Beta-Cyanoalanine Synthase as a Molecular Marker for Induced Resistance by Fungal Glycoprotein Elicitor and Commercial Plant Activators

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ABSTRACT

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The biocontrol agent *Pythium oligandrum* produces glycoprotein elicitor in the cell wall fraction, designated CWP, and induces resistance to a broad range of pathogens. To understand the mechanism of CWP-induced resistance to pathogens, gene expression at the early stage of CWP treatment in tomato roots was analyzed using a cDNA array. At 4 h after CWP treatment, 144 genes were up-regulated and 99 genes were down-regulated. In the 144 up-regulated genes, nine genes exhibited about eightfold increased expression. Analysis of the response of these nine genes to three commercial plant activators indicated that a high level of one gene, beta-cyanoalanine synthase gene (*LeCAS*) encoding hydro-

gen cyanide (HCN) detoxification enzyme, was stably induced in tomato roots by such treatment. However, expression of *LeCAS* was not significantly induced in tomato roots at 4 h by abiotic stresses, whereas only a very low level of induction of such expression by cold stress was observed. This *LeCAS* expression was also induced after exogenous treatment with a low level of 1-amino-cyclopropane-1-carboxylate as the precursor of ethylene, but not with either salicylic acid or methyl jasmonate. The induction of *LeCAS* expression in CWP-treated and plant activator-treated roots is likely to be caused by the detoxification of HCN during ethylene production. Transient activation of *LeCAS* expression caused by ethylene production in tomato roots may be a general phenomenon in fungal elicitor-induced and synthetic plant activator-induced resistance. *LeCAS* seems to be useful for screening possible novel plant activators for plant protection against pathogens.

Additional keywords: elicitor-like protein, global gene expression.

Once plants are infected by a pathogen, they are able to protect themselves against further infection (8). The existence of induced resistance, which is the acquired resistance to a broad range of pathogens in host plants previously infected with a pathogen or nonpathogenic microorganisms, is well documented (12,37). There are several types of induced resistance. One well-characterized type of induced resistance is systemic acquired resistance (SAR) in host plants infected with necrotizing pathogens (12,29). At the molecular level, SAR is characterized by the expression of a large number of acidic pathogenesis-related (PR) genes and preceded by the accumulation of salicylic acid (SA). SA is thought to be a key signal molecule for SAR induction (14). Downstream of SA, *NPR1/NIM1/SAII* has been identified as an important component of SAR (7,11,16,45). *NPR1* interacts with the TGA family of basic leucine zipper transcription factors and positively regulates PR genes by either inducing a transcriptional activator or inhibiting a transcriptional repressor (12). 2,6-Dichloroisonicotinic acid (INA) is a synthetic compound that likely induces SAR via the

same signal transduction pathway as SA (11,32). However, SA and INA are not practically used in agriculture because some crops have insufficient tolerance to them.

Induced systemic resistance (ISR) in plants colonized with the nonpathogenic bacterium *Pseudomonas fluorescens*, which is another type of induced resistance response against a range of pathogens, has been well characterized at the molecular level (55, 57). Colonization of roots by *P. fluorescens* induces not only resistance to soilborne diseases but also resistance to pathogens in the whole plant. *P. fluorescens* WCS417r induces ISR via SA-independent signaling pathways and by jasmonic acid (JA) and ethylene-dependent signaling pathways, although ISR requires *NPR1* as well as SAR (36,38,56).

Two major commercial compounds, i.e., benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester CGH-245 704 (BTH; Bion) and 3-allyloxy-1,2-benzisothiazole-1,1-dioxide (PBZ; Probenazole), have been developed as plant activators for disease control and have long been used practically in agricultural production (44,59). Studies using tobacco and *Arabidopsis* treated with these plant activators suggest that the mode of action of BTH and PBZ may be at least partially related to the SA-mediated signaling pathway because they activate the same set of PR genes in SA-treated plants (13,17,26,27,63). However, the molecular mechanism(s) for induced resistance by PBZ and BTH remains to

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be determined. Recently, Ishikawa et al. (24) reported that valdamycin A (VMA) acts to control tomato wilt disease caused by the soilborne fungus *Fusarium oxysporum* f. sp. *lycopersici*. Although VMA-treated plants accumulated SA and had an elevated expression of PR genes, our understanding of the signaling pathway(s) activated by VMA is insufficient.

Signal transduction pathways conferring induced resistance may be diverse among plant activators, fungal elicitors, and microorganisms as biocontrol agents. Induced resistance has been found to be differentially impaired in certain signaling mutants of *Arabidopsis*, indicating that different signaling pathways are activated by specific plant activators, fungal elicitors, or biocontrol microorganisms (39,41). On the other hand, the coordination of defense-related gene expression and the accumulation of signal molecules (e.g., SA, JA, and ethylene) in plants treated with plant activators, fungal elicitors, and biocontrol microorganisms suggests that some parts of the signaling mechanism may be conserved (15,37).

Besides chemical plant activators and *P. fluorescens*, some microorganisms that can induce resistance to a broad range of pathogens have been identified (6). *Pythium oligandrum* Drechs induces disease resistance to a number of soilborne plant pathogens by activation of plant defense mechanisms and by microbial antagonism caused by nutrient competition, parasitism, and production of antimicrobial substances (2,3,28,30). Because the disease resistance induced by *Pythium oligandrum* is nonspecific in affording enhanced protection in different plant species against many pathogens, *Pythium oligandrum* has been focused upon as a potential biocontrol agent of soilborne plant pathogens. Several elicitor-like proteins have been identified in some *Pythium* spp. (22,25,33,39). Oligandrin is a class I elicitor-like protein with a molecular mass of ≈ 10 kDa, which is secreted by *Pythium oligandrum* (35). A conclusive study on oligandrin-induced resistance against *Phytophthora parasitica* in tomato has suggested that oligandrin triggers cytological and biochemical modification in tomato cells, thereby inducing resistance to *P. parasitica* (35). Recently, Takenaka and Kawasaki (50) have also reported two types of cell wall protein fractions with elicitor activity from *Pythium oligandrum* (CWP). The amino acid sequences deduced from their corresponding cDNA sequences of POD-1 and POD-2 (DDBJ accession nos. AB217820 and AB217821) suggest that they have elicitor signatures (S. Takenaka, Y. Nakamura, T. Kono, H. Sekiguchi, A. Masunaka, and H. Takahashi, *unpublished data*). *Pythium oligandrum* CWP had elicitor activities that induced the defense reactions against pathogens in sugar beet and wheat. In the roots of sugar beet treated with CWP, the activities of phenylalanine ammonia lyase and chitinase and the amount of cell wall-bound phenolic compounds increased (51). Induction of defense-related gene expression by CWP treatment has also been observed in tomato plants (20). However, the mechanism for induced disease resistance in root tissues treated with CWP remains to be determined.

In this report, to study the molecular mechanism for CWP-mediated induced resistance, global gene expression in CWP-treated roots of tomato was analyzed using an 11,520 nonredundant cDNA array. In the analysis of up-regulated genes in CWP-treated roots, we found that the expression of beta-cyanoalanine synthase gene (*LeCAS*) encoding hydrogen cyanide (HCN) detoxification enzyme was highly induced in tomato roots, not only by CWP treatment but also by PBZ, BTH, and VMA treatments, whereas it was not induced by several types of environmental abiotic stress. Identification of *LeCAS*, which is commonly up-regulated in tomato roots by fungal elicitors and commercial plant activators, indicates that the *LeCAS*-mediated HCN detoxification process may be associated with a conserved signaling pathway for induced resistance. In addition, *LeCAS* may be utilized as a universal marker for screening possible novel plant activators for plant protection against pathogens.

MATERIALS AND METHODS

Plant material. *Lycopersicon esculentum* cv. Micro-Tom, which is a laboratory-grown miniature tomato (46), was grown in quartz sand at 24°C in a growth chamber under continuous fluorescent light (7,000 lux) and fertilized with a thousand-fold diluted Hyponex solution at 3-day intervals (48).

Treatment with CWP of *Pythium oligandrum*. Fourteen-day-old tomato plants were carefully harvested from the plants grown in quartz sand with attention to minimizing the damage to the root tissue by injury. After the roots were rinsed with distilled water (DW) three times, the roots of one plant were dipped in 2 mg of CWP per ml and incubated at 24°C under continuous fluorescent light (7,000 lux). CWP of *Pythium oligandrum* isolate MMR2 was prepared by a previously described method (51).

cDNA array preparation. The 11,520 nonredundant expressed sequence tags (EST) were composed of two nylon membranes (8 to 12 cm each) by a MICROGRID II Robotic workstation (Beckman Instruments, Inc., Fullerton, CA) according to the manufacturer's instruction manual. Macroarray membranes were supplied by the Japanese Solanaceae Genomics Project (JSOL). The list of 11,520 nonredundant EST clones can be found using the MiBASE Micro-Tom database (available online from the Kazusa DNA Research Institute).

Preparation of probes and hybridization. Total RNA was isolated from tomato roots by an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Total RNA was reverse transcribed to synthesize [α^{32} P]dCTP-labeled cDNA probes as described previously (23). One set of macroarray membranes was prehybridized with 12 ml of 0.5 M Church phosphate buffer (9) containing 1 mM EDTA, 7% sodium dodecyl sulfate (SDS), and 12 μ g of oligo(dA)₁₈ in a hybridization bag at 55°C for 6 h. Heat-denatured 32 P-labeled cDNA was mixed with 1 ml of the Church phosphate buffer and then added to the hybridization bag. After incubation at 55°C for 20 h, membranes were washed twice with 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS at 25°C for 5 min, once with 1 $\times$ SSC containing 0.1% SDS at 65°C for 15 min, and twice with 0.1 $\times$ SSC containing 0.1% SDS at 65°C for 15 min. The membranes were wrapped with plastic film and exposed to an IP (BAS-IP SR 2040) image plate (Fuji Photo Film, Tokyo, Japan) for 3 days.

Data analysis. Signals on the IP image plates were scanned with FXPro (Bio-Rad, Hercules, CA) and quantified using Array Vision 5.1 software (IMAGING Research Inc., Ontario, Canada). Signal intensity was calculated and normalized by a previously described procedure (23). Four replicate experiments were conducted. To select differentially expressed genes between CWP- and DW-treated plants, stringent criteria were applied as described next. The average of the normalized value of the signal intensity for each gene in four replicate experiments was adopted as the expression value of the gene. When the value of gene expression was ≤ 0.3 in one of four treatments, the gene was eliminated for further analysis, because such low intensity data are less reproducible. The average of the normalized value of the signal intensity for each gene in four replicate experiments was adopted as the expression value of the gene. One-way analysis of variance of the expression values was performed to identify the genes having similar altered expression patterns in four replicate experiments. Genes whose expression level was significantly different between CWP and DW treatments were selected using Fisher's least significant difference procedure. The ratio of induction or suppression of each gene was calculated by the expression value for CWP divided by that of DW. Ratios of < 1 were transformed to $-1/\text{ratio}$. Then, when the value of gene expression increased more than threefold or decreased to less than fivefold in the CWP-treated plant, we identified the gene expression as altered reproducibly between two treatments. The data were analyzed using Microsoft Excel.

Northern hybridization analysis. Total RNA was isolated from the roots, stems, and leaves of tomato with an RNeasy Plant-Mini Kit following the instruction manual. Ten micrograms of total RNA was loaded in each lane of a 1.2% denaturing agarose gel. Northern hybridization was performed according to a published protocol (42). To detect the expression of *basic PR-2*, *LeUBQ5*, and eight CWP-inducible genes (i.e., JSOL clone nos. FA29BH06, FA22DA07, FA33AC03, FB17BD03, FA14CF03, FA28DA01, FA25BF10, and FA16CF04 [TIGR LeGI nos. TC116638, TC125475, TC116923, TC116192, TC124613, TC117846, TC126254, and TC116283], respectively), open reading frames of each gene were amplified by reverse polymerase chain reaction (PCR) with the primers 5'-CATGGATG-AGAATCTACGTACC-3' and 5'-TATAACAAAACAACTCAATAC-3' for FA29BH06 (TC116638), 5'-GGAGGGTGAAGCA-ATGAGGA-3' and 5'-CAAACCTTCTCTCCCATGAC-3' for FA22DA07 (TC125475), 5'-GGAAACCAAAGTTATTTCTA-3' and 5'-TAGTTCCTTGAAAAGTTCCAA-3' for FA33AC03 (TC116923), 5'-GGCAAATTTGTTTCATTAAGC-3' and 5'-CAATCCAAATTTCCAACCTTT-3' for FA28DA01 (TC116192), 5'-GGCTGATCTGAAGAAGAAAT-3' and 5'-TACTTCTGCCTCT-TATAAAT-3' for FA14CF03 (TC124613), 5'-GGCCAGGAAGA-AGATCAGAG-3' and 5'-TATGCAGCTGCACTGATGCA-3' for FB17BD03 (TC117846), 5'-CCATGGGAGCTGGTGGAAATAT-G-3' and 5'-CTTTCAAAGCTTGTTTTTATACC-3' for FA16CF04 (TC126254), and 5'-CATGGATGAGAATCTACGTACC-3' and 5'-TATAACAAAACAACTCAATAC-3' for FA25BF10 (TC116283). About 600-bp DNA fragments for detecting tran-

scripts from *basic PR-2* and *LeUBQ5* by northern hybridization were also amplified by reverse PCR with primers, i.e., 5'-GTGCTTCTAGGATTACTTGTGCGCCACC-3' and 5'-CTCACT-AGTGAGTGAAGAAGCAGTGC-3' for *basic PR-2* and 5'-GCA-GATCTTCGTGAAAACCC-3' and 5'-CAATCGCCTCCAGCC-TTGTT-3' for *LeUBQ5*. First-strand cDNA as a template for reverse PCR was reverse-transcribed from total RNA of tomato roots with an RT-PCR kit (Invitrogen, Carlsbad, CA). One microgram of first-strand cDNA was added to 50 µl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 2 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.2 µM of each primer, and 5 units of *Taq* DNA polymerase (Promega, Madison, WI) for PCR. The reaction ran with the following program: 30 cycles at 94°C for 1 min, 55°C for 2 min, and 73°C for 1 min. The PCR product was purified and cloned into the *EcoRV* site of pBlue-script SK+ (Stratagene, La Jolla, CA). To confirm that the expected DNA had been cloned, the nucleotide sequence of each insert was determined by the Sanger method with the automated DNA sequencer (ABI model 310A; Applied Biosystems, Foster City, CA). The eight amplified CWP-inducible genes, *basic PR-2*, and *LeUBQ5* DNA fragments were labeled with [³²P]dCTP using the Megaprime DNA labeling systems (Amersham-Pharmacia, Piscataway, NJ).

In order to analyze *LeCAS* expression in differential tissues of tomato, total RNA was isolated from leaf, stem, and root tissues at 4 h after treatment of roots with CWP and DW. *LeUBQ5* was detected as an internal control. The level of *LeCAS* expression was quantified by measuring the intensity of each band of northern

TABLE 1. Genes with up- and down-regulated expression in glycoprotein elicitor in the cell wall fraction (CWP)-treated roots of *Lycopersicon esculentum* cv. Micro-Tom

JSOL clone number ^a	Putative function ^b	e-value	Expression value in DW treatment ^c	Expression value in CWP treatment ^c	Induction fold ^d
FA33DD04	Serine carboxypeptidase II-3 precursor	0	0.79	6.00	7.6
FA52AE06	ATP:citrate lyase	0	4.68	23.57	5.0
FA14AC12	Sucrose synthase	2E-113	2.53	14.50	5.7
FA12AC03	Unknown protein	4.1E-36	0.45	2.27	5.0
FA16DB03	GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase	0	0.80	6.24	7.8
FA24AH09	Oxalyl-CoA decarboxylase	0	2.81	16.27	5.8
LA12CD06	Speckle-type POZ protein	e-124	0.57	3.21	5.6
FA16CF04	Delta12 fatty acid desaturase	0	47.13	237.28	5.0
FA29BE08	RING-H2 zinc finger protein ATL6	0	0.42	5.87	13.9
FA31DC02	Chorismate synthase 1	0	5.40	33.81	6.3
FB08CC02	Mitochondrial small heat shock protein	0	0.53	2.94	5.6
FA18BC07	Monooxygenase	0	1.36	8.04	5.9
FA13AB01	VQ motif-containing protein	0	0.49	2.80	5.7
FA29BH06	Beta-cyanoalanine synthase	0	2.32	31.27	13.5
FA17BE09	Serine/threonine protein phosphatase	0	0.59	3.53	6.0
FA09AE07	Unknown protein	5.80E-34	1.80	18.80	10.4
FA16DE02	Cytochrome P450-1	0	3.32	26.20	7.9
FA23AA08	Unknown protein	0	0.45	2.40	5.4
FA31DA04	L-Asparaginase	0	4.35	23.03	5.3
FA25AF04	Actin 101	0	1.65	10.16	6.2
FA22DG07	Vacuolar invertase/beta-fructofuranosidase	0	0.58	4.14	7.1
FA15AE11	Mitochondrial carrier protein	0	0.32	1.63	5.2
FA22DA07	3-Dehydroquinate dehydratase/shikimate 5-dehydrogenase	0	2.66	21.55	8.1
LA19CB03	NADH-glutamate dehydrogenase	0	3.88	20.76	5.4
FA18CF05	Ethylene receptor homolog	0	0.89	8.12	9.1

(Continued on next page)

^a Japanese Solanaceae Genomics Project (JSOL) numbers were assigned using the MiBASE Micro-Tom database (available online from the Kazusa DNA Research Institute).

^b Putative function of each gene product was determined by BLASTN or BLASTX homology search using The Institute for Genomic Research (TIGR) tomato genome index (available online from TIGR website) and GenBank (available online from the National Center for Biotechnology Information/National Institutes of Health website).

^c The average of the normalized value of the signal intensity for each gene in four replicate experiments was adopted as the expression value of the gene. One-way analysis of variance of the expression values was performed to identify the genes having similar altered expression patterns in four replicate experiments. The gene in which expression level was significantly different between CWP and distilled water (DW) treatments was selected using Fisher's least significant difference procedure ($P < 0.05$).

^d Induction or suppression fold of each gene in the roots 4 h after CWP and DW treatments was calculated by the expression value for CWP divided by that of DW. Ratios of <1 were transformed to $-1/\text{ratio}$. Then, when the value of gene expression increased more than fivefold or decreased less than fivefold in the CWP-treated plant, we identified the gene expression as altered reproducibly between two treatments. Genes with induced or suppressed expression in CWP or DW are divided by dashed line.

hybridization on IP (BAS-IP SR 2040) image plate using FXPro (Bio-Rad). The means of the expression level and their standard deviation (SD) in three plants were calculated in each experiment.

Plant activator, signal compound, and stress treatments. Fourteen-day-old tomato plants were used for a series of treatments. Three commercial plant activators, i.e., 3-allyloxy-1,2-benzisothiazole-1,1-dioxide (Probenazole) (59), benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) (44) and [1S-(1 α ,4 α ,5 β ,6 α)]-1,5,6-trideoxy-4-O- β -D-glucopyranosyl)-5-(hydroxymethyl)-1-[[4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]amino]-D-chiro-inocitol-1L-(1,3,4/2,6)-2,3-dihydroxy-6-hydroxymethyl-4-[(1S,4R,5S,6S)-4,5,6-trihydroxy-3-hydroxymethylcyclohex-2-enylamino]cyclohexyl-3-D-glucopyranoside (validamycin A [VMA]) (24), were supplied by Meiji Seika Kaisha, Co. Ltd. (Japan), Syngenta (USA), and Sumitomo Chemical Co. Ltd. (Japan), respectively. Tomato roots were dipped in of one of the three plant activators (10 μ g/ml), respectively, and then incubated for 4 h at 24°C.

SA, INA, and 1-amino-cyclopropane-1-carboxylate (ACC) were applied by submerging tomato roots in suspensions of formulations in DW for 4 h at 24°C. Methyl-jasmonate (MeJA) was similarly applied to roots with the formulation suspension in 0.1% ethanol for 4 h at 24°C. As the controls, tomato roots were treated with DW or 0.1% ethanol for 4 h at 24°C.

Tomato roots were treated with 10 types of environmental abiotic stresses: heat shock at 37°C for 4 h; cold shock at 4°C for 4 h; mechanical wounding by root sectioning in $\approx$ 5 mm, and then soaking in DW for 4 h; drought for 1 h; dark for 4 h; and treat-

ment with 0.2 M NaCl, 0.5 M mannitol, 2% glucose, and bovine serum albumin (BSA) at 2 mg/ml for 4 h, respectively.

Measurement of ethylene production. Each plant was treated with ACC in a gas-tight serum flask. At the time of ethylene measurement, 1-ml gas samples were withdrawn through the rubber seal. The concentration of ethylene was determined by gas chromatography (Shimazu, Osaka, Japan) as described previously (20). The means of ethylene concentration and their SD in three plants were calculated in each experiment.

RESULTS

Up- and down-regulated genes in CWP-treated tomato roots. Macroarray analysis of four independent RNA samples isolated from CWP-treated tomato roots and a DW-treated control indicated that 144 genes were up-regulated more than threefold and 99 genes were down-regulated to less than threefold in response to CWP. A list of these 243 genes and information about the altered level of their gene expression can be found at MiBASE (available online from the Kazusa DNA Research Institute). Table 1 is a list of 65 of the 243 genes that were up-regulated greater than fivefold or down-regulated to less than fivefold by CWP treatment. Defense-related genes (e.g., PR protein P14 and class II chitinase genes), oxidative stress-related genes (e.g., glutathione S-transferase and superoxide dismutase genes), and other stress-related genes are included. Other up-regulated genes in CWP-treated tomato roots were JA- and ethylene-related genes. Expression of genes encoding an ethylene receptor homolog, an

TABLE 1. (Continued from preceding page)

JSOL clone number ^a	Putative function ^b	e-value	Expression value in DW treatment ^c	Expression value in CWP treatment ^c	Induction fold ^d
FA05AC02	Amine oxidase	0	0.46	2.34	5.1
FA25BF10	Pepper esterase/VCL1	0	72.68	431.01	5.9
FB17BH07	Unknown protein	0	2.64	36.43	13.8
FA14CF03	Farnesyl pyrophosphate synthase	0	10.22	99.60	9.7
FA19DF05	Acyl-[acyl-carrier protein] desaturase	0	3.31	17.87	5.4
FA28DA01	Embryo-abundant protein	0	2.30	35.08	15.3
FB17AB07	Lipoxygenase	5.8E-76	0.51	2.71	5.3
FB18CA01	Transaldolase	3.00E-09	3.12	15.81	5.1
LA19CH06	Jasmonic acid 2	0	0.32	4.22	13.0
FA05AG12	Unknown protein	0	0.67	3.61	5.3
FA33AC03	Flavanone 3-hydroxylase-like protein	0	1.36	16.21	11.9
FB17BD03	ATP citrate lyase b-subunit	0	1.90	20.07	10.6
LA11CF04	ATP:citrate lyase	0	3.56	35.21	9.9
LC05CA12	Unknown protein	1E-21	0.39	2.14	5.5
LC02AC05	AAA-type ATPase family protein	e-135	0.39	2.10	5.3
LC17DA03	Receptor-like kinase	9E-40	0.31	1.82	5.8
LA24AH09	Beta-expansin 2 precursor-like protein	2E-81	1.05	11.58	11.0
FA06CB10	Ovule/fiber cell elongation protein Ghfe1	0	8.93	1.47	-6.1
FB03CC08	Unknown protein	0	1.56	0.31	-5.0
FA32CB06	Peptide transporter	e-160	2.29	0.36	-6.3
FA22CC10	20S proteasome alpha-subunit C (PAC1)	0.001	2.74	0.40	-6.8
FA36CE05	Unknown protein	0	3.39	0.48	-7.1
FB10CC08	Unknown protein	0	6.42	0.87	-7.4
LA20DH04	Thiazole biosynthetic enzyme chloroplast precursor	0	6.76	0.93	-7.3
FA36AF06	T-complex protein 1 epsilon subunit	0	3.33	0.54	-6.2
LA14BH04	Aquaglyceroporin	0	6.70	0.70	-9.6
FA29AD04	C-4 sterol methyl oxidase	0	1.88	0.35	-5.4
LA22CE01	Arginine N-methyltransferase-like protein	e-132	1.72	0.31	-5.5
LA26CE02	Apospory-associated protein C	0	3.06	0.31	-9.9
LB03CE07	Aquaporin-like protein	0	27.84	4.05	-6.9
LB04DD01	Serine/threonine-protein kinase orb6	0	21.35	4.07	-5.2
LC16CC11	Unknown protein	0	4.82	0.94	-5.1
LC06AG08	Receptor protein kinase CLAVATA1	0	2.88	0.39	-7.5
LA25DE02	Pectinacetylesterase	0	1.96	0.35	-5.6
LB04AF10	Drought-induced protein SDi-6/early nodulin 12B precursor (N-12B)	0	10.23	1.16	-8.8
LC10DG03	Auxin response factor 2	2E-71	2.22	0.41	-5.4
LB01DE07	Unknown protein	0	2.61	0.42	-6.3
LA24AB07	Unknown protein	0	2.04	0.34	-6.1
LC15AH02	Glycerophosphoryl diester phosphodiesterase	0	3.04	0.52	-5.8
LA28DG10	Unknown protein	5.4E-31	25.10	4.11	-6.1

ethylene responsive transcription factor, alleneoxide synthase and lipoxigenase for JA biosynthesis, and three JA-inducible transcription factors (i.e., JERF1, NAC transcription factor JA2, and MYC transcription factor JA3) was clearly induced in CWP-treated tomato roots. There are many genes encoding enzymes involved in secondary metabolism among the up-regulated genes.

Analysis of CWP-inducible gene expression in tomato roots treated with commercial plant activators. Up-regulation of nine genes expressed in tomato roots at 4 h after CWP treatment was confirmed by northern hybridization analysis (Fig. 1). Of these nine

Fig. 1. Northern blot expression analysis of the effect of glycoprotein elicitor in the cell wall fraction (CWP) and 3-allyloxy-1,2-benzisothiazole-1,1-dioxide (PBZ; Probenazole) on transcript abundance of nine genes shown by macroarray analysis to be up-regulated in tomato roots treated with CWP. Tested are J-SOL clone numbers FA29BH06 encoding beta-cyanoalanine synthase, FA22DA07 encoding shikimate 5-dehydrogenase/3-dehydroquinase dehydratase, FA33AC03 encoding flavanone 3-hydroxylase-like protein, FB17BD03 encoding ATP citrate lyase b-subunit, FA14CF03 encoding farnesyl pyrophosphate synthase, FA28DA01 encoding embryo-abundant protein, FA25BF10 encoding pepper esterase, FA16CF04 encoding delta 12 fatty acid desaturase, and basic PR-2. Expression of these nine genes and rRNA are shown 4 h after treatment with fungal glycoprotein elicitor (CWP), PBZ, and distilled water (DW).

Fig. 2. Northern blot expression analysis of a beta-cyanoalanine synthase gene in tomato roots treated with three commercial plant activators. Expression of the beta-cyanoalanine synthase gene (*LeCAS*) in tomato roots treated with fungal glycoprotein elicitor (CWP), 3-allyloxy-1,2-benzisothiazole-1,1-dioxide (PBZ), benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), validamycin A (VMA), 2,6-dichloroisonicotinic acid (INA), and distilled water (DW) was detected 4 h after treatment by northern hybridization. rRNA was detected as an internal control.

genes, the expression of three genes (i.e., Japanese Solanaceae Genomics Project [JSOL] clone numbers FA29BH06, encoding beta-cyanoalanine synthase [*LeCAS*]; FA22DA07, encoding shikimate 5-dehydrogenase/3-dehydroquinase dehydratase; and FA33AC03, encoding flavanone 3-hydroxylase-like protein) was also induced at 4 h in tomato roots after PBZ treatment (Fig. 1).

Because co-induction of these three genes by CWP and PBZ treatments suggests that there may be a conserved molecular process underlying induced resistance by these treatments, the expression of three genes in tomato roots treated with two other commercial plant activators, BTH and VMA, was analyzed. At 4 h after BTH and VMA treatments, as well as CWP and PBZ treatments, a high level of *LeCAS* transcript was reproducibly detected (Fig. 2). The expression of two other genes was also significantly up-regulated by treatment with BTH and VMA, but the level of their induction was variable in biological repetition (data not shown). *LeCAS* was not up-regulated in tomato roots treated with synthetic chemical INA (Fig. 2).

Analysis of *LeCAS* expression in tomato roots treated with several types of abiotic stress and signaling compounds. *LeCAS* transcript was abundant in roots, but it accumulated at low level in leaf and stem tissues (Fig. 3A). Although the level of induction of *LeCAS* in leaf and stem tissues was lower than that in roots, treatment of roots with CWP systemically induced a two- to fourfold increase in the expression of *LeCAS* even in leaf and stem tissues (Fig. 3A and B), indicating that the induction of *LeCAS* expression is a systemic response to CWP in tomato plants.

The expression of *LeCAS* was analyzed in tomato roots treated with BSA, NaCl, mechanical wounding, drought, heat and cold shocks, osmotic stress by mannitol and glucose, and dark treatments. Except for very low induction of *LeCAS* by cold shock, other abiotic stresses did not induce expression of *LeCAS* in tomato roots at 4 h after treatment, whereas expression of *LeCAS* was significantly induced by CWP (Fig. 4A).

Fig. 3. Northern blot expression analysis of a beta-cyanoalanine synthase gene in tomato tissues after treatment of roots with glycoprotein elicitor in the cell wall fraction (CWP). **A**, Expression of the beta-cyanoalanine synthase gene (*LeCAS*) in tomato leaf, stem, and root tissues was detected 4 h after treatment of roots with fungal glycoprotein elicitor (CWP) and distilled water (DW). Ubiquitin 5 (*LeUBQ5*) of tomato was detected as an internal control. **B**, The level of *LeCAS* expression was quantified by measuring the intensity of each band of northern hybridization on IP image plate using Bio-Rad FX (Bio-Rad, Hercules, CA). The means of the expression level and their standard deviation in three plants were calculated in each experiment.

SA, JA, and ethylene are thought to be key signaling compounds for induced resistance (15,43). It is known that commercial plant activators accelerate the SA-mediated signal transduction pathway conferring SAR. On the other hand, ISR by *P. fluorescens* is conferred by JA- and ethylene-mediated signaling pathways. To investigate if these signaling pathways are associated with up-regulation of *LeCAS* in tomato roots by CWP treatment, induction of *LeCAS* expression by SA, MeJA, and ACC treatments was analyzed. *LeCAS* expression was induced by ACC, which is a precursor of ethylene, but not by SA and MeJA (Fig. 4B). Transient activation of ethylene production and ethylene-inducible defense gene expression has been identified in tomato roots at 4 h after CWP treatment (20). The level of *LeCAS* expression also increased in tomato roots at 4 h after CWP treatment and then decreased gradually (Fig. 4C). These results indicated that elevated level of *LeCAS* expression in tomato roots at 4 h after treatment by CWP and commercial plant activators coincided with ethylene production and may be induced by it.

The amount of ethylene production in ACC-treated tomato roots depends upon the concentration of ACC (Fig. 5B). However, a high level of *LeCAS* expression was maintained even at a low concentration of ACC, resulting in a low level of ethylene production (Fig. 5A). A high level of *LeCAS* expression, which was rapidly, sensitively, and specifically induced by CWP and commercial plant activators, seems to be a general phenomenon in the response of tomato roots to fungal elicitors and plant activators.

DISCUSSION

Pythium oligandrum has been utilized as a biocontrol agent for controlling damping-off of cress caused by *Pythium ultimum* and has been commercialized as Polygardon (Polyversum) in the Slovak Republic (5,6). The production of CWP of *Pythium*

oligandrum seems to play an important role in enhancing the potency of *Pythium oligandrum* to control plant diseases, as shown by the fact that damping-off disease caused by *Rhizoctonia solani* AG2-2 in sugar beet, Fusarium head blight caused by *Fusarium graminearum* in wheat, and bacterial wilt disease caused by *Ralstonia solanacearum* in tomato were suppressed by treatment of their roots with CWP (20,51). In the present experiments, to investigate the molecular mechanism for *Pythium oligandrum*-induced resistance to pathogens, global gene expression in tomato roots treated with CWP was analyzed. Up-regulation of defense and oxidative stress-related genes may result from the activation of conserved defense mechanisms by CWP treatment because they are generally induced in plants in which induced resistance to pathogens has been activated by several plant activators and biocontrol agents (13,15,17,47,53,57,58). Elevated levels of JA and ethylene-related gene expressions were also observed in CWP-treated tomato roots. The activation of the ethylene-mediated signaling pathway has been reported in CWP-treated tomato roots (20). The activation of the JA-mediated signaling pathway is often associated with a defense response to pathogens by combination with co-activation of the ethylene-mediated signaling pathway (15). Therefore, JA- and ethylene-mediated signaling pathways seem to be activated by CWP treatment.

Except for up-regulation of genes that are commonly related to the defense response, altered expression of several genes encoding protein kinases, putative transcription factors, and many functional unknown proteins was observed in tomato roots at 4 h after CWP treatment. Altered expression of these genes may be associated with signal transduction pathways conferring defense induced resistance by CWP treatment. Furthermore, expression of

Fig. 4. Northern blot expression analysis of a beta-cyanoalanine synthase gene in response to environmental abiotic stresses and signaling compounds. **A**, Expression of the beta-cyanoalanine synthase gene (*LeCAS*) in tomato roots treated with bovine serum albumin (BSA), NaCl, mechanical wounding, drought, heat and cold shocks, and osmotic stress by mannitol and glucose was detected as described in the experimental procedure section. *LeCAS* expression was also detected in fungal glycoprotein elicitor (CWP)- and distilled water (DW)-treated tomato roots. rRNA was detected as an internal control. **B**, Induction of *LeCAS* by salicylic acid (SA), methyl-jasmonate (MeJA), and 1-aminocyclopropane-1-carboxylate (ACC), which is a precursor of ethylene, was detected 4 h after inoculation. Ubiquitin 5 (*LeUBQ5*) of tomato was detected as an internal control. **C**, Time course of the elevated level of *LeCAS* in tomato roots at 0, 2, 4, 8, 12, 24, 48, and 96 h after treatment with CWP was analyzed. rRNA was detected as an internal control.

Fig. 5. Northern blot expression analysis of a beta-cyanoalanine synthase gene and ethylene production in response to 1-aminocyclopropane-1-carboxylate (ACC) application. **A**, Expression of the beta-cyanoalanine synthase gene (*LeCAS*) in tomato roots treated with different amounts of ACC was detected by northern hybridization. rRNA (*rRNA*) was detected as an internal control. **B**, The amount of ethylene production in tomato roots treated with different amounts of ACC was measured by gas chromatography. Both experiments were conducted using tomato roots at 4 h after ACC treatment. The means of the amount of ethylene and their standard deviation in roots of three plants were calculated in each experiment.

genes encoding ATP citrate lyase β -subunit, embryo-abundant protein, and pepper esterase was specifically induced by CWP but not by PBZ (Fig. 2). Therefore, in addition to the activation of the general defense mechanism, certain signaling pathways or metabolic pathways seem to be specifically activated for induced resistance by CWP treatment.

Elucidation of the conserved defense mechanism underlying fungal elicitors and plant activator-induced resistance is likely to be important for understanding the mechanism of induced resistance. In CWP-inducible genes identified by global analysis of gene expression, three genes encoding beta-cyanoalanine synthase (*LeCAS*), shikimate 5-dehydrogenase/3-dehydroquinate dehydratase, and flavanone 3-hydroxylase-like protein, which were up-regulated more than eightfold by CWP treatment, were identified. Up-regulation of these three genes was also observed in PBZ-treated tomato roots. Co-induction of these three genes by CWP and PBZ treatments suggests that there may be a conserved molecular process underlying induced resistance by these treatments. Among these three genes, the elevated level of *LeCAS* expression was higher and more stable than that of the other two genes. Furthermore, interestingly, the expression of *LeCAS* was also similarly induced in BTH- and VMA-treated tomato roots. However, *LeCAS* expression was not induced by INA. It has been considered that INA can specifically activate the SA-dependent signaling pathway (32). Although the mode of action of PBZ, BTH, and VMA may partially overlap the SA-mediated signaling pathway (13,17,26,27,63), it is quite possible that these plant activators can activate SA-independent signaling pathways. Therefore, an elevated level of *LeCAS* expression in CWP and plant activator-treated tomato roots may be controlled by SA-independent signaling pathways.

Beta-cyanoalanine synthase (CAS) plays an important role in cyanide metabolism in plants (34). In plants, ethylene is biosynthesized from ACC. ACC is metabolized into ethylene, HCN, CO₂, and H₂O, and then HCN is rapidly detoxified by CAS, thereby recycling the reduced nitrogen of cyanide for amino acid synthesis (4,18,21,34,62). CAS encodes deduced molecular mass of a 38-kDa protein containing the conserved pyridoxal 5'-phosphate binding site. The identity of partial amino acid sequences of CAS with those of the mitochondrial isozyme of cysteine synthase (CS) suggests that CAS is likely to be functional in mitochondria (31). The nucleotide sequence of CAS has been determined in *Spinacia oleracea*, *Arabidopsis thaliana*, and *Solanum tuberosum* and the biochemical property of CAS has been well characterized in them (21,31). Their ortholog in tomato (*LeCAS*) was assigned among the ESTs of *Lycopersicon esculentum*. Phylogenetic analysis indicated that CAS and CS are the members of α -substituted alanine synthase family (21).

For more than a decade, the induction of ethylene production in cell suspension cultures of tomato and tobacco treated with elicitors from *Phytophthora* species has been reported by some research groups (1,40,52). The transiently elevated level of ethylene production in CWP-treated tomato roots has also been confirmed (20). Furthermore, flavanone 5-hydroxylase-like protein (JSOL clone no. FA33AC03), which has been identified as ACC oxidase catalyzing ACC to ethylene (19), was up-regulated more than 10-fold by CWP (Fig. 2). These findings strongly suggest that expression of *LeCAS* is induced for detoxifying HCN resulting from the elevated level of ethylene production in CWP-treated tomato roots.

High level of CAS activity seems to be caused by the general response to ethylene production in plants. However, regulation of CAS gene expression may be diverse. In the case of potato plants, ethylene stimulated CAS activity and the amount of CAS protein, but had no effect on the amount of CAS mRNA (31). This result suggests that CAS expression is regulated posttranscriptionally in potato plants. On the other hand, in birch plants, CAS transcript accumulation increases over 16-fold by exposure to ethylene as

well as in CWP-treated tomato plants (54). The mechanism of CAS induction by ethylene production may be different among plant species. Vahala et al. (54) reported that CAS transcript also accumulate in response to ozone (O₃) which induces ethylene and JA production in birch. Functional ethylene and JA signaling is likely to be required for the induction of CAS by ethylene and O₃ in birch. It is apparent that CAS expression is regulated by various signaling pathways.

The level of *LeCAS* transcript did not increase significantly in tomato roots at 4 h after mechanical wounding, chilling, or drought, although ethylene production in response to these stresses has been well documented as a general physiological phenomenon in plants (61). As shown in Figure 6, the induction of *LeCAS* seems to be very sensitive to ethylene production, because HCN is extremely toxic to plant cells. Therefore, noninduction of *LeCAS* in tomato roots treated with these types of abiotic stresses may suggest that the level of ethylene production was not elevated at least 4 h after abiotic stress treatments. On the other hand, *LeCAS* expression was significantly induced in PBZ-, BTH-, and VMA-treated tomato roots; although, to our knowledge, a significant increase of ethylene production has not been directly observed in PBZ-, BTH-, and VMA-treated plants. The up-regulation of *LeCAS* in PBZ-, BTH-, and VMA-treated tomato roots seems to indicate that ethylene production may slightly increase in tomato roots at 4 h after treatment.

Besides induction of *LeCAS* for the detoxification of HCN, the up-regulation of *LeCAS* in tomato roots treated with CWP and plant activators can be interpreted in a different way. In the last decade, the molecular mechanisms for induced resistance to pathogens have been extensively studied. However, our understanding about the signaling network for induced resistance is still insufficient. For example, spermine and beta-aminobutyric acid have been reported to be novel signaling compounds for induced resistance (10,49,60,64). Therefore, there is a possibility that uncharacterized ethylene-independent signaling pathways may control up-regulation of *LeCAS* in tomato roots treated with CWP and plant activators.

The discovery of novel plant activators and biocontrol agents that confer induced resistance against a broad range of pathogens is very important for the durable control of plant disease in agricultural production. PBZ, a common plant activator against the rice blast fungus, has long been used in Asia since 1980, but no race overcoming PBZ-induced resistance has been found. However, at present, available effective plant defense activators are limited to specific combinations of crops and pathogens. For screening a candidate with potential as a novel plant activator inducing resistance to other pathogens in other plant species, up-regulation of the *LeCAS* gene is likely to be used as an excellent molecular marker. One simple approach might be to produce tomato plants transformed with an open reading frame of green fluorescent protein under the control CAS promoter. These transgenic plants would provide a system for effective screening of the candidates for novel plant activator compound.

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