SCIENCE CHINA
Life Sciences

Tsinghua Special Issue
• RESEARCH PAPER •

January 2014 Vol.57 No.1: 145–154
doi: 10.1007/s11427-013-4590-1

Amino acid substitutions of GLY98, LEU245 and GLU543 in COI1 distinctively affect jasmonate-regulated male fertility in *Arabidopsis*

HUANG Huang, WANG CuiLi, TIAN HaiXia, SUN Yu, XIE DaoXin* & SONG SuSheng*

Tsinghua-Peking Center for Life Sciences, MOE Key Laboratory of Bioinformatics, School of Life Sciences, Tsinghua University, Beijing 100084, China

Received October 16, 2013; accepted November 20, 2013

Jasmonate (JA) regulates various plant defense and developmental processes. The F-box protein CORONATINE INSENSITIVE 1 (COI1) perceives JA signals to mediate diverse plant responses including male fertility, root growth, anthocyanin accumulation, and defense against abiotic and biotic stresses. In this study, we carried out genetic, physiological and biochemical analysis on a series of *coi1* mutant alleles, and found that different amino acid mutations in COI1 distinctively affect JA-regulated male fertility in *Arabidopsis*. All the JA responses are disrupted by the COI1 mutations W467* in *coi1-1*, Q343* (*coi1-6*), G369E (*coi1-4*), G98D (*coi1-5*), G155E (*coi1-7*), D452A (*coi1-9*) and L490A (*coi1-10*), though the *coi1-5* mutant ($COI1^{G98D}$) contains adequate COI1 protein (~60% of wild-type). Interestingly, the low basal level of $COI1^{E543K}$ in the *coi1-8* mutant (~10% of wild-type COI1 level) is sufficient for maintaining male fertility (~50% of wild-type fertility); the *coi1-2* mutant with low level of $COI1^{L245F}$ (~10% of wild-type) is male sterile under normal growth condition (22°C) but male fertile (~80% of wild-type fertility) at low temperature (16°C); however, both *coi1-2* and *coi1-8* are defective in the other JA responses (root growth, anthocyanin accumulation, and plant response to the pathogen *Pst* DC3000 infection).

COI1, *coi1-2*, *coi1-5*, *coi1-8*, jasmonate, male fertility

Citation: Huang H, Wang CL, Tian HX, Sun Y, Xie DX, Song SS. Amino acid substitutions of GLY98, LEU245 and GLU543 in COI1 distinctively affect jasmonate-regulated male fertility in *Arabidopsis*. Sci China Life Sci, 2014, 57: 145−154, doi: 10.1007/s11427-013-4590-1

Jasmonates (JAs), including jasmonic acid, methyl-jasmonate (MeJA), jasmonate-isoleucine (JA-Ile) and their oxylipin derivatives [1,2], control various aspects of plant development and growth processes, including plant fertility [3–5], root growth [6,7], trichome formation [8,9] and leaf senescence [10,11], and regulate secondary metabolism [12], such as anthocyanin accumulation [8], glucosinolate biosynthesis [13] and sesquiterpene accumulation [14]. In addition, JAs function as defense signals to mediate plant defense responses against abiotic and biotic stresses, such as ozone exposure [15], drought [16], wounding [17], herbivore attack [18–20] and necrotrophic pathogen infection [21–25].

The F-box protein CORONATINE INSENSITIVE1 (COI1) [26] forms the SCF^{COI1} complex with ASK1/ASK2, Cullin and Rbx1 [27–29], perceives JA signals [30], and recruits Jasmonate-ZIM-domain (JAZ) proteins [31–33] for ubiquitination and subsequent degradation via the 26S proteasome pathway. The N-terminal tri-helical F-box motif in COI1 binds to ASK1 [34], while the COI1 C-terminal horseshoe-shaped solenoid, consisting of the 18 LRRs and the four loops (loop-2, loop-12, loop-14 and loop-C), binds to JA-Ile, the bioactive form of JA [35], generating subsequent surface for further interaction with JAZ proteins [34].

*Corresponding author (email:songss@tsinghua.edu.cn; daoxinlab@tsinghua.edu.cn)

JAZ proteins interact with various downstream transcription factors, including transcriptional activators MYB21/MYB24 [3], MYC2/MYC3/MYC4 [36–38], WD-repeat/bHLH/MYB complex [8], EIN3/EIL1 [39], and transcriptional repressors bHLH subgroup IIId factors [40–42] to regulate their respective JA responses. JA-induced degradation of JAZ proteins releases the downstream transcriptional factors, leading to antagonistical and coordinated regulation of various JA responses.

The COI1 perceives JA signals to mediate diverse plant responses including developmental processes, secondary metabolism, and defense against abiotic and biotic stresses. In this study, we found that different JA responses require different levels of functional COI1 protein through genetic, physiological and biochemical analysis on a series of *coi1* mutant alleles which harbored various amino acid mutations including G98D in the *coi1-5* mutant, G155E (*coi1-7*), L245F(*coi1-2*), Q343* (*coi1-6*), G369E (*coi1-4*), D452A (*coi1-9*), W467* (*coi1-1*), L490A (*coi1-10*) and E543K (*coi1-8*). We showed that the amino acid mutations in the *COI1* gene have distinct effect on COI1 function in regulating male fertility: (i) the low basal level of $COI1^{E543K}$ protein (~10% of wild-type) is adequate for maintaining male fertility in the *coi1-8* mutant (~50% of wild-type fertility), but not for other JA responses including JA-regulated root growth, anthocyanin accumulation, and plant response to the pathogen *Pst* DC3000 inoculation; (ii) the amino acid substitution L245F in *coi1-2* significantly attenuates JA-regulated male fertility under normal temperature, whereas $COI1^{L245F}$ is able to restore male fertility (~80% of wild-type fertility) by low temperature treatment without affecting the COI1 protein level; (iii) all the JA responses including male fertility are completely abolished by $COI1^{G98D}$ mutation though adequate $COI1^{G98D}$ protein is retained in *coi1-5* (~60% of wild-type COI1 level), and the other remaining amino acid changes G155E (*coi1-7*), Q343* (*coi1-6*), G369E (*coi1-4*), D452A (*coi1-9*), W467* (*coi1-1*), and L490A (*coi1-10*) completely abolish the COI1 stability and disrupt all the JA responses including male fertility.

1 Materials and methods

1.1 Materials and growth conditions

The *Arabidopsis thaliana* Col-0 wild-type, *coi1-1*, *coi1-2*, *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-8*, *coi1-9* and *coi1-10* were described previously [28,30]. The *coi1-1* and *coi1-2* mutants were identified by CAPS markers using the primer pairs 5′-GGTTCTCTTTAGTCTTTAC-3′ and 5′-GCCAGAGAGTAGTAAGCCAA-3′, and 5′-CTTGAGGTTTTAAACTTCTAC-3′ and 5′-CTCGAGAACTTCCAAATTAGGAC-3′, and restriction endonucleases *Xcm* I and *Hyp188* III, respectively. The genotypes of *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-8*, *coi1-9* and *coi1-10* were verified by sequencing. The *Arabidopsis thaliana* seeds were plated on Murashige and Skoog (MS) medium (Sigma, St. Louis, MO, USA) with 3% sucrose, chilled at 4°C for 3 d, and transferred to a growth condition under a light/dark photoperiod of 16 h/8 h at 16°C or 22°C.

For observation of male fertility from the primary inflorescences, Col-0, *coi1-1*, *coi1-2*, *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-8*, *coi1-9* and *coi1-10* were grown under a light/dark photoperiod of 16 h/8 h at 22°C (for normal temperature) for 45 d, or grown under a light/dark photoperiod of 16 h/8 h at 22°C for four weeks (for normal bolting), and transferred to a growth condition with a light/dark photoperiod of 16 h/8 h at 16°C (for low temperature treatment) for another 32 d. The flowers and stamens at floral stage 13 [43] from the primary inflorescences were presented.

1.2 RT-PCR and quantitative real-time PCR

For RT-PCR or quantitative real-time PCR analysis, the *Arabidopsis* seedlings were grown under a light/dark photoperiod of 16 h/8 h at 16°C or 22°C for two weeks, then harvested for RNA extraction. Reverse transcription was performed using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Transgene Biotech, Beijing, China). For reverse transcription (RT) PCR, amplifications were performed with 25 cycles for both *ACTIN1* and *COI1*. Amplified transcripts were detected by ethidium bromide-stained agarose gels. *ACTIN1* was used as the normalization control. The primers used for RT-PCR are as follows: *Actin1:* 5′-TGTTGAGAAGAACTACGAGC-3′ and 5′-AAGCACTTCCTGTGAACAAT-3′, *COI1*: 5′-TAGAGGTCCTTGCACAGTACTG-3′ and 5′-CATCTCTAGCTTCTGTAGAT-3′.

Quantitative real-time (qRT) PCR was performed using the ABI7500 real-time PCR system and RealMasterMix (SYBR Green I) (TAKARA, Japan). *ACTIN8* was used as the internal control. All the experiments were repeated three times. The primers used for qRT-PCR analysis are as follows: *Actin8*: 5′-TCAGCACTTTCCAGCAGATG-3′ and 5′-CTGTGGACAATGCCTGGAC-3′, *COI1*: 5′-GTGTCCTAATTTGGAAGTTCTCG-3′ and 5′-CTCCATTCCTTGTTCATCTGC-3′.

1.3 Immunoblot analysis

For immunoblot analysis of COI1 protein, the *Arabidopsis* seedlings were grown under a light/dark photoperiod of 16 h/8 h at 16°C or 22°C for two weeks. The total protein was extracted from leaves of each genotype using extraction buffer (50 mmol L^{-1} Tris-Cl, pH 7.8, 100 mmol L^{-1} NaCl, 10% (v/v) glycerol, and 20 mmol L^{-1} β-mercaptoethanol), measured by Broadford method, separated by 10% SDS-PAGE gel, and transferred to PVDF membrane. Membrane was blocked by 5% milk power in PBST solution (PBST: 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1}

Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4, 0.1% Tween 20) at room temperature for 2 h, incubated with the primary antibody (1∶500 dilution) overnight at 4°C and secondary antibody (1∶1000 dilution) at room temperature for 2 h. The protein bands were detected by chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China). The relative abundance of COI1 was calculated by the software from FluorChem M MultiFluor System (Alpha).

1.4 Measurement of root length and anthocyanin content

For measurement of root length and anthocyanin content, seeds were grown on MS medium with 0, 1, 5, 10 or 25 μmol L^{-1} MeJA, chilled at 4°C for 3 d, and transferred to a growth condition with a light/dark photoperiod of 16 h/8 h at 16°C or 22°C. The 11-day-old seedlings for each genotype were used for measurement of root length or anthocyanin content with methods as described previously [40]. The relative root length was presented as a percentage of root length on MS medium. The anthocyanin content was presented as $(A_{535}-A_{650})$/g fresh weight. The experiments were repeated three times.

1.5 Infection with bacterial pathogen

The *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 bacteria were cultured overnight, harvested at 2000×*g* for 3 min, washed three times using 10 mmol L^{-1} $MgCl_2$, and finally suspended in 10 mmol L^{-1} $MgCl_2$. Twenty-four 4-week- old plants were sprayed with 1×10^8 (colony-forming units)/mL bacteria with 0.02% Silwet L-77. The infection symptoms were observed at three days after infection. The number of bacterial population was measured as previously described [44]. The experiment was repeated three times.

2 Results

2.1 Mutations in COI1 attenuate JA-regulated plant responses

Previous genetic screening identified a series of *coi1* mutant alleles from ethyl methanesulfonate-mutagenized *Arabidopsis* seeds [28,30]. The *coi1-2*, *coi1-4*, *coi1-7*, *coi1-8*, *coi1-9*, *coi1-10* and *coi1-5* mutant alleles respectively harbored an amino acid substitution L245F, G369E, G155E, E543K, D452A, L490A and G98D (Figure 1A and B), while the amino acid W467 and Q343 of COI1 were respectively replaced by an early stop codon in the *coi1-1* and *coi1-6* mutants (Figure 1A and B). The amino acid changes are presented in the different positions of the LRRs or loops in the structural ribbon diagram of COI1 (Figure 1B). The *Xcm* I- and *Hyp188* III-based CAPS markers were developed to efficiently identify the *coi1-1* and *coi1-2* allele respectively (Figure 1C) [26].

To examine the effect of mutations in COI1 on JA-inhibitory root growth, we compared root growth among these *coi1* mutants and wild-type seedlings grown on Murashige and Skoog (MS) medium supplied with different concentrations of MeJA for 11 d. As shown in Figure 2A and B, root length of all the *coi1* mutant seedlings was not obviously inhibited by MeJA (data not shown), though the root length of *coi1-2* and *coi1-8* was slightly attenuated. MeJA exhibited obvious inhibition on the root growth of wild-type seedlings, as expected. These results demonstrate that all the amino acid changes of W467*, Q343*, L245F, G369E, G155E, E543K, D452A, L490A and G98D in COI1 significantly attenuate the JA-inhibitory root growth.

To test the effect of mutations in COI1 on JA-induced anthocyanin accumulation, we measured the anthocyanin content in the 11-day-old seedlings of wild-type and *coi1* mutants grown on MS medium with different concentrations of MeJA. Consistent with previous observations [40], the wild-type seedlings significantly accumulated anthocyanin (~3, 8, 14 or 20-fold) when treated with MeJA (1, 5, 10 or 25 μmol L^{-1}) (Figure 2C and D). However, MeJA treatment was unable to significantly induce anthocyanin accumulation in all the *coi1* mutant seedlings though *coi1-2* and *coi1-8* displayed a weak increase of anthocyanin accumulation in response to JA treatment (Figure 2C and D, data not shown). These data suggest that the amino acid changes of W467*, Q343*, L245F, G369E, G155E, E543K, D452A, L490A and G98D in COI1 severely attenuate the JA-induced anthocyanin accumulation.

A previous study showed that the *coi1-16* mutant [45], in addition to the L245F mutation in COI1, harbored the *pen2-4* mutation in the *PENETRATION2* (*PEN2*) gene that is closely linked to the *COI1* gene, which may alter the non-host resistance of the *coi1* mutant [46]. We found that the *coi1-2* mutant (L245F), *coi1-5* (G98D) and *coi1-8* (E543K) did not harbor *pen2-4* mutation using the *pen2-4* mutation-specific CAPS marker (data not shown). We further inoculated the bacterial strain *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 onto four-week-old plants of *coi1* mutants and wild type. We found that three days after spray with *Pst* DC3000, significant bacterial propagation and strong disease syndrome occurred in wild-type plants (Figure 2E and F), which is consistent with previous results [47]. All the *coi1* mutants displayed much reduced bacterial propagation and leaf disease syndrome though *coi1-2* and *coi1-8* exhibited a little more susceptible symptoms (Figure 2E and F, data not shown). These experiments show that JA-mediated plant response to the *Pst* DC3000 inoculation is obviously affected by the amino acid changes of W467*, Q343*, L245F, G369E, G155E, E543K, D452A, L490A and G98D in COI1.

Taken together, these results demonstrate that all the amino acid changes in COI1, including W467*, Q343*, L245F, G369E, G155E, E543K, D452A, L490A and G98D, significantly attenuate the COI1 function in regulating the

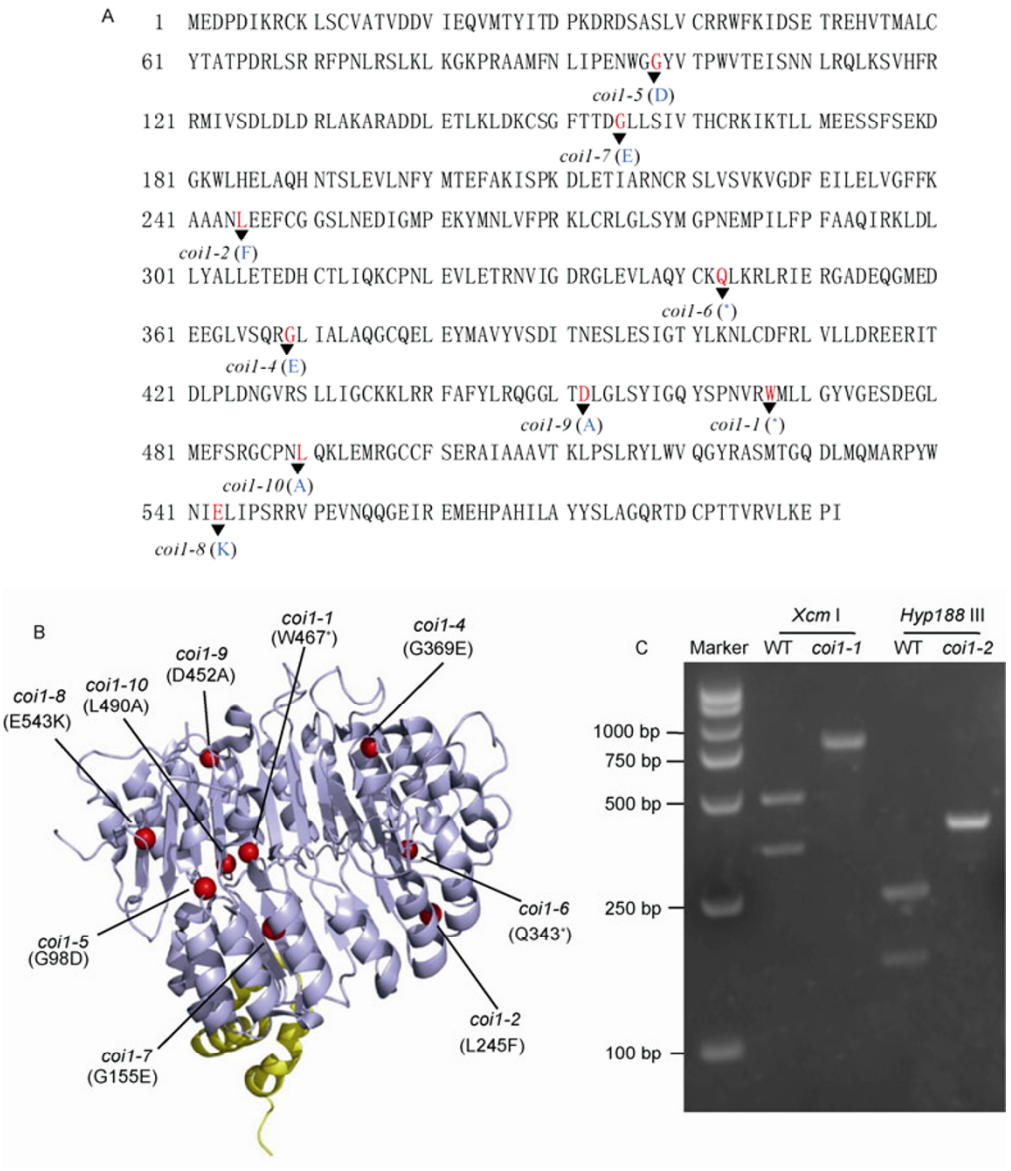

Figure 1 Distribution of the amino acid mutations in COI1. A, Amino acid sequence of COI1. The amino acid mutations are marked by arrows. The asterisks indicate the early translation stop. B, The amino acid mutations G98D in loop-2 in *coi1-5* mutant, G155E (4th LRR, *coi1-7*), L245F (7th LRR, *coi1-2*), Q343* (11th LRR, *coi1-6*), G369E (12th LRR, *coi1-4*), D452A (15th LRR, *coi1-9*), W467* (15th LRR, *coi1-1*), L490A (16th LRR, *coi1-10*) and E543K (18th LRR, *coi1-8*) are marked by red space fill in the COI1 ribbon diagram that is generated according to previous structural data [34]. The asterisks indicate the early translation stop. C, Verification of *coi1-1* and *coi1-2* by CAPS markers. The PCR-amplified 810-bp fragment of *COI1* from WT and *coi1-1*, or 393-bp fragment from WT and *coi1-2* was digested respectively by restriction endonucleases *Xcm* I or *Hyp188* III (see Materials and methods for detail).

JA-inhibitory root growth, JA-induced anthocyanin accumulation, and JA-mediated plant response to the pathogen *Pst* DC3000 inoculation.

2.2 G98, L245 and E543 in COI1 play distinct roles in JA-regulated plant male fertility

To investigate whether the mutations in COI1 affect the JA-regulated male fertility, we compared the filament length, anther dehiscence, pollen viability and fertile siliques in the primary inflorescence of the wild-type and *coi1* mutant plants grown at 22°C. We found that the *coi1-1*, *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-9* and *coi1-10* plants were completely male sterile. All the flowers in these plants exhibited unelongated filaments, indehiscent anthers and unviable pollen grains (Figure 3C, data not shown). These plants were not able to produce siliques with seeds (Figure 3A and B).

Interestingly, when grown at 22°C, the *coi1-2* mutant plants exhibited severe reduction in male fertility. Most of the flowers from *coi1-2* produced unelongated filaments, indehiscent anthers and unviable pollen grains (Figure 3C,

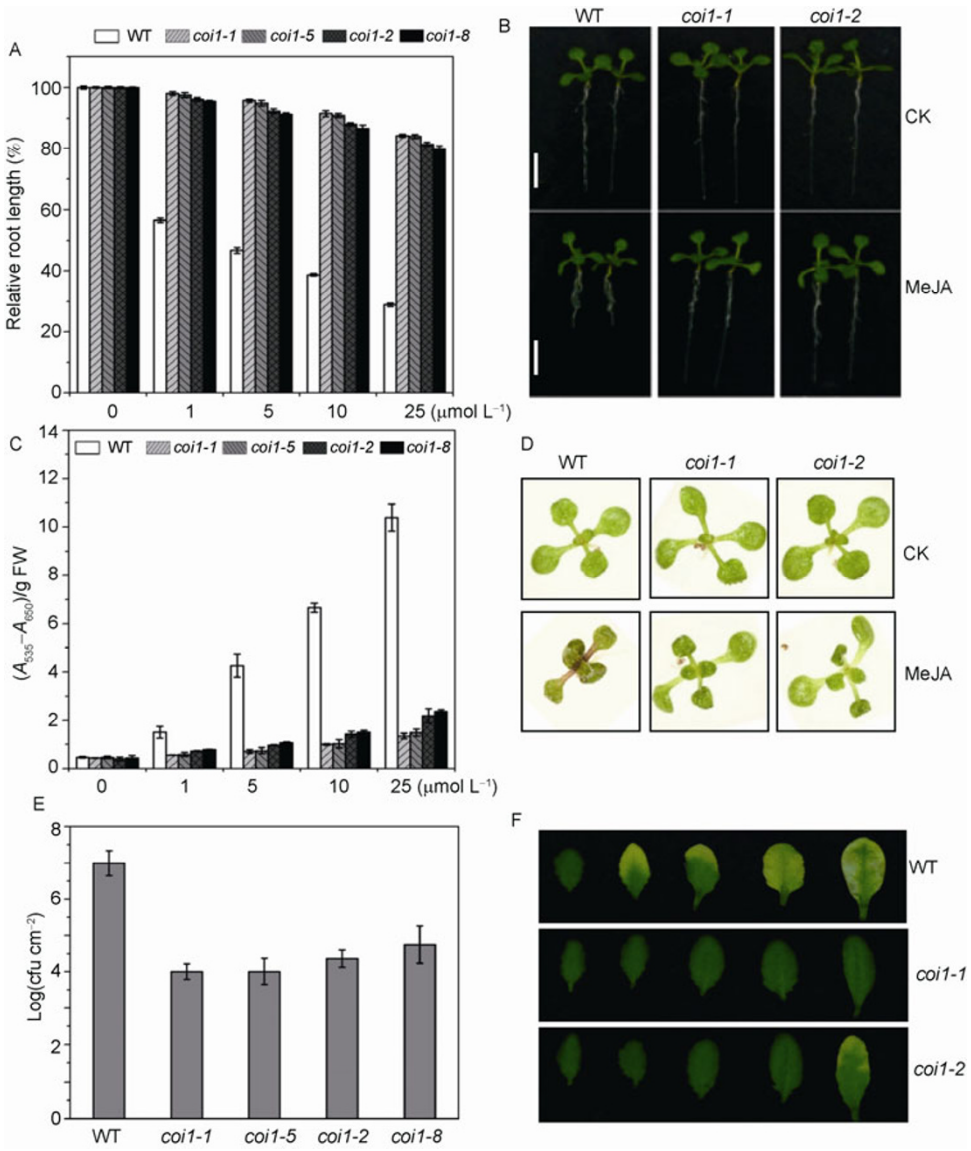

Figure 2 JA-mediated root growth, anthocyanin accumulation and plant response to the pathogen *Pst* DC3000 inoculation in the *coi1* mutants. A, Relative root length for 11-day-old seedlings of Col-0 (WT), *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* grown on MS medium with 0, 1, 5, 10 or 25 μmol L^{-1} MeJA at 22°C. Relative root length is expressed as a percentage of root length on MS medium. Error bars represent SE (n=15). B, Root phenotypes of 11-day-old seedlings for WT, *coi1-1* and *coi1-2* grown on MS medium without (CK) or with 25 μmol L^{-1} MeJA at 22°C. Scale bar, 5 mm. C, Anthocyanin contents in the 11-day-old seedlings of WT, *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* grown on MS medium containing 0, 1, 5, 10 or 25 μmol L^{-1} MeJA at 22°C. FW, fresh weight. Error bars represent SE (n=3). D, Seedling phenotypes of 11-day-old seedlings for WT, *coi1-1* and *coi1-2* grown on MS medium without (CK) or with 25 μmol L^{-1} MeJA at 22°C. E, Bacterial population of *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 in the leaves of WT, *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* at day 3 post-spray inoculation with 1×10^8 colony-forming units mL^{-1} (cfu mL^{-1}) of *Pst* DC3000. Error bars indicate SE (n=3). F, Disease symptoms of leaves from WT, *coi1-1* and *coi1-2* at day 3 after spray inoculation.

data not shown). Occasionally, few flowers in *coi1-2* displayed elongated filament and dehiscent anthers with viable pollen grains, and less than ~5% siliques from the primary inflorescence of *coi1-2* could set few seeds (Figure 3A and B).

The *coi1-8* mutant plants were partially fertile, in contrast to the *coi1-1*, *coi1-2, coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-9* and *coi1-10*. More than ~50% of the *coi1-8* siliques could set seeds (Figure 3A and B). About half of the flowers in *coi1-8* could produce normally elongated filament and dehiscent anthers with viable pollen grains (Figure 3C).

We further verified the COI1 level in these *coi1* mutant plants grown at 22°C. Immunoblot analysis with anti-COI1 antiserum revealed that *coi1-1*, *coi1-4*, *coi1-6*, *coi1-7*, *coi1-9* and *coi1-10* contained no COI1 protein at all (Figure 3D, data not shown), while the *coi1-5* with $COI1^{G98D}$ substitution contained ~60% of wild-type COI1 level (Figure 3D). Both *coi1-2* ($COI1^{L245F}$) and *coi1-8* ($COI1^{E543K}$) re-

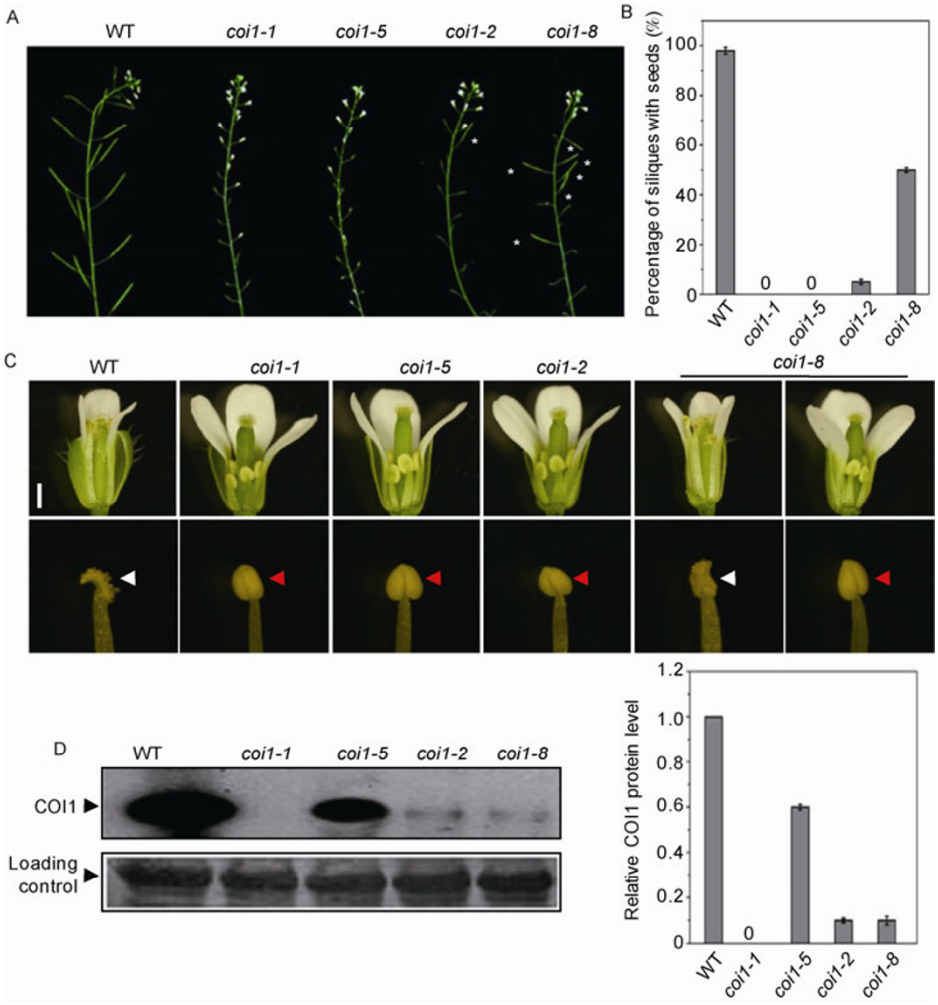

Figure 3 The phenotypic observation of male fertility in the *coi1* mutants. A, Primary inflorescences of WT, *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* at 22°C. White asterisks indicate fertile siliques of *coi1-2* and *coi1-8*. B, Percentage of siliques with seeds in the primary inflorescences of WT, *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* at 22°C. Error bars represent SE (n=15). C, Flowers (top panel) and anthers (bottom panel) at floral stage 13 from WT, *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* at 22°C. Scale bar (top panel), 0.5 mm. White arrowheads represent dehiscent anthers, and red arrowheads indicate indehiscent anthers. D, Immunoblot (left panel) and quantitative analysis of COI1 protein level (right panel) in *coi1* mutants at 22°C. Total proteins were extracted from leaves, separated by 10% SDS-PAGE, and immunoblotted with COI1 antiserum. The PVDF membrane was stained by Memstain to serve as loading control. The abundance of COI1 in wild type was set to 1.0 for calculating the relative abundance of COI1 protein in other *coi1* mutants.

tained very low level of COI1 protein (~10% of the wild-type COI1 level) (Figure 3D).

In summary, our results demonstrate that mutations in the *COI1* gene have distinct effect on COI1 function in regulating male fertility: (i) the amino acid changes W467*, G369E, G98D, Q343*, G155E, D452A and L490A completely disrupt the COI1 function in regulating male fertility. These mutant plants *coi1-1*, *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-9* and *coi1-10* are male sterile though *coi1-5* contains adequate $COI1^{G98D}$ (~60% of wild-type); (ii) the amino acid substitution L245F significantly attenuates the JA-regulated male fertility in *coi1-2*; (iii) the E543K substitution has less influence on plant fertility, and the *coi1-8* plants still maintain ~50% of wild-type fertility though only ~10% of $COI1^{E543K}$ protein is retained.

2.3 Low temperature treatment restores male fertility in *coi1-2* ($COI1^{L245F}$)

We next investigated whether treatment of low temperature affected JA-regulated root growth and anthocyanin accumulation in *coi1-1*, *coi1-2*, *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-8*, *coi1-9* and *coi1-10* mutant plants. We found that similar to the seedlings grown at 22°C, these mutants were still insensitive to JA-regulated root growth and anthocyanin accumulation when grown at 16°C (Figure 4A and B, data not shown).

We next tested whether treatment of low temperature was able to enhance male fertility in *coi1-1*, *coi1-2*, *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-8*, *coi1-9* and *coi1-10* mutant plants (see Materials and methods). We found that the *coi1-1*, *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-9* and *coi1-10*

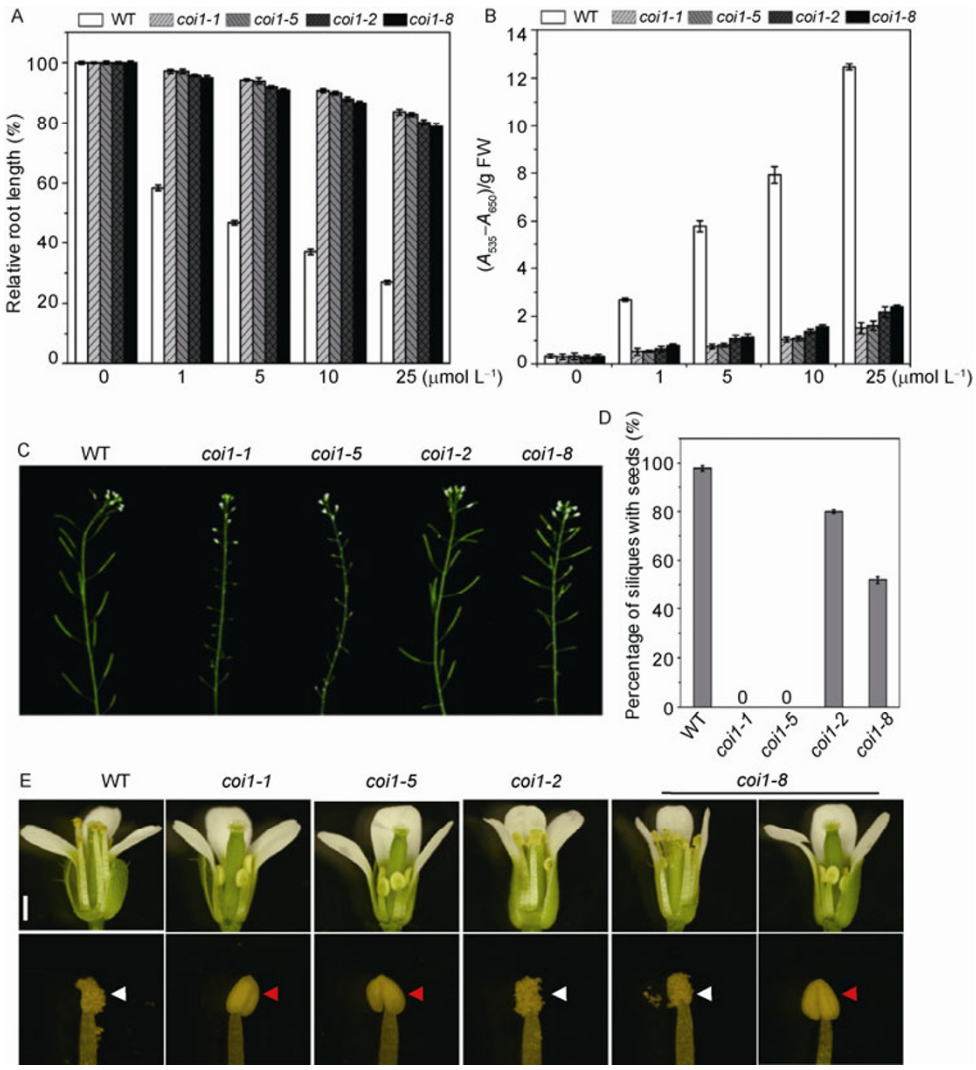

Figure 4 The low temperature treatment restores male fertility in *coi1-2*. A, Relative root length of the 11-day-old seedlings grown on MS medium with the indicated concentrations of MeJA at 16°C. Error bars represent SE (n=15). B, Anthocyanin contents in the 11-day-old seedlings grown on MS medium containing different concentrations of MeJA at 16°C. FW, fresh weight. Error bars represent SE (n=3). C, Phenotypes of the primary inflorescences of WT, *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* at 16°C. D, Percentage of siliques with seeds in the primary inflorescences of WT, *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* at 16°C. Error bars represent SE (n=15). E, Phenotypes of flowers (top panel) and anthers (bottom panel) at floral stage 13 from WT, *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* at 16°C. Scale bar (top panel), 0.5 mm. White arrowheads represent dehiscent anthers, and red arrowheads indicate indehiscent anthers.

were completely male sterile under treatment of low temperature (16°C) (Figure 4C, data not shown), similar to the phenotypes at 22°C (Figure 3A). Flowers in these mutant plants were deficient in filament elongation, anther dehiscence and pollen maturation (Figure 4E, data not shown).

Phenotypic observation of plant fertility revealed that treatment of low temperature (16°C) significantly promoted male fertility in *coi1-2* but not in *coi1-8* plants (Figure 4C and D). ~80% of siliques in the 16°C-treated *coi1-2* plants set seeds whereas only ~5% of siliques set seeds at 22°C (Figures 3A and B, 4C and D). Consistently, most flowers from the 16°C-treated *coi1-2* exhibited elongated filaments, dehiscent anthers and viable pollen grains (Figure 4E, data not shown). However, under treatment of low temperature (16°C), male fertility of the *coi1-8* plants was not obviously enhanced compared with the 22°C-treated *coi1-8* plants (Figure 3A and B, 4C and D).

Having demonstrated that low temperature recovered the fertility in *coi1-2*, we investigated whether low temperature enhanced the COI1 level in *coi1-2* and other *coi1* mutants. RT-PCR and qRT-PCR analysis showed that the expression level of *COI1* was similar in the wild type, *coi1-1*, *coi1-2*, *coi1-4*, *coi1-5*, *coi1-6*, *coi1-7*, *coi1-8*, *coi1-9* and *coi1-10* at either 16°C or 22°C (Figure 5A, data not shown). The immunoblot analysis using COI1-antiserum displayed that COI1 protein level was not obviously increased in the 16°C-treated *coi1-2*, *coi1-8* and *coi1-5* (Figure 5B). Similar to the data from the 22°C-treated plants, COI1 protein was not detectable in *coi1-1*, *coi1-4*, *coi1-6*, *coi1-7*, *coi1-9* and *coi1-10* (Figure 5B, data not shown).

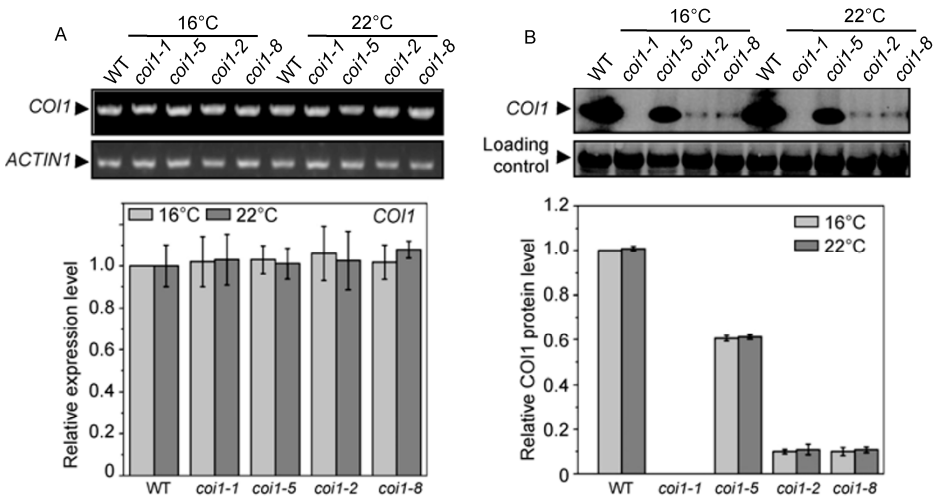

Figure 5 The low temperature treatment cannot influence the COI1 expression. A, RT-PCR (top panel) and quantitative real-time (qRT) PCR (bottom panel) analysis of *COI1* transcript level in the indicated seedlings grown at 16°C or 22°C. For RT-PCR analysis, *Actin1* was used as the normalization control. For qRT-PCR analysis, *Actin8* was used as the internal control. Error bars represent SE (n=3). B, Immunoblot (top panel) and quantitative analysis of COI1 protein level (bottom panel) in WT, *coi1-1*, *coi1-5*, *coi1-2* and *coi1-8* at 16°C or 22°C. The PVDF membrane was stained by Memstain to serve as loading control. Error bars represent SE (n=3). The abundance of COI1 in wild type at 16°C was set to 1.0 for calculating the relative abundance of COI1 protein in the 22°C-treated WT and all the indicated *coi1* mutants treated with 16°C or 22°C.

In summary, we show that JA-regulated root growth and anthocyanin accumulation of all these tested *coi1* mutants are not obviously affected by low temperature, and the treatment of low temperature has no obvious influence on the COI1 protein level in these *coi1* mutants. However, low temperature treatment is able to obviously restore male fertility in *coi1-2* ($COI1^{L245F}$), but unable to enhance male fertility in *coi1-8*.

3 Discussion

The structure of the ASK1-COI1-(JA-Ile)-JAZ1 degron complex revealed that the COI1 C-terminus consists of 18 LRRs and four loops (loop-2, loop-12, loop-14 and loop-C) forming a binding surface for the bioactive JA-Ile [34]. Here, we carried out biochemical analysis on a series of *coi1* mutant alleles which harbored various amino acid mutations distributed in the COI1 C-terminal LRRs and loops (Figure 1B). We found that the different amino acid mutations exhibited different effect on COI1 stability: the $COI1^{G98D}$ in the *coi1-5* mutant was retained at ~60% of wild-type COI1 level (Figure 3D); the $COI1^{L245F}$ in *coi1-2* and $COI1^{E543K}$ in *coi1-8* were maintained at ~10% of wild-type COI1 level (Figure 3D); the remaining COI1 mutations, including $COI1^{W467*}$ (*coi1-1*), $COI1^{G369E}$ (*coi1-4*), $COI1^{Q343*}$ (*coi1-6*), $COI1^{G155E}$ (*coi1-7*), $COI1^{D452A}$ (*coi1-9*) and $COI1^{L490A}$ (*coi1-10*), completely abolished COI1 stability (Figure 3D, data not shown).

Further analysis on correlation of the $COI1^{L245F}$ (*coi1-2*), $COI1^{E543K}$ (*coi1-8*) and $COI1^{G98D}$ (*coi1-5*) protein level with male fertility phenotype revealed that $COI1^{L245F}$, $COI1^{E543K}$ and $COI1^{G98D}$ protein exerted distinct effect on male fertility: *coi1-5* ($COI1^{G98D}$) was completely male sterile and the $COI1^{G98D}$ was not functional in JA signaling though ~60% of $COI1^{G98D}$ protein was retained (Figure 3); male fertility in the *coi1-8* ($COI1^{E543K}$) mutant was relatively less affected (~50% of wild-type fertility), though only ~10% of protein was detectable (Figure 3); the $COI1^{L245F}$ displayed strong effect on both the protein stability (similar protein level with $COI1^{E543K}$) and male fertility in the 22°C-grown *coi1-2* plants (less than ~5% of wild-type fertility level) (Figure 3). It would be interesting to investigate the molecular mechanism in stabilizing these COI1 proteins $COI1^{L245F}$ (*coi1-2*), $COI1^{E543K}$ (*coi1-8*) and $COI1^{G98D}$ (*coi1-5*), and in regulating their function in male fertility.

COI1 was shown to mediate diverse JA responses such as root growth, stamen development, anthocyanin accumulation and response to the bacterial pathogen *Pst* DC3000 infection. Evidence is limited about whether the various COI1-mediated JA responses require different abundance of the COI1 protein. In this study, we showed that different from other JA responses, JA-regulated male fertility just required low level of COI1 protein: the $COI1^{E543K}$ and $COI1^{L245F}$ protein (~10% of wild-type COI1 level) was enough for maintaining male fertility (~50% of wild-type fertility) or temperature-sensitive fertility (~80% of wild-type fertility), but not for regulating the JA-inhibitory root growth, anthocyanin accumulation and response to the bacterial pathogen *Pst* DC3000 infection (Figures 2–4). It remains to be elucidated which COI1 dosage is required for regulating other JA responses, such as leaf senescence, trichome formation and defense against insect attack and necrotrophic pathogen infection. We found that all our tested *coi1* mutants including *coi1-2* ($COI1^{L245F}$) and *coi1-8* ($COI1^{E543K}$) did not contain the *pen2-4* mutation (data not

shown), which is different from the previously reported *coi1-16* ($COI1^{L245F}$) mutant with the additional *pen2-4* mutation [45,46]. As the *pen2-4* mutation in the *PEN2* gene may alter the non-host resistance [46], our *coi1* mutants without the *pen2-4* mutation would be useful for the analysis of JA-regulated plant defense responses.

Low temperature treatment significantly rescued male sterility in *coi1-2* ($COI1^{L245F}$), whereas male fertility in *coi1-8* ($COI1^{E543K}$) was not affected (Figure 4). Two putative reasons might account for the dramatic recovery of male fertility in *coi1-2* by low temperature treatment: firstly, low temperature may modulate the function of $COI1^{L245F}$ (e.g., configuration change of $COI1^{L245F}$) for JA-regulated stamen development; secondly, low temperature may affect the property of the signaling components (e.g., MYB21/MYB24) [48,49] downstream of COI1 for stamen development. However, the low temperature did not enhance male fertility in *coi1-8* ($COI1^{E543K}$) that regulates the same downstream signaling components of *coi1-2* ($COI1^{L245F}$), which may exclude the second putative reason. It would be interesting to investigate whether and how low temperature modulates the configuration of $COI1^{L245F}$, generating the functional form of $COI1^{L245F}$ for regulation of male fertility.

This work was supported by the National Natural Science Foundation of China (31230008 and 91017012) and Ministry of Agriculture of China (2013ZX08011-006).

1 Browse J. Jasmonate passes muster: a receptor and targets for the defense hormone. Annu Rev Plant Biol, 2009, 60: 183–205
2 Wasternack C, Hause B. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in annals of botany. Ann Bot, 2013, 111: 1021–1058
3 Song S, Qi T, Huang H, Ren Q, Wu D, Chang C, Peng W, Liu Y, Peng J, Xie D. The Jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect jasmonate-regulated stamen development in *Arabidopsis*. Plant Cell, 2011, 23: 1000–1013
4 McConn M, Browse J. The critical requirement for linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis* mutant. Plant Cell, 1996, 8: 403–416
5 Song S, Qi T, Huang H, Xie D. Regulation of stamen development by coordinated actions of jasmonate, auxin and gibberellin in *Arabidopsis*. Mol Plant, 2013, 6: 1065–1073
6 Staswick PE, Su WP, Howell SH. Methyl jasmonate inhibition of root-growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc Natl Acad Sci USA, 1992, 89: 6837–6840
7 Feys BJF, Benedetti CE, Penfold CN, Turner JG. *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male-sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell, 1994, 6: 751–759
8 Qi T, Song S, Ren Q, Wu D, Huang H, Chen Y, Fan M, Peng W, Ren C, Xie D. The Jasmonate-ZIM-domain proteins interact with the WD-repeat/bHLH/MYB complexes to regulate jasmonate-mediated anthocyanin accumulation and trichome initiation in *Arabidopsis thaliana*. Plant Cell, 2011, 23: 1795–1814
9 Yoshida Y, Sano R, Wada T, Takabayashi J, Okada K. Jasmonic acid control of GLABRA3 links inducible defense and trichome patterning in *Arabidopsis*. Development, 2009, 136: 1039–1048
10 Ueda J, Kato J. Isolation and identification of a senescence-promoting substance from wormwood (*Artemisia absinthium* L.). Plant Physiol, 1980, 66: 246–249
11 Shan X, Wang J, Chua L, Jiang D, Peng W, Xie D. A role of *Arabidopsis* rubisco activase in jasmonate-induced leaf senescence. Plant Physiol, 2011, 155: 751–764
12 De Geyter N, Gholami A, Goormachtig S, Goossens A. Transcriptional machineries in jasmonate-elicited plant secondary metabolism. Trends Plant Sci, 2012, 17: 349–359
13 Schweizer F, Fernandez-Calvo P, Zander M, Diez-Diaz M, Fonseca S, Glauser G, Lewsey MG, Ecker JR, Solano R, Reymond P. *Arabidopsis* basic helix-loop-helix transcription factors MYC2, MYC3, and MYC4 regulate glucosinolate biosynthesis, insect performance, and feeding behavior. Plant Cell, 2013, 25: 3117–3132
14 Hong GJ, Xue XY, Mao YB, Wang LJ, Chen XY. *Arabidopsis* MYC2 interacts with DELLA proteins in regulating sesquiterpene synthase gene expression. Plant Cell, 2012, 24: 2635–2648
15 Rao MV, Lee H, Creelman RA, Mullet JE, Davis KR. Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. Plant Cell, 2000, 12: 1633–1646
16 Seo JS, Joo J, Kim MJ, Kim YK, Nahm BH, Song SI, Cheong JJ, Lee JS, Kim JK, Choi YD. OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice. Plant J, 2011, 65: 907–921
17 Robson F, Okamoto H, Patrick E, Harris SR, Wasternack C, Brearley C, Turner JG. Jasmonate and phytochrome A signaling in *Arabidopsis* wound and shade responses are integrated through JAZ1 stability. Plant Cell, 2010, 22: 1143–1160
18 Howe GA, Jander G. Plant immunity to insect herbivores. Annu Rev Plant Biol, 2008, 59: 41–66
19 McConn M, Creelman RA, Bell E, Mullet JE, Browse J. Jasmonate is essential for insect defense in *Arabidopsis*. Proc Natl Acad Sci USA, 1997, 94: 5473–5477
20 Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA. The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell, 2004, 16: 126–143
21 Lorenzo O, Chico JM, Sanchez-Serrano JJ, Solano R. *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. Plant Cell, 2004, 16: 1938–1950
22 Thaler JS, Humphrey PT, Whiteman NK. Evolution of jasmonate and salicylate signal crosstalk. Trends Plant Sci, 2012, 17: 260–270
23 Vijayan P, Shockey J, Levesque CA, Cook RJ, Browse J. A role for jasmonate in pathogen defense of *Arabidopsis*. Proc Natl Acad Sci USA, 1998, 95: 7209–7214
24 Hu P, Zhou W, Cheng Z, Fan M, Wang L, Xie D. JAV1 controls jasmonate-regulated plant defense. Mol Cell, 2013, 50: 504–515
25 Robert-Seilaniantz A, Grant M, Jones JD. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annu Rev Phytopathol, 2011, 49: 317–343
26 Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG. *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science, 1998, 280: 1091–1094
27 Ren CM, Pan JW, Peng W, Genschik P, Hobbie L, Hellmann H, Estelle M, Gao B, Peng JR, Sun CQ, Xie DX. Point mutations in *Arabidopsis* Cullin1 reveal its essential role in jasmonate response. Plant J, 2005, 42: 514–524
28 Xu LH, Liu FQ, Lechner E, Genschik P, Crosby WL, Ma H, Peng W, Huang DF, Xie DX. The SCF^{COI1} ubiquitin-ligase complexes are required for jasmonate response in *Arabidopsis*. Plant Cell, 2002, 14: 1919–1935
29 Yan J, Li H, Li S, Yao R, Deng H, Xie Q, Xie D. The *Arabidopsis* F-box protein CORONATINE INSENSITIVE1 is stabilized by SCF^{COI1} and degraded via the 26s proteasome pathway. Plant Cell, 2013, 25: 486–498

30 Yan J, Zhang C, Gu M, Bai Z, Zhang W, Qi T, Cheng Z, Peng W, Luo H, Nan F, Wang Z, Xie D. The *Arabidopsis* CORONATINE INSENSITIVE1 protein is a jasmonate receptor. Plant Cell, 2009, 21: 2220–2236
31 Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R. The JAZ family of repressors is the missing link in jasmonate signalling. Nature, 2007, 448: 666–671
32 Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J. JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. Nature, 2007, 448: 661–665
33 Yan YX, Stolz S, Chetelat A, Reymond P, Pagni M, Dubugnon L, Farmer EE. A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell, 2007, 19: 2470–2483
34 Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu FF, Sharon M, Browse J, He SY, Rizo J, Howe GA, Zheng N. Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature, 2010, 468: 400–405
35 Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C, Solano R. (+)-7-iso-jasmonoyl-l-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol, 2009, 5: 344–350
36 Niu Y, Figueroa P, Browse J. Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in *Arabidopsis*. J Exp Bot, 2011, 62: 2143–2154
37 Fernandez-Calvo P, Chini A, Fernandez-Barbero G, Chico JM, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, Pauwels L, Witters E, Puga MI, Paz-Ares J, Goossens A, Reymond P, De Jaeger G, Solano R. The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell, 2011, 23: 701–715
38 Cheng Z, Sun L, Qi T, Zhang B, Peng W, Liu Y, Xie D. The bHLH transcription factor MYC3 interacts with the Jasmonate ZIM-domain proteins to mediate jasmonate response in *Arabidopsis*. Mol Plant, 2011, 4: 279–288
39 Zhu Z, An F, Feng Y, Li P, Xue L, Mu A, Jiang Z, Kim JM, To TK, Li W, Zhang X, Yu Q, Dong Z, Chen WQ, Seki M, Zhou JM, Guo H. Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. Proc Natl Acad Sci USA, 2011, 108: 12539–12544
40 Song S, Qi T, Fan M, Zhang X, Gao H, Huang H, Wu D, Guo H, Xie D. The bHLH subgroup IIId factors negatively regulate jasmonate-mediated plant defense and development. PLoS Genet, 2013, 9: e1003653
41 Sasaki-Sekimoto Y, Jikumaru Y, Obayashi T, Saito H, Masuda S, Kamiya Y, Ohta H, Shirasu K. Basic helix-loop-helix transcription factors jasmonate-associated MYC2-LIKE1 (JAM1), JAM2, and JAM3 are negative regulators of jasmonate responses in *Arabidopsis*. Plant Physiol, 2013, 163: 291–304
42 Nakata M, Mitsuda N, Herde M, Koo AJ, Moreno JE, Suzuki K, Howe GA, Ohme-Takagi M. A bHLH-type transcription factor, ABA-INDUCIBLE BHLH-TYPE TRANSCRIPTION FACTOR/JA-ASSOCIATED MYC2-LIKE1, acts as a repressor to negatively regulate jasmonate signaling in *Arabidopsis*. Plant Cell, 2013, 25: 1641–1656
43 Smyth DR, Bowman JL, Meyerowitz EM. Early flower development in *Arabidopsis*. Plant Cell, 1990, 2: 755–767
44 Katagiri F, Thilmony R, He SY. The *Arabidopsis thaliana*-pseudomonas syringae interaction. Arabidopsis Book, 2002, 1: e0039
45 Ellis C, Turner JG. A conditionally fertile *coi1* allele indicates cross-talk between plant hormone signalling pathways in *Arabidopsis thaliana* seeds and young seedlings. Planta, 2002, 215: 549–556
46 Westphal L, Scheel D, Rosahl S. The *coi1-16* mutant harbors a second site mutation rendering PEN2 nonfunctional. Plant Cell, 2008, 20: 824–826
47 Yang DL, Yao J, Mei CS, Tong XH, Zeng LJ, Li Q, Xiao LT, Sun TP, Li J, Deng XW, Lee CM, Thomashow MF, Yang Y, He Z, He SY. Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. Proc Natl Acad Sci USA, 2012, 109: E1192–1200
48 Mandaokar A, Thines B, Shin B, Lange BM, Choi G, Koo YJ, Yoo YJ, Choi YD, Choi G, Browse J. Transcriptional regulators of stamen development in *Arabidopsis* identified by transcriptional profiling. Plant J, 2006, 46: 984–1008
49 Cheng H, Song S, Xiao L, Soo HM, Cheng Z, Xie D, Peng J. Gibberellin acts through jasmonate to control the expression of *MYB21*, *MYB24*, and *MYB57* to promote stamen filament growth in *Arabidopsis*. PLoS Genet, 2009, 5: e1000440