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Isolation and characterization of formaldehyde-responsive genes from golden pothos (*Epipremnum aureum*)

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Abstract Plants absorb and metabolize formaldehyde, a C-1 compound that is one of the main indoor air pollutants. To elucidate the molecular mechanism of formaldehyde metabolism in plants, we isolated formaldehyde-responsive genes from golden pothos by means of GeneFishing PCR. We focused on the immediate-early response genes following formaldehyde treatment. Two full length cDNA sequences corresponding to a putative class II chitinase and a hypothetical novel protein, which we termed DEG2, were generated by rapid amplification of cDNA ends (RACE). The chitinase, which we designated *EaCHI1*, was up-regulated in the leaves and stems, whereas *DEG2* was up-regulated in the leaves and roots of formaldehyde-treated golden pothos. Phylogenetic analysis showed that putative class II chitinases were split into two groups, monocot and dicot. EaCHI1 belonged to the former group, occupying the most basal level among the monocot chitinases analyzed. The identification of chitinase as a formaldehyde-responsive gene suggests a novel physiological role for this enzyme in plant carbon metabolism and environmental responses. The DEG2 sequence was not similar to any known protein sequence.

Key words: Chitinase, formaldehyde, GeneFishing, golden pothos, RACE.

Formaldehyde is one of the main indoor air pollutants, present in tobacco smoke, furniture, industrial adhesives, and varnishes. It has been classified as a mutagen and suspected carcinogen (Wippermann et al. 1999). Foliage plants, including golden pothos (*Epipremnum aureum*), can reportedly purify the air of various atmospheric chemicals, including formaldehyde (Wolverton et al. 1984). Genetic engineering to improve the ability of indoor foliage plants to detoxify such chemicals would provide a convenient way to purify indoor air. Exogenous formaldehyde can be incorporated into the metabolism of photosynthetic cells and be used as a carbon source (Achkor et al. 2003). Biochemical and genetic studies in several eukaryotes indicate that the main enzyme responsible for the metabolism of formaldehyde is the glutathione-dependent formaldehyde dehydrogenase (FALDH). This enzyme is universally present in animal and plant tissues and catalyzes the NAD-dependent formation of *S*-formylglutathione from *S*-hydroxymethylglutathione, which forms spontaneously from formaldehyde and glutathione (Uotila and Koivusalo 1989). *S*-formylglutathione is then hydrolyzed to formate and glutathione by *S*-formylglutathione hydrolase. Formate can be oxidized to CO_2 by formate dehydrogenase (Cossins 1964). The genes for formaldehyde dehydrogenase in *Arabidopsis* and rice have been isolated (Dolferus et al. 1997). Enhanced formaldehyde detoxification through the over-expression of glutathione-dependent formaldehyde dehydrogenase in *Arabidopsis* has also been reported (Achkor et al. 2003).

To date, there have been few reports on the effects of formaldehyde on gene expression in plants, although a formate dehydrogenase gene in *Arabidopsis* has been reported to be responsive to formaldehyde (Fukusaki et al. 2000). To investigate the effect of formaldehyde on gene expression in plants, we searched for formaldehyde-responsive genes in golden pothos using differential screening, and identified two genes. One of them encoded a putative class II chitinase, and the other one was comprised of an unknown open reading frame. Recently, chitinase has been implicated in both the plant defense response and the response to abiotic stresses such as cold, freezing, heat, salt, drought and strong light (Kwon et al. 2007; Takenaka et al. 2009). Here, we report the identification of chitinase and another gene encoding a hypothetical protein differentially expressed in golden pothos during the early response to

Abbreviations: ACP, annealing control primer; RACE, rapid amplification of cDNA ends; RT, Real-time quantitative reverse transcriptase
This article can be found at http://www.jspcmb.jp/

formaldehyde treatment.

Materials and methods

Formaldehyde treatment

A monocot plant, golden pothos (*Epipremnum aureum*), was cultivated in pots of soil in a growth chamber at 25ºC under 12 h light/12 h dark conditions. Vines with five to six leaves were cut from potted plants and cultured in 1000-fold diluted "hyponex" (HyponexJapan, Tokyo, Japan) for two to three weeks before formaldehyde treatment. For formaldehyde treatment, pothos vines with roots were put into a small airtight chamber (27×26×37 cm) containing 5 µl of a 37% formaldehyde solution in a sample tube, into which a strip of filter paper was placed to facilitate evaporation. As controls, an empty chamber and a chamber with water were also supplemented with formaldehyde. Changes in the formaldehyde concentration in all three chambers were measured. Air in the chamber was intermittently circulated by a propeller driven by a battery-powered motor. The formaldehyde concentration was measured with a gas detector tube (Komyo, Kanagawa, Japan). The leaves, stems and roots of formaldehyde-treated plants were sampled at time zero, and at 1, 3, 6 and 24 h after exposure to formaldehyde.

RNA isolation and First-strand cDNA synthesis for GeneFishing

Leaves of golden pothos treated with or without formaldehyde for 1 h were harvested. Total RNA was isolated from the leaf samples using an RNeasy Plant Mini kit (QIAGEN, Tokyo Japan). Total RNA was used as a template for the synthesis of first-strand cDNA. Reverse transcription was carried out for 1.5 h at 42ºC in a final reaction volume of 20 µl. Reactions contained 3 µg of purified total RNA, 4 µl of 5× reaction buffer (Promega, Madison, WI, USA), 5 µl of dNTPs (each 2 mM), 2 µl of 10 µM dT-ACP1 (5′-CTGTGAATGCTGCGA-CTACGAT$_{18}$-3′), 0.5 µl of RNasin® RNase Inhibitor (40 U µl^{-1}; Promega, Tokyo, Japan), and 1 µl of Moloney murine leukemia virus reverse transcriptase (200 U µl^{-1}; Promega). First-strand cDNA was diluted by the addition of 80 µl of ultra-purified water prior to GeneFishing PCR.

Annealing control primer (ACP)-based PCR (GeneFishing)

To identify putative formaldehyde-responsive genes, we employed an ACP-based PCR method (Kim et al. 2004) using a GeneFishing DEG kit (Seegene, Seoul, Korea). Briefly, second-strand cDNA synthesis was carried out at 50ºC in one cycle of first-stage PCR in a final reaction volume of 20 µl containing 3–5 µl (approximately 50 ng) of diluted first-strand cDNA, 1 µl of dT-ACP2 (10 µM), 1 µl of 10 µM arbitrary ACP, and 10 µl of 2× Master Mix (Seegene). The protocol for second-strand synthesis was one cycle of 94ºC for 1 min, followed by 50ºC for 3 min, and then 72ºC for 1 min. After second-strand DNA synthesis, second-stage PCR amplification was carried out for 40 cycles of 94ºC for 40 s, followed by 65ºC for 40 s, 72ºC for 40 s, and then a 5 min final extension at 72ºC. Amplified PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Bands of interest were extracted from the agarose gel using a QIAEXII Gel Extraction kit (QIAGEN) and then cloned into a pCR2.1 vector (Invitrogen, Tokyo, Japan). Clones were sequenced using an ABI3100 sequencer (Applied Bio Systems, Tokyo, Japan).

RACE

5′-RACE of the cloned sequences was carried out using a Marathon cDNA amplification kit (Clontech, CA, USA), according to the manufacturer's protocol. The following 5′-RACE primers for DEG2 and DEG3, respectively, were designed based on the sequences of the initial clones: DEG2R1, 5′-CACTTCTACTGCTGTCACACGCACA-C-3′; DEG3R1, 5′-GCTTTCAGCTTCAACTTGTGCTGTAC-3′. Amplified fragments were cloned into pCR2.1 and sequenced. For additional RACE PCR to confirm the full length sequence of DEG2, the following DEG2-specific primers were designed: DEG2R2, 5′-TCTGGAGAGATGCTC-CATGGTCGTAGATG-3′; DEG2R3, 5′-CGGTTGGCGTTGA-GTGATCAGTCTAG-3′; and DEG2R4, 5′-TAAGCAAAACC-AGTGCAGTGGCAGTCG-3′.

Sequence analysis

Nucleotide and amino acid sequences were analyzed using Genetyx version 9 software (Genetyx, Tokyo, Japan). Phylogenetic analysis of the amino acid sequences of EaCHI1, the stress related chitinases AtCTL1 (At1g05850), AtchiA (At5g24090), AtchiB (At3g12500) and AtchiV (At3g54420) from *Arabidopsis thaliana*, and putative chitinase proteins from *A. thaliana* (At3g16920), *Brassica rapa* (EU186371), *Ricinus communis* (XM_002515618), *Pyrus pyrifolia* (FJ589784), *Sorghum bicolor* (XM_002460419), *Zea mays* (EU959576), *Oryza sativa* (EU605859), *Hordeum vulgare* (AK249826), and *Triticum aestivum* (AK331884) was performed using the neighbor-joining (NJ) method of Genetyx. Prediction of the subcellular localization sites of proteins was performed using WoLF PSORT (http://wolfpsort.org/). Prediction of signal peptide was also performed using Genetyx and SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

Real-time quantitative reverse transcriptase (RT)-PCR

Total RNA was isolated from leaves, stem and roots of golden pothos using an RNeasy Plant Mini kit. Single strand cDNA was synthesized from total RNA using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) and a LightCycler (Roche Diagnostics, Basel, Switzerland). The primers used for RT-PCR were as follows; EaCHI1F, 5′-CCTCGACTTGATGGGAGTTGG-3′; EaCHI1R, 5′-TATTG-GCGGCTTAGGAGGTTG-3′; DEG2F, 5′-ACGCCAACCGC-AACGTCATC-3′; DEG2R, 5′-CAGCACTCCTTGGGCAAG-CA-3′. A partial *actin* sequence from golden pothos (accession no. AB539724) was amplified as an internal control using PCR primers based on six conserved *actin* sequences from rice (AY212324), *Arabidopsis thaliana ACT2* and *ACT8* (NM_112764 and NM_103814), pea (PSU81047), *Hordeum vulgare* (AY145451), and *Elaeis guineensis* (AY550991). The sequences of the primers were as follows: 5′-ATCTGGCATC-ACACTTTCTAC-3′ and 5′-TCCACATCTGTTGGAAAGTG-C-3′. The primers for real-time quantitative RT-PCR of golden

pothos *actin* gene were as follows; actinF, 5′-CCAAGACCAG-CTCATCTGTG-3′; actinR, 5′-GATGGCTGGAACAGAACC-TC-3′.

Results

Formaldehyde treatment

Changes in the formaldehyde concentration in the chambers respectively containing the hydrocultured golden pothos and water along with the empty chamber, were measured. As the formaldehyde gradually vaporized, the formaldehyde concentration peaked (at approximately 12.5 ppm) 1 h after the application and then gradually decreased under all three conditions, most rapidly in the chamber containing the golden pothos, followed by the chamber with water, and then by the empty chamber. The concentration of formaldehyde in the chamber with golden pothos was undetectable after 96 h, but was still greater than 1 ppm in the chamber containing water and in the control chamber. These results indicated that golden pothos absorbs formaldehyde, and suggested that physiological changes had occurred in the plants. The results also indicated that gaseous formaldehyde dissolved in water.

Isolation of formaldehyde-responsive genes from golden pothos by ACP-based PCR

ACP-based PCR products from formaldehyde-treated and untreated golden pothos were separated by agarose gel electrophoresis. Approximately fifty thousand independent bands were detected from the treated and untreated plants. We identified two bands (DEG2 and DEG3) that exhibited increased signal intensity and one band (DEG1) that exhibited lower signal intensity in formaldehyde-treated golden pothos compared with the untreated samples (Figure 1). The results were reproducible in repeated experiments (data not shown). The bands corresponding to DEG2 and DEG3 were extracted from the gel, cloned into pCR2.1, and then sequenced. The nucleic acid sequences of *DEG2* and *DEG3* are presented in Figure 2. *DEG1* were also cloned, but not used for further study.

Cloning of full-length cDNAs

The full-length sequences of *DEG2* and *DEG3* were isolated by RACE. Primers specific for *DEG2* and *DEG3* were used to clone the 5′ ends of the cDNAs, and fragments of approximately 0.45 and 1.2 kb, respectively, were obtained by 5′-RACE. The composite nucleotide sequence and deduced amino acid sequence of the DEG3 cDNA are shown in Figure 2A. Sequence analysis revealed that the gene encoded a polypeptide of 305 amino acids, with an estimated molecular mass of 33.5 kDa and a theoretical isoelectric point (PI) of 7.05. A search of the nucleotide database revealed significant

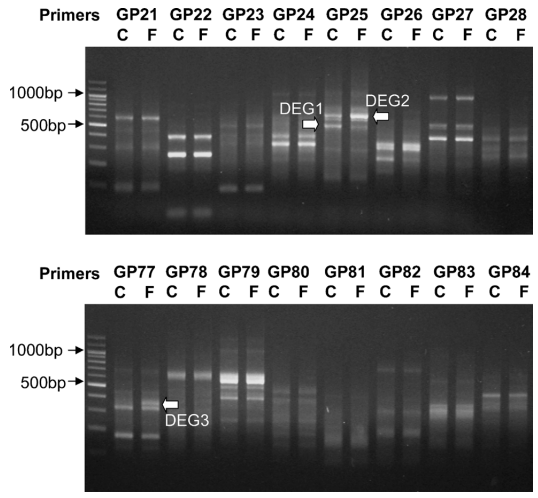

Figure 1. Differentially expressed genes identified by ACP-based PCR. PCR was performed using dT-ACP2 and an arbitrary ACP as the primers. Amplified PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. C, control; F, formaldehyde-treated plant.

similarity between the DEG3 gene product and the products of putative class II *chitinase* genes. We named this sequence *Epipremnum aureum chitinase 1* (*EaCHI1*). *EaCHI1* exhibits the highest similarity to the rice *chitinase*-like gene (EU605859), with an E-Value of 2e-135. Six highly conserved amino acid residues that are believed to play an essential role in chitinase catalytic activity or substrate binding are conserved in EaCHI1 (Zhong et al. 2002). Another notable feature of EaCHI1 is a putative signal peptide sequence (24 amino acids) at the N-terminus (Figure 2A), which was predicted to target this protein to the extracellular space using WoLF PSORT, Genetyx, and SignalP. The composite nucleotide sequence and deduced amino acid sequence of the DEG2 cDNA are shown in Figure 2B. The gene encoded a polypeptide of 96 amino acids, with an estimated molecular mass of 10.2 kDa and a theoretical PI of 6.28. Analysis of the amino acid sequence of DEG2 indicated the presence of a putative N-terminal signal peptide (18 amino acids) (Figure 2B), which was predicted to target the protein to the extracellular space, chloroplasts, or vacuoles, also using WoLF PSORT, Genetyx, and SignalP. There was no significant homology between the nucleotide/amino acid sequence of DEG2 and any other registered nucleotide/protein. To confirm that the *DEG2* sequence represented the full-length cDNA, we carried out additional 5′-RACE experiments using three different primers (Figure 2B). The products from each reaction were the same sequence and similar in length (data not shown). These results strongly suggest that the *DEG2* sequence corresponded to the full-length cDNA. The nucleotide sequence data for *EaCHI1* and *DEG2* have been deposited into the DDBJ database (accession numbers AB539722 and AB539723, respectively).

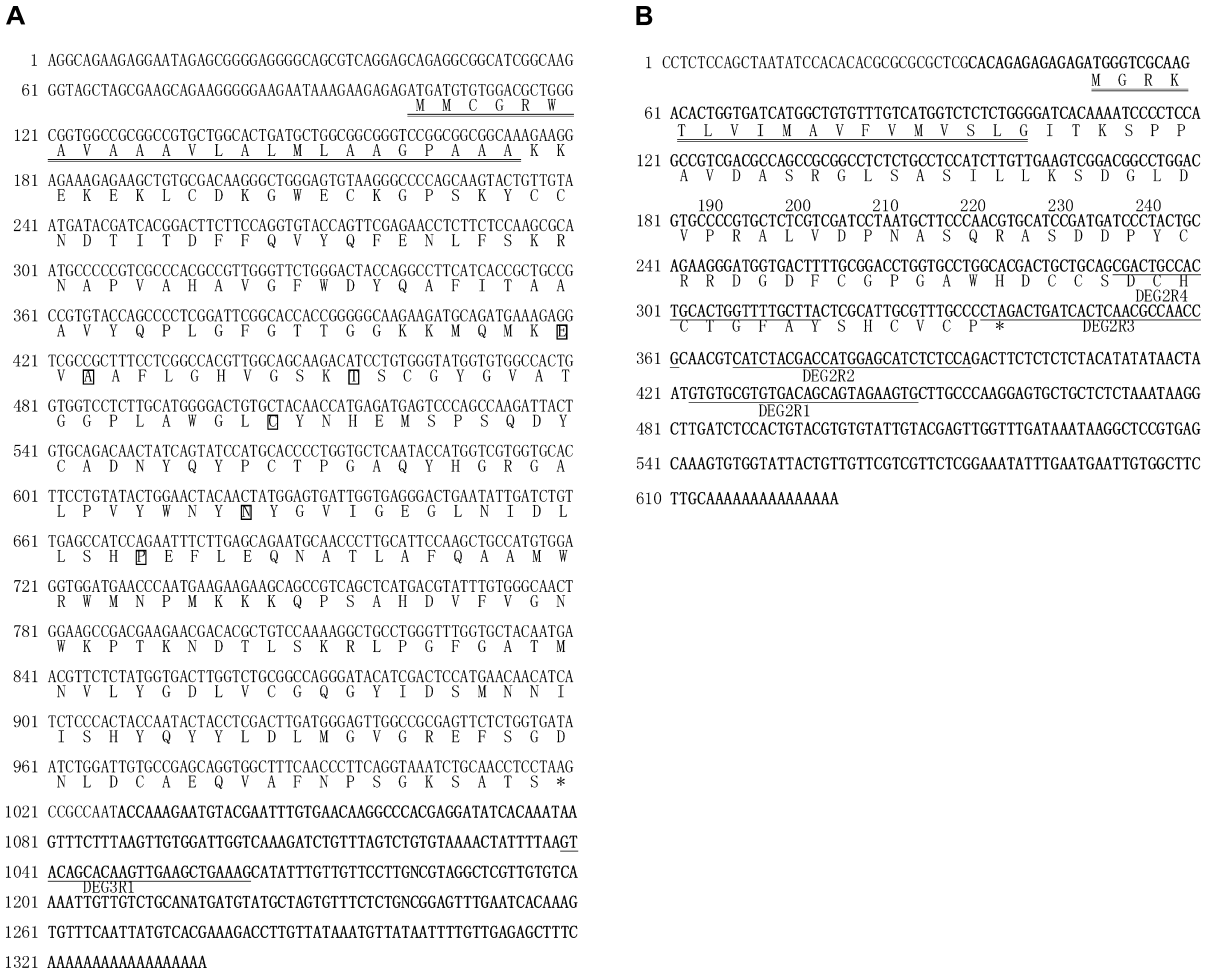

Figure 2. Nucleotide and amino acid sequences of EaCHI1/DEG3 (A) and DEG2 (B). The regions that were isolated by ACP-based PCR are in bold-face type. Putative signal peptide sequences are double-underlined. (A) The RACE primer sequence of DEG3R1 is underlined. Six highly conserved amino acid residues in plant class II chitinases are boxed. (B) The RACE primer sequences of DEG2R1, DEG2R2, DEG2R3, and DEG2R4 are underlined.

Expression of EaCHI1 and DEG2 in formaldehyde-treated golden pothos

To investigate the formaldehyde response of *EaCHI1* and *DEG2*, we carried out real-time quantitative RT-PCR analysis of the transcript levels in leaves, stems, and roots of golden pothos at various time points after formaldehyde treatment (Figure 3). Pothos *actin* was used as an internal control. *EaCHI1* expression was up-regulated in leaves 1 h after treatment, then decreased at 3 h, and increased again 6–24 h after formaldehyde treatment. In stems, *EaCHI1* transcripts were unchanged for up to 6 h after exposure, and then increased 24 h after exposure. In roots, a significant decrease in transcript levels was detected 24 h after treatment. *DEG2* expression in the leaves of formaldehyde-treated golden pothos increased significantly 1 h after treatment, decreased 3 h after treatment, and then gradually recovered after 24 h. In stems, *DEG2* transcripts were down-regulated for at least 24 h after exposure. In roots, transcript levels were increased more than 1.5 fold 1 after exposure and then gradually decreased up to 24 h, at which point they were elevated 0.3 fold.

Phylogenetic analysis

Figure 4 shows the results of the phylogenetic analysis of EaCHI1, the stress-related chitinases AtCTL1, AtchiA, AtchiB, and AtchiV from *A. thaliana*, and putative chitinase proteins from *A. thaliana*, *B. rapa*, *R. communis*, *P. pyrifolia*, *S. bicolor*, *Z. mays*, *O. sativa*, *H. vulgare*, and *T. aestivum*. EaCHI1 homologues were classified into two groups, dicot and monocot. EaCHI1 belonged to the monocot group, but was the most distant of the monocot chitinases analyzed. Among the stress-related chitinases, EaCHI1 was distant from AtchiA, AtchiB and AtchiV (Figure 4), which exhibit stress-responsive expression patterns in *A. thaliana*, and from AtCTL1 (Figure 4), which is not up-regulated by stress conditions, but is essential for heat, salt and drought tolerance.

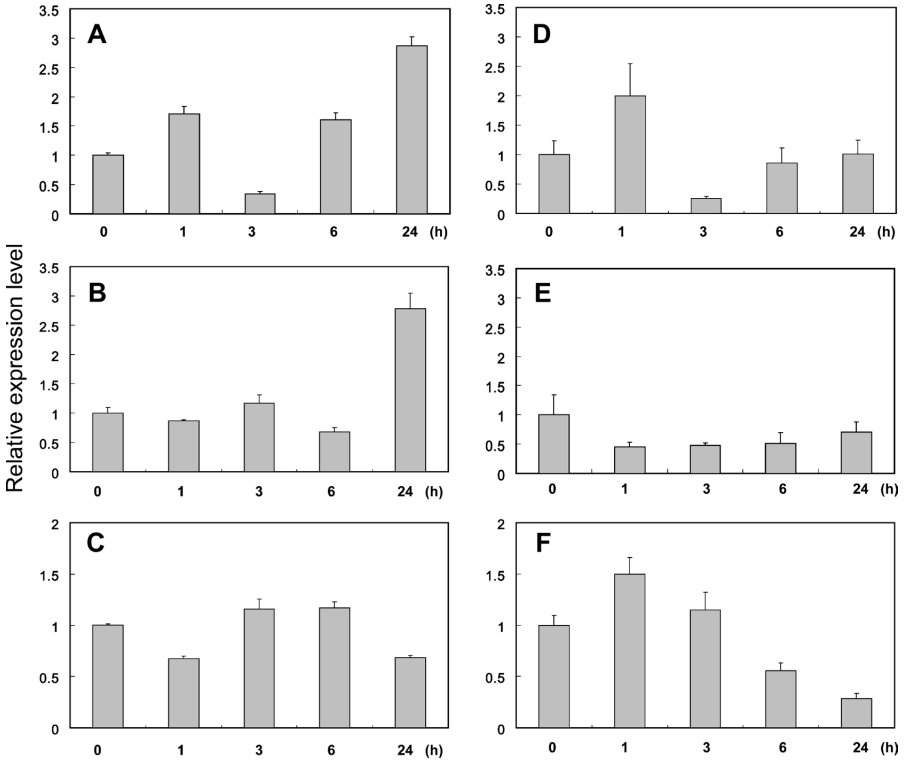

Figure 3. Expression of *EaCHI1* and *DEG2* in golden pothos. Total RNA extracted from formaldehyde-treated leaves, stems and roots at time zero, and 1, 3, 6, and 24 h after exposure to formaldehyde, was subjected to quantitative RT-PCR. All transcript levels were normalized to actin, and are represented as relative values to these at time zero (1.0). Data are the average of three independent experiments ± standard deviation. (A–C) *EaCHI1*, (D-F) *DEG2*. (A, D) Leaves, (B, E) stems, (C, F) roots.

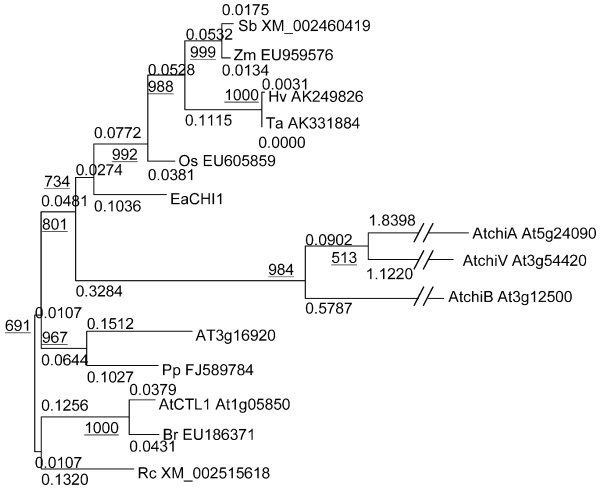

Figure 4. Phylogenetic analysis of plant chitinases. The evolutionary tree was generated using the NJ method and the deduced amino acid sequences of EaCHI1, AtCTL (At1g05850), AtchiA (At5g24090), AtchiB (At3g12500) and AtchiV (At3g54420) from *Arabidopsis thaliana*, and putative chitinases from *A. thaliana* (At3g16920), *Brassica rapa* (Br EU186371), *Ricinus communis* (Rc XM_002515618), *Pyrus pyrifolia* (Pp FJ589784), *Sorghum bicolor* (Sb XM_002460419), *Zea mays* (Zm EU959576), *Oryza sativa* (Os EU605859), *Hordeum vulgare* (Hv AK249826), and *Triticum aestivum* (Ts AK331884). The underlined figures show the bootstrap values.

Discussion

There have been several reports of detoxification of air pollutants by indoor plants (Dingle et al. 2000; Godish and Guindon 1989; Wolverton et al. 1984); however, there are few reports on the effects of formaldehyde on gene expression in plants. Differential screening for genes that responded to formaldehyde treatment using GeneFishing PCR (Kim et al. 2004) resulted in the isolation of two putative formaldehyde-responsive genes, a putative *chitinase* (*EaCHI1*), and an unknown ORF (*DEG2*) from golden pothos. This study focused on the immediate-early responsive genes that are activated immediately following formaldehyde treatment. This may be the reason why a lower number of genes (including EaCHI1 and DEG2) were stimulated by formaldehyde. Although a greater number of differentially displayed PCR products were found at the later stages of formaldehyde treatment, might correspond to stress-related genes which are not related to the sensing or metabolism of formaldehyde, because formaldehyde-treated plants suffer severe physiological damage, such as the shrinking and dehydration of young leaves, just 3 h after treatment.

In formaldehyde-treated leaves, *EaCHI1* and *DEG2* were up-regulated 1 h after exposure to formaldehyde, and then expression decreased after 3 h. This down-

regulation at 3 h may be due to the damage inflicted by formaldehyde, since formaldehyde is known to have physiologically detrimental effects on plants.

Chitinases are classified into either family 18 (class I, II, and IV) or family 19 (class III) proteins, based on the amino acid homology in their catalytic domains and the catalytic mechanism (Davies and Henrissat 1995; Henrissat 1991). Because plants do not contain chitin, it is generally assumed that plant chitinases play a role in plant by attacking chitin, which is a common constituent of the cell walls of fungi and by itself can elicit plant defense (Kwon et al. 2007). Several lines of evidence indicate that the transgenic expression of genes for chitinases results in increased resistance to fungal and bacterial pathogens (Ebel 1998; Jach et al. 1995; Nandakumar et al. 2007). In addition to their putative role in plant defense, recent studies have implicated plant chitinases in plant interactions with symbiotic bacteria (Xie et al. 1999), developmental processes, including flower development and leaf senescence (Leung 1992; Lotan et al. 1989), and tolerance to abiotic stress, including cold, freezing, heat, salt, drought and strong light (Kwon et al. 2007; Takenaka et al. 2009). Chitinases have also been shown to stimulate embryonic development (De Jong et al. 1992; Van Hengel et al. 2001) and seed development (Van Damme *et al.* 1999). All of the chitinase genes that have been identified as stress responsive genes thus far belong to classes I and II of the family 19 chitinases, with the exception of *AtChiA* (Takenaka et al. 2009). *EaCHI1* belongs to class II, and was up-regulated in the leaves and stems, but not the roots, of formaldehyde-treated golden pothos (Figure 3). This is a novel finding in terms of the formaldehyde-responsive expression of a chitinase. EaCHI1 homologs are known to be present in monocot and dicot plants; however, their function in plants remains unclear. *Arabidopsis* chitinases AtchiA (class III), AtchiB (class I) and AtchiV (class IV) are up-regulated by environmental stress (Takenaka et al. 2009), but EaCHI1 is very distant from these chitinases based on phylogenetic analysis (Figure 4). EaCHI1 is rather similar to the class II chitinase AtCTL1 (Figure 4), which is essential for heat, salt and drought tolerance in *Arabidopsis*, but is not up-regulated by stress treatment. These results suggest that *EaCHI1* has a distinct role from these other chitinases in the stress response, and provide novel insight into the physiological roles of chitinase in plants.

The sequence of *DEG2* was not homologous to any known sequence in the nucleotide/protein databases; thus, it encodes a novel protein. Functional analysis of DEG2 may provide important insights into the molecular mechanisms underlying folmaldehyde metabolism in plants.

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