Mutational loss of the prohibitin AtPHB3 results in an extreme constitutive ethylene response phenotype coupled with partial loss of ethylene-inducible gene expression in Arabidopsis seedlings

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Abstract

The eer3-1 loss-of-function mutant, which was identified by screening for Arabidopsis mutant seedlings with an enhanced ethylene response, has both increased sensitivity and profound exaggeration of response to ethylene when visually assessed, yet exhibits partial ethylene insensitivity at the molecular level. The eer3-1 mutation represents a conditional allele with an ethylene-dependent phenotype that results from an amino acid substitution in the previously uncharacterized prohibitin, AtPHB3, with complete loss of EER3 function resulting in an extreme constitutive ethylene response in air. Prohibitins in other organisms have diverse roles including transcriptional regulation, with loss of prohibitin function in this capacity associated with tumour formation in mammals. Subcellular localization of AtPHB3 indicates that it is found in several cellular locations, including the nucleus and throughout the cytoplasm. Genetic analysis demonstrates that EER3 functions downstream of EIN2, since an ein2-5;eer3-2 double mutant has the same profound hypocotyl inhibition phenotype seen with the eer3-2 mutant. Based on the presented work, AtPHB3 probably functions as a positive regulator of expression of a subset of ethylene-regulated genes along with a group of genes required to maintain growth in the presence of ethylene.

Key words: Arabidopsis, AtPHB3, EER3, ethylene, prohibitin, triple response.

Introduction

Ethylene, which is a simple unsaturated gaseous hydrocarbon considered to be one of the five classic plant hormones, has profound effects on the growth, development, and environmental response of plants (Abeles et al., 1992; Bleecker and Kende, 2000). Examples of processes that ethylene regulates include fruit ripening, senescence, response to pathogens, and the intensively studied triple response of dark-grown seedlings, which is characterized by inhibition of hypocotyl elongation coupled with the formation of a pronounced apical hook following ethylene signalling. Although not of great agricultural importance, the seedling triple response phenotype, which consists of ethylene-dependent shortening and thickening of the hypocotyls combined with apical hook formation, has been extensively utilized for identification of Arabidopsis mutants that have altered responses to ethylene, thus giving invaluable insight into how ethylene is perceived in plants in general. Historically, two primary classes of ethylene signalling mutants have been focused on due to the relative ease with which they could be isolated. These include Arabidopsis mutants with ethylene insensitivity (etr and ein class), which present long hypocotyls in saturating ethylene, and mutants with a constitutive ethylene response (ctr and ebf class), members of which have a pronounced triple response phenotype even in the absence of ethylene. Through the identification and characterization of these mutants, a basic understanding of how ethylene promotes a response has been generated.

An ethylene signalling event initiates through a direct association of the carbon–carbon double bond of ethylene with a copper moiety found in members of the ethylene
receptor family (Schaller and Bleecker, 1995; Rodriguez et al., 1999), which consists of five distinct members (ETR1, ETR2, EIN4, ERS1, and ERS2). Although each of these has varying degrees of similarity to bacterial two-component regulators, it remains unclear whether any of the receptors participate in a His→Asp phosphorelay as part of their respective functions in the signalling mechanism (Gamble et al., 1998; Wang et al., 2003). Besides serving to bind ethylene, the ethylene receptors also associate with a prominent negative regulator of the ethylene signalling pathway, CTR1 (Kieber et al., 1993; Clark et al., 1998; Cancel and Larsen, 2002). The ethylene receptors function to maintain CTR1, which is a Ser/Thr kinase with homology to mammalian Raf, in an active state in the absence of ethylene (Hua and Meyerowitz, 1995). Loss of CTR1 function relieves repression of the ethylene signalling pathway and causes a growth phenotype in air that is similar to what is seen for wild-type plants grown long term in the presence of ethylene. Currently, no biochemical target of CTR1 has been identified, although it is speculated that since CTR1 has characteristics of a MAPKKK, it may function at the head of a MAP kinase cascade.

EIN2 is an enigmatic factor that functions in the ethylene signalling pathway at a point downstream of CTR1, with loss-of-function mutations in this factor causing complete ethylene insensitivity (Alonso et al., 1999). Little is known about how EIN2 propagates the ethylene signal, although homology analysis suggests that this membrane-localized protein has structural similarity to the N-Ramp family of ion transporters. EIN2 can be divided into two domains, including a membrane-associated domain that probably forms a membrane-spanning pore, possibly for ion movement, and a cytoplasmic domain of unknown function. Overexpression in an ein2-5 background of the cytoplasmic domain, referred to as the CEND, results in a profound phenotype in adult plants that is strikingly similar to adult ctrl loss-of-function mutants and adult wild-type plants that have been grown long term in the presence of saturating ethylene. Surprisingly, this severe reduction in size seen for the ein2-5:CEND plants is not correlated with increased abundance of ethylene-regulated genes, leaving it unclear as to how EIN2 or the CEND functions in propagating the ethylene signal.

It has been argued that, depending on the length of exposure, the consequences of ethylene signalling are promoted in distinctly different manners. Until recently, it was believed that the ethylene response exclusively relied on a transcriptional cascade mediated by EIN3 and EIL1 (Chao et al., 1997; Alonso et al., 2003b), both of which are transcription factors required for induction of ethylene-responsive genes, including ERF1 (Solano et al., 1998). Studies of short-term ethylene-dependent inhibition of hypocotyl elongation, however, have shown that brief exposure to ethylene actually slows growth in a manner independent of EIN3 and EIL1, with an ein3;eill double mutant behaving similarly to the wild type in terms of the initial inhibitory response to ethylene (Binder et al., 2004). It remains to be seen how the proposed EIN3- and EIL1-independent mechanism is actually mediated since no components of this have been described to date.

As part of an effort to continue to elucidate the ethylene signalling pathway in Arabidopsis, the focus of mutant screening has recently shifted in the direction of identification and characterization of mutants with an enhanced ethylene response. Mutants with an enhanced ethylene response probably represent defects in factors required for opposing or resetting the ethylene signalling mechanism. Representatives of this group include mutants with lesions in the ethylene receptor ETR1 (etr1-7) (Cancel and Larsen, 2002) and the PP2a A regulatory subunit, RCN1 (eer1/rcn1) (Larsen and Chang, 2001; Larsen and Cancel, 2003), both of which are probably involved in activation/reactivation of CTR1 for suppression of the ethylene signalling pathway. Additionally, loss-of-function mutations have been found in two F-box factors, EBF1 and EBF2 (ebf1-1 and ebf2-1), both of which are required for proper turnover of the ethylene-responsive transcriptional activators, EIN3 and EIL1 (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Binder et al., 2007). Finally, work has recently been reported regarding the role of EER4, which is a component of the TAFIID complex, in opposing ethylene signalling (Robles et al., 2007). EER4, which is also known as TAF12b, probably functions as a bridge between specific events such as ethylene-dependent gene transcription and the general transcriptional machinery of the TFIID complex. This has been demonstrated by a direct interaction between EER4 and EIN3, with an eer4 loss-of-function mutation resulting in elimination of ERF1 expression following ethylene signalling. Interestingly, in conjunction with the observed selective loss of gene expression, the eer4 mutant has a severe enhanced ethylene response and, when combined with ein3-1 as a double mutant, the eer4 mutation at least partially restores ethylene responsiveness. From these results, it can be argued that EER4 not only mediates transcription of a subset of positive regulators of ethylene response, such as ERF1, but is also required for transcription of components of a previously undocumented mechanism required for resetting or dampening the ethylene signalling pathway.

Further identification of mutants with an enhanced ethylene response, which are characterized as having increased sensitivity and/or exaggeration of response to ethylene, should continue to help elucidate this critically important pathway, especially with regard to how the ethylene signalling pathway is dampened following a signalling event. In this report, the requirement of an Arabidopsis prohibitin for proper ethylene signalling is documented, with loss of this factor in the eer3 mutant resulting in extreme ethylene responsiveness in etiolated seedlings.
Materials and methods

Growth conditions
For all seedling growth experiments, seeds were surface-sterilized and cold stratified at 4 °C for 4 d in the dark to synchronize germination. Seeds were then suspended in 0.15% (w/v) agarose and sown on PNS medium (Larsen and Chang, 2001). For triple response experiments, the medium was supplemented with either 5 μM AgNO₃ (Sigma Chemical Co., St Louis, MO, USA) or 5 μM aminoethoxyvinylglycine (AVG) (Sigma) as required. Ethylene and propylene dose–response analyses along with analysis of jasmonic acid (JA) responsiveness were all done as previously described (Cancel and Larsen, 2002; Larsen and Cancel, 2003).

All adult plants in this study were grown in soil under a 24 h light cycle at 20 °C in a plant growth room supplemented with Sylvania Gro-Lite fluorescent bulbs. For treatments of leaves for RNA extraction, adult plants were grown for 4 weeks in air and then treated with either air or 100 μl l⁻¹ ethylene for 24 h. Immediately after treatment, leaf tissue was collected and quick-frozen for RNA extraction.

Measurement of ethylene production
Measurement of ethylene production was conducted as previously described (Larsen and Cancel, 2003).

Northern analysis
RNA analysis was performed as previously described (Larsen and Cancel, 2003).

Map-based cloning of eer3-1
For generation of a mapping population, eer3-1 (male; ecotype Col-0) was crossed to Ws-0 wild-type (female), and F₂ seedlings that displayed the eer3-1 phenotype in the presence of 100 μl l⁻¹ ethylene were isolated and planted in soil for subsequent collection of leaf tissue and isolation of genomic DNA. Genomic DNA was prepared as described (Larsen and Cancel, 2003) and used as a template for PCR-based mapping.

To narrow the map position of eer3-1, novel CAPS (cleaved amplified polymorphic sequence) markers were developed in a previously described manner (Larsen and Cancel, 2003) for the region to which eer3-1 was localized. CAPS markers generated and used for map-based cloning of eer3-1 were as follows. For the MNF13.20 marker, primers used were 5'-TTGATGAAGTTGG-3' and 5'-ATTAATATATCATGTTCTG-3'. MboI digestion resulted in five DNA fragments for Col-0 and four for Ws-0. For the MNF13.24 marker, primers used were 5'-GATCATCTCATGTTCTG-3' and 5'-CAGAAC-GCATATATATGTTCTG-3'. AflIII digestion resulted in two DNA fragments for Col-0 and one for Ws-0.

Candidate genes within the genetic window were identified and amplified by PCR with Pfu Turbo (Stratagene) and primers designed to the predicted 5' and 3' untranslated regions (UTRs), subcloned into pGemT-easy (Promega, Madison, WI, USA), and sequenced by the University of Florida DNA Sequencing Core Laboratory. Sequences were compared with the published Arabidopsis genomic sequence to identify the eer3-1 mutation.

Generation of transgenic plants
Functional complementation was performed by generation by PCR of a genomic construct representing 1 kb of upstream sequence, the complete coding sequence for At5g40770, and its predicted 3' UTR. This construct was subcloned into pFGCS941 and introduced into the eer3-1 mutant by Agrobacterium-mediated transformation. Primary transformants were selected by screening for those that were Glufosinate-ammonium (Sigma Chemical Co.) resistant, with T₃ plants subsequently analysed by growth in the dark for 4 d in 100 μl l⁻¹ ethylene, after which they were assessed for manifestation of the exaggeration of ethylene response seen for eer3-1.

Genetic analysis
Double mutants were generated by crossing eer3-1 (male) to cvr1-3 (female) and eer3-2 (male) to either ein2-5 or ein3-1 (females). F₂ progeny from the crosses with ein2-5 and ein3-1 were grown in the dark for 4 d with 100 μl l⁻¹ ethylene to isolate ethylene-insensitive individuals homozygous for ein2-5 or ein3-1. Isolated individuals were subsequently genotyped by PCR to identify those that were homozygous for the eer3-1 mutation. For PCR analysis, 5'-CGGATTTGATGATGATGTG-3' and 5'-GTGGTAGGTTC-GAGAACATACATC-3' were used to amplify a portion of the EER3 genomic DNA, after which the PCR products were cut with Rsal and BstI, with only wild-type DNA being cut by BstI at nucleotide 404 of the PCR product.

For generation of the eer3-1/cvr1-3 double mutant, F₂ seedlings were screened in the absence of ethylene for those that exhibited a constitutive triple response. Identified individuals were subsequently genotyped by PCR to identify those that were homozygous for the eer3-1 mutation.

GFP analysis of protein localization
An EER3-GFP (green fluorescent protein) translational fusion was made by combining the At5g40770 genomic construct, represented by 1 kb of upstream sequence, the complete coding sequence for At5g40770 with the both the intron and exons without the native stop codon, with the full coding sequence of rsGFP. This construct was cloned into pBl101, transferred to Agrobacterium, and infiltrated into Nicotiana benthamiana leaves. After 3 d, leaves were harvested and treated with 5 μM AgNO₃ or 100 μl l⁻¹ ethylene for 24 h, stained with 15 mg ml⁻¹ 4’,6-diamidino-2-phenylindole (DAPI) for 30 min, and visualized using a Leica SP-2 confocal microscope. As a control, Agrobacterium carrying an unfused rsGFP construct under the control of the Arabidopsis ACT2 promoter (An et al., 1996) was infiltrated into tobacco leaves.

Results

eer3-1 mutant seedlings are hypersensitive to ethylene and propylene
In order to isolate Arabidopsis mutants with enhanced ethylene response (eer), ethylmethane sulphonate (EMS)-mutagenized M₂ Col-0 seedlings were grown in the dark for 4 d in the presence of 100 μl l⁻¹ ethylene, after which seedlings with extreme hypocotyl shortening compared with the Col-0 wild type were isolated as putative eer mutants. Following isolation, putative eer mutants that showed growth comparable with the wild type in the presence of either ethylene perception or biosynthesis inhibitors, yet extreme response to saturating ethylene, were selected for further analysis. For this report, the recessive mutant eer3-1 was chosen for characterization and subsequent map-based cloning of the respective mutation.
Initially, eer3-1 was compared with Col-0 wild type with regard to its capacity for growth in the absence or presence of increasing concentrations of exogenous ethylene. For this analysis, eer3-1 and Col-0 wild-type seedlings were grown for 4 d in the dark in the presence of either 5 μM AgNO₃, which is an ethylene perception inhibitor, or 5 μM AVG, which is an ethylene biosynthesis inhibitor, with the latter treatment supplemented with increasing concentrations of exogenous ethylene. From this analysis, it was found that in the absence of ethylene signalling, as represented by treatment with AgNO₃, the eer3 mutant was capable of hypocotyl elongation similar to the wild type, with eer3-1 hypocotyls approximately 65% the height of Col-0 wild type (Fig. 1A). Addition of increasing concentrations of ethylene had a greater inhibitory effect on the elongation

![Fig. 1. Growth of etiolated eer3-1 seedlings in the presence of ethylene or propylene. An ethylene dose–response analysis was performed for Col-0 wild-type and eer3-1 seedlings. For this analysis, seedlings were grown for 4 d in the dark with either 5 μM AgNO₃, in order to block ethylene perception, or 5 μM AVG, in order to reduce ethylene biosynthesis, with increasing concentrations of ethylene ranging from 0 μl l⁻¹ to 100 μl l⁻¹. Following growth, hypocotyl lengths for each treatment were determined. (A) The top panel represents actual hypocotyl length. (B) The middle panel shows relative hypocotyl length (length/length at 5 μM AgNO₃), with the concentration of ethylene causing 50% inhibition denoted by (—). The inset photograph shows 4-day-old dark-grown seedlings of Col-0 wild type and eer3-1 exposed to 100 μl l⁻¹ ethylene. (C) The bottom panel shows the ratio of eer3-1 hypocotyl length to Col-0 wild-type hypocotyl length for each ethylene concentration, with (—) denoting the predicted ratio if the eer3-1 mutant was not hyper-responsive to ethylene. Mean ±SE values were determined from approximately 30 seedlings. A dose–response analysis using the ethylene agonist propylene was performed to demonstrate further the enhanced ethylene response phenotype of the eer3-1 mutant. As with the ethylene dose–response analysis, Col-0 and eer3-1 seedlings were grown for 4 d in the dark with either 5 μM AgNO₃ or 5 μM AVG, with increasing concentrations of propylene ranging from 0 ml l⁻¹ to 10 ml l⁻¹. Following growth, hypocotyl lengths for each treatment were determined. (D) The top panel represents actual hypocotyl length. (E) The middle panel shows relative hypocotyl length (length/length at 5 μM AgNO₃), with the concentration of propylene causing 50% inhibition denoted by (—). (F) The bottom panel shows the ratio of eer3-1 hypocotyl length to wild-type hypocotyl length for each propylene concentration, with (—) denoting the predicted ratio if the eer3-1 mutant was not hyper-responsive to propylene. Mean ±SE values were determined from approximately 30 seedlings.](https://academic.oup.com/jxb/article-abstract/58/8/2237/556019)
of eer3-1 hypocotyls compared with Col-0 wild type, with Col-0 wild type requiring upwards of 3-fold higher ethylene to achieve 50% inhibition of hypocotyl elongation compared with eer3-1 (Fig. 1B). Most striking was the profound difference in hypocotyl elongation when comparing Col-0 wild type with eer3-1 at saturating levels of exogenous ethylene, with eer3-1 hypocotyls being <20% the height of Col-0 wild type (Fig. 1C). It should be noted that neither of these phenotypic differences could be accounted for by eer3-1 overproducing ethylene since AVG was included in this dose–response analysis, effectively limiting endogenous ethylene, and the most severe phenotypic difference seen between eer3-1 and Col-0 wild type occurs in the presence of saturating ethylene.

In order to confirm that eer3-1 is indeed ethylene hypersensitive, a dose–response analysis using the ethylene agonist propylene was performed. For this analysis, eer3-1 and Col-0 wild-type seedlings were grown for 4 d in the dark in the presence of either 5 μM AgNO3 or 5 μM AVG, the latter of which was supplemented with increasing concentrations of propylene. Following incubation, hypocotyl length was measured, which revealed that eer3-1 had similar growth characteristics when exposed to propylene in comparison with ethylene treatment (Fig. 1D). From this analysis, it was found that the lengths of eer3-1 hypocotyls were comparable with those of Col-0 wild type in the presence of AgNO3, with eer3-1 approximately 85% the height of Col-0 wild type. Treatment with propylene revealed that there was upwards of a 3-fold increase in the concentration of propylene required to cause 50% inhibition of hypocotyl elongation for Col-0 wild type compared with eer3-1 (Fig. 1E). Finally, there was a profound exaggeration of response for eer3-1 compared with Col-0 wild type to saturating concentrations of propylene (Fig. 1F), with eer3-1 seedlings <10% the height of Col-0 wild type.

The eer3 mutant has increased ethylene production and reduced rosette size

The eer3-1 mutant was also examined for additional phenotypic manifestations in other tissues. From this analysis, it was found that eer3-1 roots have a reduced capability to grow in an ethylene-independent manner (Fig. 2A). Dose–response analysis with increasing concentrations of ethylene did not reveal either increased sensitivity or exaggeration of response to ethylene, suggesting that the limited growth capacity found for eer3-1 roots is due either to a general growth defect or to a constitutive ethylene response. Additionally, eer3-1 mutants demonstrated a significant reduction in rosette size compared with Col-0 wild-type plants. Total leaf area was measured for 4-week-old Col-0 wild-type and eer3-1 plants and, from this analysis, it was determined that eer3-1 adult plants are approximately 50% smaller than Col-0 wild-type plants in the absence of exogenous ethylene (Fig. 2D).

In order to determine if the eer3-1 mutation causes an increase in ethylene production, ethylene was collected for 12 h from 4-d-old dark-grown Col-0 wild type and eer3-1 seedlings and the collected gas was subsequently measured using a gas chromatography system. As shown in Fig. 2D, it was found that eer3-1 mutant seedlings produce approximately four times more ethylene than Col-0 wild type, although this cannot be considered to be the cause of the eer3-1 dark-grown seedling phenotype since the aforementioned growth analyses were done in the presence of an ethylene biosynthesis inhibitor and the exaggerated phenotype for eer3-1 was observed following treatment with saturating ethylene.

The eer3-1 mutation negatively impacts ethylene-regulated gene expression

It was also of interest to determine if the eer3-1 mutation had an effect on ethylene-inducible gene expression, with the expectation being that in conjunction with the severe effect on growth seen for the mutant, there would be a concomitant increase in induction of ethylene-regulated genes.
genes. For this analysis, expression of ethylene-responsive reporter genes was assessed in both ethylene-treated seedlings and leaves of eer3-1 and Col-0 wild type.

Several genes have been reported to be excellent indicators of the ethylene response in etiolated seedlings, including an ACC oxidase, ACO2 (Chao et al., 1997), an ethylene receptor, ETR2 (Sakai et al., 1998), and an AP2-like transcription factor, AtEBP (Buttner and Singh, 1997). For this analysis, Col-0 wild-type and eer3-1 seedlings were grown in the dark for 4 d in either the presence or absence of 5 μM AgNO₃ or 100 μl L⁻¹ ethylene, after which total RNA was isolated from collected tissue. Northern analysis revealed that for this limited sample of ethylene-regulated genes, the eer3-1 mutation either had no effect or a deleterious effect on ethylene-dependent induction of gene expression. As shown in Fig. 3A, ethylene treatment resulted in normal induction of ETR2 in eer3-1 compared with Col-0 wild type, whereas both ACO2 and AtEBP were not up-regulated in the mutant to the same level as seen for Col-0 wild type. Further analysis of AtEBP expression in seedlings exposed to a variety of conditions, including 5 μM AgNO₃, air, 500 nl l⁻¹ ethylene, and 100 μl L⁻¹ ethylene revealed a profound reduction in AtEBP expression in the eer3-1 mutant compared with Col-0 wild type in response to ethylene (Fig. 3B).

Ethylene-responsive gene expression was also assessed for eer3-1 leaves using several genes that have been demonstrated to be excellent indicators of the ethylene response. The reporter genes chosen included the ethylene- and JA-inducible AP-2 like transcription factor, ERF1, the ethylene receptor, ETR2, and the defence-related genes, basic chitinase (chiB) and PDF1.2. For this analysis, 4-week-old plants of eer3-1 and Col-0 wild type were exposed to either air or 100 μl L⁻¹ ethylene for 24 h, after which leaf tissue was collected for isolation of total RNA. For both ERF1 and ETR2, there was no apparent difference in ethylene inducibility when comparing Col-0 wild type and eer3-1. In contrast, the eer3-1 mutation has a significant deleterious effect on the expression of both basic chitinase and PDF1.2 following ethylene treatment compared with Col-0 wild type, suggesting that normal EER3 function is required for proper ethylene inducibility of these particular genes. Treatment with 100 μM +/- JA resulted in proper induction of both basic chitinase and PDF1.2 in eer3-1 (Fig. 3D), suggesting that the mutant phenotype of eer3-1 is probably specific to ethylene signalling.

The eer3-1 phenotype results from an amino acid substitution in an Arabidopsis prohibitin

A mapping population was generated by crossing eer3-1 (Col-0 background) to Ws wild type and subsequently selecting F₂ progeny that displayed exaggerated responsiveness to saturating ethylene. From this analysis, eer3-1 was localized to the bacterial artificial chromosome (BAC) MNF13 and subsequently to the gene At5g40770, which has previously been reported to encode an Arabidopsis prohibitin of unknown function, AtPHB3 (Fig. 4A, B). The eer3-1 mutation represents a single nucleotide change (G to A at nucleotide 493) in the coding sequence of At5g40770, which leads to substitution of N for D at position 165 in the AtPHB3 protein.

Total RNA was isolated from Arabidopsis roots, leaves, stems, and flowers in order to determine the tissue-specific expression pattern of EER3. Total RNA was also extracted from 4-d-old dark-grown Col-0 wild-type

![Fig. 3. The eer3-1 mutation limits induction of a subset of ethylene-regulated genes in both seedlings and leaves. (A) Ethylene-dependent gene expression in dark-grown seedlings of Col-0 wild type and eer3-1. Col-0 wild-type and eer3-1 seedlings were grown in the presence or absence of 5 μM AgNO₃ or 100 μl L⁻¹ ethylene in the dark for 4 d, after which tissue was collected for isolation of total RNA. For each sample, 10 μg of total RNA was used for northern analysis to test the expression of genes known to be ethylene responsive in seedlings including ACO2, ETR2, and AtEBP. Tomato 18S rDNA was used to judge loading accuracy. (B) Expression of AtEBP in Col-0 wild-type and eer3-1 dark-grown seedlings was checked over a range of ethylene concentrations. For this experiment, Col-0 wild-type and eer3-1 seedlings were grown for 4 d in either 5 μM AgNO₃ or 10 μM AVG in the presence of air, 500 nl L⁻¹ ethylene, or 100 μl L⁻¹ ethylene, after which tissue was collected for isolation of total RNA. For northern analysis, 10 μg of total RNA were used to test the expression of AtEBP, with tomato 18S rDNA being used to judge loading accuracy. (C) For analysis of ethylene-responsive gene expression in leaves, 4-week-old adult plants of Col-0 wild type and eer3-1 were exposed to air (A) or 100 μl L⁻¹ ethylene (E) for 24 h, after which leaf tissue was collected and total RNA was isolated. For each sample, 10 μg of total RNA were used for northern analysis to test the expression of the ethylene-responsive genes ERF1, ETR2, chiB, and PDF1.2, along with tomato 18S rDNA. (D) In order to test the effects of the eer3-1 mutation on jasmonate-inducible gene expression, 13-d-old plants of Col-0 wild type and eer3-1 grown hydroponically were treated with either air (A) or 100 μM (+/-) jasmonic acid (JA) for 24 h, after which rosette tissue was collected and total RNA was isolated. For each sample, 10 μg of total RNA were used for northern analysis to test the expression of the jasmonate-responsive genes ERF1, chiB, and PDF1.2, along with tomato 18S rDNA.](https://academic.oup.com/jxb/article-abstract/58/8/2237/556019)
seedlings treated with either 5 \( \mu M \) AgNO\(_3\) or 100 \( \mu M \) ethanol and from eer3-1 seedlings treated with 100 \( \mu M \) 1-ethanol. EER3 was expressed from low to moderate levels in all tissues tested, but was not found to be ethylene inducible (Fig. 4C, D). The eer3-1 mutation did not significantly reduce the stability of the EER3 transcript (Fig. 4D).

A genomic construct consisting of 1 kb of the upstream promoter sequence, the 5' UTR, two exons and an intron, and the 3' UTR was generated in pFGC5941 and introduced into eer3-1 by Agrobacterium-mediated transformation. Glufosinate-resistant T2 progeny were subsequently analysed for growth capability in the presence of saturating ethylene, with dark-grown 4-d-old transgenic lines demonstrating ethylene-responsive growth that was identical to Col-0 wild type instead of eer3-1 (Fig. 4E), indicating that the observed nucleotide change is indeed the cause of the mutant phenotype. Over-expression of EER3 in Arabidopsis did not cause any observable phenotypic changes (data not shown).

**A strong loss-of-function allele of eer3 results in severe constitutive ethylene response**

Since it was not clear whether eer3-1 represented a complete loss-of-function mutation, it was necessary to identify a loss-of-function insertion allele. For this, a T-DNA line (Alonso et al., 2003a) that represented an

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**Fig. 4. eer3-1 represents a loss-of-function mutation that results in an amino acid substitution in the Arabidopsis prohibitin, AtPHB3.** (A) Physical mapping of eer3-1. Thick bars represent the order of bacterial artificial chromosomes (BACS) from the 16.3–16.4 Mb region of chromosome 5. The BAC that contains the EER3 gene and the recombination frequencies at each generated CAPS marker surrounding the eer3-1 mutation are indicated. All CAPS markers generated are described in the Materials and methods. The genomic structure of Arabidopsis EER3 is depicted by boxes that represent exons and intervening lines that represent either non-coding intragenic regions or introns. The eer3-1 and eer3-2 mutations are indicated. (B) Comparison of the protein sequence of EER3/AtPHB3 with other known prohibitins from the indicated species. The eer3-1 mutation, which represents a substitution of N for D at position 159, is specified. (C) Pattern of EER3 expression in various Arabidopsis tissues. For northern analysis, 10 \( \mu g \) of total RNA from roots, leaves, stems, and flowers were electrophoretically separated, blotted, and probed with either EER3 or tomato 18S rDNA. (D) EER3 expression is not ethylene regulated. Col-0 wild-type seedlings were grown in the dark in the presence of either 5 \( \mu M \) AgNO\(_3\) or 100 \( \mu M \) 1-ethanol for 4 d, after which tissue was collected and total RNA was isolated. eer3-1 seedlings were grown in the dark only in the presence of 5 \( \mu M \) AgNO\(_3\), after which tissue was collected for RNA isolation. For northern analysis, 10 \( \mu g \) of total RNA from each sample were electrophoretically separated, blotted, and probed with either EER3 or tomato 18S rDNA. (E) Functional complementation of the eer3-1 mutation. A genomic construct of EER3 consisting of the promoter, 5' UTR, coding sequence, and 3' UTR was introduced into the eer3-1 mutant by Agrobacterium-mediated transformation. Dark-grown Col-0 wild-type, eer3-1, and T2 progeny from eer3-1 transformed with wild-type EER3 were tested for manifestation of an enhanced ethylene response phenotype following incubation in the dark with 100 \( \mu M \) 1-ethanol for 4 d.
insertion in exon 2 of \textit{At5g40770} was obtained and homozygous progeny were generated, with this line being referred to as \textit{eerr3-2} for the remainder of the report. Growth of \textit{eerr3-2} for 4 d in the dark in the presence of 5 \textmu M AgNO\textsubscript{3} produced an extreme constitutive ethylene response that was identical to ethylene-treated \textit{eerr3-1}, including a pronounced apical hook (Fig. 5A), thus suggesting that \textit{eerr3-1} represents an ethylene conditional allele since \textit{eerr3-1} seedlings grow relatively normally in the absence of ethylene. In the presence of 100 \textmu M ethylene, the phenotypes of dark-grown \textit{eerr3-1} and \textit{eerr3-2} were indistinguishable, with both exhibiting the same extreme ethylene response phenotype. The growth of adult \textit{eerr3-2} plants in the absence of exogenous ethylene was also phenotypically identical to that of \textit{eerr3-1} plants (data not shown).

Northern analysis was performed to determine the effect of the T-DNA insertion on expression of \textit{At5g40770} in \textit{eerr3-2}. For this analysis, Col-0 wild-type and \textit{eerr3-2} seedlings were grown in the dark for 4 d, after which tissue was collected for RNA analysis. The T-DNA insertion in \textit{eerr3-2} was found to cause both a significant increase in \textit{At5g40770} transcript size and a reduction in transcript abundance in comparison with Col-0 wild type (Fig. 5B).

Northern analysis also revealed that ethylene-dependent gene expression patterns were altered in \textit{eerr3-2} similarly to \textit{eerr3-1}. Total RNA was extracted from 4-d-old dark-grown Col-0 wild-type and \textit{eerr3-2} seedlings treated with either 5 \textmu M AgNO\textsubscript{3} or 100 \textmu M ethylene, and this was used for northern analysis with the ethylene-inducible gene \textit{AtEBP} (Fig. 5C). From this analysis, expression of \textit{AtEBP} following ethylene treatment was severely reduced in \textit{eerr3-2} compared with Col-0 wild type. Gene expression was also analysed for 4-week-old Col-0 wild-type and \textit{eerr3-2} leaves that were treated with either air or 100 \textmu M ethylene for 24 h (Fig. 5D). Northern analysis focused on \textit{PDF1.2}, with expression of this being severely reduced in the \textit{eerr3-2} mutant compared with Col-0 wild type following ethylene treatment, thus verifying that \textit{eerr3-2} is a \textit{bona fide} loss-of-function allele.

Double mutants between \textit{eerr3} and mutants of known ethylene signalling components

As part of an effort to understand the role of \textit{AtPHB3} in ethylene signalling, double mutants were made between \textit{eerr3} loss-of-function mutants and mutants representing defects in known components of the ethylene signalling pathway. The \textit{ctr} loss-of-function mutant has a strong constitutive ethylene response even in the absence of ethylene. Because of this, an \textit{eerr3-1;ctr1-3} double mutant was constructed in order to determine if the \textit{eerr3-1} phenotype could be elicited in this double mutant in the absence of ethylene. For this analysis, Col-0 wild-type, \textit{eerr3-1}, \textit{ctr1-3}, and \textit{eerr3-1;ctr1-3} seedlings were grown in the dark in air for 4 d, after which seedling height was assessed (Fig. 6A). The \textit{eerr3-1;ctr1-3} double mutant had an extreme hypocotyl inhibition phenotype that was nearly identical to ethylene-treated \textit{eerr3-1}, suggesting that \textit{eerr3-1} is required to oppose the activation of the ethylene signalling pathway that arises upon loss of \textit{CTR1} function. Additionally, there was a moderate reduction in the size of adult \textit{eerr3-1;ctr1-3} plants compared with either \textit{ctr1-3} or \textit{eerr3-1}.

The \textit{ein2-5} loss-of-function mutation results in complete ethylene insensitivity due to a lesion in \textit{EIN2}, which encodes a component required for propagation of an ethylene signal. In order to determine if the \textit{eerr3-2} mutation is epistatic to the \textit{ein2-5} mutation, an \textit{eerr3-2;ein2-5} double mutant was constructed and it was assessed as to whether the \textit{ein2-5} mutation can mask the severe constitutive ethylene response associated with the \textit{eerr3-2} mutation. Col-0 wild-type, \textit{ein2-5}, \textit{eerr3-2}, and
eer3-2;ein2-5 seedlings were grown in the dark in air for 4 d, after which seedlings were assessed for presentation of a severe triple response phenotype. As shown in Fig. 6B, whereas seedlings of Col-0 wild type and ein2-5 had long hypocotyls in air, both eer3-2 and the eer3-2;ein2-5 double mutant had the extreme hypocotyl shortening phenotype associated with eer3-2. Since ein2-5 represents a mutation that results in complete blockage of ethylene signalling, this indicates that EER3 functions at or below EIN2 in this pathway.

The ein3-1 mutation, which is a loss-of-function mutation in the gene encoding the transcription factor EIN3, results in partial ethylene insensitivity in etiolated seedlings. An eer3-2;ein3-1 double mutant was constructed in order to determine if the ein3-1 mutation could block the manifestation of the extreme constitutive ethylene response phenotype seen for eer3-2 in air. For this analysis, Col-0 wild-type, eer3-2, ein3-1, and eer3-2;ein3-1 seedlings were grown in the dark in air for 4 d, after which hypocotyl length was assessed. As shown in Fig. 6C, the ein3-1 mutation was incapable of blocking manifestation of the eer3-2 phenotype in the eer3-2;ein3-1 double mutant, suggesting that EER3 functions at or below the level of EIN3-mediated transcription in the ethylene signalling pathway.

**GFP localization of EER3**

In order to determine the subcellular location of EER3, a translational fusion between EER3 and rsGFP (PROEER3:EER3:rsGFP) was constructed and the localization of this was compared with that of GFP alone (PROACT2:rsGFP). Leaves of *N. benthamiana* were infiltrated with *Agrobacterium* carrying either PROACT2:rsGFP or PROEER3:EER3:rsGFP, and, after 3 d, samples were examined for fluorescence using a confocal microscope. Analysis revealed a normal pattern of cytoplasmic fluorescence for rsGFP, with little or no signal within the nucleus (Fig. 7). For EER3:rsGFP, fluorescence was widely distributed throughout the epidermal cells expressing this fusion protein, including large patches throughout the cytoplasm along with areas within and surrounding the nucleus, as demonstrated by counterstaining with DAPI. This type of complex pattern of localization is consistent with what has been reported for prohibitins in other biological systems.

**Discussion**

In order to further the understanding of the mechanisms underlying ethylene signalling, a unique approach of identifying and characterizing mutants that have enhanced response to ethylene has been undertaken in recent years. To date, several factors that have either increased
sensitivity or exaggeration of response to ethylene have been identified, suggesting that these factors are responsible for opposing or resetting the ethylene signalling pathway. As part of this effort to identify new components of this pathway, an Arabidopsis mutant with extreme exaggeration of response to ethylene has been identified, with the *eer3-1* mutation negatively affecting a previously uncharacterized Arabidopsis prohibitin, AtPHB3.

*eer3-1* is a mutant with an increase in sensitivity to moderate levels of ethylene coupled with severe hypocotyl shortening in the presence of saturating ethylene. In the absence of ethylene, *eer3-1* seedlings are capable of near normal growth, as evidenced by treatment with the ethylene perception inhibitor, AgNO₃, strongly suggesting that the phenotype of *eer3-1* is conditionally dependent on ethylene signalling and that *eer3-1* does not represent a mutant with a general growth defect. Surprisingly, analysis of a complete loss-of-function allele, *eer3-2*, revealed that absence of AtPHB3 function results in a constitutively profound exaggerated ethylene response, with almost all AgNO₃-treated seedlings being fully hooked yet a fraction of the size of ethylene-treated Col-0 wild-type seedlings. From the present analysis of *eer3-2*, it can be argued that AtPHB3 functions in part as a critical negative regulator of the ethylene signalling pathway.

Surprisingly, even though both *eer3* loss-of-function alleles demonstrate profound exaggeration of response to ethylene at the visual level, the present analysis has not revealed positive changes in ethylene-regulated gene expression that are concomitant with the severity of the suppression of growth seen for both. In contrast, for each mutant allele, expression of a subset of ethylene-regulated genes is actually negatively impacted, including the AP2-like transcription factor, AtEBP, and the defensin, PDF1.2. This suggests that AtPHB3 in part also plays a positive role in expression of ethylene-regulated genes following ethylene signalling. The findings regarding the role of AtPHB3 in promoting ethylene-responsive gene expression are not inconsistent with the phenotype of the *eer3-2;ein3-1* double mutant, which argues that AtPHB3 functions either below or in parallel to EIN3-mediated transcription. It should be noted that although *eer3* loss-of-function mutants are phenotypically similar to the *ebf1-2;ebf2-1* double mutant (Guo and Ecker, 2003), there is no apparent effect of the *eer3-1* or *eer3-2* mutations on *EBF1* and *EBF2* expression (MJC and PBL, data not shown).

Although paradoxical, it is likely that AtPHB3 actually serves in some capacity that is related to transcription of both genes required for promotion of a subset of ethylene responses, some of which may be required to oppose the manifestation of the ethylene response, and those required to sustain growth, thus resulting in the profound inhibition of hypocotyl elongation in the mutant. Based on this model, ethylene signalling may result in not only the induction of genes responsible for progression of response but also the suppression of expression of genes required for maintenance of growth. Consistent with this proposed role for AtPHB3 in directly regulating gene expression, localization patterns indicate that among the multiple locations where it is found in the cell, there is a distinct population of AtPHB3 located in the nucleus.

Reports from other systems have demonstrated that some prohibitin proteins participate in formation of transcriptional complexes, resulting in either activation or repression of target genes (Fusaro et al., 2003; Rastogi et al., 2006). This is best evidenced by activation of transcription of p53 targets through a direct interaction between prohibitin and p53, with loss of prohibitin function resulting in tumour formation due to failure to initiate cellular senescence (Fusaro et al., 2003). In conjunction with activation of p53-regulated senescence, prohibitin in mammalian cells is also responsible for the repression of E2F1-regulated gene expression through chromatin remodelling. E2F1 activity, which is a factor required for cell cycle progression and proliferation, is repressed upon stimulation of cell cycle arrest by environmental stresses or DNA damage. This repression of E2F1 activity is co-ordinated through recruitment of heterochromatin protein 1 by prohibitin to form heterochromatic foci that lead to suppression of E2F1-regulated gene expression (Rastogi et al., 2006). Loss of prohibitin function in mammalian cells results in failure to initiate a programmatic switch that is required for repression
of genes involved in growth concomitant with induction of genes required for promotion of senescence, thus resulting in inappropriate cell proliferation following stress.

Although interacting partners for AtPHB3 have not been reported, it is possible that AtPHB3 functions similarly to mammalian prohibitin to form transcriptional complexes for either promoting expression of a subset of ethylene-regulated genes, some of which may be required for resetting ethylene response, or maintaining or inducing transcription of genes required to sustain growth during or following ethylene signalling. In support of this, it was found that AtPHB3 interacts strongly in the yeast two-hybrid assay with a component of the TAFIID complex, Taf12b, with the eer3-1 mutation almost completely disrupting this association. Mutational loss of Taf12b also results in a severe enhanced ethylene response phenotype (Robles et al., 2007), suggesting that both AtPHB3 and Taf12b are required for regulation of a common subset of genes necessary for opposing the ethylene signalling pathway or maintenance of growth in the presence of ethylene (LM Robles and PB Larsen, unpublished results).

From the present analyses, the prohibitin AtPHB3 functions as a key regulator of ethylene signalling in Arabidopsis, serving to dampen the magnitude of ethylene signalling probably by participating in a subset of transcriptional processes that are required for the proper manifestation of the ethylene response. Future work will focus on determining the molecular role of prohibitin in these transcriptional processes, including defining its biochemical partners and transcriptional targets. By continuing to screen for and analyse factors that are required to oppose the ethylene response in Arabidopsis, a better understanding should develop regarding how this pathway is regulated, thus giving new means to control the manifestation of ethylene-mediated phenomena in plants in general.

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References
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