Analysis of the Ethylene Response in the *epinastic* Mutant of Tomato

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Ethylene can alter plant morphology due to its effect on cell expansion. The most widely documented example of ethylene-mediated cell expansion is promotion of the "triple response" of seedlings grown in the dark in ethylene. Roots and hypocotyls become shorter and thickened compared with controls due to a reorientation of cell expansion, and curvature of the apical hook is more pronounced. The *epinastic* (*epi*) mutant of tomato (*Lycopersicon esculentum*) has a dark-grown seedling phenotype similar to the triple response even in the absence of ethylene. In addition, in adult plants both the leaves and the petioles display epinastic curvature and there is constitutive expression of an ethylene-inducible chitinase gene. However, petal senescence and abscission and fruit ripening are all normal in *epi*. A double mutant (*epi/epi;Nr/Nr*) homozygous for both the recessive *epi* and dominant ethylene-insensitive *Never-ripe* loci has the same dark-grown seedling and vegetative phenotypes as *epi* but possesses the senescence and ripening characteristics of *Never-ripe*. These data suggest that a subset of ethylene responses controlling vegetative growth and development may be constitutively activated in *epi*. In addition, the *epi* locus has been placed on the tomato RFLP map on the long arm of chromosome 4 and does not demonstrate linkage to reported tomato *CTR1* homologs.

The gaseous plant hormone ethylene participates in the regulation of many developmental processes throughout the life cycle of plants (Abeles et al., 1992). The growth and expansion of cells is just one area where ethylene has been shown to have an influence. The most widely documented example of the effect of ethylene on cell expansion is the triple-response phenotype exhibited by dicot seedlings grown in the dark in the presence of ethylene. In the absence of ethylene, dark-grown seedlings have an etiolated morphology consisting of an elongated, slender root and hypocotyl with the development of a hypocotyl hook. In the presence of ethylene, seedlings develop a short, thickened root and hypocotyl with enhanced curvature of the apical hook due to ethylene inhibition of cell elongation (Guzman and Ecker, 1990). In contrast, in deepwater rice and other semiaquatic plants, ethylene acting in conjunction with gibberellins has been shown to stimulate internode cell elongation (Kende et al., 1998).

The triple response phenotype has been used extremely successfully as a screen for the isolation of components of the ethylene signal transduction pathway in Arabidopsis. Mutants found to be insensitive to ethylene display a normal etiolated phenotype in response to the gas, whereas a constitutive triple response (*ctr*) mutant was also identified that showed the triple response phenotype in the absence of ethylene (Guzman and Ecker, 1990). A combination of molecular and genetic approaches has elucidated the identities of a number of the components of the pathway and the order in which they act (for review, see Chang and Shockey 1999; Stepanova and Ecker, 2000). Briefly, ethylene is perceived by a family of integral membrane receptors with similarity to two-component His kinases and function as negative regulators. The *CTR1* gene is also a negative regulator of the pathway acting downstream of the receptors and encodes a protein with similarity to the Raf family of Ser/Thr protein kinases (Kieber et al., 1993). *CTR1* may interact directly with the receptors (Clark et al., 1998) and has been proposed to represent the head of a putative mitogen-activated protein kinase cascade (Chang and Shockey 1999). The signaling events from *CTR1* to the nucleus are unclear but appear to involve EIN2, a novel integral membrane protein with homology to mammalian natural resistance-associated macrophage protein metal ion transporters (Alonso et al., 1999). Ethylene signaling in the nucleus is mediated by the EIN3 family of transcriptional regulators, which act directly upon ethylene response factors to activate ethylene-inducible gene

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expression (Chao et al., 1997; Solano et al., 1998). Based upon homology to the Arabidopsis model, a number of components of the ethylene response pathway have now been isolated from tomato (Lyco-persicon esculentum), including receptors (Lashbrook et al., 1998; Tieman and Klee, 1999) and CTR-like genes (Lin et al., 1998; Zegzouti et al., 1999; L.C. Adams and J.J. Giovannoni, unpublished data). Furthermore, utilization of the same triple response screen used in Arabidopsis led to the discovery that the tomato fruit-ripening mutant Never-ripe (Nr) was insensitive to ethylene (Lanahan et al., 1994). Additionally, the delayed petal senescence, flower abscission, and fruit-ripening phenotypes of Nr were also shown to be the result of ethylene insensitivity. Wilkinson et al. (1995) showed that Nr was caused by a mutation in a member of the tomato ethylene receptor gene family (Wilkinson et al., 1995).

The epinastic (epi) mutant of tomato displays characteristics indicative of altered cell expansion similar to wild-type plants treated with ethylene. For example, the stems and petioles are thicker than in wild-type plants and petioles display epinastic curvature. Leaves also have a twisted epinastic morphology, and ethylene production is increased above the level of wild-type plants (Fujino et al., 1988). Dark-grown epi/epi seedlings show characteristics of the triple-response phenotype, as indicated by a shortening and thickening of the hypocotyl, and therefore resemble wild-type seedlings treated with ethylene (Fujino et al., 1989). Furthermore, inhibitors of ethylene synthesis and action were unable to rescue the epi phenotype, leading to the suggestion that the mutation may result in a constitutive ethylene response. This hypothesis is supported by the similarity of the epi seedling phenotype to that of the Arabidopsis ctr1 mutant seedling, the phenotype of which also does not revert when grown on ethylene inhibitors (Kieber et al., 1993). We have investigated the putative constitutive ethylene response of epi with the aid of a double mutant, including the ethylene-insensitive Nr allele (epi/epi;Nr/Nr). Our results indicate that, unlike the ctr1 mutant of Arabidopsis, epi does not demonstrate a global constitutive ethylene response. Alternative models are proposed that suggest a role for epi either in the regulation of a subset of ethylene responses regulating cell expansion or in an independent pathway required for normal growth that cross-talks with the ethylene response pathway.

RESULTS

The epi Phenotype and the Relationship with Ethylene

The vegetative phenotypes of Ailsa Craig (AC; nr/ nr; normal nearly isogenic control for the dominant Nr/Nr mutant), VFN8 (Epi/Epi; normal nearly isogenic control for the recessive epi/epi mutant), Nr/ Nr, epi/epi, and the epi/epi;Nr/Nr double mutant are shown in Figure 1A. AC, VFN8, and Nr/Nr displayed normal vegetative growth, whereas epi/epi showed the characteristic stunted growth and twisted leaf morphology. The epi-like phenotype was also shown by the epi/epi;Nr/Nr double mutant. The senescence and abscission of petals from normal wild-type tomato flowers occur within a few days of anthesis and are enhanced by successful pollination (Llop-Tous et al., 2000). These processes have been shown to be mediated by ethylene, as both are greatly delayed in the ethylene-insensitive Nr mutant, with petals remaining attached to the developing fruit long after fertilization has occurred (Lanahan et al., 1994; Llop-Tous et al., 2000). Pets of epi/epi flowers had the same rate of senescence as those of wild-type plants (data not shown), whereas those of double-mutant plants clearly showed the same delayed petal senescence phenotype that is characteristic of Nr (Fig. 1B). The ripening of fruit was also the same in double mutant and Nr plants (Fig. 1C). Although epi/epi fruit ripened normally when compared with VFN8 and AC, both Nr/Nr and epi/epi;Nr/Nr fruit remained firm and accumulated less lycopene.

Other than the obvious effects on vegetative growth, phenotypes commonly associated with an altered ethylene response remained unaltered in the epi/epi mutant. No differences in leaf and petal senescence or abscission were observed, and both the rate of fruit ripening and the time from anthesis to the onset of ripening remained the same for both genotypes. Small increases in the transcript abundance of the ethylene-regulated genes E8, PG, and PSY1 were observed in epi fruit compared with those of VFN8 throughout ripening. This was probably due to the small increase in ethylene synthesis observed in epi fruit during ripening (data not shown).

The phenotypes of dark-grown seedlings of single and double mutants grown in the presence or absence of 1-aminoacyclopropane-1-carboxylic acid (ACC) are shown in Figure 2A. As previously described (Fujino et al., 1989) dark-grown epi seedling hypocotyls showed reduced elongation and enhanced swelling even in the absence of ACC. epi/epi;Nr/Nr seedlings showed the same phenotype as the epi/epi parent. Growth on 20 μM ACC caused dramatic hypocotyl shortening and swelling in the AC and VFN8 cultivars and in epi/epi. The growth inhibition effect of ACC was reduced in Nr/Nr and double-mutant seedlings. The effect of the ethylene action inhibitor 1-MCP on seedling growth on ACC is shown in Figure 2B. 1-MCP alleviated the effect of ACC on all of the genotypes but did not revert epi or the double mutant.

Ethylene-regulated gene expression was examined in the leaves of light-grown plants held in air or treated with 20 μL L⁻¹ ethylene using probes for the basic chitinase genes CH19 (Danhash et al., 1993) and E4 (Lincoln and Fischer, 1988; Fig. 3). Chitinase expression was elevated in untreated epi/epi and
double-mutant plants when compared with normal cultivars and Nr, but was slightly higher in epi/epi. E4 transcripts remained at a constant low level in all five genotypes. The abundance of both transcripts increased in all ethylene-treated samples, regardless of genotype.

Leaf Epidermal Cell Shape Is Altered in epi

Plant growth is determined by cell expansion, which in turn is controlled by numerous environmental and genetic mechanisms (van Volkenburgh, 1999). Ethylene has been shown to have both positive and negative effects on cell growth and expansion, depending on the species and process that is under investigation. In Arabidopsis, the ethylene-insensitive mutants ctr1 and ein2 both have larger leaves compared with wild-type controls (Bleecker et al., 1988; Guzman and Ecker, 1990). In contrast, the ctr1 mutant, which displays a constitutive ethylene response, has smaller leaves due to reduced cell expansion (Kieber et al., 1993). The cell morphology of epidermal cells from the adaxial surface of wild-type tomato cultivars AC and VFN8 along with those of Nr/Nr, epi/epi, and the epi/epi;Nr/Nr double mutant were examined by scanning electron microscopy (Fig. 4). Epidermal cells of AC and VFN8 typically had a convoluted and irregular morphology. This appeared more pronounced in Nr/Nr cells. However, in epi/epi the convoluted phenotype was greatly reduced, with cells having a more rounded, swollen appearance. Addition of the Nr mutant into the epi background in the double mutant partially suppressed the swollen phenotype and the cells appeared slightly more convoluted but had not regained the appearance of the normal parents. Cells of the abaxial surface were also examined, and a trend similar to that described above was observed (data not shown). However, it should be noted that this surface is not amenable to study in tomato. Whereas the adaxial surface is relatively planar, the abaxial surface is highly convoluted due to the presence of vascular tissue.

Figure 1. Phenotypes of the epi;epi;Nr/Nr double mutant. A, Vegetative growth; note the epinastic growth of double-mutant plants (genotypes from left to right are as follows: AC [nr/nr], Nr/Nr, VFN8 [Epi/Epi], epi;epi, and epi;epi;Nr/Nr. B, Delayed petal senescence and abscission in epi;epi;Nr/Nr plants; note epinastic curvature of leaves. C, Fruit ripening is impaired in the double mutant. Genotypes are indicated as follows: a, AC (nr/nr); b, Nr/Nr; c, VFN8 (Epi/Epi); d, (epi/epi); and e, epi;epi;Nr/Nr. All fruit were of equivalent age and were harvested when normal ripening lines reached the red ripe stage. The difference in fruit locule number is due to cultivar differences. Fruit of AC typically have two locules, whereas those of the VFN8 cultivar are multilocular.
Genetic Mapping of the epi Mutant

In order to assess the possibility of genetic linkage between the epi locus and candidate genes, including two previously mapped tomato CTR1-like sequences (Giovannoni et al., 1999), an F₂ population of 31 individuals was generated from a cross between the epi/epi mutant (L. esculentum) and the wild species Lycopersicon cheesemanii (accession no. LA483). Sixty RFLP markers spaced approximately 20 cM apart throughout the tomato genome were analyzed for linkage to the mutant phenotype. This analysis revealed linkage to the marker CT50 that is located on the long arm of chromosome 4. Further analysis revealed the location of the mutant to lie within a 12-cM region on chromosome 4 between CT133 and TG163. The map position was subsequently refined to a 5-cM interval between TG22 and CT239 using an F₂ population of 139 individuals generated from a cross between epi and the more divergent wild species Lycopersicon pennellii (accession no. LA716; Fig. 5). In our F₂ populations and in the cross performed to generate the epi/epi; Nr/Nr double mutant, we have found that the epi locus segregated as a recessive trait. This observation is contradictory to the published literature (Fujino et al., 1988), where the mutation is reported to be dominant.

DISCUSSION

As previously reported, the epi mutant shows characteristics of a constitutive ethylene response mutant. For example, epi tissues produce abnormally high levels of ethylene and have an epinastic growth habit (Fujino et al., 1988; Fig. 1), and the dark-grown seedling phenotype is similar to that of wild-type seedlings treated with ethylene (Fujino et al., 1989; Fig. 2). Furthermore, these phenotypes are not reverted by inhibitors of ethylene synthesis or action (Fujino et al., 1989; Fig. 2).

We have examined the relationship between ethylene signaling and the epi mutant with the aid of a double mutant constructed with the ethylene-insensitive Nr mutant. Double-mutant, or epistasis, analysis has been used extensively in studies of the...
ethylene signal transduction pathway in order to resolve the order of individual components within the pathway. For example, epistasis analysis was used to position the Arabidopsis ctr1 mutant downstream of the ethylene receptor mutant etr1 (Kieber et al., 1993). Using the Arabidopsis system as a model, we may expect that if epi displays a whole-plant constitutive ethylene response, then a double mutant constructed with Nr (which displays dominant ethylene insensitivity) would have an identical phenotype to that of epi. The results shown in Figure 1 indicate that this is not the case. Whereas vegetative growth clearly resembles that of epi in the double mutant (Figs. 1A and 2), petal senescence and abscission and fruit ripening phenotypes are distinctly Nr in origin (Fig. 1, B and C). These observations suggest that epi may be constitutively activating a subset of ethylene responses affecting vegetative development, whereas ethylene responses controlling petal senescence and abscission and fruit ripening are unaffected by epi. Phenotypes indicative of a whole-plant constitutive response to ethylene have previously been reported in tomato. For example, transgenic plants constitutively expressing high levels of the ACC synthase gene, LEACS2, produce elevated levels of ethylene, resulting in leaf epinasty and rapid senescence and abscission of flowers (Lee et al., 1997). Similarly, antisense inhibition of the ethylene receptor LEETR4 results in a constitutive ethylene response phenotype that includes leaf epinasty, premature senescence and abscission of flowers, and early fruit ripening (Tieman et al., 2000). However, the phenotype of epi differs from that seen in these other studies in that only a subset of ethylene responses affecting vegetative growth appears to be modified.

The ethylene response of epi was monitored at the molecular level by examining the expression of the ethylene-inducible genes CHI9 and E4 (Fig. 3). Chitinase gene expression was elevated in the epi/epi and double-mutant leaves in the absence of ethylene, suggesting a constitutive ethylene response. However, E4 expression was at a low basal level in all the genotypes examined, indicating that there is not global constitutive ethylene-inducible gene expres-
Ethylene Response of the Tomato epi Mutant

Figure 5. Placement of the epi locus on the tomato RFLP map. An F₂ population segregating for epi and RFLP loci was generated from a cross between L. esculentum (epi/epi) × L. pennelli (Epi/Epi). A total of 139 individuals were scored on the basis of the epi phenotype, and linkage to markers was determined as described in “Materials and Methods.” The tomato RFLP linkage map of Tanksley et al. (1992) is shown to demonstrate conservation of marker order.

Figure 6. Models proposing the action of the EPI protein in tomato. A, EPI lies within the ethylene signal transduction pathway to regulate a subset of ethylene responses that lead to normal cell expansion. Mutation of EPI results in abnormal cell expansion and leads to elevated ethylene synthesis (+ve). B, EPI acts to control normal cell expansion and growth in a separate pathway to the ethylene response pathway. Mutation of EPI leads to abnormal cell expansion that in turn activates the ethylene response pathway, causing elevated ethylene synthesis.

Ethylene synthesis, whereas other ethylene responses, including senescence, ripening, and abscission, remain unaffected. However, an alternative interpretation is that EPI is not an integral component of the ethylene signaling pathway but functions in a separate pathway required for normal cell expansion and vegetative growth that can influence the ethylene signaling pathway (Fig. 6B). In this model, a mutation in EPI causes abnormal cell growth and expansion, leading to activation of the ethylene response pathway. Components of this second hypothetical pathway are obviously unknown, but recent data obtained from mutant screens and antisense experiments have revealed that disruption of cell-wall-modifying enzymes in Arabidopsis yields phenotypes similar to those of epi. For example, antisense inhibition of the Arabidopsis expansin gene AtEXP10 resulted in a curled leaf phenotype similar to that seen in epi (Cho and Cosgrove, 2000). In addition, the Arabidopsis mutant korrigan has the same dark-grown seedling phenotype as epi, and the cotyledon epidermal cells have a similar swollen shape to the epi epidermal cells (Fig. 4; Nicol et al., 1998; Zuo et al., 2000). KORRIGAN has been shown to encode an E-type endo-1,4-β-glucanase (Nicol et al., 1998; Zuo et al., 2000) highly similar to the tomato Cel3 gene (Brummell et al., 1997). A search of the Institute for Genomic Research tomato gene index (http://www.tigr.org/tdb/lgi/) revealed that the tomato Cel3 gene is identical to the RFLP marker CT70 that maps to locations on chromosomes 1 and 5. Therefore, as epi maps to the long arm of chromosome 4 (Fig. 5), it does not correspond to cel3. Additionally, due to the similarity of epi to the Arabidopsis ctr1 mutant, we have mapped two tomato CTR homologs, tCTR1 and tCTR2, to chromosomes 2 and 1, respectively.


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(Giovannoni et al., 1999), indicating that neither gene represents the mutation at the epi locus.

We have investigated the constitutive ethylene response phenotype of the tomato epi mutant. Our data suggest that the epi mutant does not result in a whole-plant constitutive activation of the ethylene response, but that it may affect a subset of ethylene responses required for normal vegetative growth and development. In addition, we have shown that epidermal cell shape is highly altered in epi and we have mapped the mutation onto the long arm of chromosome 4. A gene expression profile of epi is currently being generated using micro-array technology and efforts are under way to identify the EPI gene via positional cloning. These experiments will help us to determine the molecular nature of the link between cell growth and expansion and the ethylene response.

MATERIALS AND METHODS

Plant Material and Treatments

Homozygous Nr/Nr seed and the parental cultivar AC (nr/hr) were originally obtained from the Glasshouse Crops Research Institute (Littlehampton, UK). Seed for the homozygous epi/epi mutation and parental cultivar VFN8 (Epi/Epi) were obtained from the Tomato Genetics Resource Center (University of California, Davis), as were the wild tomato species Lycopersicon cheesmannii (accession no. LA483) and Lycopersicon pennellii (accession no. LA716). Plants were grown under standard greenhouse conditions at Texas A&M University (College Station) and Cornell University (Ithaca, NY). Experiments on dark-grown seedlings were performed as follows. Surface-sterilized seeds were sown on 1.5% (w/v) water agar in the presence or absence of 20 μM ACC and 2 μL L⁻¹ 1-MCP and incubated in the dark for 2 weeks. Ethylene treatment of light-grown plants was performed by sealing plants in an air-tight chamber and injecting ethylene to a final concentration of 20 μL L⁻¹ for 8 h.

RNA Isolation and Gel Blot Analysis

Three grams of frozen leaf material was ground to a powder with liquid nitrogen using a mortar and pestle. The powder was transferred to a tube containing 10 mL of extraction buffer (4% [w/v] p-aminosalicylic acid, 1% [w/v] 1, 5-napthalenedisulfonic acid, and disodium salt), to which 150 μL of β-mercaptoethanol had been added. Ten milliliters of water-saturated phenol, and 10 mL of chloroform was added to the contents of the tube and mixed. The phases were separated by centrifugation for 20 min at 10,000 rpm at 4°C. The aqueous phase was removed to a new tube and an equal volume of chloroform added. The contents were mixed well and centrifuged as above. The aqueous phase was removed and placed in a clean tube, and 2.5 mL of 10 M LiCl and 200 μL of β-mercaptoethanol was added. Precipitation was allowed to progress overnight on ice, and the RNA pellet was recovered by centrifugation for 20 min at 10,000 rpm. The pellet was resuspended in 1 mL of diethyl pyrocarbonate-treated sterile water, divided into two 500-μL aliquots, and 13 μL of 20% (w/v) SDS was added and mixed well. The mixture was extracted once with chloroform:isoamyl alcohol (24:1). The phases were clarified by centrifugation at high speed in a microcentrifuge for 10 min. The aqueous phase was removed and placed in a clean tube, and the RNA was precipitated by addition of 25 μL of 5 M NaCl and 2 volumes of ethanol. After storage at −20°C for 1 h, the RNA was collected at high speed in a microcentrifuge for 30 min at 4°C. The pellet was washed in 60% (v/v) ethanol, air dried, and resuspended in 100 μL of sterile diethyl pyrocarbonate-treated water.

Twenty micrograms of total RNA was fractionated through 1% (w/v) agarose gels containing 15% (v/v) formaldehyde. Gels were blotted onto Hybond N nylon membrane (Amersham-Pharmacia Biotech, Uppsala) according to the manufacturer’s instructions. Filters were hybridized at 65°C to [³²P]-labeled random primed probes, synthesized as described by Feinberg and Vogelstein (1983), in a buffer containing 5× SSC, 0.5% (w/v) SDS, 50 mM Na-P (pH 7.5), and 5× Denhardt’s solution. Hybridizations were performed for at least 16 h, after which the filters were washed in 2× SSC, 0.1% (w/v) SDS and then 1× SSC, 0.1% (w/v) SDS at 65°C. Signal intensity was visualized by autoradiography using XAR-S film (Kodak, Rochester, NY) with two intensifying screens at −80°C.

Scanning Electron Microscopy

Leaf samples were fixed in an aqueous solution of 2.5% (v/v) glutaraldehyde for 1 h and rinsed three times for 1 h each in sterile water. After fixation, samples were dehydrated in a graded ethanol series, critical-point dried in carbon dioxide, coated with gold, and examined under a scanning electron microscope (JEOL-JSM-6400, JEOL, Dallas).

DNA Analysis and Genetic Mapping

Genomic DNA was isolated from expanding tomato leaves and analyzed by DNA gel-blot hybridization as described previously ( Tanksley et al., 1992). Two populations of F₂ seed segregating for epi and RFLP loci were generated from crosses between Lycopersicon esculentum (epi/epi) × L. cheesmannii (Epi/Epi) and L. esculentum (epi/epi) × L. pennellii (Epi/Epi). All RFLP markers used had been previously localized onto the tomato map (Tanksley et al., 1992). Mapping in the L. esculentum (epi/epi) × L. pennellii (Epi/Epi) population was performed on the basis of knowledge obtained from the L. esculentum (epi/epi) × L. cheesmannii (Epi/Epi) population. A total of 139 individuals from the L. pennellii population were hybridized to the markers TG443 and TG163 that flank the epi locus. Thirty-seven recombinant individuals were identified and these were used as a subpopulation to identify linkage with nearby markers (Fig. 5).
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