COI1, a jasmonate receptor, is involved in ethylene-induced inhibition of Arabidopsis root growth in the light

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Abstract

Plant response to stress is orchestrated by hormone signalling pathways including those activated by jasmonates (JAs) and by ethylene, both of which stunt root growth. COI1 is a JA receptor and is required for the known responses to this hormone. It was observed that the coi1 mutant, which is largely unresponsive to growth inhibition by JAs, was also partially unresponsive to growth inhibition by ethylene and by its immediate precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), in the light but not in the dark. Although COI1 was required for this response to ACC, other components of the JA signal perception pathway were not. Mutants selected for insensitivity to ethylene, including etr1, ein2, and ein3, showed greater ACC-induced root growth inhibition in the light than in the dark. However, the double mutants etr1;coi1, ein2;coi1, and ein3;coi1, and coi1 seedlings treated with silver ions to block the ethylene receptors showed almost complete unresponsiveness to ACC-induced root growth inhibition in the light. The light requirement for the COI1-mediated growth inhibition by ACC was for long photoperiods, and the ACC response was not abolished by mutations in the known photoreceptors. The complementation assay indicated that SCF complex assembly was not required for COI1 function in the ACC response, in contrast to the JA response. It is concluded that COI1 is required for the light-dependent, JA-independent, root growth inhibition by ethylene.

Key words: Arabidopsis, COI1, ethylene, jasmonate, light, root growth inhibition.

Introduction

In Arabidopsis, jasmonates (JAs) inhibit root growth (Staswick et al., 1992), and regulate pollen development, anther dehiscence (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000), defence against pests and pathogens (McConn et al., 1997; Thomma et al., 1999), and response to mechanical wounding (Devoto et al., 2005). A screen for Arabidopsis mutants unresponsive to growth inhibition by a bacterial toxin and the JA homologue, coronatine, revealed coronatine insensitive1 (coi1-1), a null mutant insensitive to JAs, which produces infertile pollen (Feys et al., 1994). COI1 is required for most JA responses studied so far (Feys et al., 1994; Benedetti et al., 1995; Vijayan et al., 1998; Xie et al., 1998; Reymond et al., 2000). COI1 encodes a 67 kDa protein containing an N-terminal F-box motif and leucine-rich repeats (LRRs) (Xie et al., 1998), and forms a functional E3 ubiquitin ligase, SCFCOI1(SKP1, CDC53p/CUL1 F-box protein) complex (Devoto et al., 2002; Xu et al., 2002). COI1 directly binds to the jasmonyl-isoleucine (JA–Ile) conjugate (Yan et al., 2009) and SCFCOI1 targets JASMONATE ZIM-DOMAIN (JAZ) proteins for degradation to activate downstream JA signalling in Arabidopsis (Chini et al., 2007; Thines et al., 2007). COI1 has 33% identity to the amino acid sequence of the F-box protein TIR1, an auxin receptor, which forms the SCFTIR1 ubiquitin ligase that regulates response to auxin (Ruegger et al., 1998; Xie et al., 1998; Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

Previous studies have defined plant signalling pathways through mutations that suppress response to a particular perturbation or hormone. Recent findings strongly suggest that hormone signalling is more complicated than the summation of linear pathways, and that mutants isolated
for altered response to one hormone might therefore have an altered response to others. Examples of such mutants include the auxin-insensitive mutant, axr1, which is also less sensitive to JA (Tiryaki and Staswick, 2002), and the DELLLA protein mutants selected for insensitivity to gibberellin, which are also less sensitive to 1-aminocyclopropane-1-carboxylic acid (ACC) (Achard et al., 2003; Fu and Harberd, 2003).

The plant hormone ethylene inhibits root growth and regulates developmental processes including germination, fruit ripening, programmed cell death, and responsiveness to stress and pathogens (Johnson and Ecker, 1998; Bleecker and Kende, 2000). Ethylene triggers the ‘triple response’ of etiolated dicotyledonous seedlings, characterised by inhibition of hypocotyl and root cell elongation, radial swelling of the hypocotyl, and exaggerated curvature of the apical hook (Neljubow, 1901; Guo and Ecker, 2004). Ethylene also induces prolific root hair formation (Tanimoto et al., 1995). Many of the genes of the ethylene signalling pathway have been identified (Wang et al., 2002; Guo and Ecker, 2004; Chen et al., 2005; Benavente and Alonso, 2006; Etheridge et al., 2006). These include five genes for the ethylene receptors ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and ETHYLENE INSENSITIVE4 (EIN4). Dominant mutations in these genes abolish the response to ethylene (Bleecker et al., 1988; Hua et al., 1995, 1998; Roman et al., 1995; Sakai et al., 1998). The ethylene receptors regulate the activity of CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), which in turn negatively regulates the downstream signalling pathway (Kieber et al., 1993; Chen et al., 2002; Guo et al., 2003; Huang et al., 2003). Key components of this include EIN2, a novel protein similar to members of the Nram metal-ion transporter family (Guzman and Ecker, 1990; Alonso et al., 1999), and EIN3, a transcription factor (Roman et al., 1995; Chao et al., 1997). EIN3 in turn regulates expression of other transcription factors including ETHYLENE RESPONSE FACTOR1 (ERF1), an integration between the ethylene and JA pathways (Solano et al., 1998; Lorenzo et al., 2003).

The interaction between JA and ethylene is complex. Both JA and ethylene biosynthesis are induced by wounding, pests, and pathogens (Creelman et al., 1992; O’Donnell et al., 1996; Kuc, 1997). The two hormones synergistically induce transcription of defence-related genes encoding PATHOGENESIS-RELATED5 (PR5), PLANT DEFENSIN1.2 (PDF1.2), a chitinase (CHI-B), and a hevein-like (HEL) protein (Xu et al., 1994; Penninckx et al., 1998; Norman-Setterblad et al., 2000). Both JA and ethylene pathways are constitutively activated in a cellulose synthase-defective mutant, constitutive expression of VSP1 (cev1) (Ellis and Turner, 2001; Ellis et al., 2002), and are required simultaneously to activate ERF1 and ORA59 which mediate defence responses against pathogens (Lorenzo et al., 2003; Pre et al., 2008). In contrast, ethylene antagonises expression of some other JA-responsive genes encoding vegetative storage proteins (VSPs) and a thionin (Thi1.2) (Rojo et al., 1999; Norman-Setterblad et al., 2000; Ellis and Turner, 2001), and JA antagonises ethylene-induced hypocotyl hook formation in the triple response, in a COI1-dependent manner (Ellis and Turner, 2002).

In the dark, coil-16 shows a wild-type response to root growth inhibition when exposed to ethylene, indicating that this response is COI1 independent (Ellis and Turner, 2002). However, it is reported here that in the light, coil-16 shows reduced response to ethylene-induced root growth inhibition. Significantly, this COI1-dependent response is independent of JA, and apparently does not require components of the known ethylene perception-response pathway. It is shown that the mechanism for regulation of ethylene-induced root growth inhibition differs depending on the light conditions. It was also found that the mechanism for regulation of ethylene-induced root growth inhibition is different from that of root hair elongation. These results are discussed in a new model for interaction of JA and ethylene signalling.

Materials and methods

Plant materials and growth conditions

The Arabidopsis thaliana accessions Col gl, Ws-0, and No-0, and the mutants jad1-1, aos, etr1-1, etr1-2, ein2-1, ein3-1, ein4, phyA-211, phyB-9 (Col background), opr3 (Ws-0 background), and ers2-1 (No-0 background) were obtained from the NASC (Loughborough, UK). coil-16 (Col gl/ background) was from the authors’ laboratory. etr2-1, ers1-1, jin1, 35S::ERF1 (Col background), and blue light receptor mutants (cry1, cry2, phot1, Col background; and phot2, Ws-0 background) were gifts from Dr Paul Larsen, Dr Chi-Kuang Wen, Dr John Mullet, Dr Roberto Solano, and Dr Tatsuya Sakai, respectively.

Seeds were surface-sterilised, spread on solidified 1% Murashige and Skoog (MS; Duchefa, Haarlem, The Netherlands) medium in square dishes, stratified at 4 °C for 2 d, and transferred to continuous light (CL) growth cabinets (Sanyo, Illinois, USA) or growth chambers at 22–23 °C with a light intensity of 70–90 μmol m−2 s−1 unless stated otherwise. For some experiments the medium was supplemented with the following growth regulators: methyl jasmonate (MeJA) was purchased from Bedoukian Research (Connecticut, USA), silver nitrate, sodium thiosulphate, ACC, ethylene, salicylic acid (SA), and epi-brassinolide (EBR) from Sigma-Aldrich (Dorset, UK), and 6-benzylaminopurine (BAP) from Duchefa. For ethylene treatment, square dishes were placed in a bell jar containing 5 ppm ethylene gas. For dark treatment, square dishes were wrapped with two layers of aluminium foil.

Construction of double mutants

etr1-1 was used as the male parent in crosses to the male-sterile coil-16. In the F2 population, the homozygous etr1-1 and coil-16 were identified by PCR. For etr1-1 mutation, the PCR and digestion were performed as described by Hua and Meyerowitz (1998). The amplification conditions were modified to 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The cycle was repeated 35 times, preceded by 2 min at 94 °C, and followed by 5 min at 72 °C. For coil-16 mutation, three primers were designed as SNAP (single nucleotide amplified polymorphism) markers (Drenkard et al., 2000): COI1-16 P2 (5’ GACAACTTTTGAAGATAGTGCAGCTCAGTACA), COI1-16 P3 (5’ AACTAGTTGGTCTTAAAGGCTGCAGCTATTA), and COI1-16 P4 (5’ AACTAGTTGGTCTTAAAGGCTGCAGCTATTC). The amplification conditions were 94 °C for 30 s, 68 °C for 1 min, and
was separated on a 1% agarose gel; the wild-type allele gave a band of 340 bp for the primer combination P2 and P4, and the coi1-16 allele gave a band of 340 bp for the primer combination P2 and P3.

For ers1-1;coi1-16, ers2-1;coi1-16, ein2-1;coi1-16, and ein3-1;coi1-16, F2 populations were grown in CL on MS agar supplemented with 50 μM MeJA and 4 μM ACC, and seedlings with long roots and less/no root hair were selected. Each of them was confirmed for double homozygous mutations in its F3 population.

Construction of transgenic lines

The transgenic lines LRR and W44 contained the constructs pΔFbox and pCOI1W44 respectively, as previously described (Devoto et al., 2002). These were introduced into the vector pPily and introduced into plants by Agrobacterium-mediated transformation. The F-box of the transgenic lines contained the construct pCOI1F-box which was obtained by replacing an Ncol–SmaI fragment of pLexA-COI1 (Devoto et al., 2002) with a purified and digested PCR fragment amplified with the primers s3 (5’-GATCTACCATGGAGGATCTGATATC) and a38b (5’-TAGCTACCC AGGAGTCA-CATGCTCTCTGTC). This was introduced into the vector pPily and introduced into plants by Agrobacterium-mediated transformation. The expression level of each transgene was verified by immunoblot analysis. All the constructs carry a kanamycin-resistant marker. Each construct line was used as the male parent in crosses to the male-sterile coi1-16 and coi-1. The homozygous coi1 lines with each transgene were selected for MeJA and kanamycin resistance in F2 and F3 populations.

Root growth assay

Seedlings were grown on agar surfaces at 75 ° to the horizontal. Seedlings were removed and the root lengths were measured directly on a ruler wetted with 50% (v/v) glycerol. Relative root growth was calculated according to the formula, $y = x - 100$, where $x$ was the average of root lengths of untreated seedlings and $y$ was the root lengths of each treated seedling, and was averaged. Statistics was performed, and $P$-values were calculated using SigmaPlot (Systat Software Inc, California, USA) and SPSS (http://spss.com/) programmes.

qRT-PCR analyses

Wild-type and coi1-16 seedlings were grown for 7 d in CL on MS agar supplemented or not with 4 μM ACC. Ten to 15 seedlings were harvested per sample and total RNA was extracted. Prior to quantitative real-time reverse transcription-PCR (qRT-PCR), total RNA was treated with DNase I (NEB, Massachusetts, USA) and synthesised into cDNA using SuperScript II reverse transcriptase (Invitrogen, California, USA) according to the manufacturer’s instructions. The reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems), a 7900HT Fast Real-Time PCR system (Applied Biosystems), and the standard curve method. The amplification conditions were 95 °C for 15 s and 60 °C for 1 min. The cycle was repeated 40 times, preceded by 95 °C for 2 min and followed by a dissociation programme to create melting curves. Three technical replicates for each sample were run. The β-tubulin gene (TUB2) was used as a reference gene. The primers designed by Primer Express version 3.0 (Applied Biosystems, California, USA) were as follows: ETR2 forward 5’-GGTTGCGGTGCTGCTATT A and reverse 5’-TCCTAGTCTCCATTAATAATCCGAAA; ERS2 forward 5’-GCCACACGGAGGCATTCA and reverse 5’-TGCTCAATGGAGCACCACACA; and TUB2 forward 5’-AAAGGCTTTCTGCAATTGGTACA and reverse 5’-CCGCTTCTGTAATTCACCTCCTC.

Results

The coi1-16 mutant shows reduced response to ACC-induced root growth inhibition

Seedlings of Arabidopsis accession Col gl (wild-type) and the mutant coi1-16 were grown in CL on media containing a range of compounds at concentrations that inhibit root growth, including MeJA, ACC, EBR, SA, and the cytokinin BAP, and their root growth was compared. coi1-16 displayed significantly less response than the wild-type to root growth inhibition induced by MeJA and by ACC (Figs 1, 2A). These results suggest that COI1 is required for part of the ACC-induced root growth inhibition and for most if not all of the MeJA-induced root growth inhibition. The role of COI1 on root growth in response to ACC was investigated.

ACC-induced, COI1-mediated root growth inhibition is via ethylene and does not require JA biosynthesis and perception

ACC is the direct precursor of ethylene. Tests were therefore carried out to determine whether ethylene had similar effects to ACC on root growth of wild-type and coi1-16 seedlings. Ethylene, like ACC, induced root growth inhibition in the wild-type but to a lesser extent in coi1-16 (54.4% root growth for the wild-type and 69.5% for coi1-16, $P < 0.001$).

Fig. 1. Effect of various root growth inhibitors on coi1-16. Seedlings were grown for 7 d in continuous light (CL) on Murashige and Skoog (MS) agar supplemented with either methyl jasmonate (MeJA), 1-aminocyclopropane-1-carboxylic acid (ACC), epi-brassinolide (EBR), salicylic acid (SA), or the cytokinin 6-benzylaminopurine (BAP) at the indicated concentration. Root lengths of treated seedlings compared with that of control seedlings are expressed as the mean of relative root growth (%). Bars indicate the standard error (SE). A significant difference ($P < 0.001$) compared with the wild-type is indicated with asterisks.
This suggests that the reduced response of coi1-16 to ACC is due to its reduced response to ethylene. COI1 is required for most JA responses reported, including JA-induced root growth inhibition. If ethylene enhanced JA biosynthesis, as is found in tomato (Sivasankar et al., 2000), then this could account for the reduced response of coi1-16 to ACC-induced root growth inhibition. To test this possibility, experiments were conducted to examine whether ACC also inhibited root growth in the JA biosynthesis mutants allene oxide synthase (aos) (Park et al., 2002; von Malek et al., 2002) and 12-oxophytodienoate reductase3 (opr3) (Stintzi and Browse, 2000). Both mutants showed a wild-type (Col gl for aos, and Ws-0 for opr3) response to ACC-induced root growth inhibition (Table 1). This indicates that JA synthesis is not required for the ACC-induced, COI1-mediated response.

COI1 is required for part of ACC- and ethylene-induced root growth inhibition. Experiments were therefore carried out to investigate whether the ethylene receptor ETR1 was also required for this response. A dominant mutant, etr1-1, was less responsive to ACC-induced root growth inhibition in continuous darkness (CD), as reported (Chang et al., 1993), but was only marginally less responsive than the wild-type in the light (Table 1; Figs 2B, 3A). In contrast, coi1-16 had a wild-type response to ACC in the dark (Table 1; Fig. 3A) as reported (Ellis and Turner, 2002). To test whether COI1 and ETR1 contributed additively to the ACC-induced, light-dependent root growth inhibition, the etr1-1;coi1-16 double mutant was constructed. etr1-1;coi1-16 showed reduced response to MeJA both in the light and in the dark, and was unresponsive to ACC in the dark (Fig. 3A), as expected from the phenotypes of the parental single mutants. However, the double mutant was less responsive than either coi1-16 or etr1-1 to ACC in terms of root growth in the light (Table 1; Figs 2B, 3A). This shows that the coi1-16 and etr1-1 mutations contribute additively to unresponsiveness to ACC in the light. Since this coi1-16 mutant line, from which the double mutant was constructed, was later found to contain a second mutation, pen2 (Westphal et al., 2008), seedlings containing a single coi1-16 mutant allele were treated with silver thiosulphate (STS) solution to inhibit ethylene perception in the light. STS restored root growth of ACC-treated wild-type and coi1-16 to 62.3±3.0% and 85.0±3.1%, respectively. Evidently, in the light, when the ethylene signalling is abolished by the etr1 mutation, or by silver ions, there remains a response to ethylene which requires COI1.

An additional interaction between COI1 and ETR1 was observed: on media containing MeJA, and in the dark, the percentage germination of the wild-type, etr1-1, and etr1-1;coi1-16 was 96, 3.3, and 86.7%, respectively (Table 2).
Table 1. Relative root growth of various mutants on ACC and their gene requirement for the ACC-induced root growth inhibition pathway

Seedlings were grown for 7 d in CD or CL on MS agar supplemented with 4 μM ACC. Root lengths of treated seedlings compared with that of control seedlings are expressed as the mean of relative root growth ± SE (%). Those genotypes showing significant differences (P <0.05) between light and dark treatments are indicated with asterisks. Data from wild-type controls, which correspond to specific experiments for each mutant, are indicated with matching alphabetical letters. If the gene is/is not required in each light condition this is indicated as +/-.

| Genotype | Description          | Relative root growth on ACC (%) |
|----------|----------------------|---------------------------------|
|          | CD                   | CL                             |
|          | Gene requirement     | Gene requirement               |
| Col g/   | Control              | 42.0±2.1 a                     | 50.9±2.9 A                     |
|          |                      | 53.7±2.9 b                     | 44.2±1.3 B                     |
|          |                      | 54.6±1.6 c                     | 42.0±1.1 C                     |
|          |                      | 62.2±2.7 d                     | 44.5±1.2 D                     |
|          |                      | 55.8±4.6 e                     | 53.6±2.4 E                     |
|          |                      | 39.3±2.9 f                     | 43.3±1.4 F                     |
|          |                      | 45.0±1.8 g                     | 41.6±1.2 G                     |
|          |                      | 49.4±4.6 h                     | 48.4±1.5 H                     |
|          |                      | 50.0±4.8 i                     | 41.7±1.8 I                     |
|          |                      | 57.1±10.7 j                    | 46.7±2.3 J                     |
|          |                      | 39.0±2.2 k                     | 47.5±1.4 K                     |
|          |                      |                                 | 47.1±1.3 L                     |
| Ws-0     | Control              | 32.4±5.7 l                     | 43.8±3.5 M                     |
|          |                      | 47.0±2.5 m                     | 46.1±2.0 N                     |
| coi1-16* | JA-insensitive mutant| 51.2±2.8 a                     | 70.2±2.5 A                     |
|          |                      | 46.0±4.6 b                     | 41.9±1.6 B                     |
|          |                      | 53.0±4.4 c                     | 43.6±2.1 C                     |
|          |                      | 40.3±1.9 b                     | 38.1±1.7 B                     |
|          |                      | 34.6±3.1 l                     | 42.6±1.7 M                     |
|          |                      | 106.5±8.6 d                    | 56.4±2.8 D                     |
|          |                      | 82.8±3.2 e                     | 65.3±3.8 E                     |
|          |                      | 66.7±4.9 c                     | 49.2±3.0 C                     |
|          |                      | 94.9±4.5 f                     | 71.4±3.1 F                     |
|          |                      | 83.9±3.7 m                     | 66.1±4.5 N                     |
| ctr1*    | Constitutive ethylene response mutant | 69.8±7.4 g                     | 88.4±5.8 G                     |
|          |                      | 99.2±4.4 h                     | 86.2±3.5 H                     |
|          |                      | 77.2±5.2 i                     | 65.2±4.1 I                     |
| 35S::ERF1| JA- and ethylene-inducible gene overexpressor | 37.0±4.8 c | 32.4±6.1 C |
| etr1-1;coi1-16 | Double mutant | 96.4±5.6 d | 83.0±6.0 K |
| etr1-1;coi1-16* | Double mutant | 77.0±2.8 b | 91.1±3.0 J |
| ers2-1;coi1-16 | Double mutant | 81.2±3.4 j,m | 82.7±3.5 J,N |
| ein3-1;coi1-16 | Double mutant | 92.8±4.5 h | 95.6±3.3 H |
| ein3-1;coi1-16 | Double mutant | 81.6±4.2 k | 92.7±4.0 L |

A similar, though less extreme, pattern was observed in the light. Evidently, the ability of ETR1 to bind ethylene and, in turn, activation of its downstream pathway, antagonises the effect of JA-induced, COII-mediated germination inhibition.

COII is not required for ACC-induced root hair elongation or gene expression

Exogenous ethylene or ACC increases the number and length of root hairs in Arabidopsis, in the light and in the dark (Tanimoto et al., 1995). Tests were conducted to determine whether this response required COII. ACC induced root hair elongation in both wild-type and coi1-16 seedlings in the light and in the dark (Fig. 3B, data not shown). In contrast, etr1-1 and etr1-1;coi1-16 seedlings showed no visible root hair elongation in the presence or absence of ACC (Fig. 3B). The ethylene-insensitive mutants listed in Table 1 were germinated in the light and in the dark on MS and on ACC. All had shorter root hairs compared with the wild-type, except ein4, as previously reported (Cho and Cosgrove, 2002) (data not shown). Together, these results indicate that ACC-induced root growth inhibition in the light requires both the COII and ETR1 pathways, whereas ACC-induced root hair elongation requires the ETR1 pathway but not the COII pathway.

Expression of ethylene-responsive genes was also investigated in coi1-16. Genes that are induced by ethylene but not
differentially regulated by JAs were selected according to a previous report (Hua et al., 1998) and Genevestigator (Hruz et al., 2008). Seedlings were grown in the light on MS and on ACC for 7 d, and expression of ETR2 and ERS2 was determined by qRT-PCR. In the wild-type, ETR2 and ERS2 were induced >2-fold by ACC (Fig. 4). In coi1-16, induction was marginally less compared with that of the wild-type (Fig. 4); however, the difference was not statistically significant, indicating that COI1 is not required for ACC-induced expression of ETR2 and ERS2.

**Involvement of other ethylene perception-response components in ACC-induced, light-dependent root growth inhibition**

To identify other ethylene perception-response genes involved in ACC-induced, light-dependent, COII-mediated root growth inhibition, a range of mutants was tested for light-induced response to ACC (Table 1). The reasoning behind this was that if the mutations defined genes that were in the signalling pathway for ACC-induced, light-dependent, COII-mediated root growth inhibition, then the mutant plants would show less response to ACC-induced root growth inhibition in the light than in the dark; that is, their phenotype would be similar in this respect to that of the coi1-16 mutant. The ethylene receptor mutants, ers1-1, ers2-1, etr2-1, and ein4 were less responsive to ACC than the wild-type, both in the light and in the dark, and were less responsive to ACC in the dark than in the light, similar to etr1-1. The constitutive ethylene response mutant ctr1-1 was partially responsive to ACC in the dark and in the light. ein2-1 and ein3-1 were less responsive to ACC than the wild-type in the light and in the dark, and were less responsive to ACC in the dark than in the light.

| CD | CL |
|----|----|
| MS | 50 µM MeJA | 4 µM ACC | MS | 50 µM MeJA | 4 µM ACC |
| Col/gf | 97.3 | 96.0 | 98.7 | 92.0 | 100 | 97.3 |
| coi1-16 | 100 | 93.3 | 93.3 | 93.3 | 100 | 100 |
| etr1-1 | 66.7 | 3.3 | 76.7 | 86.7 | 26.7 | 86.7 |
| etr1-1;coi1-16 | 86.7 | 86.7 | 86.7 | 93.3 | 80.0 | 80.0 |

**Fig. 3.** Effect of MeJA and ACC on growth of wild-type, coi1-16, etr1-1, and etr1-1;coi1-16 seedlings. (A) Root growth of the wild-type, coi1-16, etr1-1, and etr1-1;coi1-16 in the light and in the dark. Seedlings were grown for 7 d in continuous darkness (CD) or continuous light (CL) on Murashige and Skoog (MS) agar supplemented with 50 µM MeJA or 4 µM ACC. Root lengths of treated seedlings compared with that of control seedlings are expressed as mean of relative root growth (%). n.d. indicates no data. (B) Images of roots following ACC treatments. Seedlings were grown for 7 d in CL on MS agar supplemented or not with 4 µM ACC and observed at ×5 magnification under a brightfield microscope attached to a CCD camera. The scale bar indicates 200 µm.

Table 2. Germination ratio of wild-type, coi1-16, etr1-1, and etr1-1;coi1-16 seedlings

Seedlings were grown for 7 d in CD or CL on MS agar supplemented with 50 µM MeJA or 4 µM ACC. At least 15 seeds were sown per genotype. The germination ratio is shown as a percentage (%).

| Treatment | CD | CL |
|-----------|----|----|
| MS 50 µM MeJA | 97.3 | 96.0 | 98.7 | 92.0 | 100 | 97.3 |
| 4 µM ACC | 100 | 93.3 | 93.3 | 93.3 | 100 | 100 |
| 50 µM MeJA | 66.7 | 3.3 | 76.7 | 86.7 | 26.7 | 86.7 |
| 4 µM ACC | 86.7 | 86.7 | 86.7 | 93.3 | 80.0 | 80.0 |
A transgenic line overexpressing ERF1, 35S::ERF1, showed a slightly enhanced response to ACC to a similar extent in the light and in the dark, suggesting the existence of a positive feedback of this pathway. These results suggest that ERF1 could be required for both ACC-induced COII-mediated and ETR1-mediated root growth inhibition pathways, whereas ETR2, EIN4, ERS1, ERS2, EIN2, EIN3, and possibly CTR1 are on the same pathway as ETR1.

Results in Fig. 3A show that the ctr1-1;coi1-16 double mutant was less responsive to ACC-induced root growth inhibition than the single mutants. The double mutants ers1-1;coi1-16, ers2-1;coi1-16, ein2-1;coi1-16, and ein3-1;coi1-16 also showed less response than the single parental mutants to ACC-induced root growth inhibition in the light (Table 1). These results indicate that COII functions additively with the known ethylene signalling pathway in ACC-induced root growth inhibition in the light. The double mutant ctri-1;coi1-16 was severely stunted and infertile.

ACC-induced, COII-mediated root growth inhibition is independent of individual photoreceptors, but dependent on photoperiods

The results indicate that ACC-induced, COII-mediated root growth inhibition requires light. To investigate whether the known photoreceptors were required, photoreceptor mutants were tested for ACC-induced root growth inhibition in the light. These included the far-red/red light receptor mutants, phytochromeA (phyA) and phyB, and the UV-A/blue light receptor mutants, cryptochrome1 (cry1), cry2, phototropin1 (phot1) and phot2, the double mutants cry1;cry2 and phot1;phot2, and the quadruple mutant cry1;cry2;phot1;phot2. All of the tested mutants showed a wild-type response to ACC in the light (data not shown).

These results indicate that none of the known photoreceptors appears to be individually required for ACC-induced, COII-mediated root growth inhibition. Therefore, the effect of different photoperiods, including CD, short day (SD, 8/16 h light/dark), long day (LD, 16/8 h light/dark) and CL, on ACC-induced root growth inhibition of the wild-type and coi1-16 was examined (Table 3). coi1-16 showed significantly less response (P < 0.001) to ACC than the wild-type in CL and LD (Table 3), coi1-16 was significantly less responsive than the wild-type to MeJA-induced root growth inhibition in all light conditions, and a tendency for a greater response to MeJA in the longer photoperiods was suggested (Table 3).

MeJA and ACC inhibit root cell elongation

MeJA and ACC inhibit root growth in light-grown seedlings, wholly or partially through COII. To investigate whether this was through reducing cell elongation or reducing cell division, seedlings were grown for 7 d in the light on MS agar containing each inhibitor, and the lengths of mature root epidermal cells were measured under a brightfield microscope. Treatment of wild-type seedlings with MeJA reduced mature cell length and root length to 46.7% and 26.3% of those of controls, respectively (Table 4). Therefore, the reduction in cell length could account for 72% of the observed reduction in root length. In contrast, treatment of coi1-16 seedlings with MeJA reduced mature cell length only to 92.1% of that of controls, although root length was reduced to 79.1%. This means that the reduction in cell length could only account for 38% of the observed reduction in root length, indicating that COII plays a major role in the MeJA-induced inhibition of cell elongation. Treatment of wild-type seedlings with ACC reduced cell length and root length to 48.0% and 56.2% of those of controls, respectively, and treatment of coi1-16 seedlings with ACC reduced cell length and root length to 90.7% and 76.5%, respectively (Table 4). Therefore, reduction in cell length could account for most if not all of
the observed reduction in root length in the wild-type and 40% of the observed reduction in root length in coi1-16.

**ACC-induced, COI1-mediated root growth inhibition does not require SCF complex assembly but requires the LRR domain of COI1**

The previous section described the possibility of different mechanisms by which MeJA- and ACC-induced COI1-mediated root growth inhibition might be regulated. To investigate this point further, the coi1-16 mutant was examined after various COI1 constructs had been introduced. The plants carrying the F-box or LRR region of COI1 and the COI1 gene with a substitution at Trp44 to alanine (W44), all fused to the cauliflower mosaic virus 35S promoter in the Col background (Devoto et al., 2002), were crossed to coi1-16. The F3 lines that were confirmed to be homozygous for coi1-16 with each transgene expressed were used for the analyses. The parental transgenic lines F-box, LRR and W44 showed a wild-type response to MeJA and ACC in the light and in the dark (Fig. 5). The transgenic lines in the coi1-16 background (coi1-16;F-box, coi1-16;LRR, and coi1-16;W44) showed a statistical difference compared with the wild-type in response to MeJA, indicating that none of these transgenes is sufficient to complement completely the reduced response of coi1-16 to MeJA. In contrast, in response to ACC in the light, coi1-16;F-box showed a coi1-16-like response, and coi1-16;LRR and coi1-16;W44 showed a wild-type response, indicating that transgenes encoding the intact LRR domain are sufficient to complement the reduced response of coi1-16 to ACC in the light. Interestingly, the W44 transgene partially restored infertility of the coi1 null mutant, but not the F-box or LRR transgenes.

**Discussion**

Many environmental stimuli and developmental cues activate responses through signalling pathways, of which the ethylene signalling pathway is one of the most thoroughly characterised (Wang et al., 2002; Guo and Ecker, 2004; Chen et al., 2005; Benavente and Alonso, 2006; Etheridge et al., 2006). A significant finding of this study was therefore the identification of COI1, a JA receptor, which regulates plant response to stress (Xie et al., 1998; Yan et al., 2009), as a novel component of the ethylene signalling pathway. The key supporting evidence was that the coi1-16 mutation reduced growth inhibition by ethylene and its immediate precursor, ACC (Figs 1, 2A). Although it might be expected that the previous exhaustive mutation screens for components of the ethylene signalling pathway would have identified all non-redundant genes in ethylene signalling, these screens have been conducted in the dark where the response to ethylene is well characterised. However, the contribution of COI1 to ethylene signalling was observed exclusively in the light, and screens in the dark would therefore have missed the coi1 mutation.

Several other genes appear to function in more than one signalling pathway, including AUXIN RESISTANT1 (AXR1), mutations in which render plants less responsive to indole-3-acetic acid (IAA), MeJA, ACC, BAP, EBR, and abscisic acid (ABA) (Tiryaki and Staswick, 2002). Unlike axr1, however, coi1-16 is specifically less responsive to MeJA and ACC, but not to other growth inhibitors tested (Fig. 1). Cytokinin has previously been shown to inhibit plant growth partly through stimulation of ethylene biosynthesis by stabilizing ACC synthases (Cary et al., 1995; Su and Howell, 1995; Hansen et al., 2009). These studies, however, mainly focus on etiolated seedlings and hypocotyl response in the light, and ethylene production is not necessarily stimulated by BAP alone in the light-grown plants (Arteca and Arteca, 2008). This could be why a wild-type response of coi1-16 to BAP-induced root growth inhibition in the light was observed (Fig. 1). Also, cytokinin resistant 1 (ckr1), which is allelic to ein2, has been reported to be less responsive to BAP-induced root growth inhibition in the light (Su and Howell, 1992), suggesting that cytokinin or cytokinin-induced ethylene signalling is transmitted via a pathway containing EIN2.

ACC induces JA synthesis in tomato (Sivasankar et al., 2000), and if this was also the case in Arabidopsis then this could explain the requirement of COII for ACC-induced root growth inhibition. However, the JA biosynthesis mutants, aos and opr3, displayed wild-type-like root growth inhibition when treated with ACC (Table 1). The JA-insensitive mutants, jar1-1 and jin1, also showed a wild-type response to ACC (Table 1). These results suggest that neither JA biosynthesis nor the components of the JA perception-response pathway are involved in ACC-induced, COII-mediated root growth inhibition. Apparently, therefore,
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**Fig. 5.** Complementation of coi1-16 with various COI1 constructs. (A) Diagrams of COI1 and the different constructs used for complementation studies. An asterisk indicates a substitution of Trp44 to alanine. (B) Root growth of the wild-type, coi1-16, and coi1-16 with each construct in the light and in the dark. Seedlings were grown for 7 d in continuous darkness (CD) or continuous light (CL) on Murashige and Skoog (MS) agar supplemented with 50 µM MeJA or 4 µM ACC. Root lengths of treated seedlings compared with that of control seedlings are expressed as the mean of relative root growth (%). Bars indicate the standard error (SE). A significant difference (P <0.05) compared with the wild-type is indicated with asterisks.

**COI1** is required for the response not only to JA, but also to ethylene-induced root growth inhibition.

**ETR1** encodes one of the five ethylene receptors. The dominant mutant *etr1-1* encodes a protein that has no measurable ethylene binding, and it is unresponsive to root growth inhibition by this hormone. The phenotypes of all dominant ethylene receptor mutants were characterised by a lack of ethylene-induced triple response in the dark but, with a few exceptions (e.g. Bleecker et al., 1988; Smalle et al., 1997), their phenotypes, especially root growth, in the light have not been thoroughly characterised. In the dark, *etr1-1* was unresponsive to ACC-induced root growth inhibition, but, unexpectedly in the light, *etr1-1* was substantially responsive to ACC (Figs 2B, 3A). Likewise, other ethylene receptor mutants, *etr2-1*, *ers1-1*, *ers2-1*, and *ein4*, and the downstream ethylene-insensitive mutants, *ein2-1* and *ein3-1*, were more responsive to ACC-induced root growth inhibition in the light than in the dark (Table 1). These results indicate that in the light, an additional pathway for response to ACC, independent of the known ethylene signalling pathway, is activated. Loss-of-function *etr1;ers1* double mutants have been reported to show a more severe constitutive ethylene response in the light than in the dark (Hall and Bleecker, 2003; Qu and Schaller, 2004). This may be due to activation of an additional ethylene pathway involving **COI1** in the light. Of the ethylene perception-response mutants tested, only *etr1-1* was not more responsive to ACC in the light than in the dark (Table 1). However, CTR1 is a negative regulator of the ethylene pathway, and mutations in the gene give a constitutive ethylene response, not unresponsiveness. Moreover, CTR1 is predicted to bind to endoplasmic reticulum-bound ETR1 (Gao et al., 2003); therefore, it is assumed to be operating in the **ETR1** pathway, but not in the **COI1** pathway.

In contrast to the ethylene perception-response mutants, in the dark, *coi1-16* showed a wild-type response to ACC in terms of root growth (Fig. 3A; Table 1). This is consistent with previous observations (Ellis and Turner, 2002), and indicates that ACC-induced root growth inhibition requires **COI1** in the light, but not in the dark. To test whether **COI1** and the known ethylene signalling operated in the same or in different pathways, the double mutants *etr1-1;coi1-16*, *ers1-1;coi1-16*, *ers2-1;coi1-16*, *ein2-1;coi1-16*, and *ein3-1;coi1-16* were constructed. In the light, all the double mutants were significantly less responsive to ACC-induced root growth inhibition than single parental mutants (Fig. 3A; Table 1). Although this *coi1-16* mutant, from which the double mutants were constructed, was later found to contain a second mutation, the phenotype of these double mutants was reproduced by treating a single *coi1-16* mutant with silver ions, which blocked the ethylene receptors. The observed additive contribution of the ethylene perception-response genes and **COI1** indicates that the two pathways are independent. It is concluded that both the known ethylene signalling pathway and **COI1** contribute to the response to ACC-induced root growth inhibition in the light, whereas only the known ethylene signalling pathway contributes to the response to ACC in the dark.

Additionally, **ERF1** may be shared on the **ETR1** and **COI1** pathways since the transgenic line 35S::**ERF1** showed a similar response to ACC-induced root growth inhibition in the light and in the dark (Table 1), in agreement with the previous finding that **ERF1** integrates JA and ethylene pathways in defence signalling (Lorenzo et al., 2003). Ethylene and JAs synergistically induce expression of **ERF1**
regulated directly or indirectly by light. 2002; Feng et al. 2003.) It is therefore possible that the ETR1 and COII pathways jointly regulate ACC-induced root growth inhibition through the GCC-box even though JAs themselves are not involved. These results are summarized in Fig. 6.

COII-mediated root growth inhibition by ACC requires light. However, each of the UV-A/blue, red, and far-red light receptor mutants showed a wild-type response to ACC, indicating that these photoreceptors are not involved. Instead, the requirement was for long photoperiods, indicating that there might be a dose dependency of light to activate the COII pathway in response to ACC (Table 3). Although coi1-16 had reduced response to ACC in the longer photoperiods, it had enhanced response to MeJA in the longer photoperiods (Table 3). These results may imply that in ACC-induced root growth inhibition, the COII pathway compensates for the loss of function of the ETR1 pathway in the light so that the proportional contribution of each pathway shifts as photoperiods increase. The interaction between COI1 and light has previously been suggested: COI1 interacts and binds a target protein/substrate (Fig. 5). However, the finding that the COI1 protein incapable of making a SCF complex was sufficient to recover the reduced response of coi1-16 to ACC (Fig. 5). coi1-16 has a point mutation in the LRR region of COI1. JA–Ile and coronatine have been shown to bind to the LRR domain of COI1 (Katsir et al., 2008; Yan et al., 2009). It may be speculated that the LRR domain of COI1 also interacts with ethylene-related components to regulate root growth without forming an SCF complex. In contrast, MeJA-induced root growth inhibition requires the intact COI1 protein which is capable of making a SCF complex and binding a target protein/substrate (Fig. 5). However, the finding that the COI1 protein incapable of making a SCF complex could partly recover male sterility of coi1-1 suggests that there may be a COII-mediated fertility mechanism which does not require SCF complex assembly.

The complexity of the interaction of JA- and ACC-induced signalling is revealed by three further findings. First, etr1-1 had a very low germination ratio on MeJA, whereas the double mutant etr1-1;coi1-16 showed a wild-type germination ratio (Table 2). ETR1 is a negative regulator of the ethylene pathway and etr1-1 is a dominant mutant incapable of binding ethylene and activating the downstream pathway. This may suggest that activation of the ETR1 pathway by binding of ETR1 to basal or MeJA-induced ethylene inhibits JA-induced, COII-mediated germination inhibition. JAs inhibit germination in *Brassica napus* and *Linum usitatissimum* (Wilen et al., 1992; Ellis and Turner, 2002) in *Arabidopsis*, however, contradictory results have been reported: JAs synergize (Trusov et al., 2006) or antagonise (Staswick et al., 1992; Ellis and Turner, 2002) ABA-induced germination inhibition. Secondly, although COII was required for ACC-induced root growth inhibition, it was not required for ACC-induced root hair elongation (Fig. 3B), indicating that ACC-induced root hair elongation is regulated entirely through the ETR1 pathway. This supports the notion that the ETR1 pathway is active both in the light and in the dark, since the extent of ACC-induced root hair elongation for the ethylene-insensitive mutants tested (except *ein4*) and for etr1-1;coi1-16 was similar in the light and in the dark (data not shown). Therefore, the effects of ACC on root growth inhibition and root hair elongation are regulated differently. These conclusions are summarized in

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(Lozano et al., 2003), which encodes a GCC-box-binding protein (Solano et al., 1998). JAs alone or together with ethylene are known to induce expression of genes containing a GCC-box in their promoters such as *PDF1.2* and *Thi2.1* (Brown et al., 2003). It is therefore possible that the ETR1 and COII pathways jointly regulate ACC-induced root growth inhibition through the GCC-box even though JAs themselves are not involved. These results are summarized in Fig. 6.

**Fig. 6.** Model pathway. ACC/ethylene-induced root growth inhibition is mediated by the COI1 pathway in a light-dependent and JA-independent manner, in parallel with the conventional ethylene pathway. However, the COI1 pathway is not involved in ACC-induced root hair formation. It is also suggested that JA-induced COI1-mediated germination inhibition is antagonised by the ETR1 pathway.

Evidence is presented that MeJA induces root growth inhibition through the reduction of root cell length (Table 4) and probably of growth rate of root cells, and COII is mainly involved in inhibition of cell length. In contrast, ACC-induced root growth inhibition is predominantly regulated by cell elongation inhibition, and COII is mainly involved in this process (Table 4). These results suggest that the mechanisms of root growth inhibition by MeJA and ACC may be different, and COII is required and acts differently in response to each signal. This notion was also supported by the results from the assay of COII transgenic plants. Constitutive expression of the transgenes encoding the LRR domain of COI1 or the COI1 protein incapable of making a SCF complex was sufficient to recover the reduced response of coi1-16 to ACC (Fig. 5). coi1-16 has a point mutation in the LRR region of COII (Devoto et al., 2002; Ellis and Turner, 2002). These findings indicate that ACC-induced root growth inhibition does not require SCF complex assembly but does require the LRR domain of COI1. JA–Ile and coronatine have been shown to bind to the LRR domain of COI1 (Katsir et al., 2008; Yan et al., 2009). It may be speculated that the LRR domain of COI1 also interacts with ethylene-related components to regulate root growth without forming an SCF complex. In contrast, MeJA-induced root growth inhibition requires the intact COI1 protein which is capable of making a SCF complex and binding a target protein/substrate (Fig. 5). However, the finding that the COI1 protein incapable of making a SCF complex could partly recover male sterility of coi1-1 suggests that there may be a COII-mediated fertility mechanism which does not require SCF complex assembly.

The complexity of the interaction of JA- and ACC-induced signalling is revealed by three further findings. First, etr1-1 had a very low germination ratio on MeJA, whereas the double mutant etr1-1;coi1-16 showed a wild-type germination ratio (Table 2). ETR1 is a negative regulator of the ethylene pathway and etr1-1 is a dominant mutant incapable of binding ethylene and activating the downstream pathway. This may suggest that activation of the ETR1 pathway by binding of ETR1 to basal or MeJA-induced ethylene inhibits JA-induced, COII-mediated germination inhibition. JAs inhibit germination in *Brassica napus* and *Linum usitatissimum* (Wilen et al., 1992; Ellis and Turner, 2002) in *Arabidopsis*, however, contradictory results have been reported: JAs synergize (Trusov et al., 2006) or antagonise (Staswick et al., 1992; Ellis and Turner, 2002) ABA-induced germination inhibition. Secondly, although COII was required for ACC-induced root growth inhibition, it was not required for ACC-induced root hair elongation (Fig. 3B), indicating that ACC-induced root hair elongation is regulated entirely through the ETR1 pathway. This supports the notion that the ETR1 pathway is active both in the light and in the dark, since the extent of ACC-induced root hair elongation for the ethylene-insensitive mutants tested (except *ein4*) and for etr1-1;coi1-16 was similar in the light and in the dark (data not shown). Therefore, the effects of ACC on root growth inhibition and root hair elongation are regulated differently. These conclusions are summarized in
Fig. 6. Though JAs were shown to induce root hair formation synergistically with ethylene (Zhu et al., 2006), the effect of ethylene or ACC on root hair elongation of coi1-16 has not previously been reported. Finally, independence of the COII pathway from the ETR1 pathway was confirmed by expression of ethylene-responsive genes. ACC induced expression of ETR2 and ERS2 in the wild-type and coi1-16 (Fig. 4), indicating that COII is not required for ACC-induced expression of these genes. This suggests an as yet unknown ethylene response mechanism that involves COII in the light. Further investigation of how COII interacts with the conventional ethylene pathway is awaited.

A significant finding reported here is that the root growth response of Arabidopsis to ACC requires the COII gene, and is light dependent and JA independent. This has important implications for the use of ethylene perception-response mutants to investigate the role of ethylene signalling in plant development and adaptation in the light: mutants that block response to ACC and to ethylene in the dark do not necessarily do so in the light, and at least some of these responses require COII. Likewise, coi1 mutants are widely used to study response to JA but we show that their response to ethylene is also reduced. Since none of the known ethylene receptors is involved in the COII-mediated pathway, the existence of a new receptor or perception mechanism is suggested. The study focused on an ACC response in root growth of 7-d-old seedlings; however, studies on other ethylene responses in different developmental stages might provide further insights. It will also be interesting to know whether ethylene interacts with the novel JAZ proteins through COII. Since JAs and ethylene together are known to coordinate many responses to environmental stresses, these findings raise the possibility that COII functions as an integration point for JA and ethylene signalling.

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