Ethylene Signaling Influences Light-Regulated Development in Pea[1][OPEN]

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Plant responses to light involve a complex network of interactions among multiple plant hormones. In a screen for mutants showing altered photomorphogenesis under red light, we identified a mutant with dramatically enhanced leaf expansion and delayed petal senescence. We show that this mutant exhibits reduced sensitivity to ethylene and carries a nonsense mutation in the single pea (*Pisum sativum*) ortholog of the ethylene signaling gene *ETHYLENE INSENSITIVE2* (*EIN2*). Consistent with this observation, the *ein2* mutation rescues the previously described effects of ethylene overproduction in mature phytochrome-deficient plants. In seedlings, *ein2* confers a marked increase in leaf expansion under monochromatic red, far-red, or blue light, and interaction with *phytochromeA*, *phytochromeB*, and *long1* mutants confirms that *ein2* enhances both phytochrome- and cryptochrome-dependent responses in a *LONG1*-dependent manner. In contrast, minimal effects of *ein2* on seedling development in darkness or high-irradiance white light show that ethylene is not limiting for development under these conditions. These results indicate that ethylene signaling constrains leaf expansion during deetiolation in pea and provide further evidence that down-regulation of ethylene production may be an important component mechanism in the broader control of photomorphogenic development by phytochrome and cryptochrome.

The plant hormone ethylene is well known for its effects on fruit ripening and senescence (Klee and Giovannoni, 2011; Graham et al., 2012), but it also has a wide range of other effects on plant development. These include regulation of the apical hook in etiolated seedlings (Mazzella et al., 2014), modulation of gravitropism (Buer et al., 2006), and a generally inhibitory effect on cell elongation and growth of roots, stems and petioles (Vandenbussche et al., 2012). However, it can also promote elongation in some species and tissues (e.g. Arabidopsis [*Arabidopsis thaliana*] hypocotyls; Smalle et al., 1997) and especially, in response to inundation (Voesenek and Sasidharan, 2013), implying a complex regulatory system specifically adapted to suit particular growth circumstances. Ethylene also plays an important role in plant responses to external abiotic and biotic factors, including oxidative stress and pathogen attack (Bailey-Serres et al., 2012; Savatin et al., 2014).

Ethylene is synthesized from Met through two intermediates, *S*-adenosyl-Met and 1-aminocyclopropane-1 carboxylic acid (ACC), through the consecutive action of three enzymes: *S*-adenosyl-Met synthetase, ACC synthase, and 1-aminocyclopropane-1 carboxylic acid oxidase (ACO; Lin et al., 2009). This synthesis is tightly regulated through control of ACC synthase and ACO gene expression at transcriptional and posttranscriptional levels (Merchante et al., 2013). Ethylene is perceived by a family of copper-containing receptors located in the endoplasmic reticulum membrane, which are negative regulators of the ethylene response. These ETHYLENE RECEPTOR (ETR) proteins interact physically with the kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) and the metal transporter-like protein ETHYLENE INSENSITIVE2 (EIN2; Lin et al., 2009; Merchante et al., 2013). Ethylene binding results in inactivation of CTR1 and dephosphorylation of EIN2. This exposes EIN2 to proteolytic cleavage, and a C-terminal fragment is released to enter the nucleus, where it inhibits degradation of the EIN3/ETHYLENE INSENSITIVE3-LIKE1 transcription factors and promotes the expression of ethylene-responsive genes (Qiao et al., 2012).

Perhaps more so than for other hormones, functional analysis of ethylene synthesis and signaling pathways has largely been restricted to the Arabidopsis system. Work in rice (*Oryza sativa*) has confirmed the conservation of several key components, including *CTR1* and *EIN2* (Ma et al., 2013), and tomato (*Solanum lycopersicum*) has also provided a useful system for the study of ethylene effects on fruit ripening (Klee and Giovannoni,

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2011). However, beyond these species, the most significant example is the legume *Medicago truncatula*, where a mutant for the *EIN2* ortholog has highlighted an additional role for ethylene in the development of root symbioses (Penmetsa et al., 2008).

Ethylene has also long been implicated in plant responses to light. Early studies in pea (*Pisum sativum*) and bean (*Phaseolus vulgaris*) showed that ethylene production was repressed by red light (Goeschl et al., 1967; Vangronsveld et al., 1988), and light-induced apical hook opening was shown to coincide with a reduction in ethylene (Kang et al., 1967). More recently, opposite effects of ethylene on stem elongation in light and dark have been shown in Arabidopsis, where ethylene inhibits elongation of etiolated seedlings but stimulates elongation in light-grown seedlings (Vandenbussche et al., 2012). Interpretation of ethylene effects has often been complicated by the fact that, in many growth responses, ethylene acts together with and is sometimes subsidiary to other hormones, and this may also be relevant for understanding light responses. For example, ethylene may influence apical hook dynamics through modifying auxin action (Abbas et al., 2013) and participates in elongation responses to shade or flooding in part through regulation of gibberellin synthesis and/or signaling (Pierik et al., 2004).

We previously reported evidence for a link between ethylene and light-regulated development in pea (Foo et al., 2006). However, despite the wide use of the pea system for studies of hormone biology, including gibberellins (Weston et al., 2008), auxins (Tivendale et al., 2012), brassinosteroids (Jager et al., 2007), and strigolactones (Gomez-Roldan et al., 2008), mutants specific for ethylene have yet to be reported. Here, we show that a unique pea mutant with exaggerated responses to light is a mutant for the pea *EIN2* ortholog and examine its effects on photomorphogenic development through the use of specific photoreceptor and light-signaling mutants.

RESULTS

A Pea Mutant with Enhanced Light-Induced Leaf Expansion Is Ethylene Insensitive

During screening of M2 seedlings derived from an ethyl methanesulfonate mutagenesis of pea 'Torsdag' under red light, we isolated a mutant (isolation name AF145) with distinctly larger leaflets than the wild type, a phenotype that segregated as a Mendelian recessive trait. However, when plants were grown in darkness, the mutant was more similar to the wild type, and only a subtle difference in leaflet size was apparent (Fig. 1A), suggesting that mutant seedlings are hypersensitive to red light for leaflet expansion. The photomorphogenic phenotype of AF145 was clearly distinct from previously described *light-independent photomorphogenesis1* (*lip1*) and *phytochromeA* (*phyA-3D*) mutations (Sullivan and Gray, 2000; Weller et al., 2004), which both cause increased leaflet expansion under red light but also, have a substantial effect on stem elongation (Fig. 1A).

Figure 1. Isolation of a unique mutant showing light-hypersensitive leaflet expansion and impaired petal senescence. A, Two-week-old seedlings grown under continuous red light at 15 μmol m^{-2} s^{-1} (left) or in complete darkness (right). The constitutively photomorphogenic *lip1* mutant and the light hypersensitive *phyA-3D* mutant are shown for comparison. B, Flower senescence and pod growth. Plants were grown in the greenhouse under an 18-h extended natural photoperiod. WT, Wild type.

Upon reaching the reproductive stage, AF145 plants also showed a marked delay in petal senescence and abscission, which is illustrated in Figure 1B. Whereas petals on wild-type plants began to senesce within 3 d of anthesis and are generally shed from the plant within 1 week, petals on AF145 plants persisted without any obvious senescence for up to 2 weeks and were retained at the base of developing pods for more than 4 weeks postanthesis. In a proportion of AF145 flowers, the developing pod became temporarily trapped within the fused keel petal from 4 to 6 d after anthesis, causing it to buckle before eventually forcing through the keel and regaining a normal orientation. The shape of AF145 pods was also notably different from the wild type: shorter overall with a rounded upper edge, a blunter tip, and a slight indentation at the distal end.

Ethylene is a well-known regulator of flower senescence (Rogers, 2013), and mutants with impaired ethylene production or signaling show delayed flower and fruit senescence (Graham et al., 2012), suggesting that the AF145 phenotype might reflect a general insensitivity to ethylene. To test this, we applied the ethylene-releasing compound ethephon to seeds at planting. The triple response to ethylene was first described in pea seedlings

and originally defined as an inhibition of stem elongation, an increase in stem diameter, and a deviation from upright growth (Goeschl et al., 1966). Figure 2 shows that dark-grown wild-type pea seedlings exhibited several classic ethylene responses when treated with the ethylene-releasing compound ethephon (Yang, 1969). These included a significant reduction in epicotyl length and a significant increase in stem width and apical hook angle compared with control wild-type plants (all comparisons $P < 0.001$). In contrast, ethephon-treated AF145 seedlings showed no significant reduction in epicotyl length and no significant increase in stem width or hook angle compared with untreated seedlings. Ethephon treatment also caused a 50% inhibition of root length in wild-type seedlings ($P < 0.001$) but inhibition of only 15% in the AF145 mutant ($P < 0.05$). The lack of a significant response to ethephon treatment in the AF145 mutant is further shown in the dose-response experiment shown in Supplemental Figure S1.

Ethylene Insensitivity Is Associated with a Mutation in the Pea Ortholog of *EIN2*

In view of reduced ethylene sensitivity of the AF145 mutant, we examined genes with a positive role in ethylene signaling as potential candidates. Bioinformatic searches of the *M. truncatula* genome (Mt3.5) identified orthologs of *EIN2* (*Sickle*; Medtr7g101410), *EIN3* (Medtr5g087790), and *EIN5* (Medtr7g114570) with positions that predicted locations for pea orthologs in linkage groups 5 and 1. The AF145 locus was mapped in an F2 progeny ($n = 90$) from a cross between AF145 and cv Terese and found to be weakly linked to markers on the top one-half of linkage group 5, including *EIN5*, although the presence of 44 recombinant individuals showed that AF145 was distinct from *EIN5*. Scoring of additional markers for this cross refined the position of the AF145 locus, showing that it was also distinct from another potentially ethylene-related candidate *PHYTOCHROME-INTERACTING FACTOR1* (*PIF1*; 15 recombinant individuals) and close to the inferred position of *PsEIN2* (Fig. 3A). We, therefore, proceeded to isolate and map *PsEIN2*.

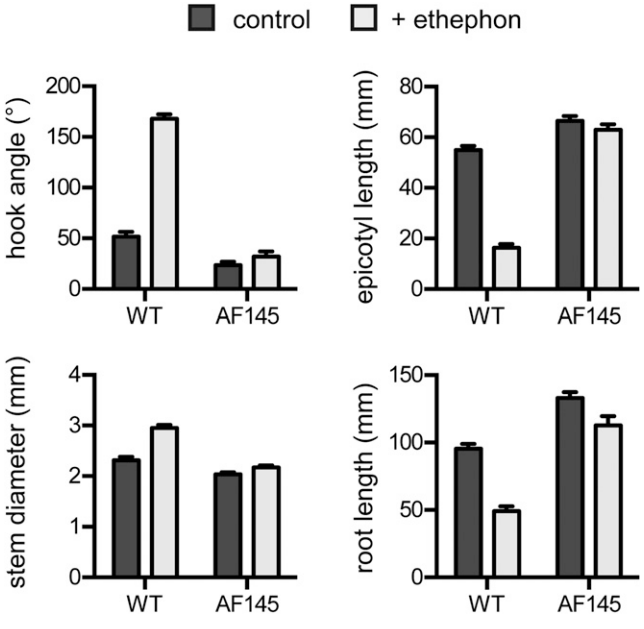

Figure 2. AF145 mutant seedlings are insensitive to ethylene. Wild-type (WT) and AF145 seeds were treated with 25 μg of ethylene-releasing compound ethephon and germinated in darkness for 7 d. Values represent means ± SE for $n = 16$ to 20.

As expected, the PsEIN2 protein is very similar in structure to MtEIN2/Sickle and predicted EIN2-like proteins from other legumes. In contrast to *MtEIN2*, the *PsEIN2* gene has seven rather than six exons, with an additional intron interrupting the large sixth exon of *MtEIN2* near the 3′ end of the coding sequence (Fig. 3C). A phylogenetic analysis of *EIN2*-like genes in sequenced legume genomes revealed two clades, *EIN2a* and *EIN2b* (Fig. 3B), consistent with a recent report of multiple legume *EIN2* genes (Miyata et al., 2013). *PsEIN2* and *MtEIN2* fall within the *EIN2a* clade, whereas the *EIN2b* clade was only represented in *Lotus japonicus*, soybean (*Glycine max*), and bean. In soybean, only one gene in the *EIN2a* clade was present, with no evidence for a second soybean homeolog.

Mapping of *PsEIN2* revealed no recombinants with the AF145 locus in the mapping population, and sequencing of *PsEIN2* complementary DNA from the AF145 mutant identified a single-nucleotide substitution directing a nonsense mutation of Trp 360 located in the 9th of 12 transmembrane helices within the N-terminal domain (Fig. 3C; Alonso et al., 1999). This predicts elimination of the entire C-terminal domain that, in Arabidopsis, is essential for transmission of the ethylene signal to the nucleus (Wen et al., 2012). Together with the evidence that *EIN2* is a single-copy gene in the temperate legume clade, this implies that the AF145 mutation causes complete loss of *PsEIN2* function.

Impaired Ethylene Signaling Moderates the Effect of Phytochrome Deficiency in Mature Plants

We previously outlined a role for ethylene in expression of pleiotropic consequences of phytochrome deficiency in mature pea plants (Foo et al., 2006). Double *phyA phytochromeB* (*phyB*) mutants have a shoot phenotype with features characteristic of ethylene action, which is associated with elevated ethylene levels and can be overcome by application of ethylene inhibitors. We used the AF145/*ein2* mutant to examine this interaction genetically, constructing double and triple mutants with *phyA* and *phyB* mutants. Figure 4 shows that, compared with wild-type plants, *ein2* plants grown in the greenhouse under an extended natural photoperiod showed a consistent small increase in internode elongation compared with the wild type, with a 15% increase in stem length between internodes 1 and 12 ($P < 0.01$). The *ein2* mutation also slightly increased elongation in the *phyA* mutant ($P = 0.02$) but had a marginal effect on elongation

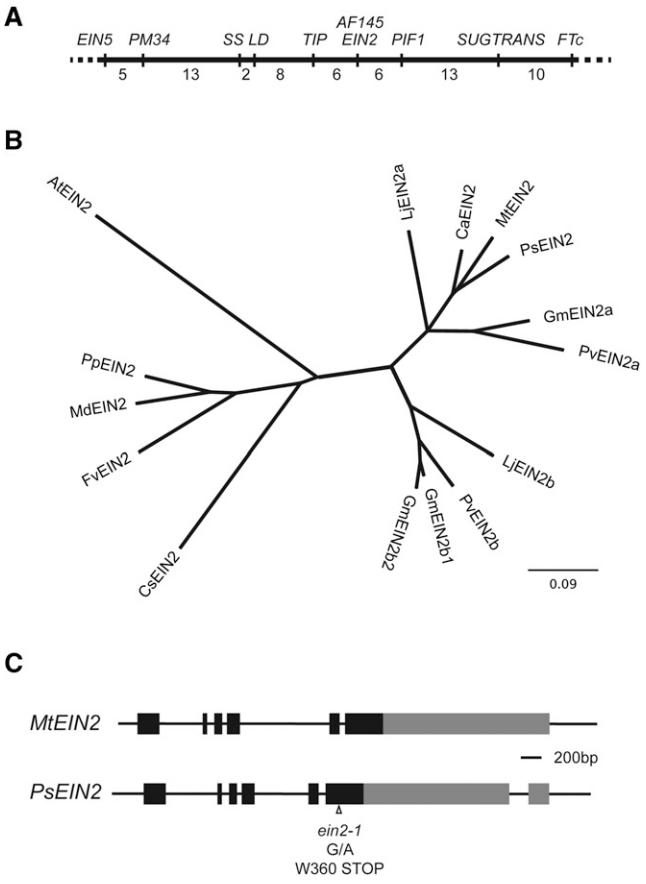

Figure 3. AF145 is an *EIN2* mutant. A, Location of the AF145 locus and the *PsEIN2* gene in pea linkage group V. B, Phylogenetic relationships of *EIN2* genes in legumes. Details of all sequences and the corresponding alignment are presented in Supplemental Figure S3. C, Diagram showing the structure of *PsEIN2* and *MtEIN2* genes and the location of the *ein2* G to A (G/A) mutation. Boxes and connecting lines represent exons and introns, respectively. Exon regions shaded gray correspond to those encoding the cleaved C-terminal fragment of AtEIN2 (Qiao et al., 2012). Cs, *Cucumis sativus*; FTc; Fv, *Fragaria vesca*.

in the *phyB* mutant background ($P = 0.048$). However, introduction of the *ein2* mutation dramatically increased internode elongation of *phyA phyB* double-mutant plants by over 70%, such that the length between nodes 1 and 12 in the triple mutant was indistinguishable from that in the *phyB* single mutant ($P = 0.66$). As in the case of ethephon treatment (Foo et al., 2006), the *ein2* mutation also reverted the characteristic pale, thickened, twisted stem and reduced leaf expansion of the *phyA phyB* double mutant (Fig. 4, B and C). These results are consistent with the conclusions that multiple features of the *phyA phyB* mutant phenotype do, indeed, result from elevated levels of ethylene and hence, that phyA and phyB are important in regulating ethylene levels in mature light-grown plants.

PsEIN2 Influences Seedling Photomorphogenesis

Seedlings of the *ein2* mutant displayed no obvious growth defects when grown under natural daylight in the greenhouse, and both internode elongation and leaf expansion were very similar between the wild type and *ein2* (Fig. 4C). However, the initial hypersensitivity of the *ein2* mutant for leaflet expansion in response to red light led us to examine its deetiolation phenotypes under other wavelengths. Figure 5 confirms that the *ein2* mutant grown in darkness displayed an essentially normal etiolated appearance. Internode elongation was not significantly different from the wild type ($P = 0.30$), and although the mutant showed a slight increase in leaflet size at this age, leaflets remained fully closed. The *ein2* mutation also had little effect on internode elongation under red ($P = 0.07$) or blue ($P = 0.40$) light but showed a significant increase relative to the wild type under far-red light ($P < 0.001$). Interestingly, under either blue or far-red light, the *ein2* mutant showed the same dramatic increase in leaflet expansion and opening as noted in the initial screen under red light, with leaflets fully unfolded and expanded. In contrast, the application of ethylene to wild-type seedlings under monochromatic light caused a significant decrease in leaflet expansion (Supplemental Fig. S2).

To test the involvement of phytochrome with these deetiolation phenotypes, we also examined the *ein2 phy* double and triple mutants described above. Figure 6 shows that the increased leaflet expansion and fully deetiolated appearance of the *ein2* mutant under red light (Fig. 1) was also clearly seen in the *phyA* and *phyB* single-mutant backgrounds. In the *phyA phyB* double-mutant background, however, the *ein2* mutant showed an etiolated appearance very similar to the *phyA phyB* double mutant, with leaflets remaining small and folded. Leaflets were slightly larger in the red light-grown *ein2 phyA phyB* mutant than in *phyA phyB* (Fig. 6; $P < 0.001$), similar to the effects seen in the *ein2* single mutant in darkness (Fig. 5). However, it is notable that the proportional effect of *ein2* in plants grown under red light was lower in this *phyA phyB* background (92% increase) than in the wild type, *phyA*, or *phyB* backgrounds (135%, 216%, and 163% increases, respectively).

In contrast, under blue light, the *ein2* effect on leaflet expansion was largely independent of phyA and phyB, with a similar proportional increase in leaflet area on a wild-type or *phyA phyB* background (Fig. 6). In addition, ethylene treatment significantly inhibited leaflet expansion to a similar extent in both the *phyA phyB* mutant under blue light and the *phyB* mutant under red light (Supplemental Fig. S2). In view of our previous demonstration that nonphytochrome deetiolation responses to blue light are predominantly controlled by cryptochrome 1 (cry1; Platten et al., 2005), these results indicate that ethylene may repress light signaling for leaf development downstream of both phytochrome and cryptochrome photoreceptors.

We also observed a subtle but nevertheless, significant effect of *ein2* on internode elongation under blue light. As previously noted, phyA clearly acts to inhibit internode elongation under low-irradiance blue light or at higher irradiances in the absence of cry1 (Platten

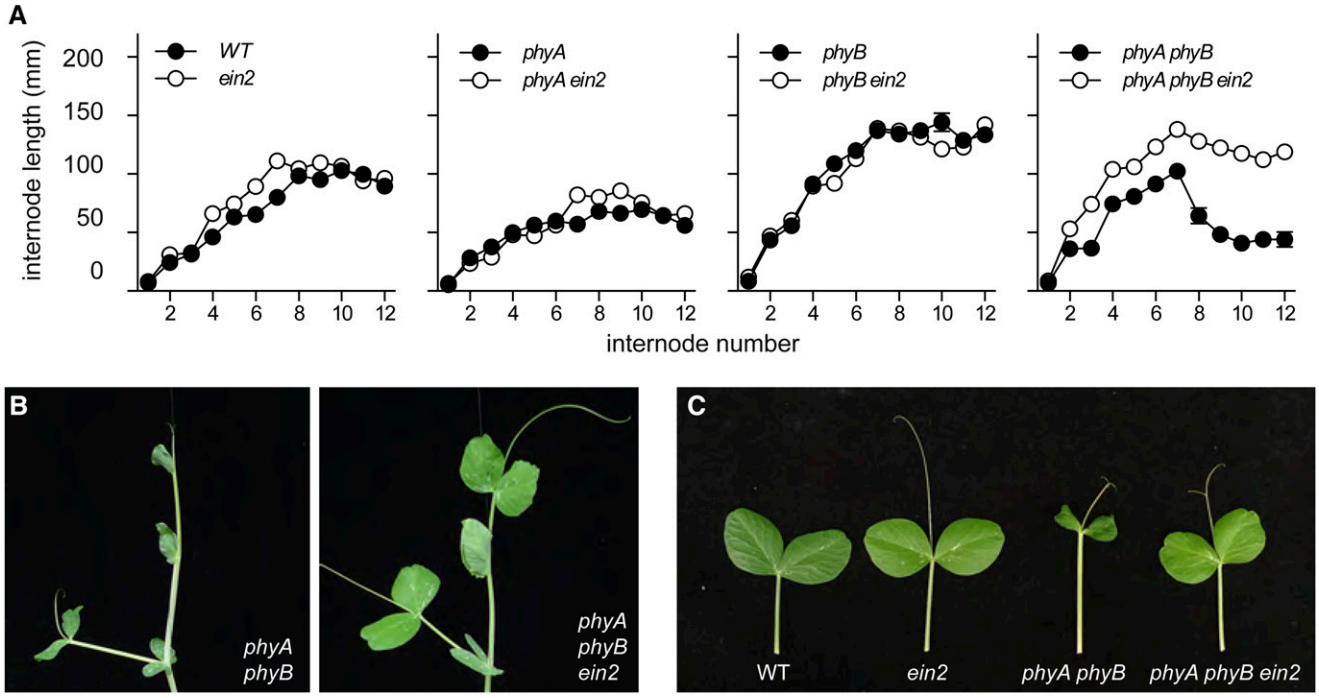

Figure 4. The *ein2* mutation overcomes phenotypes associated with ethylene overproduction in the *phyA phyB* double mutant. A, Effect of the *ein2* mutation on internode lengths in wild-type (WT) and *phyA*, *phyB*, and *phyA phyB* mutant backgrounds. Values represent means ± SE for $n = 8$ to 12. B, Shoot apex of 4-week-old plants. C, Representative leaf from node 5 of a 4-week-old plant. Plants were grown in the greenhouse under a natural daylight photoperiod extended to 18 h.

et al., 2005). However, under higher irradiance blue or white light, *phyA phyB* double mutants have shorter internodes than the *phyB* single mutant (Weller et al., 2001). Because both of these genotypes show normal growth in darkness, this result indicates that phyA can also act to promote stem elongation. The results in Figure 6 confirm that both *phyA* and *phyA phyB* genotypes have shorter internodes than their corresponding *PHYA* genotypes ($P < 0.001$ for both comparisons) and show that this difference is overcome by introduction of the *ein2* mutation. This implies that the increased inhibition of elongation in *phyA* and *phyA phyB* genotypes under blue light is likely to reflect increased ethylene production. It also suggests that, at least under these conditions, phyA normally acts to limit ethylene production.

Genetic Interaction of *EIN2* with Light-Signaling Components

In Arabidopsis, one important light-signaling mechanism involves the CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1)/ELONGATED HYPOCOTYL5 (HY5) module (Lau and Deng, 2012), and we previously characterized the effects and interaction of the respective pea orthologs of these genes, *LIP1* and *LONG1* (Weller et al., 2009). Because the leaflet phenotype of the *ein2* mutant under red and blue light superficially resembled that of *lip1*, we examined the genetic interactions of *ein2* with both *lip1* and *long1*. Figure 7A shows that the effect of *ein2* on leaflet expansion was substantially reduced by the *long1* mutation from a 4.7-fold increase in a wild-type background to a 2.1-fold increase on an *long1* background in seedlings under blue light. A similar difference was also seen in seedlings grown under red light. This shows that the *ein2* leaflet phenotype is partially dependent on *LONG1* and that *LONG1*-dependent signaling is enhanced by ethylene. However, the significant residual effect of *ein2* in the *long1* background indicates that other factors act in parallel with *LONG1* to regulate leaf expansion through ethylene. Both *ein2* and *long1* had only minor effects on internode elongation under red and blue light, and these seemed to be essentially additive in the *ein2 long1* double mutant. The only unanticipated feature of the double *ein2 long1* mutant was a significant reduction in stem thickness relative to the wild type and single mutants, which was clearly seen under both red and blue light.

The interaction of *ein2* with *lip1* also revealed an unexpected phenotype. In *lip1* F3 families segregating for the *ein2* mutation, we observed that approximately one-quarter of seeds showed apparently delayed germination. Subsequent examination 1 week after sowing showed that these seeds had germinated but that the shoot had grown under the soil in a twisted manner and failed to emerge through the soil (Fig. 7B; Supplemental Fig. S4). Molecular genotyping confirmed the identity of these seedlings as *lip1 ein2* double mutants. Closer inspection of germinating *lip1 ein2* seedlings revealed that

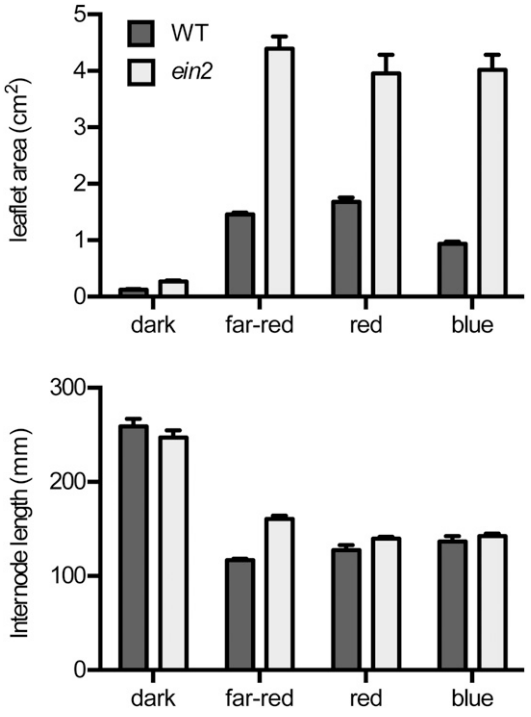

Figure 5. The *ein2* mutation enhances leaflet expansion under multiple wavelengths of monochromatic light. Wild-type (WT) and *ein2* seedlings were grown from sowing for 12 d under monochromatic far-red, red, or blue light (all 15 μmol m^{-2} s^{-1}) or in complete darkness. Leaflet area was estimated as the product of length and width of the larger leaflet from the first true foliage leaf (node 3). Internode length was measured as the length between nodes 1 and 3. Values represent means ± SE for n = 8 to 12.

the shoot tip remained trapped between the cotyledons, preventing normal internode elongation and upward growth. However, if the shoot tip was manually freed from the cotyledons a few days after imbibition and the seed was exposed to light, these seedlings were able to resume a normal growth orientation and ultimately, did not differ substantially in appearance from the *lip1* single mutant. Significantly, the addition of the *long1* mutation reverted this phenotype, and *long1 lip1 ein2* triple mutants that segregated from *lip1 ein2* double-mutant families showed normal emergence and an elongated appearance similar to *long1 ein2* double mutants (Fig. 7C).

DISCUSSION

The developmental changes that occur in response to light exposure are complex and reflect the regulation of multiple plant hormones at the level of synthesis, transport, or signaling. The most prominent of these are gibberellin and auxin (Casal, 2013), but ethylene has also been implicated in plant responses to light, including the regulation of hook opening, cotyledon expansion, and stem elongation (Vandenbussche et al., 2012). However, apart from detailed work on Arabidopsis hypocotyls (Yu et al., 2013), relatively few studies have examined the interaction of light and ethylene. Our characterization of an ethylene signaling mutant in pea together with the availability of several light perception and signaling mutants have provided a unique opportunity to examine this interaction in a species with hypogeal germination. We have identified a mutant with a delay in petal senescence and insensitivity to applied ethylene (Figs. 1 and 2) that carries a nonsense mutation in the pea *EIN2* gene (Fig. 3). The tight linkage of the mutant locus to *PsEIN2* and similarity of the mutant to *ein2* mutants in Arabidopsis and *M. truncatula* (Alonso et al., 1999; Penmetsa et al., 2008) strongly support the conclusion that the mutation in *PsEIN2* is the cause of the observed phenotypic defects, and we thus refer to the mutation and mutant line as *ein2*.

In Arabidopsis, ethylene-induced cleavage of endoplasmic reticulum-localized EIN2 releases a C-terminal fragment that moves to the nucleus and provides the critical link between ethylene perception and transcriptional regulation of ethylene response genes (Qiao et al., 2012). The pea *ein2* mutation characterized here is predicted to truncate the PsEIN2 protein within the membrane-spanning region upstream of the C-terminal fragment, and the resultant protein would therefore be expected to have minimal ability to regulate transcription. In Arabidopsis, *ein2* mutants cause complete insensitivity to all measured ethylene responses, suggesting that there is no alternative pathway for

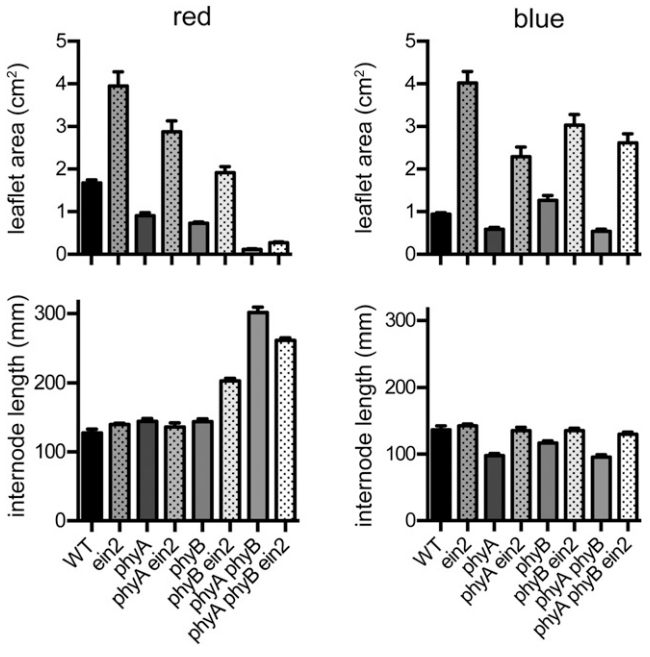

Figure 6. The *ein2* mutation enhances the response to both phytochromes and cryptochromes. Seedlings of the wild type (WT), *ein2*, *phyA*, *phyB*, and their double- and triple-mutant combinations were grown from sowing for 12 d under monochromatic red or blue light (15 μmol m^{-2} s^{-1}). Leaflet area was estimated as the product of length and width of the larger leaflet from the first true foliage leaf (node 3). Internode length was measured as the length between nodes 1 and 3. Values represent means ± SE for n = 7 to 12.

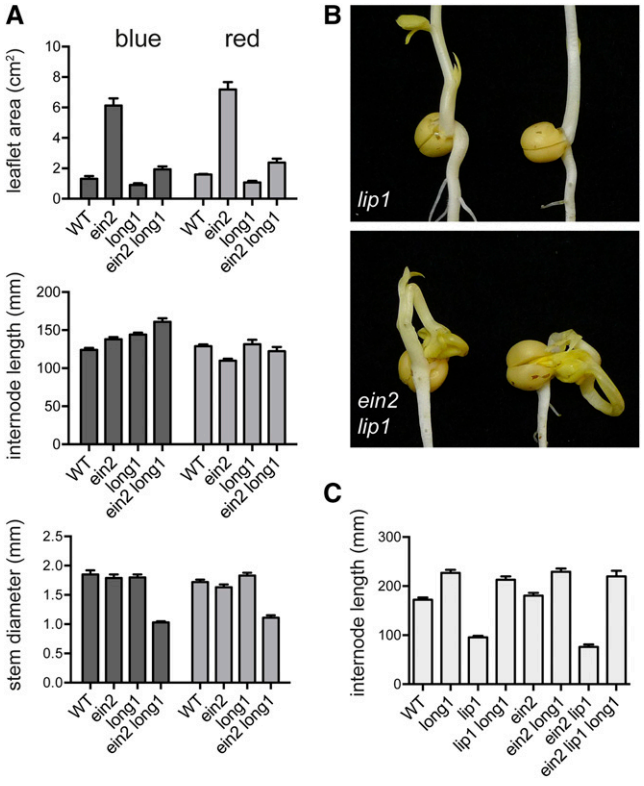

Figure 7. The effects of *ein2* are partly mediated through the *LIP1/LONG1* module. A, Interaction of *ein2* and *long1* in control of internode length, leaf expansion, and stem thickness. Internode length was measured as the length between nodes 1 and 3. Leaflet area was estimated as the product of length and width of the larger leaflet from the first true foliage leaf (node 3). Values represent means ± SE for $n = 8$ to 12. B, Comparison of 12-d-old *lip1* and *ein2 lip1* double-mutant plants grown in darkness. C, Reversion of *lip1 ein2* elongation phenotype by *long1* in plants grown under natural daylight in the greenhouse. Internode length was measured as the length between nodes 1 and 6. Values represent means ± SE for $n = 7$ to 10. WT, Wild type.

ethylene-regulated gene expression, and EIN2 is thus considered to play an indispensable role in ethylene signaling (Merchante et al., 2013). Recent reports have shown that the legumes *L. japonicus* and soybean both possess two distinct *EIN2* orthologs (Miyata et al., 2013), raising the possibility of redundancy for *EIN2* function in legumes. However, our phylogenetic analyses show that a duplication of *EIN2* is also present in bean, another warm-season legume, but in the temperate legumes pea, *M. truncatula*, and chickpea (*Cicer arietinum*), *EIN2* is single copy (Fig. 3B). The pea *ein2* mutation is therefore likely to eliminate all EIN2 function, making it a useful tool for studies examining the role and interactions of ethylene in a wide range of developmental processes.

Although the pea *ein2* mutant has several classic ethylene response phenotypes, it was initially isolated on the basis of a partial hypersensitivity to monochromatic red light. An enhanced response to light has not been reported as a conspicuous feature of Arabidopsis or *M. truncatula ein2* mutants, and we therefore investigated photomorphogenic aspects of the pea *ein2* phenotype in more detail. Loss of *EIN2* has little effect in dark-grown pea seedlings, suggesting that only a basal level of ethylene signaling is sustained under these conditions (Figs. 1, 2, and 4). The *ein2* mutation also had little effect on either internode length or leaf expansion in seedlings grown under white light (Fig. 4), indicating that, at saturating irradiances of white light, ethylene signaling also has a minimal role in pea seedling development. This contrasts with Arabidopsis, where ethylene causes a distinct promotion of hypocotyl elongation in light-grown seedlings that is blocked in ethylene signaling mutants *ein2* and *etr1* (Smalle et al., 1997). The effects of ethylene in light-grown plants are known to be variable across species (Vandenbussche et al., 2012), and it thus seems that in stems of light-grown pea seedlings (unlike in Arabidopsis hypocotyls) ethylene is not limiting for stem elongation.

In contrast to the lack of effect on stem elongation, loss of *EIN2* had a marked effect on leaf expansion under monochromatic light conditions (Fig. 5), indicating that ethylene normally opposes the effects of light on leaf expansion. The fact that leaf expansion of the *ein2* mutant under monochromatic red, blue, or far-red light is effectively similar to that of wild-type seedlings grown under white light (Fig. 5; Platten et al., 2005) provides a clear indication that that *ein2* enhances signaling from multiple photoreceptors. Consistent with this observation, we found that removal of the two pea phytochromes dramatically reduced the effect of the *ein2* mutation on leaf expansion under red light (Fig. 6), implying that *ein2* acts to enhance phytochrome signaling for leaf expansion. In addition, under blue light, the *ein2* mutation was still able to strongly promote leaflet expansion, despite the absence of phyA and phyB (Fig. 6). This shows that *ein2* can also enhance cryptochrome signaling for leaf expansion, because the residual response to blue light in the *phyA phyB* double mutant is almost entirely attributed to cry1 action (Platten et al., 2005). Conversely, ethylene application was observed to inhibit leaflet expansion in wild-type or photoreceptor mutant seedlings grown under monochromatic light (Supplemental Fig. S2).

Together, these observations show that the *ein2* mutation enhances and the ethylene application inhibits leaf expansion in seedlings where photoreceptor activation is subsaturating and suggest that the ethylene signaling pathway may moderate or oppose the effects of light at one or more points downstream of both phytochrome and cryptochrome photoreceptors. In Arabidopsis, two distinct regulatory modules play prominent roles in downstream photoreceptor signaling: the COP1/SUPPRESSOR OF PHYA-105 ubiquitin ligase complex and the PIF family of basic helix-loop-helix transcription factors (Leivar and Quail, 2011; Lau and Deng, 2012). Recent reports in Arabidopsis have highlighted potential interactions between these factors and ethylene signaling, predominantly focusing on the stimulation of hypocotyl elongation by ethylene. In

this response, ethylene is thought to promote elongation by enhancing the retention of COP1 in the light, thus increasing its activity and the degradation of its proteolytic target HY5 (Yu et al., 2013). This action of ethylene is downstream of EIN3 and therefore, EIN2, and it is possible that a similar mechanism could explain the antagonistic interaction of light and EIN2-dependent ethylene signaling on pea leaf expansion.

We previously showed that the divergent pea *HY5* ortholog *LONG1* is required for light-induced leaflet expansion downstream of both phytochrome and cryptochrome photoreceptors (Weller et al., 2009) and show here that the *long1* mutation significantly reduces the effect of *ein2* on leaflet expansion under both red and blue light (Fig. 7). The incomplete dependence of the *ein2* phenotype on *LONG1* is likely to reflect redundancy for *LONG1* action caused by the presence of a second gene orthologous to Arabidopsis *HY5 HOMOLOG* (*HYH*; Weller et al., 2009) in view of the significant overlap in function between *HY5* and *HYH* genes in Arabidopsis (Sibout et al., 2006). Nevertheless, it suggests that *LONG1* is important for the effect of *ein2* and that the *ein2* effect is substantially achieved through an interaction with the *LIP1/LONG1* pathway. This could reflect negative regulation by ethylene of LONG1 itself or negative regulation by LONG1 of ethylene biosynthesis or signaling. The former explanation seems less likely, because in that case, the *ein2* mutation would be expected to influence all LONG1-dependent processes to an equivalent extent, whereas it clearly affects leaf expansion much more strongly than stem elongation. A third interpretation could be that a reduced level of ethylene signaling is permissive to processes farther downstream in light-regulated leaf expansion, which may include ion transport activity and/or auxin signaling (Cluis et al., 2004; Fuchs et al., 2006). Lastly, several of the PIF proteins have been implicated in the interaction of light and ethylene signaling in Arabidopsis. A mutant for PIF5 shows mild ethylene deficiency symptoms (Khanna et al., 2007), whereas *pif1* mutant seedlings show an enhanced cotyledon opening under blue light (Castillon et al., 2009), and PIF3 is activated by EIN3 to promote hypocotyl elongation under white light (Zhong et al., 2012). Although a role for PIF proteins has yet to be shown in legumes, it is possible that they may also form another common target for light and ethylene.

Isolation of the *ein2* mutant also allowed us to further test the role of ethylene in other aspects of photomorphogenic development. We previously reported that older pea plants deficient in phytochrome activity show deregulated expression of ethylene biosynthesis genes, accumulate ethylene, and display a strong inhibition of stem elongation, greening, and leaf expansion (Foo et al., 2006). These phenotypes can be largely overcome by the *ein2* mutation (Fig. 4), consistent with previous experiments using a chemical inhibitor of ethylene biosynthesis (Foo et al., 2006). One simple model for these observations could be that phytochromes directly inhibit ethylene production, but an alternative explanation is that cryptochromes directly or indirectly stimulate ethylene production; this response is inhibited by phytochromes. This is consistent with our previous observation that removal of cry1 in a *phyA* mutant background leads to a decrease in expression of the ethylene biosynthesis gene *ACO1* in pea seedlings (Foo et al., 2006). In either case, these results suggest that growth changes in response to quality of light may also reflect adjustment of ethylene production, which was also reported in tobacco (*Nicotiana tabacum*) and *Sorghum bicolor* (Finlayson et al., 1999; Pierik et al., 2004). Although there is also evidence in the literature to suggest that light may influence ethylene sensitivity (Bours et al., 2013), the fact that blocking ethylene biosynthesis can rescue *phyA phyB* internode-length phenotypes in mature plants under white light (Foo et al., 2006) suggests that the effects of light on ethylene production may dominate in this system.

The hypothesis that phytochrome action may oppose a cry-dependent stimulation of ethylene production is also consistent with certain developmental phenotypes observed at the seedling stage. For example, blue light acting through cry1 is significantly more effective for inhibition of elongation in phytochrome-deficient seedlings than in the wild type (Platten et al., 2005), but this is not the case when ethylene signaling is blocked by *ein2* (Fig. 6). Although this response is more subtle than the dramatic *phyA phyB* mature plant phenotype, the fact that it occurs in seedlings, where energy for growth is still provided by the cotyledons and can be directly contrasted with the baseline etiolated condition, makes it clearer to interpret as a modification of normal photomorphogenic response. Thus it seems that phyA can either promote or inhibit stem elongation depending on the developmental context and light conditions. How these two responses relate to each other and whether they represent alternative or parallel modes of phyA action remain to be determined.

Overall, our results show that ethylene signaling has a significant influence on light-dependent leaf expansion and suggest that modulation of ethylene synthesis may be an important mechanism in this response, although the possibility that ethylene may also regulate light signaling cannot be excluded. It is notable that leaf expansion in the *ein2* mutant is largely decoupled from inhibition of stem elongation during deetiolation, suggesting that ethylene action on these two processes may have different thresholds or act through distinct mechanisms. Detailed studies examining possible cross regulation between light signaling and ethylene biosynthesis/signaling will also be needed to clarify the nature of these interactions. It will also be of interest to examine how ethylene signaling may influence other light-regulated processes, such as maintenance and opening of the apical hook. Beyond these questions, our characterization of an ethylene response mutant in pea adds to the extensive range of genetic tools already available to examine the biology of plant hormones in this species. In other plant species, ethylene has important roles in a wide range of processes, including senescence, response to pathogens and herbivores, and

regulation of symbiotic associations (Penmetsa et al., 2008), and in the future, it will be interesting to see how these effects are manifested in the pea system. The availability of the *ein2* mutant will also help dissect the network of hormone interactions in pea, in which the involvement of ethylene in the effects of brassinosteroid deficiency has been highlighted (Ross and Reid, 1986).

MATERIALS AND METHODS

Plant Material, Mutagenesis, Growth Conditions, and Treatments

The AF145 mutant line carrying the *ein2* mutation was generated by ethyl methanesulfonate mutagenesis of cv Torsdag in the same population that yielded the *long1* mutant (Weller et al., 2009). All plants grown for mapping and analysis of mature plant phenotypes (Figs. 1B, 4, and 7B) were grown in a temperature-limited greenhouse under a natural photoperiod extended to 18 h. All plants for seedling experiments (Figs. 1A, 2, 5, 6, and 7, A and C) were grown in growth cabinets at 20°C. Monochromatic and white light sources used in cabinet experiments have been described previously (Hecht et al., 2007). To assess the ethylene response of the *ein2* mutant (Fig. 2; Supplemental Fig. S1), a small portion of the seed coat was removed from dry seeds, and the required amount of the ethylene-releasing compound ethephon was applied to the cotyledon surface in 5 μL of ethanol. Control plants received 5 μL of ethanol alone.

Sequence Isolation, Mapping, and Molecular Markers

Mapping was conducted in the F2 of a cross between the AF145 (*ein2*) line and cv Terese. Selected markers on pea (*Pisum sativum*) LG V described by Aubert et al. (2006) were supplemented by newly designed markers for pea orthologs of *Medicago truncatula* genes in the relevant region of chromosome 7 (*M. truncatula* genome v3.5). Partial gene sequences for pea *EIN2*, *EIN5*, and *PIF1* genes were isolated designing primers on *Medicago* spp. sequences *MtEIN2* (EU709495; Medtr7g101410; Penmetsa et al., 2008), *MtEIN5* (Medtr7g114570), and *MtPIF1* (Medtr7g099540). Marker details are in Supplemental Table S1. Markers used for genotyping of *phyA-1*, *phyB-5*, and *long1-1* mutations have been described previously (Platten et al., 2005; Weller et al., 2009). Analyses of segregation data were performed using JoinMap 4 (Kyazma).

Full-length genomic and complementary DNA sequences of the pea *EIN2* gene were isolated using PCR, RACE, and genome-walking approaches as previously described (Hecht et al., 2011), which used specific primers designed on an initial DNA fragment obtained with *M. truncatula*-specific primers. All PCR fragments were sequenced at Macrogen. Primer details are given in Supplemental Table S1. For phylogenetic analysis, amino acid sequences of EIN2 proteins from legumes and several other rosid species were aligned in Geneious using ClustalW (alignment shown in Supplemental Fig. S3), and a phylogenetic tree (Fig. 3B) was generated using the neighbor-joining distance clustering method.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession number KP202149.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Ethylene dose-response in AF145 mutant.

Supplemental Figure S2. Effect of ethylene on leaflet expansion.

Supplemental Figure S3. EIN2 protein alignment.

Supplemental Figure S4. Interaction of *lip1* and *ein2* mutations.

Supplemental Table S1. Details of markers.

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