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An *Arabidopsis* mutant *cex1* exhibits constant accumulation of jasmonate-regulated *AtVSP*, *Thi2.1* and *PDF1.2*

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Abstract Jasmonates (JA) act as a regulator in plant growth as well as a signal in plant defense. The *Arabidopsis* vegetative storage protein (AtVSP) and plant defense-related proteins thionin (Thi2.1) and defensin (PDF1.2) have previously been shown to accumulate in response to JA induction. In this report, we isolated and characterized a novel recessive mutant, *cex1*, conferring constitutive JA-responsive phenotypes including JA-inhibitory growth and constitutive expression of JA-regulated *AtVSP*, *Thi2.1* and *PDF1.2*. The plant morphology and the gene expression pattern of the *cex1* mutant could be phenocopied by treatment of wild-type plants with exogenous JA, indicating that *CEX1* might be a negative regulator of the JA response pathway. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Jasmonate; Signal transduction; *cex1*

1. Introduction

Jasmonic acid and its methyl ester, methyl jasmonate, are endogenous plant compounds (collectively referred to as jasmonates, JA) that synthesized from linolenic acid in a lipoxygenase-dependent biosynthetic pathway. They are widely distributed in most organs of the plant kingdom, high levels of JA in plants are found in young tissues, flowers and pericarp tissues of developing reproductive structures. Recently, JA are well known to act as a regulator in plant growth and development as well as a signal in plant defense and wound responses. They are involved in regulating the expression of numerous genes and influencing diverse processes including pollen development, fruit ripening, root growth, tendril coiling, wound responses, and resistance against insects and pathogens (for reviews, see [1–6]).

JA inhibited *Arabidopsis* seedling growth [7] and induced the expression of the *Arabidopsis* vegetative storage protein (AtVSP) and plant defense-related proteins thionin (Thi2.1) and defensin (PDF1.2) [9–11]. The *Arabidopsis* JA-insensitive mutants, *jar1* [7,12], *coi1* [13], *jin1* and *jin4* [14], were previously identified by screening mutagenized M2 seedlings for JA-insensitive root elongation on Murashige and Skoog (MS) medium containing a high concentration of JA. The *coi1* mutant was resistant to both JA and coronatine, a phytotoxin that is structurally related to JA [13], and exhibited loss of JA-induced expression of *AtVSP* [15], *Thi2.1* [16] and *PDF1.2* [17,18]. The *jar1*, *jin1* and *jin4* mutants showed decreased sensitivities to the JA-inhibitory root growth and a reduced level of *AtVSP* expression in response to the JA treatment [7,14]. The signal transduction pathway that modulates JA action has been investigated through analysis of these JA-insensitive mutants.

In this work, we screened ethyl methanesulfonate (EMS)-mutagenized *Arabidopsis* M2 seeds on MS medium for mutants that constitutively exhibited phenotypes observed in plants treated with JA. We have isolated and characterized *cex1*, an *Arabidopsis* mutant with *c*onstant *ex*pression of JA-inducible genes *AtVSP*, *Thi2.1* and *PDF1.2*.

2. Materials and methods

2.1. Mutant screening

The *Arabidopsis* M2 seeds (ecotype Columbia) used in mutant screening were purchased from Lehle Seeds Company (http://www.arabidopsis.com/, stock no. M2E-02-03). The seeds were treated by EMS and grown in soil to allow self-fertilization (referred to as the M1 population). The seeds collected from these M1 plants were referred to as the M2 population.

Seeds were routinely sterilized by treating with 20% bleach for 15–30 min, washing with five changes of sterile water, and sown in Petri dishes on MS medium (Sigma cat. no. M-5519) supplemented with 2% sucrose and 0.8% agar. The Petri dishes were treated at 4°C for 2 days and then incubated at 22°C under continuous fluorescent illumination. Seedlings with short root and stunt growth were selected from 7 to 15 days after plating. The selected plants were grown to maturity for M3 seeds in soil.

The back cross between the wild-type (wt) plant and *cex1-1* or *cex1-2* was performed using the wt plant as the female parent. The cross of *cex1-1*×*cex1-2* was performed using *cex1-1* as the female parent. The genetic cross between *coi1-1* and *cex1-1* was performed using *coi1-1* as the female parent. Seeds collected from the F1 plants were referred to as the F2 population.

Methyl jasmonate used in the JA treatment was purchased from Aldrich (cat. no. 39270-7).

2.2. Northern analysis

Probes for the detection of *AtVSP*, *Thi2.1* or *PDF1.2* transcripts were PCR-amplified with primers of ATVSP.5′ (5′-ACGTCCAGTCTTCGGCATCC-3′)/ATVSP.3′ (5′-GAGCTTAAAAACCCTTCCAG-3′), Thi.5′ (5′-GTGATCAAACAAGTAAACCAT-3′)/Thi.3′ (5′-AACAAACCTTCTACGACACAT-3′), or PDF.5′ (5′-AAGTTTGCTTCCATCATCACC-3′)/PDF.3′ (5′-ATACACACGATTTAGCACCAA-3′), respectively. The gene-specific probes were labeled by the PCR DIG Probe Synthesis kit according to the manufacturer's instruction (Roche Molecular Biochemicals cat. no. 1-636-090).

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Plants were grown on MS medium for 2 weeks. For the JA treatment, 2-week-old seedlings were transferred to MS medium containing 25 μM JA and grown for an additional 24 h. The plant materials were frozen in liquid nitrogen immediately after harvest. Total RNA was isolated using Trizol reagent (Gibco BRL cat. no. 15596). 20 μg of RNA was separated on 1.3% formaldehyde agarose gel and transferred onto Hybond N^+ membrane (Amersham). RNA blots were hybridized with the DIG-labeled probes in the DIG Easy hybridization solution according to manufacturer's instruction (Roche Molecular Biochemicals cat. no. 1-603-558).

2.3. *PThi2.1::GUS construct and plant transformation*

PThi2.1::GUS construct was made by fusing the *Thi2.1* promoter region (*PThi2.1*) with the GUS reporter gene into plasmid pBI101.3 (Clontech). The *PThi2.1::GUS* construct was transferred into *Agrobacterium tumefaciens* and subsequently mobilized into the plant genome by in plant *Agrobacterium*-mediated vacuum infiltration. The homozygous plant transgenic for *PThi2.1::GUS* was used in GUS staining experiments [19].

3. Results

3.1. *Isolation of Arabidopsis mutants*

We screened about 80 000 *Arabidopsis* M2 seedlings for mutants in which seedling growth was inhibited on MS medium without JA supplementation. Seeds were germinated on MS plates and grown for 7–15 days, 27 plants with short root and stunt growth were selected and grown in soil to produce M3 seeds. To examine the expression pattern of JA-inducible genes in the 27 selected plants, total RNA was extracted from 2-week-old M3 seedlings grown on MS medium and probed with JA-inducible gene *Thi2.1*. Out of the 27 selected plants, two mutants (*cex1-1* and *cex1-2*), derived from the same M2 seed-lot (lot no. M2E-02-03-80), showed constant expression of *Thi2.1* (Fig. 1). The *cex1-1* and *cex1-2* mutants grown on MS medium exhibited stunt growth phenotype similar to the wt plants grown on the medium containing methyl jasmonate (Fig. 2A,B). Root elongation for the *cex1-1* and *cex1-2* mutants on MS medium was comparable to the JA-treated wt (Fig. 2C).

The plant hormone ethylene was shown to regulate the expression of some JA-responsive genes including *PDF1.2* [17,18]. Constitutive ethylene-responsive mutants, the *ctr1* mutant and *etr1/etr2/ein4* triple mutant [20,21], exhibited stunt growth and constitutive expression of ethylene-inducible genes in the absence of ethylene. The *cex1* mutant was identified by monitoring constitutive JA-responsive phenotypes including stunt growth and constant expression of JA-inducible genes. Morphology of the *cex1* seedling and adult plant might be similar to the constitutive ethylene-responsive phenotypes. However, air-grown etiolated seedlings of the constitutive ethylene-responsive mutants exhibited a strong ethylene response and had very short hypocotyls and roots [20,21]. We germinated and grew the *cex1-1* and *cex1-2* mutants on MS plates in the dark for 1 week. As shown in Fig. 3, dark-grown seedlings of the *cex1-1* and *cex1-2* mutants had normal hypocotyls and roots observed in wt, indicating that *cex1* did not exhibit constitutive ethylene responses.

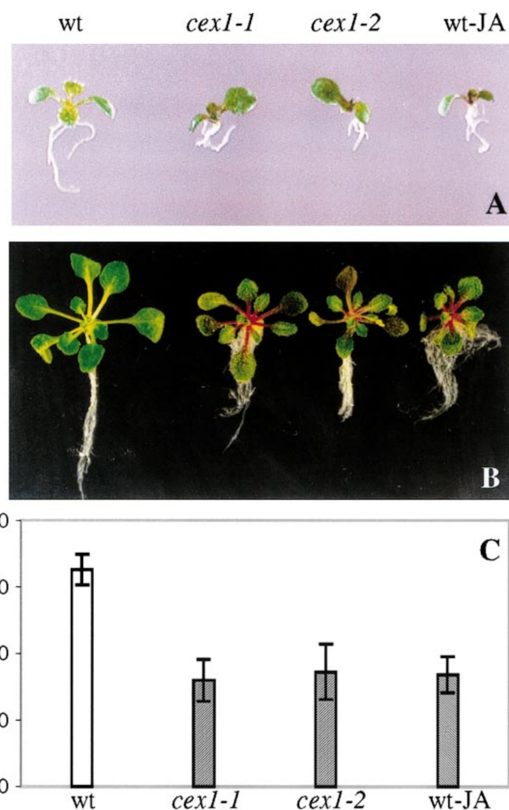

Fig. 2. The *cex1* growth phenotype. A: 6-day-old seedlings. B: 3-week-old plants. C: Root length was measured 2 weeks after germination, bars show S.E.M. $n = 15$. The *Arabidopsis* wt, *cex1-1* and *cex1-2* mutants were grown on MS medium. Wt grown on the MS medium containing 0.5 μM methyl jasmonate was used as a control (wt-JA), it is interesting to note that the 3-week-old wt-JA had a high number of lateral roots.

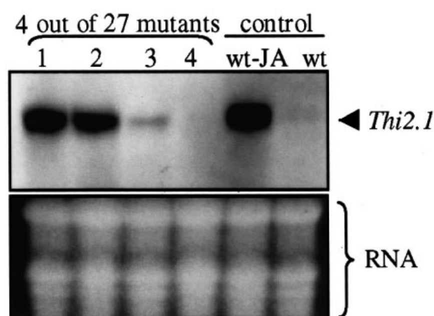

Fig. 1. Accumulation of *Thi2.1* transcript in *cex1-1* (lane 1) and *cex1-2* (lane 2). Four out of the identified 27 mutants were selected to show in lanes 1, 2, 3 and 4. Mutants in lane 1 and lane 2 were designated as *cex1-1* and *cex1-2*, respectively. Wt plants without JA (wt) or with JA treatment (wt-JA) were used as controls. Total RNA stained with ethidium bromide was shown as the RNA loading control.

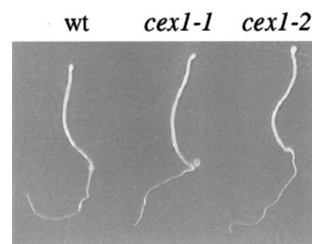

Fig. 3. Dark-grown *cex1-1*, *cex1-2* and wt on MS medium at 22°C for 1 week.

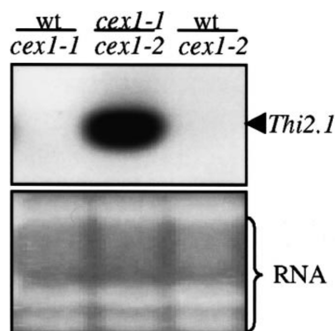

Fig. 4. Northern analysis of the F1 progeny from the crosses of wt×*cex1-1*, wt×*cex1-2* and *cex1-1*×*cex1-2*.

Table 1
Segregation analysis

Cross	F2 progeny	*Thi2.1* expression		χ^{2a}
		No	Yes	
wt×*cex1-1*	119	94	25	$P>0.5$
wt×*cex1-2*	135	98	37	$P>0.5$
cex1-1×*cex1-1*	35	0	35	–

[a]Chi-square test for the expected ratio of 3 wt:1 *cex1*.

3.2. Genetic analysis of the cex1 mutant

Analysis of progeny from the crosses of wt×*cex1-1* and wt×*cex1-2* demonstrated that the phenotypes of *cex1-1* and *cex1-2* mutants were inherited as single recessive Mendelian markers. The F1 progeny of the crosses between wt and two mutants exhibited wt phenotypes with the loss of constant expression of *Thi2.1* (Fig. 4). The F2 progeny derived from the two crosses were scored for the segregation pattern of the constant expression of *Thi2.1*, the Chi-square test for the expected ratio of 3 wt:1 mutant was $P>0.5$ (Table 1).

The cross of *cex1-1*×*cex1-2* was made to test whether the two mutants were allelic. The F1 progeny of *cex1-1*×*cex1-2* exhibited the mutant phenotype with the constant expression of *Thi2.1* (Fig. 4), and no recombination was detected in the F2 progeny (Table 1). Therefore, the two mutants were allelic at the *cex1* locus. Because the two mutants were identified from the same M2 seed-lot, it is possible that the *cex1-1* and *cex1-2* mutants were derived from the same mutation. Further work was therefore concentrated on the *cex1-1* mutant.

3.3. The constant expression of JA-inducible genes in the cex1 mutant

To further examine the expression of *Thi2.1* in *cex1* mutant, we made *Arabidopsis* transgenic plants expressing GUS reporter gene under the control of a *Thi2.1* promoter (*PThi2.1*). In the absence of methyl jasmonate, strong expression of *PThi2.1::GUS* was detected in the transgenic *cex1-1* mutant, but not in the transgenic wt plant except the expression in the flower buds (Fig. 5). After treated with methyl jasmonate, the transgenic wt plant strongly expressed the *PThi2.1::GUS*.

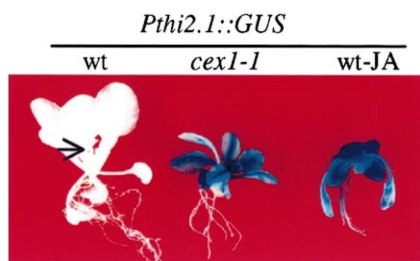

Fig. 5. Histochemical staining of GUS activity in 3-week-old transgenic plants of *cex1-1*, wt and JA-treated wt (wt-JA). The arrow pointed the GUS staining in wt.

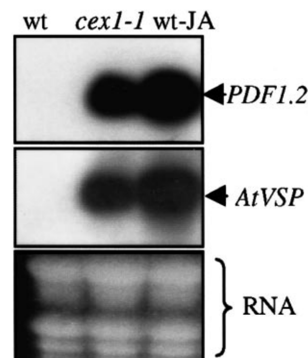

Fig. 6. The constitutive expression of *AtVSP* and *PDF1.2* in *cex1-1* but not in wt. JA-treated wt was used as a control (wt-JA). Total RNA stained with ethidium bromide was shown as the RNA loading control.

It was reported that JA induced the expression of numerous genes [22]. In particular, the expression of *AtVSP*, *PDF1.2* as well as *Thi2.1* was strongly activated by JA [7–10]. We therefore tested whether *AtVSP* and *PDF1.2* were constitutively expressed in the *cex1* mutant. As shown in Fig. 6, *AtVSP* and *PDF1.2* were highly expressed in the *cex1* mutant similar to the JA-treated wt plant. Thus, the *cex1* mutant exhibited the constant expression of JA-regulated *Thi2.1*, *AtVSP* and *PDF1.2*.

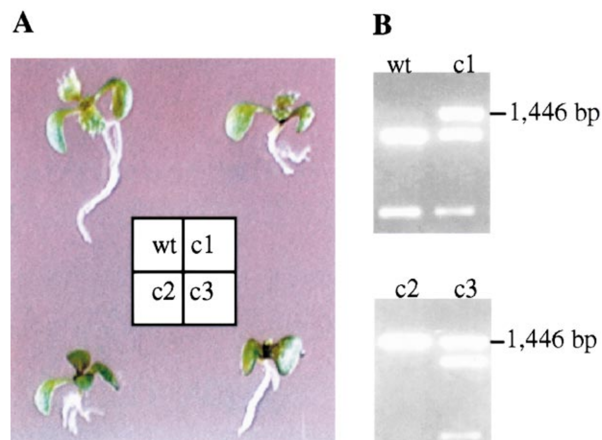

Fig. 7. Isolation and characterization of the *cex1/coi1-1* double mutant. A: 1-week-old *cex1* homozygous seedlings, c1, c2 and c3, derived from the F2 progeny of *cex1*×*coi1-1*. B: A 1446-bp fragment containing part of *COI1* or *coi1-1* was PCR-amplified by the primers p1 (5′-GGTTCTCTTTAGTCTTTAC-3′) and p2 (5′-CAGACAACTATTTCGT TACC-3′) [16]. *Xcm*I cleaved the 1446-bp fragment from the wt DNA, but not from *coi1-1* in which the *Xcm*I recognition site CCA-9N-TGG was altered by the *coi1-1*^{G1401A} mutation to CCA-9N-TGA [16]. The 1446-bp fragment from c2 was not cleavable by *Xcm*I, indicating that this plant was homozygous for the *coi1-1* mutation and was therefore the *cex1/coi1* double mutant.

3.4. Location of cex1 in the JA signal pathway

The location of *cex1* in the JA signal transduction chain can be defined by analysis of double mutants between *cex1* and other JA-responsive mutants including *coi1*, *jar1*, *jin1* and *jin4*. The *COI1* was previously suggested to be upstream of *JIN1* and *JIN4* in the JA signal transduction chain [14,23]. Because the *COI1* gene was previously cloned and the *coi1-1* genotype can be monitored by a *coi1-1*-specific CAPs marker [16], it is possible to isolate the *cex1/coi1* double mutant from the cross of *cex1-1*×*coi1-1*. Therefore, we crossed the *cex1-1* to *coi1-1* mutant and isolated homozygous *cex1-1* mutants from the F2 progeny by screening mutant phenotypes of stunt growth and short roots. Three homozygous *cex1-1* mutants, the c1, c2 and c3 plants, were shown in Fig. 7A in comparison with the wt phenotype. The restriction analysis of the three homozygous *cex1-1* mutants with the *coi1-1*-specific CAPs marker demonstrated that the c2 plant was homozygous for *coi1-1* mutation (Fig. 7B). Using the same method, we have identified 17 *cex1/coi1* double mutants. The identified *cex1/coi1* double mutants, such as the c2 plant shown in Fig. 7A, exhibited *cex1* phenotypes of stunt growth and short roots, indicating that *CEX1* acts downstream of *COI1* in the JA signal transduction pathway.

4. Discussion

Selection of plant mutants for resistance to high concentrations of applied hormones is a powerful approach for studying hormone signal transduction [24]. This approach has led to the isolation of four JA-insensitive mutants, *coi1*, *jar1*, *jin1* and *jin4*, in *Arabidopsis thaliana* [7,12–14]. To isolate more JA-resistant mutants for further dissection of the JA signal transduction pathway, we have recently used MS medium containing a high concentration (50 μM) of JA to screen the *Arabidopsis* M2 population, representing 200 000 M1 population mutagenized by EMS, γ-ray or fast neutron irradiation. Nineteen JA-resistant mutants were isolated. However, genetic analysis of the F1 progeny from the crosses between *coi1-1* and each of the 19 mutants demonstrated that all of the 19 plants were *coi1* alleles (unpublished data), indicating that it might be difficult to use this approach for identification of additional JA-insensitive mutants.

In this experiment, we germinated *Arabidopsis* M2 seeds on MS medium and screened for mutants conferring sensitive phenotypes similar to plants grown on JA medium. We preliminarily picked up more than 600 candidate plants and reduced the total numbers to 27 plants after close examination of plant growth on MS medium in comparison to JA-treated wt plants. The recessive *cex1* mutant was isolated and characterized.

The *cex1* mutant exhibited constitutive JA-responsive phenotypes. The plant morphology and the gene expression pattern of the *cex1* mutant were phenocopied by treatment of wt plants with exogenous methyl jasmonate, indicating that *CEX1* is a negative regulator of the JA response pathway. It awaits a direct measurement of the endogenous JA to determine whether *cex1* accumulates a high level of JA. If the JA level in *cex1* remains similar to wt, *CEX1* would be a JA-signaling repressor. If *cex1* displays an elevated JA level, *CEX1* would act as a JA biosynthesis repressor. The *cex1* mutation within the negative regulator gene would constitutively activate the JA signal pathway, the JA-inducible genes including *Thi2.1*, *PDF1.2* and *AtVSP* were therefore expressed constantly. A mutant with the similar elevated AtVSP expression phenotype, albeit preliminary data (Ellis and Turner, 6th International Congress of Plant Molecular Biology, abstract no. S35-6), displays enhanced resistance to powdery mildew. It is expected that the JA-regulated plant resistance against pathogen infection and insect attack [25–27] would constitutively be maintained in the *cex1* mutant. Future research on molecular cloning of the *CEX1* gene will be very useful for dissection of the JA signal transduction pathway and understanding of the mechanism of JA-regulated plant defense.

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