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Title: *Brassica carinata* CIL1 mediates extracellular ROS production during auxin- and ABA-regulated lateral root development

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Abstract

In the cellular milieu of plants, signals from multiple sources interact to control development. Lateral root development is controlled by the antagonistic action of the hormones auxin and abscisic acid (ABA). Auxin stimulates the separation of pericycle initials and cell expansion. Absciscic acid, while required for lateral root initiation and elongation, acts as an antagonist through auxin-dependent pathways. These hormones in turn mediate reactive oxygen species (ROS) accumulation in growing roots, promoting cell expansion. We describe the isolation and characterization of a copper- and auxin-induced gene *COPPER INDUCED in LEAVES (CIL1)* from *Brassica carinata*.

Transgenic *B. carinata* expressing antisense *CIL1* was used to determine the biological function of this gene. Lines with reduced *CIL1* expression showed a decrease in lateral root development, as well as auxin and ABA insensitivity. Steady-state analysis of redox components showed a decrease in NADPH oxidase, superoxide dismutase, and catalase activity with a large increase in hydrogen peroxide concentration. GFP-tagged CIL1 accumulated at the plasma membrane and in the apoplast indicating CIL1 is likely extracellular. From these data we propose that CIL1 is an extracellular protein involved in ROS cycling mediating auxin and ABA signals.

Keywords: Auxin - Absciscic acid - Lateral roots - Reactive oxygen species – *Brassica carinata* – glucosylphosphatidylinositol-anchored protein

Introduction

The hormone auxin regulates a range of developmental pathways during the life of a plant. Perception of auxin is well understood, and causes degradation of AUX/IAA transcriptional repressors by ubiquitination, enabling transcription of auxin-regulated genes (Ruegger et al. 1997; Gray et al. 2001; reviewed in Quint and Gray, 2006). Lateral root initiation involves auxin-stimulated separation of pericycle founder cells followed by seven distinct cell division stages, resulting in emergence of the primordium (Reviewed in Casamiro et al. 2003; Swarup et al. 2008). Separation of pericycle initials occurs after the production of cell-wall remodeling enzymes and is promoted by auxin influx via *LAX3* (*LIKE AUX1*; Swarup et al. 2008). Initiation, emergence, and elongation of lateral roots is believed to involve the action of multiple hormones including auxin (Malamy and Benfey, 1997; Casamiro et al. 2001; Himanen et al. 2002; Dubrovsky et al. 2008), cytokinins (Dasharath et al. 2004; Laplaze et al. 2007), and more recently, abscisic acid (ABA; De Smet et al. 2003; Deak and Malamy, 2007; Guo et al. 2009).

Absciscic acid is a key regulator of root development, stomatal function, and response to abiotic stressors including drought, cold, and heat. Expression of *AUXIN RESPONSE FACTOR 10* (*ARF10*) resistant to negative regulation by microRNA160 caused hypersensitivity to ABA, greatly reducing germination frequency in mutant Arabidopsis seeds grown on the hormone (Liu et al. 2007). Additionally, initiation and elongation of lateral roots requires abscisic acid (ABA) signaling through *LATERAL ROOT DEVELOPMENT 2* (*LRD2*), acting as an antagonist to auxin (Deak and Malamy, 2007). Later work, however demonstrated that ABA-mediated repression of lateral root development can proceed through an auxin-independent pathway using the Arabidopsis catalytic subunit of protein phosphatase 2A (PP2Ac-2; Pernas et al. 2007). These studies point toward both auxin-dependent and -independent ABA signaling pathways operating during development. Although specific genes involved in lateral root development have been identified (Deak and Malamy, 2007; Swarup et al., 2008), the transduction mechanism for auxin and ABA signals are unknown.

Reactive oxygen species (ROS) are oxygen-containing molecules with unpaired electrons (Haliwell, 2006). In plants, they are capable of acting as signaling molecules produced in response to hormones such as ABA (Pei et al. 2000; Zhang et al. 2001a; Mori and Schroeder, 2004; Kwak et al. 2006; Vilela et al. 2007) and auxin (Joo et al. 2001; Foreman et al. 2003; Li et al. 2005; Jones et al. 2007) because of their ability to accept single electrons in a step-wise series of reduction reactions. Interaction of ROS and ABA during control of stomatal aperture function has been well documented. ROS, specifically hydrogen peroxide (H_2O_2), produced downstream of ABA treatment, is able to inhibit K^+ transport, promoting cytosolic Ca^{2+} accumulation in *Arabidopsis* and bean (*Vicia faba*) guard cells, accelerating stomata closure during drought stress (Pei et al. 2000; Zhang et al. 2001b). Though a large body of work examining ROS production in response to ABA has focused on guard cells as a model, limited evidence suggests that H_2O_2 is also involved in mediating the ABA-repressive effect on primary root growth in *Arabidopsis* (Liang et al. 2007).

The effect of ABA-mediated H_2O_2 production during root development contrasts with increasing evidence of auxin-regulated ROS production. Indole-3 acetic acid (IAA) treatment of maturing maize embryos, germinating seeds, or true leaves correlated with an increase in catalase gene expression (Lingqiang and Scandalios, 2002). Auxin-induced growth inhibition of maize roots is mediated through the reduction of superoxide (O_2^-), a precursor of the hydroxyl radical ($OH^\cdot$), causing cell wall loosening and allowing cell expansion to occur (Schopfer, 2001; Liskay et al. 2004). In growing *Arabidopsis* roots, auxin-induced gravitropic responses are promoted by the asymmetrical distribution of ROS through the action of phosphatidylinositol 3-kinase and its putative association with the NADPH oxidase (NOX) enzyme complex (Joo et al., 2005). Demonstration of positive regulation between ABA and auxin signaling with NOX (Foreman et al. 2003; Kwak et al. 2003; Li et al. 2005; Lin et al. 2009) suggests this enzyme complex may represent a point of convergence between the two hormones, and that ROS produced by this complex may be involved in transmitting signals from these hormones during development.

In the course of identifying copper-induced transcripts in *Brassica carinata* (Zheng et al. 2001), we identified *COPPER INDUCED in LEAVES1 (CIL1)*. Here, we show homozygous antisense *cil1* lines exhibit decreased lateral root production and reduced response to abscisic acid (ABA). Since lateral root development and response to ABA involve ROS, we investigated the steady state redox composition of the antisense plants. CIL1 is predicted to be a glucosylphosphatidylinositol (GPI) anchored protein. Expression of *GFP-CIL1* in *Arabidopsis thaliana* demonstrates extracellular protein localization. We hypothesize CIL1 is an extracellular negative regulator of ROS production and lateral root development in *B. carinata*.

Materials and Methods

Plant Material and Growth

Brassica carinata (breeding line C90-1163), received from the Agriculture and Agri-Food Canada Research Station (Saskatoon, Saskatchewan, Canada) was used for these studies. Seed was sown in pots of Terra-Lite Redi-Earth with Nutricote 14-14-14 fertilizer (Chisso-Asahi, Nagasaki, Japan) at an approximate density of two seeds cm⁻². Plants were grown in growth chambers at 50% relative humidity, 21/17°C and 16/8 day and night with a light intensity of 216 $\mu\text{mol m}^{-2} \text{s}^{-1}$. All seedling photographs are representative of at least 3 independent biological replicates consisting of 6 seeds per replicate. Plant photographs are representative of 3 independent biological replicates consisting of 4 plants per replicate.

All root growth assays consisted of three independent biological replicates and were analyzed using two-tailed Student's t-test. To analyze root growth on solid agar media, seeds were surfaced sterilized according to Babic (1993). For all assays, 12 seeds (6 wild type and 6 AC) were sown on 15 cm Petri dishes. Plates were oriented vertically to allow roots to grow on the surface of the media in a Conviron C1114 growth chamber at 22°C,

16 h light, 8 h dark with a light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$. After 8 days, plates were photographed. Primary root length was measured by hand and the total lateral root number was counted. Control medium was composed of ½ Murashige and Skoog basal medium (MS; Sigma-Aldrich, St. Louis, MO, USA, cat. no. M5519). For abscisic acid (ABA) germination assays, control medium was supplemented with $3.5 \mu\text{M}$ ABA (Sigma-Aldrich, cat. no. A1049) dissolved in ethanol. Equivalent volumes of ethanol were added to control medium.

cDNA Library Construction, Screening and Sequence Analysis

The library construction and differential screening procedure were described previously (Zheng et al., 2001). Sequences were analyzed using the Editseq program (DNASTAR Inc., Madison, WI, USA). Sequence comparisons were performed using the Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/BLAST/>.) The predicted cellular targeting and signal peptide cleavage of CIL1 was obtained using TargetP 1.1 at <http://www.cbs.dtu.dk/services/TargetP/>. Examination of CIL1 for potential GPI anchor attachment was done using the BIG-PI plant predictor tool (http://mendel.imp.ac.at/gpi/plant_server.html).

Antisense CIL1 Plant Generation

The full length sequence of the cDNA corresponding to *CIL1* was amplified using a primer designed to add a 5' *Pac* I site (Underlined)

5' – NNN TTAATTAA CAG AAC TCA ACT CAG CCA TGG – 3'

The 3' primer was designed to add a terminal *Spe* I site (Underlined):

5' – NNN ACTAGT ACT AAA ACG ATA AAA CAA ACA – 3'

The amplified *CIL1* product was subcloned into the pCR2.1 TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) and subsequently digested with *Pac* I and *Spe* I restriction enzymes. The excised fragment was then ligated into the pRD400 binary vector (Datla et al., 1992) with a modified multiple cloning site containing *Pac* I and *Spe* I restriction sites directly following the dual 35S promoter system, generating pRDCIL.

After transformation of *Agrobacterium tumefaciens* MP90 with pRDCIL, the construct was used to transform *Brassica carinata* C90-1163 cotyledon petioles according to Babic et al. (1998).

RNA Isolation and Analysis

All gene expression analysis experiments consisted of three independent biological replicates. To minimize variability, all tissue was harvested at the same time of day after the same stage of plant growth had been reached. Two hundred milligrams (fresh weight) of plant material was frozen in liquid N₂, ground with a pestle to a fine powder and extracted with Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's directions. Total RNA was dissolved in DEPC-treated H₂O and stored at -80°C.

For real-time qRT-PCR, the axillary bud (AB) of the third leaf, the third leaf, shoot apex (SA), stem, and all roots were collected from both transgenic and wild type *B. carinata*. All samples were collected at the same time of day from plants that had reached the fourth-leaf stage of growth. qRT-PCR analysis was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) with the following modifications. Prior to the RT reactions, 1.25 µg of total RNA was treated with 2 U of RNase-free DNase I (Amersham, Little Chalfont, Buckinghamshire, UK) for 10 min at 37°C in 1X first-strand buffer (Invitrogen, Carlsbad, CA, USA). Subsequently, each reaction utilized 1X SYBR Green Master Mix containing 50 nM each primers CIL1770F and CIL1770R (*CIL1770F* 5'-TTGGTTATGTTGGCTTCAGT-3'; *CIL1770R* 5'-GAGAGATAGAGATAGGTGGA-3'), or 50 nM *BcActL* and *BcActR* (*BcActL* 5'-ACTACGAGCAGGAGATG-3'; *BcActR* 5'-GAGCACAATGTTACCGT-3'), 0.5 µL QuantiTect RT Mix, 1X fluorescein, and RNase-free water to 48 µL per sample. The RT reactions were completed at 50°C for 30 min then the samples were transferred to a 96-well plate and amplified in an iCycler with an optical module (Bio-Rad, Hercules, CA, USA). Cycling conditions were as follows, an initial denaturation step at 95°C for 5 min followed by 40 cycles of 15 s at 94°C, 30 s at 58°C, and 30 s at 72°C. At the end of the 40 cycles, a melt curve was generated by holding the samples at 58°C for 30 s and

increasing the temperature by 0.5°C every cycle for 74 cycles; the final cycle was at 92°C. The results of the qRT-PCR were analyzed using the $\Delta\Delta C_T$ formula, where “ C_T ” is the threshold cycle at which the DNA being amplified by PCR enters the log stage of replication (Pfaffl, 2001; Livak and Schmittgen, 2001). The standard error calculation was performed according to Vandesompele et al. (2002).

Plasma Membrane Preparation

Approximately 40 g of leaves were harvested from 56-day old *B. carinata* wild type and *cil1* plants. Leaves were homogenized in a Polytron after addition of 4 volumes of 0.5 M sucrose, 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH (pH 7.5), 5 mM ascorbic acid, 1 mM DTT, 0.6% (w/v) polyvinylpyrrolidone, and 1 mM phenylmethanesulphonylfluoride. The homogenate was filtered through 4 layers of cheesecloth followed by centrifugation at 10,000g for 15 min at 4°C. The supernatant was transferred to new tubes and centrifuged at 48,400g for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended in 8 mL of 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate (pH 7.8) by pipetting and vortexing.

Phase separation was completed as described by Sagi et al. (2001) with the following modifications. The entire resuspended pellet was phase separated, then diluted 5-fold with ice-cold Tris-HCl dilution buffer (10 mM, pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 1 mM ascorbate, and 1 mM PMSF. The suspension was centrifuged at 120,000g for 30 min at 4°C. Pellets were then resuspended in Tris-HCl dilution buffer and used for the NOX assay.

Crude Leaf Protein Preparation

The following extraction method was used for assays involving catalase, glutathione reductase, ascorbate peroxidase, superoxide dismutase, and ascorbate peroxidase: Leaves were harvested from 56-day old *B. carinata* plants. One gram (fresh weight) leaf pieces were homogenized in 10 mL of 50 mM potassium-phosphate, 1 mM EDTA (pH 7.0), 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 15,000g for 20 min at 4°C and the supernatant used in assays.

Enzymatic Assays

All assays were performed with three independent biological replicates. A two-tailed Student's t-test was used to statistically analyze the data.

NADPH oxidase activity of plasma membranes

NADPH oxidase activity was assayed in membrane preparations by a modified assay based on the reduction of sodium 3' [1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) by O_2^- radicals (Sagi and Fluhr, 2001), with modifications. The assay reaction mixture contained 1 mL 50 mM Tris-HCl (pH 7.5), 0.5 mM XTT, 100 μ M NADPH, and 15-20 μ g membrane protein preparation. The reaction was initiated by the addition of NADPH and XTT reduction was followed at 470 nm for 5 min. Background corrections were performed with 50 U SOD and the rate of O_2^- generation was calculated using an extinction coefficient of $2.16 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Catalase

Catalase activity was determined by assaying the decomposition of H_2O_2 at 240 nm (Aebi, 1984), with modifications. The assay mixture was composed of 10 μ L protein extract, 30 mM H_2O_2 and a final volume of 1 mL. To initiate the assay, H_2O_2 was added and mixed well with a plastic paddle. The decrease in absorbance was then followed at 300 nm for 30 sec.

Glutathione Reductase

Glutathione reductase activity was determined by following the oxidation of NADPH at 340 nm (Schaedle and Bassham, 1977) with modifications. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.15 mM NADPH, 0.5 mM GSSG, and 3 mM $MgCl_2$. The reaction was initiated by the addition of 12.5 μ g crude protein and the oxidation of NADPH was followed at 340 nm for 5 min. An extinction coefficient of β -NADPH of 6.22 mM at 340 nm was used.

Ascorbate peroxidase

Ascorbate peroxidase activity was determined by measuring the decrease in absorbance at 290 nm (an absorbance coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) as ascorbate was oxidized (Nakano and Asada, 1981), with modifications. The reaction mixture consisted of 50 mM potassium-phosphate, 0.1 mM EDTA (pH 7.0), 0.5 mM ascorbate, and 0.1 mM H_2O_2 . The reaction was initiated with the addition of 0.3 mg protein and the absorbance decrease was recorded from 10-30 s after initiation.

Superoxide Dismutase

The supernatant was used in a modified assay based on Giannopolitis and Ries (1977). The reaction volume was 3 mL and contained 1.3 μM riboflavin, 13 mM methionine, 63 μM NBT, 0.05 M sodium carbonate, pH 10.2, and 0-100 μl protein extract. The reactions were performed in disposable cuvettes with 75 μL of extract. The cuvettes were placed in an aluminum foil-lined box and the reaction was initiated by illumination for 15 min with a Phillips F15T08/CW fluorescent bulb. The reaction was terminated by removing the light source. The rate of the reaction was determined by the increase in absorbance at 560 nm over 5 min.

Ascorbate Oxidase

Ascorbate oxidase activity was measured by measuring the oxidation rate of ascorbate at 265 nm (de Pinto et al., 2004).

Determination of Redox Constituents

Leaf O_2^- production

Determination of O_2^- production was completed according to Able et al. (1998). Leaves were harvested from 41-day old *B. carinata* plants and 1 g (fresh weight) of leaf pieces were homogenized in 5 ml of 50 mM Tris-HCL (pH 7.5) and centrifuged at 5,000g for 10 min. The reaction mixture contained 50 μ g of leaf supernatant protein, 0.5 mM XTT, and 50 mM Tris-HCL (pH 7.5) to 1 mL. The reduction of XTT was followed at 470 nm for 5 min. Corrections for background were performed with 50 units SOD. Rates of O_2^- generation were calculated using an extinction coefficient of $2.16 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

H₂O₂ Content

Hydrogen peroxide levels were determined according to Shi et al. (2005), with slight modifications. Leaves were harvested from 41-day old *B. carinata* plants and 0.5 g (fresh weight) of leaf pieces were homogenized in liquid nitrogen. After homogenization 5 mL 0.1% (w/v) trichloroacetic acid was added. The homogenate was centrifuged at 12,000g for 15 min followed by combining 0.5 mL of supernatant with 0.5 mL K-phosphate buffer (pH 7.0) and 1 mL 1 M KI. The content of H_2O_2 was calculated by measuring the absorbance at 390 nm based on a standard curve.

Glutathione Content

Leaves of 41-day old *B. carinata* plants were harvested and 1 g of leaf pieces was homogenized according to DeRidder et al. (2002) for the preparation of crude extract to use for the spectrophotometric detection of glutathione and glutathione disulfide (Griffith, 1980).

Ascorbic Acid Content

Leaves were collected from 56-day old *B. carinata* and flash-frozen in liquid N_2 . Frozen leaf material (0.5 g) was prepared according to Kampfenkel et al. (1995), with modifications. To the homogenized material 10 mL 5% trichloroacetic acid was added and ground into the powdered leaf material. The homogenate was centrifuged at 15,600g

for 5 min at 4°C and the supernatant was colourimetrically assayed for ascorbate (Arakawa et al., 1981).

NAD⁺, NADH, NADP⁺, and NADPH Content

Leaf pieces (1 g) of 56-day old *B. carinata* was ground in liquid nitrogen and were extracted and assayed according to Queval et al. (2007) with slight modifications. For NAD⁺ and NADH, alcohol dehydrogenase (ADH) was freshly dissolved in 0.1 M HEPES and 2 mM EDTA (pH 7.5) to a concentration of 2,500 U mL⁻¹. To a cuvette containing 100 µL neutralized supernatant, 0.5 mL of 0.1 M HEPES, 2 mM EDTA (pH 7.5), 100 µL 1.2 mM DCPIP, 50 µL of 20 mM PMS, 125 µL water, and 50 µL ADH. The reaction was initiated by the addition of 75 µL absolute ethanol. After shaking, the decrease in A₆₀₀ was recorded for 5 min. Contents were calculated by reference to a constructed standard curve. To assay NADP⁺ and NADPH glucose 6-phosphate dehydrogenase (G6PDH) was freshly prepared by centrifugation of a (NH₄)₂SO₄ suspension and resuspension of the pellet to 200 U mL⁻¹ in 0.1 M HEPES, 2 mM EDTA (pH 7.5). To a cuvette containing 150 µL neutralized supernatant, 0.5 mL 0.1 M HEPES, 2 mM EDTA (pH 7.5), 100 µL 1.2 mM DCPIP, 50 µL of 20 mM PMS, 50 µL glucose 6-phosphate, and 150 µL of water were added. The reaction was initiated with the addition of 50 µL G6PDH. After shaking, the decrease in A₆₀₀ was recorded for 5 min and contents were calculated by reference to a constructed standard curve.

Isolation of CIL1 and generation of plasmid pCR8-CIL

We amplified the full-length intron-less open reading frame (ORF) of *CIL1* using *B. carinata* genomic DNA and primers designed to add a consensus translational initiation sequence (Kozak, 1984) to the 5' end of the amplified fragment. All primers were designed manually using the Lasergene software suite (DNASTar, Inc., Madison, WI, USA) and synthesized by the Gene Synthesis facility at the Plant Biotechnology Institute

(Saskatoon, SK, USA). The sense primer introduced a consensus translational initiation sequence (underlined):

5'-CACC GCA ATG GCT TCA AAC GCT TCT CTC-3'

The 3'-antisense primer did not modify the *CIL1* ORF sequence:

5'-TCA GAA TAT GAA AAC TGA AGC-3'

The amplified *CIL1* fragment was subcloned into *pCR8/GW-TOPO* (Invitrogen, Carlsbad, CA, USA) to generate pCR8-CIL.

CIL1 subcloning and plasmid generation

We amplified the modified emerald GFP sequence using plasmid pPT157 (Teerawanichpan et al., 2007) and primers designed to introduce *Hpa I* and *Stu I* restriction sites to the amplified fragment via PCR amplification. The 5' sense primer containing the *Hpa I* site (underlined) was:

5'-TTT GTT AAC GTG AGC AAG GGC GAG GAG CTG-3'

The 3'-antisense primer contained the *Stu I* site (underlined):

5'-TTT AGG CCT CTT GTA CAG CTC GTC CAT GCC-3'

The amplified *GFP* ORF was immediately cloned into the pCR2.1 TOPO-TA cloning vector (Invitrogen, Carlsbad, CA, USA).

pCR8-CIL was digested with *Oli I* (Fermentas, Burlington, ON, CAN) cleaving the *CIL1* ORF in-frame 1 aa downstream of the predicted site of the N-terminal signal sequence as predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). Next, *pCR-GFP* was digested with *Hpa I* and *Stu I* to excise *GFP* from the *pCR2.1* vector. The *GFP* fragment was then ligated with the linearized *pCR8-CIL* plasmid resulting in the cassette shown in Figure 4. The fusion cassette was then recombined with pER310, an *Agrobacterium tumefaciens* binary vector modified as a Gateway destination vector, pK7WG2 (E. Risseuw and R. Datla, unpublished). This resulted in an expression cassette of

“CaMV35S promoter – CIL1 N-signal – GFP – CIL1 ORF – CaMV35S terminator”. The resulting *A. tumefaciens* cells were used to stably transform *A. thaliana* Landsberg *erecta* wild type plants by floral dipping (Clough and Bent, 1998).

perGFP

The modified emerald GFP, pPT157 (Teerawanichpan et al., 2007) in the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) was Gateway recombined with the previously described pER310 binary vector. The resulting *A. tumefaciens* cells were used to stably transform *A. thaliana* Landsberg *erecta* wild type plants as described.

Microscopy

To visualize the CIL1 fusion protein and GFP in stably transformed *A. thaliana* roots, fluorescence microscopy was performed using a Carl Zeiss Meta510 laser-scanning confocal microscope. Six independent lines were examined for fluorescence localization. GFP was detected using an argon laser a 488 nm excitation filter and an emission range of 505-530 nm. The red fluorescent dye propidium iodide was detected using a HeNe laser with an excitation of 543 nm and an emission of 617 nm. Data were captured by the Carl Zeiss LSM 510 software. For some experiments, roots were plasmolysed with 0.8 M sorbitol or 2% NaCl for 5 min immediately prior to imaging.

Results

We report on *CIL1* (*COPPER INDUCED in LEAVES 1*), isolated from a *Brassica carinata* cDNA library generated from copper-treated leaves (Zheng et al., 2001). The full-length cDNA representing the *CIL1* gene is 1030 bp with an open reading frame of 810 bp (Genbank accession EF493830). Comparison of the *CIL1* derived amino acid sequence to database entries revealed similarity (69% identity) to a protein from *Arabidopsis thaliana* designated AIR12 (*AUXIN INDUCED in ROOTS 12*).

Homozygous single copy antisense *CIL1* lines (determined by Southern blot analysis; Data not shown) were generated in *B. carinata*. Expression of *CIL1* in antisense and wild type plants was determined using real-time quantitative RT-PCR and the $\Delta\Delta C^T$ method (Livak and Schmittgen, 2001). In wild type *B. carinata* the vegetative organs with the highest and lowest *CIL1* expression were the 3rd leaves and axillary buds, respectively (Fig. 1a). The greatest reduction of *CIL1* expression occurred in the whole root of *cil1 B. carinata* antisense line 6 (AC), where it is 10% of wild type (Fig. 1b). AC line 6 showed the greatest and most consistent suppression of *CIL1* transcript and was chosen for further analysis.

Lateral root production and length is decreased in antisense *cil1 B. carinata*

Wild type and AC seeds were surface-sterilized and incubated on ½ MS plates oriented vertically for 8 days. At this time the lateral root number was counted and lengths of the primary root and all lateral roots were measured. AC seedlings produced 50% fewer lateral roots than wild type (Fig. 2a) but show no difference in primary root length (Fig. 2b). Lateral root elongation is controlled by antagonistic action between the inhibitory and permissive effects of abscisic acid (ABA) and auxin, respectively (Deak and Malamy, 2005) and application of copper chloride, an abiotic stressor correlates with expression increases of ABA response genes in rice (Sudo et al. 2008). Since auxin and ABA act antagonistically during lateral root development we examined the effects of auxin and ABA on root elongation of *B. carinata* seedlings. When germinated on medium containing ½ MS, 400 nM α -NAA, wild type seedlings develop a shorter primary root and produce additional lateral roots compared to control medium (Fig. 2c). While AC seedlings show proliferative lateral root development, unlike wild type seedlings, they retain primary root identity. When germinated on a medium containing ½ MS and 3.5 μ M ABA, wild type seedlings germinate, but have reduced primary root elongation while AC seedlings appear to be insensitive to ABA and elongate (Figs. 2d, e). On media containing both 400 nM α -NAA and 3.5 μ M ABA germination of wild type seedlings is completely abolished while AC seedlings are able to germinate and develop a

primary root that is further reduced compared to when ABA alone is added to the medium (Fig. 2f).

Antisense *CIL1* *B. carinata* exhibit altered steady state ROS levels

Since ROS accumulate in response to auxin and AC plants in the vegetative stage show a phenotype indicative of mild decreased apical dominance and increased branching during reproductive growth (Figure 4) we were interested in inferring the redox state of AC plants. Leaves were harvested from 56-day old *B. carinata* plants, homogenized and used in assays to determine activity of 8 enzymes involved in the generation and turnover of ROS: NADPH oxidase (NOX), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbate oxidase (AOX), dehydroascorbate reductase (DHAR), and glutathione peroxidase (GPX). Enzyme activity in *cil1* plants was normalized setting wild type activity at 100%. Of the enzymes examined, CAT, SOD, and NOX activity was greater in AC and GR activity was reduced (Fig. 4a). We determined abundance of constituents of the ROS generation/detoxification pathway: superoxide, hydrogen peroxide, oxidized/reduced glutathione, ascorbate, dehydroascorbate, NADP⁺, NADPH, NAD⁺, and NADH. Reductions in superoxide and hydrogen peroxide concentration were detected in AC, while the concentration of ascorbate was increased compared to wild type *B. carinata* (Fig. 4b). Reduction in GR activity did not correlate with a decrease in total (oxidized and reduced) glutathione, however the method used could not differentiate between the two forms of glutathione. Increased SOD activity corresponds with a decrease in superoxide concentration. However, the increase in catalase activity does not result in a similar decrease in hydrogen peroxide concentration, raising the possibility that increased catalase activity is a consequence of elevated H₂O₂.

***CIL1* is a glucosylphosphatidylinositol anchored protein present at both the plasma membrane and the apoplast**

Analysis of the deduced CIL1 amino acid sequence using the BIG-PI tool (Eisenhaber et al. 2003) predicted both N- and C-terminal hydrophobic signal domains, and a site for GPI anchor attachment. Proteins containing a glucosylphosphatidylinositol (GPI) anchor are extrinsic membrane proteins, post-translationally modified to attach a GPI anchor to the C-terminal end (Elortza et al. 2006). This anchor targets proteins to the extracellular leaflet of the plasma membrane in eukaryotic cells. To investigate the subcellular localization of CIL1 in greater detail, emerald GFP (Teerawanichpan et al. 2007) was sandwiched between the N- terminal hydrophobic domain and the rest of the CIL1 ORF (Fig. 5a). The construct design was similar to the method used by Sun et al (2004) to examine localization of a tomato arabinogalactan GPI-AP and was placed under the control of the cauliflower mosaic virus 35S promoter. *p35S::GFP* was used as a control. Stably transformed *A. thaliana* root and leaf cells immersed in water show GFP distributed throughout the nucleus and cytoplasm (Fig. 5b) while GFP-CIL1 localizes to the cell margins (Fig. 5c). Upon plasmolysis in 800 mM sorbitol, green fluorescence is present at the plasma membrane and in the apoplast and is clearly separate from the red fluorescence from the cell wall-specific propidium iodide stain (Fig. 5d), indicating CIL1 release from the plasma membrane. When plasmolyzed with a salt concentration of 2% GFP-CIL1 localization is evident in hechtian strands (Sun et al. 2004; Sardar et al. 2006) along the membranes of cells (Figs 5e, f).

Discussion

CIL1 is a GPI-anchored protein involved in ABA response during seed germination, development of lateral roots, and production or scavenging of ROS. Previously, CIL1 was classified as a *B. carinata* ortholog of *A. thaliana*, AIR12 (Preger et al. 2009). However, the copper treatment used during the isolation of *CIL1* suggests that genes from this small family are not only induced by auxin, but by copper as well. *CIL1* transcript is not only present in the root; it is found in the stem, leaves, axillary buds, and at the shoot apex.

The *Glycine max* orthologue of CIL1 was identified as a cytochrome *b561* isoform that binds a single heme *in vitro* and was postulated to interact with members of the plasma membrane redox system (Preger et al. 2009; Iyer et al. 2008; Aravind, 2001). In mammals, cytochrome *b561* is involved with ascorbate regeneration across membranes (Su et al. 2006) during the conversion of dopamine to norepinephrine in the chromaffin granules of adrenal glands (Aravind, 2001). Steady state analysis of *cil1* redox enzymes and molecules show a decrease in $O_2^{\cdot -}$ and H_2O_2 concentration accompanied by an increase in Asc, SOD, and CAT activity in leaves. This could represent an overall increase in redox detoxification. Alternatively, the loss of the cytochrome *b561* CIL1 may prevent proper extracellular electron cycling, leaving a larger pool of Asc and preventing developmental responses that rely on extracellular redox cycling from occurring. GFP-tagged CIL1 localizes to the plasma membrane and in plasmolyzed cells is present in the apoplast. GPI-anchored proteins are capable of being cleaved from the GPI anchor inserted in the plasma membrane by phospholipase C (Borner et al. 2003; Eisenhaber et al. 2003). This work represents the first *in planta* localization of this class of GPI-anchored proteins and the first description of a phenotype associated with the cytochrome *b561* family.

Reactive oxygen species are integral for signal transmission in response to developmental and abiotic stressor signals. Accumulation of ROS likely occurs at multiple stages of root development, initially in response to transient cytosolic calcium increases as a result of mechanical stimulus applied to a growing root tip (Monshausen et al., 2009); and later in response to auxin during cell elongation and lateral root initiation (Joo et al., 2005). The effect of ABA on root-specific ROS accumulation in response to developmental or abiotic stress signals is not well described. Based on evidence from NOX-derived ROS production during ABA-mediated guard cell closure (Li et al., 2006), the antagonistic action of ABA and auxin in lateral root development (De Smet et al., 2003), and the reduced ABA response in AC plants it is likely there is a redox-dependent mechanism involving NOX, to coordinate root development. Plants NOXs are enzyme complexes

composed of respiratory burst oxidative homologue (Rboh) proteins (Foreman et al. 2003; Sagi et al. 2004; Monshausen et al. 2007; Chung et al. 2008). NOXs contribute to the rapid rise of ROS activated by the response to biotic and abiotic stressors (Bolwell et al. 1998, Grant et al. 2000; Torres and Dangl, 2005; Mazel et al. 2004; Einset et al. 2007) and in response to plant hormones (Rodriguez et al. 2002; Schopfer et al. 2002; Kwak et al. 2003; Li et al. 2006). Plant NOXs are plasma membrane bound complexes that bind NADPH and use the iron core to reduce extracellular oxygen to superoxide (Torres et al. 1998; Vignais et al. 2002; Lambeth, 2004). Tomato (*Lycopersicon esculentum*) *rboh* plants demonstrated a reduction in H₂O₂, a highly branched phenotype, fasciated reproductive organs, and seedling insensitivity to ABA (Sagi et al. 2004). AC plants share many of these phenotypes, including increases in branching, however CIL1 does not have any significant homology to any known RBOH. Based on our observations, we hypothesize CIL1 acts extracellularly during the early stages of ROS production.

The reduction in lateral root number in AC plants is likely mediated through auxin and ROS signaling. During lateral root initiation auxin originating from the pericycle induces expression of genes encoding cell wall remodeling enzymes in adjacent endodermal cells, causing cell separation (Swarup et al. 2008). Non-enzymatic cell wall loosening occurs when OH[•] production increases following perception of the auxin signal in growing root primordia, cleaving cell wall polysaccharides allowing expansion to occur (Schopfer, 2001; Liskay et al. 2004). Extracellular OH[•] is produced by Fe²⁺/Cu⁺-mediated H₂O₂ reduction in Fenton-type reactions (Halliwell, 2006). The enhanced catalase activity of *cil1* plants may represent an increased rate of H₂O₂ turnover leaving a smaller pool of H₂O₂ available to react with Fe²⁺/Cu⁺, decreasing lateral root growth. Actively growing regions of roots such as root tips and initiating lateral roots show high concentrations of ROS (Monshausen et al. 2007; Dunand and Penel, 2007). A decrease in O₂^{•-} concentration as seen in *cil1* plants may contribute to a reduced rate of lateral root initiation or elongation by perturbing cell expansion.

The response of AC plants to ABA during germination and early root development may be the result of reduced steady state ROS production. A delicate balance of ROS exists in cells: a high concentration of ROS or depleted antioxidants results in oxidative stress and cell death, while a low concentration of ROS causes the molecules to act as molecular signals (Scandalios 2005; Halliwell, 2006). Many auxin and ABA events require $O_2^{\cdot-}$ and H_2O_2 , and small changes in the concentrations of these molecules may perturb cellular processes. NADPH oxidase is a required component of both auxin and ABA signaling (Foreman et al., 2003; Sagi et al. 2004; Li et al. 2006) and may represent a convergent point for both pathways during root development. Auxin and ABA signaling both require MAP kinase activity and downstream ABA signaling requires additional H_2O_2 generated through NOX (Strader et al. 2008; Lin et al. 2009). During abiotic stress, ABA signals are thought to cause an increase of phospholipase C, a protein capable of cleaving the anchor of GPI-APs, releasing them from the plasma membrane (Hirayama et al. 1995; Kopka et al. 1998; Cousson, 2009). It is possible that CIL1 is only active while anchored to the membrane, and that cleavage in response to abiotic stress reduces the hypothesized electron transfer activity, leaving a larger pool of electrons for NOX to utilize to resist the stressor (Chung et al. 2008; Fig. 6). CIL1 represents a member of a novel component of both auxin and ABA signaling that intersects with plasma membrane redox proteins or possibly apoplastic proteins during lateral root growth in *B. carinata*. This presents an intriguing phenomenon where a dual membrane-apoplastic protein affects both development and environmental responses via ROS production, either separate, or in conjunction with NADPH oxidases.

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Figure Legend

Fig 1 Expression profile of *CIL1* in 31-day old *Brassica carinata* organs. For all graphs, n=3, error bars represent SE. leaf, 3rd leaf; root, whole root; apex, shoot apex; AxB, 3rd axillary bud; stem, primary stem. **a** Real-time quantitative RT-PCR analysis of *CIL1*

expression in wild type organs. *B. carinata* actin was used as the constitutively expressed reference gene. Threshold cycles were analyzed using the GeneX Microsoft Excel macro supplied by Bio-Rad and normalized to root expression. **b** Real-time quantitative RT-PCR analysis of *CIL1* expression in *cil1* antisense plants normalized to the equivalent wild type organ

Fig 2 Altered root architecture of antisense *cil1* (AC) *Brassica carinata* plants versus wild type (WT) and response to auxin and abscisic acid (ABA). For all graphs, $n=3$, bars represent SE. $*P < 0.05$, $**P < 0.005$, as compared to wild type plants using a two-tailed Student's t-test. All plants were grown vertically for the indicated time. All photographs are representative images of at least 15 plants. **a** Mean lateral root number of 8-day old *B. carinata* seedlings grown on half strength MS medium. **b** Primary root of seedlings grown for 8 days on half strength MS medium. **c** Primary and lateral roots of seedlings grown for 13 days on half strength MS supplemented with 400 nM α -NAA. **d** Seedlings grown half strength MS supplemented with 3.5 μ M $\pm$ ABA for 13 days, scale bars represent 1 cm. **e** Primary root length of seedlings grown for 13 days on medium containing half strength MS supplemented with 3.5 μ M $\pm$ ABA. **f** Germination of seedlings after 13 days on media containing 400 nM α -NAA and 3.5 μ M $\pm$ ABA

Fig 3 Development of wild type (WT) and antisense *cil1* (AC) plants. **a** Vegetative development of 38 day-old AC plants demonstrating decreased apical dominance. Leaf proliferation (numbers) at the 3rd axillary bud (arrow) indicated. **b** Vegetative development of 38 day-old WT plants demonstrating apical dominance at the 3rd axillary bud (arrow). **c** Reproductive shoot development of 4-month old *Brassica carinata* plants

Fig 4 Redox state of 46 day old *Brassica carinata* plants. **a** Steady-state activity of enzymes involved in generation and elimination/turnover of ROS. NOX: NADPH oxidase; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; GR: glutathione reductase; AOX: ascorbate oxidase; DHAR: dehydroascorbate reductase; GPX: glutathione peroxidase. **b** Relative steady-state abundance of molecules involved in generation and elimination of reactive oxygen species (ROS). GSH+GSSG: oxidized and reduced glutathione; ASC: ascorbate; DHASC: dehydroascorbate. For all graphs: bars indicate SE, $n=3$. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, as compared to wild type plants using a two-tailed Student's t-test

Fig 5 Domain structure and subcellular localization of *p35S::GFP-CIL1* fusion constructs in stably transformed Arabidopsis. **a** Structure of *GFP-CIL1* fusion construct used to transform Arabidopsis. The construct consists of the N-terminal CIL1 signal peptide, an inserted emerald GFP domain, the CIL1 open reading frame, and a C-terminal GPI anchor attachment domain. The construct is under the control of the cauliflower mosaic virus CaMV35S promoter. **b** Detection of GFP fluorescence using confocal microscopy in cells expressing *p35S::GFP*. **c-e** Detection of GFP fluorescence using confocal microscopy in *A. thaliana* cells expressing *p35S::GFP-CIL1* in water **c**, after plasmolysis with 0.8 M sorbitol and staining with 2 mg/mL propidium iodide (PI) for 2 min (**d**), or after plasmolysis with 2 % NaCl for 2 minutes (**e**, **f**) Red fluorescence indicates PI

localization in the cell wall (d). Circled region in (e) represents the area examined in (f), where Hechtian strands are clearly visible. PM, plasma membrane; CW, cell wall

Fig 6 Proposed auxin and abscisic acid regulation of extracellular redox enzymes mediated by CIL1. ABA, abscisic acid; Pi-PLC, phospholipase C; NOX, NADPH oxidase; SOD, superoxide; CAT, catalase