is a nearly isogenic line in *L. esculentum* MH1. An F_2 population segregating and scored for both ethylene insensitivity and fruit ripening resulted from self-pollinated F_1 progeny derived from the progenitor cross Ailsa Craig (*Nr/Nr*) × Ailsa Craig (*nr/nr*). A second F_2 population scored only for ethylene insensitivity resulted from self-pollinated F_1 progeny derived from a progenitor *L. esculentum* (cv Ailsa Craig; *Nr/Nr*) × *L. cheesmannii* (University of California, Davis; *nr/nr*) cross. Homozygous *L. esculentum rin*, *nor*, and *alcobaca* (*alc*) mutants that are nearly isogenic lines in Rutgers and *L. esculentum* homozygous for the *Nr* mutation that is a nearly isogenic line in Pearson were used for all other experiments and were kindly provided by C. Rick (University of California, Davis).

Ethylene Overproducing Tomato Lines

The transgenic tomato line 7776 contains a 1-aminocyclopropane-1-carboxylate (ACC) synthase cDNA that is expressed constitutively by the cauliflower mosaic virus (CaMV) 35S promoter. The ACC synthase cDNA was obtained by using a Perkin Elmer Cetus kit for polymerase chain reaction amplification of mRNA. Oligonucleotide primers were synthesized based on the published sequence of a ripening-related tomato ACC synthase gene (Van Der Straeten et al., 1990). The primer 5′-CCCTTTTAATTAAGATCTTAACGAAC-3′ was used for first strand cDNA synthesis from RNA obtained from red tomato fruit. The primer 5′-CCAGATCTAAATGGGATTTGAGATTG-3′ was used in conjunction with the first primer for polymerase chain reaction amplification of the cDNA. The coding region of the cDNA was placed under transcriptional control of the CaMV 35S promoter and introduced into tomato plants via Agrobacterium-mediated transformation. Numerous lines were screened for ethylene overproduction by gas chromatography (Ward et al., 1978), and line 7776 was identified as one of the lines that produced high levels of ethylene (>50 nL/g/hr).

Ethylene Treatments

Seeds were surface sterilized with 5.25% sodium hypochlorite, rinsed in sterile water, and then germinated in 4-inch plastic boxes for 12 days in the dark on 1.5% agar with or without ACC (A-0430; Sigma).

Infiltrations of ACC (and *Pseudomonas syringae* pv tomato DC3000) into leaves were performed by forcing 20 μL of 20 mM ACC solution (or a 1:100 dilution of overnight culture in 10 mM $MgCl_2$) into the underside of a leaf using a 1-cc syringe. Visual determinations of epinasty were made after 16 to 18 hr. Determinations of ethylene synthesis rates were performed by gas chromatography as described previously (Klee et al., 1991) using freshly harvested leaves.

Ethylene treatment of tomato inflorescences was accomplished by placing freshly harvested flowers (stems in distilled water) in a desiccation jar and injecting 100% ethylene to a final concentration of 50 μL/L. The desiccation jar was left on the benchtop for 3 days and abscission was scored.

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