Characterization of the early response of the orchid, *Phalaenopsis amabilis*, to *Erwinia chrysanthemi* infection using expression profiling

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*Erwinia chrysanthemi* is a devastating bacterial pathogen in *Phalaenopsis amabilis* and causes soft-rotting disease by secretion of cell wall-degrading enzymes. However, the molecular mechanisms underlying the interaction of *P. amabilis* with *E. chrysanthemi* remain elusive. In this study, early molecular events of the plant in response to the pathogen attack were investigated. The alteration in reactive oxygen species accumulation and peroxidase activity occurred at the site of infection. Subsequently, a systematic sequencing of expressed sequence tags (ESTs) using suppression subtractive hybridization (SSH) was performed to obtain the first global picture of the assembly of genes involved in the pathogenesis. The majority of the SSH clones showed a high identity with genes coding for proteins that have known roles in redox homeostasis, responses to pathogens and metabolism. A notable number of the SSH clones were those encoding WRKY, MYB and basic leucine zipper transcription factors, indicating the stimulation of intracellular signal transduction. An orchid gene encoding *trans*-2-enoyl-CoA reductase (ECR) was the most abundant transcripts in the EST library. ECR is an enzyme catalyzing the very long chain fatty acids (VLCFAs) biosynthesis, and the full-length cDNA of the ECR gene (*PaECR1*) was obtained. Functional analysis of *PaECR1* was conducted by virus-induced gene silencing to knock down the gene expression in *P. amabilis*. The *PaECR1*-silenced plants were more susceptible to *E. chrysanthemi* infection, implying potential roles for VLCFAs in the pathogenesis. In summary, the pathogen-responsive gene expression profiles facilitated a more comprehensive view of the molecular events that underlie this economically important plant–pathogen interaction.

**Abbreviations** – ACC, acetyl-coenzyme A carboxylase; ADH, short chain alcohol dehydrogenase protein; AGI, *Arabidopsis* genome initiative; bZIP, basic leucine zipper transcription factors; CAT, catalase; CaMBP, calmodulin-binding family protein; CDPK, calcium-dependent protein kinase; CF, culture filtrates; CFU, colony-forming units; CHS, chalcone synthase; CymMV, *cymbidium mosaic virus*; DAPI, 4′,6-diamidino-2-phenylindole; ECR, *trans*-2-enoyl-CoA reductase; EST, expressed sequence tags; FDR, false discovery rates; GFP, green fluorescent protein; GR, glutathione reductase; GST, glutathione S-transferase; HR, hypersensitive response; JA, jasmonic acid; MS, Murashige and Skoog; ORF, open-reading frame; PAMPs, pathogen-associated molecular patterns; PE, *Phalaenopsis-Erwinia*; POX, peroxidase; PR10c, pathogenesis-related protein 10c; RACE, 5′ and 3′-rapid amplification of cDNA ends; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SSH, suppression subtractive hybridization; VIGS, virus-induced gene silencing; VLCFA, very long chain fatty acid.
**Introduction**

As plants are confined to the place where they grow, they have to develop a broad range of defense responses to cope with microbial pathogen attack such as bacteria, fungi and viruses. Activation of the defense response is controlled by a complex transcriptional regulation and signal transduction network. One of the best characterized resistance responses is the gene-for-gene type of incompatible interactions (Baker et al. 1997, Jones and Dangl 2006, Collier and Moffett 2009). The response is triggered by a race/cultivar-specific recognition event where resistance depends on the recognition of an avirulence (avr) gene from the pathogen and a corresponding resistance (R) gene from the plant host (Hammond-Kosack and Parker 2003). In plant cultivars carrying R genes, the hypersensitive response (HR) is often the initial defense response to infection by pathogens. The HR results in localized cell and tissue death at the site of infection, which constrains further spread of the infection. Subsequently, the local HR can induce a long lasting systemic acquired resistance that primes the plant for resistance against a broad spectrum of pathogens (Gozzo 2003, McDowell and Woffenden 2003). In the absence of an appropriate avr–R gene combination, the pathogen is able to spread systemically and the host–pathogen interaction is compatible. The pathogenesis is not detected efficiently by the host in a compatible interaction (Nomura et al. 2005). Therefore, the activation of defense mechanisms is weak or even suppressed by the pathogens, granting successful infection and causing disease symptoms (Nomura et al. 2005, Tyler 2009).

In addition to the cultivar-specific resistance mediated by R genes, plants exhibit broader and more basal defense responses, which can be activated by a range of unspecific biotic and abiotic elicitors (Nicaise et al. 2009). Plant bacterial pathogens, and in general other pathogens, reveal themselves to the host defense system through molecules called pathogen-associated molecular patterns (PAMPs). PAMPs that act as general elicitors in plants include flagellin and lipopolysaccharides from bacteria, and chitin and ergosterol from fungi. Plants have evolved specialized cell-surface pattern recognition receptors to detect conserved features of PAMPs and activate basal defense (Hou et al. 2009). These basal defense responses include production of reactive oxygen species (ROS) and ethylene, transcriptional induction of a large suite of defense genes, including pathogenesis-related (PR) genes and post-transcriptional suppression of the auxin-signaling pathway (Navarro et al. 2006, Hou et al. 2009). The basal defense systems are generally sufficient to halt the colonization of pathogens, and minimize their ability to damage the plant’s normal growth, development and dispersal. As the activation of basal defense depends on the perception of PAMPs, it is also called PAMP-triggered immunity (Jones and Dangl 2006, Nicaise et al. 2009).

The oxidative burst, a rapid production of ROS is described as one of the earliest responses to pathogen infection and is generally associated with HR (Torres 2010). Apoplastic generation of superoxide (O$_2^-$), or its dismutation product hydrogen peroxide (H$_2$O$_2$), has been documented following recognition of a variety of pathogens at the site of attempted invasion (Torres et al. 2006). ROS have direct antimicrobial activities and can therefore reduce pathogen viability. In addition, ROS are responsible for strengthening of host cell walls via cross-linking of glycoproteins, or lipid peroxidation and membrane damage (Montillet et al. 2005, Torres 2010). It is also apparent that plant cells generate such reactive species as signaling molecules, produced at controlled levels and leading to defense responses (Asai and Yoshikoga 2008). Along with the production of ROS, plant cells deploy enzymatic and non-enzymatic antioxidative systems to facilitate the removal of such reactive molecules. The formation of ROS can be removed through the action of enzymes such as catalase (CAT), peroxidase (POX), superoxide dismutase, glutathione S-transferase (GST) and glutathione reductase (GR) (Hancock et al. 2002). The induction of an oxidative burst and the antioxidative system may work in concert, not only to adjust the level of ROS formation but also to mediate redox signal transduction during pathogenesis (Averyanov 2009).

The soft rot Erwinia, Erwinia carotovora subsp. atroseptica, E. carotovora subsp. carotovora and E. Erwinia chrysanthemi are destructive to a wide variety of plants world wide. These highly virulent necrotrophic pathogens attack a variety of host tissues, including blossoms, actively growing shoots and fruits. These pathogens are also capable of rapid systemic movement downward through stem tissue, resulting in infection and killing of rootstock crowns (Barras et al. 1994, Toth et al. 2003). Pathogenicity results from the secretion of extracellular enzymes responsible for the disorganization of the plant cell wall. These include pectin methyl esterases, pectin acetyl esterases, exo- and endopectate lyases and exopolygalacturonases (Kazemi-Pour et al. 2004). These enzymes cause maceration of plant tissues, cell separation and cell death, indicating that the pectinases are important virulence determinants (Bell et al. 2004, Zhao et al. 2005). However, no genetically defined resistance to Erwinia has been described, and the pathogen does not appear to contain typical avr genes nor does it normally cause HR in plants. Resistance to this pathogen has been observed in Solanum brevidens (Austin et al. 1988, Bains et al. 1999, Tek et al. 2004), a wild and
non-tuber bearing potato species, but the genetic basis for this resistance remains elusive. Therefore, development of resistance to this pathogen appears to rely on a generally induced defense mechanism.

The cell wall-degrading enzymes secreted by the soft rot *Erwinia* have been proposed to have dual roles: in addition to being the main virulence determinants of the pathogen, they also display elicitor properties that trigger basal defense responses (Norman-Setterblad et al. 2000, Hammerschmidt 2004). For example, treatment of tobacco plants with the cell wall-degrading enzymes has been shown to induce both local and systemic expression of several genes encoding PR proteins (Palva et al. 1993, Vidal et al. 1997, 1998). Moreover, these enzymes are able to induce defense responses both locally and systemically to decrease the susceptibility to *E. carotovora* in tobacco plants (Palva et al. 1993, Vidal et al. 1998). Similarly, *E. carotovora* culture filtrates (CF) containing cell wall-degrading enzymes and oligogalacturonides, which are the breakdown products of pectin, mediate expression of defense-related genes in non-host *Arabidopsis* plants (Norman et al. 1999, Norman-Setterblad et al. 2000). Several lines of evidence suggest that ethylene and jasmonic acid (JA) pathways may play a central role in regulating defense gene induction and resistance triggered by cell wall-degrading enzymes from *E. carotovora* (Norman et al. 1999, Norman-Setterblad et al. 2000, Brader et al. 2001, Fagard et al. 2007). In addition, a gene coding for calcium-dependent protein kinase (CDPK) from orchids was found to be induced after infection with *E. chrysanthemi* and the corresponding CF (Tsai et al. 2007). Recent study indicated that microbial siderophores secreted by *E. chrysanthemi* can modulate plant defenses through an antagonistic mechanism between SA and JA signaling cascades (Dellagi et al. 2009). Thus, the soft-rotting *Erwinia* species produce enzymes that appear to function both as factors needed for successful infection as well as indirect inducers of defenses that should stop further development of the bacteria in host tissue (Wegener and Olsen 2004).

*Phalaenopsis* spp., a monocotyledon plant species belonging to the Orchidaceae, is a commercially valuable ornamental crop for cut flowers or potted plants. Soft-rotting disease caused by *E. chrysanthemi* is one of the most devastating diseases encountered among economically important orchids (Liu et al. 2003, Sjahril et al. 2006). The outbreak of soft-rotting disease in orchid floriculture is favored by high relative humidity and temperature during cultivation or transportation to markets. The use of phytochemicals has harmful effects on environment, and therefore it is essential to control disease by having extensive information of the plant-pathogen interactions. Although the knowledge of the molecular

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**Materials and methods**

**Plant materials and inoculation with *E. chrysanthemi***

*P. amabilis* orchid at five-leaf stage (24-month-old) ‘TS97K’ (*P. amabilis* W1–10 × *P. amabilis* W1–22) was kindly provided by the Taiwan Sugar Company (Tainan, Taiwan). The plants were grown in a greenhouse under natural light and controlled conditions. The temperature ranged from 23 to 27°C, and the relative humidity was 70%. For inoculation of orchid leaves with *E. chrysanthemi*, the bacterial cells were grown overnight on Luria-Bertani liquid medium and harvested by centrifugation at 4000 g for 10 min. The bacterial pellets were resuspended in 0.9% sodium chloride solution, and then adjusted to an absorbance (600 nm) of 1.0 containing approximately 8–9 × 10^7 colony-forming units (CFU). A newly emerging leaf with approximately 10 cm in length in the orchid plant was selected for inoculation. Subsequently, the 1-cm leaf apex of the orchid plant was transversally sliced off with a sterile razor blade. The remaining part of the leaf blade was subjected to inoculation with *E. chrysanthemi*. The cross section of leaf blade was then rubbed gently with 30 μl of the bacterial suspension. After inoculation, the orchid plants were incubated for the indicated time periods at 28°C and high humidity to facilitate bacterial growth. Mock inoculation was performed in parallel by wounding the leaves without the bacterial suspension. Leaf samples containing the regions 1 cm from the excised (or inoculated) sites were collected and frozen in liquid nitrogen.
immediately. For each treatment, at least three individual leaf samples were harvested from the orchid plants.

Chemical applications and CF preparation

CF from *E. chrysanthemi* was prepared as previously described (Vidal et al. 1997). Supernatant from overnight cultures, corresponding to $10^7$ CFU ml$^{-1}$, was obtained by centrifugation of the bacterial suspension (10 min at 4000 g) and was filter sterilized. Treatment of the plants with chemicals was performed with a method similar to leaf inoculation. An aliquot (30 μl) of freshly prepared CF or methyl-JA (50 μM) was spotted onto the cross section of the leaf blade. Plant samples were collected at the indicated time after treatment.

Detection of ROS accumulation in orchid plants

Leaves of orchid plants were collected at the indicated times after inoculation with *E. chrysanthemi*. The samples were subsequently incubated in 5 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetylester (CM-H2DCF-DA, Molecular Probes, Eugene, OR) for 60 min in the dark. The samples were then washed throughly with water and visualized under a microscope equipped with a green filter (Leica MPS60 fluorescent microscope, excitation 450–490 nm; emission 500–530 nm). The period of exposure was consistent throughout the experiment.

Antioxidative enzyme activity assays

Leaf samples were collected and ground into fine powder with liquid nitrogen. The protein extract was obtained by homogenization of leaf samples in 1 M phosphate buffer pH 7.0 (2:1, fresh weight/volume). After centrifugation at 120,000 g at 4°C for 20 min, the supernatant containing soluble protein was quantified with bovine serum albumin as the calibration standard by the method of Bradford (1976). POX activity was determined according to the method described by Beyer and Fridovich (1987). The protein samples (6 μg) were loaded onto 10% native-polyacrylamide gels, and electrophoresis was conducted at 150 V for 30 min in a cold room (4°C). The gels were stained with 50 mM sodium phosphate buffer pH 7.0 containing 3,3-diaminobenzidine (0.05 ml mg$^{-1}$) and hydrogen peroxide (35%) for 5 h until the dark brown bands were visible.

RNA extraction and construction of the SSH cDNA library

Total RNA was extracted from the leaf tissues of orchid plants using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). SSH was performed with a polymerase chain reaction (PCR)-Select cDNA subtraction kit (Clontech, CA) according to the manufacturer’s instructions with some modifications. The cDNA was synthesized from total RNA with a SMART cDNA synthesis kit (Clontech) using an oligo dT primer that allowed the amplification of the complete mRNA population in each sample. The cDNA prepared from the leaves 24 h after inoculation with of *E. chrysanthemi* was used as the ‘tester’ and cDNA from the control samples was used as the ‘driver’. The tester and driver cDNA populations were digested with restriction enzyme Rsal and ligated with the specific adaptors provided in the kit. The tester pool was then hybridized separately with an excess of driver cDNA. After two rounds of hybridizations, the fragments expressed in the tester but not in the driver were specifically amplified. The products were cloned into pGEM-T vector (Promega, WI) and transformed into *Escherichia coli* strain JM109. The pathogen-induced EST clones were stored and used for PCR amplification of the cDNA inserts.

DNA sequencing and gene ontology analysis

The pathogen-induced EST clones were sequenced using the T7 promotor primer. Sequence similarity searches were performed against the National Center for Biotechnology Information database using the basic local alignment search tool (BLASTX), which compared translated nucleotide sequences with protein sequences (Altschul et al. 1997). These ESTs were named according to homologous sequences in the database. Sequences with a BLASTX e value $>10^{-5}$ were designated as having no significant similarity (unknown protein). Sequences of the unique pathogen-induced ESTs were then imported into BLAST2GO, a web-based gene ontology annotation and analysis tool for subsequent analysis (Conesa et al. 2005). This involved automated retrieval of gene ontology terms associated with the hits. The ESTs were manually assigned to functional categories based on the analysis of BLAST2GO and also with the aid of information reported by the scientific literature. The sequences of ESTs and genes described in this study have all been submitted to GenBank.

Semi-quantitative RT-PCR analysis

Total RNA was reversed transcribed into cDNA by the lmProm-II™ Reverse Transcription (RT) System with a mix of oligo (dT)$_{18}$ and (dT)$_{20}$ as primers according to the manufacturer’s manual (Promega). The sequence-specific primer pairs and recommended annealing temperatures ($T_a$) corresponding to each gene are
summarized in a table (Appendix S1). The cDNA was added to the PCR mixture containing 1 U Taq DNA polymerase (Promega), 25 mM MgCl₂, 10 mM dNTPs and 1 μM of each primer pair. The PCR conditions consisted of an initial denaturation step at 94°C for 2 min, 30 cycles of amplification (94°C for 1 min, primer-specific annealing temperature for 1 min, and 72°C for 1.5 min) and a final elongation step at 72°C for 10 min (Appendix S1). The PCR products were immediately separated by electrophoresis in a 1% agarose gel.

5′ and 3′-rapid amplification of cDNA ends
The EST fragments that contain the consensus WRKY and ECR sequences were initially isolated from the E. chrysanthemi-infected orchid plants by SSH. The full-length cDNAs of the WRKY and ECR genes were subsequently obtained by 5′ and 3′-rapid amplification of cDNA ends (RACE) using the SMARTer RACE cDNA amplification kit (Clontech) according to the manufacturer’s instructions. After RT with primers and SMARTScribe Reverse Transcriptase supplied by Clontech, the first strand cDNA was used directly in 5′- and 3′-RACE PCR. Primary PCR amplification reactions to obtain individual 5′ or 3′ sequence were achieved using a proof-reading enzyme, Advantage 2 Polymerase Mix (Clontech). The WRKY and ECR gene-specific primers (Appendix S1) were designed to generate the 5′- and 3′-cDNA fragments, respectively. The obtained cDNA ends were ligated into pGEM-T vector (Promega) and sequenced. Secondary PCR amplifications were performed to combine 5′ and 3′ sequences and generate full-length cDNAs. The full-length cDNAs of the WRKY and ECR were amplified by using primers designed from the extreme 5′ and 3′ ends. The following PCR amplification parameters were used: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 2 min, and a final elongation step of 10 min at 72°C. The full length of the WRKY and ECR genes were referred to as P. amabilis WRKY1 (PaWRKY1) and PaECR1, respectively.

Protein subcellular localization
The coding region ofPaWRKY1 cDNA sequence was amplified using gene-specific primers containing BglII and SpeI restriction sites (Appendix S1). The PaWRKY1 cDNA fragment was cloned into the pCAMBIA1302 vector at the BglII and SpeI sites. The PaWRKY1-green fluorescent protein (GFP) fusion genes were driven by the 35S promoter. The fusion construct and empty-vector control were transformed into onion epidermis cells by particle bombardment using a Biolistic PDS-1000/He device (Bio-Rad, CA). After incubation of transformed onion epidermis cells for 24 h at 24°C, GFP was detected by a fluorescence microscope equipped with a fluorescence module (Leica DMR, Leica Microsystems, Wetzlar, Germany). Nuclei were stained by adding one drop of 4',6-diamidino-2-phenylindole (DAPI) staining solution (1 μg ml⁻¹) to the onion cells. The onion epidermis cells were incubated in the dark for 15 min, and DAPI fluorescence images were obtained by UV illumination of the specimen.

DNA-binding assay in a Y1H system
The interaction of PaWRKY1 protein with the DNA regulatory element was analyzed by a yeast one-hybrid (Y1H) assay according to the manufacturer’s protocol (Clontech Yeast Protocols Handbook, BD Biosciences Clontech, CA). The open-reading frame (ORF) of the PaWRKY1 gene was amplified by PCR using the forward (PaWRKY1-Y1H-F) and reverse (PaWRKY1-Y1H-R) primers containing the digestion site of the enzymes, Ncol and BamHI, respectively (Appendix S1). The ORF of the PaWRKY1 sequence was in-frame fused with the GAL4 activation domain of the one-hybrid vector, pGADT7-Rec2, forming pGR-PaWRKY1. The target cis-acting DNA fragments harboring three tandem repeats of W box or mutated W box (mW box) elements (Appendix S1) were fused into the pHIS2.1 vector under the control of the GAL1 minimal promoter. The promoter regions (approximately 300 or 700 bps) of the orchid CDPK (GenBank: EF587760) were also amplified using the promoter-specific primers (Appendix S1) and included in the DNA-binding assay. The resulting recombinant plasmids containing the target promoter were termed pHIS-W box, pHIS-mW box, pHIS-CDPK300 or pHIS-CDPK700. Subsequently, the yeast strain Y187 was co-transformed with the pGR-PaWRKY1 and pHIS-target promoter. The yeast strains transformed with the above constructs were grown on synthetic dextrose medium (SD)/-Trp/-Leu/-His selective medium in the absence or presence of 30 mM 3-amino-1,2,4-triazole (3-AT) at 30°C for 3 days.

Construction of transgenes and Arabidopsis transformation
The coding region of PaWRKY1 was amplified using Pfu DNA Polymerase (Promega) with gene-specific primers containing a HindIII and a BglII site (in-frame ATG initiation codon) (Appendix S1). The PaWRKY1 cDNA fragment was cloned into the binary plant expression vector pCAMBIA1380 under the control of the 35S promoter. The construct was then introduced into Agrobacterium
Microarray analysis and data processing

The Arabidopsis plants were grown on the medium containing MS (Murashige and Skoog) salt (Duchefa Biochemie) and 0.3% phytagel (Sigma) in long day conditions (16-h light and 8-h dark) at 22°C. Total RNA was isolated from 1-week-old seedlings of empty-vector control and transgenic 35S-PaWRKY1 Arabidopsis plants were selected by growth on Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands, Catalog number M0222.0050) and 0.3% phytagel (Sigma, MO) supplemented with hygromycin (20 mg l⁻¹). Three independent transgenic Arabidopsis lines were generated and stably expressed the transgene PaWRKY1. The second generation of the transgenic plant lines homozygous for the transgene was used for experiments. The control Arabidopsis plants transformed with the empty vector were also generated in parallel. Characterization of the transgenic Arabidopsis plants overexpressing PaWRKY1 was performed by examining the expression of transgenes using RT-PCR with the gene-specific primers (Appendix S1).

VIGS of PaECR1 in the orchid plants

Functional analysis of orchid genes by VIGS was conducted according to the method described by Lu et al. (2007) with minor modification. The partial coding region of PaECR1 was amplified using Pfu DNA Polymerase (Promega) with gene-specific primers PaECR1-VIGS-F and PaECR1-VIGS-R (Appendix S1). A 443-nucleotide fragment corresponding to 409–851 codons of PaECR1 ORF was obtained for subsequent cloning. The transcript-derived PaECR1 fragments were first cloned into pGEM-T easy vector (Promega) and subcloned into the Smal-digested pCymMV-pro60 to construct pCymMV-pro60-PaECR1. The pCymMV-pro60 VIGS vector carrying the PaECR1 was subject to DNA sequencing to ensure the success of ligation. The empty plasmid pCymMV-pro60 served as a control. The pCymMV derivative plasmids were linearized with SpeI. Infectious RNA transcripts corresponding to the constructed vectors of Cymbidium mosaic virus (CymMV) were synthesized by in vitro transcription with mMMESSAGEMACHINE T3 kit (Ambion, TX). The transcribed RNAs were resuspended in KP buffer (1 M KH₂PO₄ and K₂HPO₄, pH 7.0) and used for inoculation. Infectious RNA was rubbed onto the second emerging leaves of 24-month-old P. amabilis plants in the presence of a small amount of carborundum. The orchid plants were grown in a growth chamber at 22°C under 16/8 light/dark cycle for 2 months before assessment of suppression of host genes and susceptibility to E. chrysanthemi infection. Inoculation of the gene-silenced leaf tissues of the orchid plants with E. chrysanthemi were similar to that described in the previous section, except that the inoculation was performed by needle punctuation (size 0.4 × 13 mm). The primer pairs (PaECR1-full) were based on the rank-product algorithm (Breitling et al. 2004) implemented in the Robin software. The Benjamini and Hochberg algorithm calculates false discovery rates (FDR) that are inherently corrected for multiple testing (Benjamini and Hochberg 1995). Genes were considered as being significantly up and downregulated if the FDR value for the corresponding probe set was smaller than 0.05. To enrich for biologically relevant changes, only genes with a minimal fold change of two in all experiments were selected. Gene ontology analysis was performed with agriGO software (http://bioinfo.cau.edu.cn/agriGO/) (Du et al. 2010). The microarray data described in this study have been deposited in the Gene Expression Omnibus and are accessible through the series accession number, (GEO:GSE227766) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20034).

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corresponding to the 5' and 3' sequence of complete PaECR1 ORF were used in RT-PCR to assess the knock-down efficiency of the host gene by VIGS (Appendix S1).

Results

Production of ROS and alteration of POX enzyme activity in orchid leaves infected with *E. chrysanthemi*

To understand the early responses of the orchid plants to *E. chrysanthemi*, the development of disease symptoms of directly inoculated leaves was monitored at different periods of time. At 3- and 6-h post-inoculation, initiation of tissue maceration was first observed at the site of inoculation (Fig. 1). At 12 h, the typical tissue maceration became more obvious and gradually spread to other parts of the leaf tissues. The progression of maceration continued throughout 24 h and tissue necrosis occurred at 48 h. To examine whether plants inoculated with *E. chrysanthemi* were producing ROS, which are an important component during pathogenesis, the generation of ROS and localization was detected at 3 h after inoculation. A significant increase in ROS generation was observed around the primary site of infection when compared with control leaves mock inoculated with buffers (Fig. 2A). The results suggested that ROS production was an early event in orchid leaves in response to infection with *E. chrysanthemi*.

To further characterize if the antioxidative enzyme was involved in the response of the leaf tissues to *E. chrysanthemi* infection, POX activity was analyzed. The enzyme activity of POX was decreased at 3-h post-inoculation in comparison with control samples (Fig. 2B). The subsequent return to basal levels of POX activity was observed at 6 and 12 h. Taken together, these results suggested that alteration of ROS levels and antioxidant systems were involved in the early response of orchid plants to *E. chrysanthemi* infection.

Isolation of pathogen-induced ESTs in orchid plants by a SSH strategy

To study the molecular basis of the orchid plants during the early stage of *E. chrysanthemi* infection, the pathogen-induced ESTs were isolated at 24-h post-inoculation through the construction of a subtractive cDNA library. The cDNA populations derived from the mock-inoculated control and *E. chrysanthemi*-infected plants served as the driver and tester, respectively. After two rounds of subtractive hybridization, the enriched cDNA fragments were obtained and ranged from about 0.5 to 1.4 kbs with most fragments distributed between 0.8 and 0.9 kbps (Appendix S2). The constructed SSH

Fig. 1. Disease symptoms induced by *Erwinia chrysanthemi* on *Phalaenopsis amabilis* leaves for different time periods. The leaves of *P. amabilis* plants at five-leaf stage (24-month-old) were inoculated with an *E. chrysanthemi* suspension containing bacteria at the concentration of 8–9 × 10⁷ CFU. The inoculated plants were kept in a growth room at high humidity (80–100%) at 28°C. Healthy leaves of the same age (Healthy) and mock-inoculated ones (Mock) from the orchid plants served as controls. The photographs were taken at different time points up to 48-h post-inoculation (hpi). The scale bar represents 1 cm. The experiments were repeated more than three times, and a single representative photograph is shown.
Fig. 2. Oxidative burst and antioxidant enzyme activity after infection with *Erwinia chrysanthemi* on leaves of *Phalaenopsis amabilis* plants. (A) The orchid leaves were inoculated with *E. chrysanthemi* pathogen and kept at 28° C. Histochemical analysis of ROS in the mock- and bacterial-inoculated leaves was performed by staining the tissues with 2,7-dichlorofluorescein. The production of ROS was visualized under a microscope with UV filters at 3-h post-treatment. The same tissues under bright-field illumination are shown on the bottom panel. The scale bar represents 0.4 mm. (B) POX activity was analyzed in the orchid leaves infected with *E. chrysanthemi*. Six micrograms of protein were loaded and separated on a 10% native-polyacrylamide gel. The gels were stained for POX activity with 3,3-diaminobenzidine and hydrogen peroxide. Similar results were obtained from two independent experiments.

Fig. 3. Functional percentage distribution of the pathogen-induced ESTs in the orchid leaves at 24-h post-inoculation with *Erwinia chrysanthemi*. The ESTs were classified into functional group based on the sequence alignment using BLASTX and BLAST2GO (Conesa et al. 2005). The vast majority of annotations were involved in oxidation reduction (15%), responses to pathogens (10%) and metabolic processes (11%) (Fig. 3). Genes encoding proteins involved in lipid metabolic processes (6%), generation of precursor metabolites and energy response to stresses (5%), response to stresses (4%), lignin biosynthetic processes (4%) and cell wall-associated enzymes (4%) formed the second largest groups. The remainder was distributed among several processes, including response to metal ions (2%), signal transduction (2%), transcription factors (2%), vitamin metabolic processes (2%), cytoskeleton (2%) and response to JA stimulus (1%). Notably, the ESTs in the category of signal transduction were characterized as 14-3-3-like protein and calmodulin-binding family protein (CaMBP) (Appendix S3). Within the category of transcription factors were found basic leucine zipper, WRKY and MYB transcriptional regulators. The category of responses to pathogens consisted of one unique ESTs with homology to PR proteins. The transcript encoding ECR (PE7) was the most abundant EST and is related to the generation of precursor metabolites and energy. Despite the necrotrophic interaction between the pathogen and plant, several of the *E. chrysanthemi*-induced transcripts represented typical pathogen-responsive genes such as chalcone synthase (CHS; PE2). Therefore, these results revealed that the early response of the orchid to *E. chrysanthemi* could be largely attributed to the library containing the pathogen-induced cDNAs was defined as the *Phalaenopsis-Erwinia* (PE) EST library thereafter. In the PE library, a total of 170 SSH clones were randomly selected for sequencing and assigned with a PE library number (Appendix S3). The mean size for the sequenced ESTs from this library was 0.53 kb. The EST fragments representing the same gene were counted as a single gene, and a total of 102 unique ESTs were obtained. The sequence data were deposited in the EST database in GenBank under accession numbers (GenBank: HO059275-HO059377) (Appendix S3). By comparison with the databases using BLASTX, sequences of the 84 unique ESTs were homologous with the published sequences in the databases. A total number of 18 unique ESTs had no significant similarity and were classified as unknown proteins (Appendix S3).

To obtain a better insight into the identity and possible functional significance of the pathogen-induced ESTs, the 84 unique EST sequences were used to annotate the function of their respective genes within the software program BLAST2GO (Conesa et al. 2005). According to gene ontology analysis, each clone was distributed into 1 of 16 functional categories related to biological processes (Appendix S3). The vast majority of annotations were involved in oxidation reduction (15%), responses to pathogens (10%) and metabolic processes (11%) (Fig. 3). Genes encoding proteins involved in lipid metabolic processes (6%), generation of precursor metabolites and energy response to stresses (5%), response to stresses (4%), lignin biosynthetic processes (4%) and cell wall-associated enzymes (4%) formed the second largest groups. The remainder was distributed among several processes, including response to metal ions (2%), signal transduction (2%), transcription factors (2%), vitamin metabolic processes (2%), cytoskeleton (2%) and response to JA stimulus (1%). Noticeably, the ESTs in the category of signal transduction were characterized as 14-3-3-like protein and calmodulin-binding family protein (CaMBP) (Appendix S3). Within the category of transcription factors were found basic leucine zipper, WRKY and MYB transcriptional regulators. The category of responses to pathogens consisted of one unique ESTs with homology to PR proteins. The transcript encoding ECR (PE7) was the most abundant EST and is related to the generation of precursor metabolites and energy. Despite the necrotrophic interaction between the pathogen and plant, several of the *E. chrysanthemi*-induced transcripts represented typical pathogen-responsive genes such as chalcone synthase (CHS; PE2). Therefore, these results revealed that the early response of the orchid to *E. chrysanthemi* could be largely attributed to the
molecular networks involved in redox homeostasis, signal transduction, transcriptional regulation, energy, metabolism and responses to pathogens.

Expression validation of the pathogen-induced ESTs by semi-quantitative RT-PCR

A subset of genes were selected for semi-quantitative RT-PCR based on their putative functions and the results of the SSH differential screening. The expression analysis was to confirm the validity of the pathogen-induced ESTs and also to determine more precise timing of expression for selected genes of interest. The steady-state transcript level of ECR (PE7) and WRKY transcription factor (PE99) increased at 6-h post-inoculation and remained elevated for 48 h (Fig. 4A). To gain more insight into the roles of the pathogen-induced ESTs, the expression of the ECR and WRKY genes in response to various defense signaling molecules was investigated. The leaves of the orchid plants were treated with wounding, defense signaling molecules such as JA and CF or inoculated with E. chrysanthemi. As expected, a significant increase in the expression levels of the ECR and WRKY genes was observed at 24 h after inoculation (Fig. 4B). The results indicated that the expression of the ECR and WRKY genes was mainly influenced by the infection with E. chrysanthemi instead of wounding. Treatment of orchid leaves with CF or JA had subtle effects on the expression of the two genes (Fig. 4B). The results suggested that the induced expression of the pathogen-induced ECR and WRKY genes was specific to infection with E. chrysanthemi.

A total of 10 additional pathogen-induced ESTs corresponding to a range of functional categories were also selected for characterization (Appendix S4). The most prominent classes were those involved in oxidation reduction, including short chain alcohol dehydrogenase protein (ADH; PE5), methylenetetrahydrofolate reductase (MTHFR; PE9) and GST (PE26). The genes encoding protein with putative functions in responses to pathogens such as PR protein 10c (PR10c; PE1), CHS (PE2) and PDR-like ABC transporter (ABC; PE19) were selected. The expression of acetyl-coenzyme A carboxylase (ACC; PE97) and caffeic acid O-methyltransferase (COMT; PE12) belonging to the categories of energy and lignin biosynthetic processes were examined. The ESTs related to signal transduction (CaMBP; PE114) and basic leucine zipper transcription factors (bZIP; PE61) were also subjected to expression analysis. The gene expression
designated as PaWRKY1. The nucleotide sequence of 748 nucleotides in length was obtained and subsequently isolated using RACE. A full-length cDNA clone with 36 kDa and a calculated isoelectric point of 9.46. The protein of 310 residues with an approximate molecular mass of 9.72. The full-length PaECR1 transcript encoded a protein of 170 amino acids with a predicted molecular weight of 20 kDa and an isoelectric point of 9.72. The full-length PaECR1 transcript encoded a protein of 310 residues with an approximate molecular mass of 36 kDa and a calculated isoelectric point of 9.46. The gene sequences encoding PaWRKY1 and PaECR1 were deposited in GenBank under the accession numbers (GenBank: HM596074 and HM596076, respectively).

Sequence analysis and comparison with data banks confirmed the high similarity and identity of this orchid PaWRKY1 protein to members of group IIc WRKY proteins of higher plants (Eulgem et al. 2000). The PaWRKY1 protein sequence showed significant homology to Triticum aestivum TaWRKY (Mangelsen et al. 2008), Solanum tuberosum StWRKY1 (Dellagi et al. 2000), Fragaria ananassa FaWRKY1 (Encinas-Villarejo et al. 2009), Arabidopsis thaliana AtWRKY75 (Devaiah et al. 2007) and Brassica napus BnWRKY75 (Yang et al. 2009) with percentages of identity ranging from 77 to 86%. The multiple sequence alignment of PaWRKY1 with members of the group IIc WRKY proteins in plants was generated (Fig. 5A). There was little sequence conservation at the N-terminal region among the WRKY proteins. The C-terminal part of the coding regions was highly conserved and contained signature motifs characteristic of WRKY transcription factors. The PaWRKY1 protein consisted of one putative WRKY domain, together with a potential nuclear localization signal and a zinc finger-like motif in the C-terminal region (C-X4-C-X23-H-X1-H) (Fig. 5A). In addition, amino acid sequence alignment of PaECR1 with orthologs from Gossypium hirsutum (Song et al. 2009), A. thaliana, Oryza sativa, Nicotiana benthamiana (Park et al. 2005) and Vitis vinifera was generated (Fig. 5B). The PaECR1 protein shared a high degree of sequence identity (approximately 70%) with the ECRs from other plant species. The PaECR1 contains 82 amino acids at the C-terminal region with homology to steroid-5-α-reductase, which catalyzes the reduction of testosterone to dihydrotestosterone (Kohlwein et al. 2001). The functional residues on the enzyme active sites such as Glutamine 89, Lysine 144 and Arginine 145 (Paul et al. 2007) are conserved in the orchid PaECR1. A putative NADPH-binding domain (Song et al. 2009) is also present in the PaECR1.

Subcellular localization and DNA-binding activity of the PaWRKY1 transcription factor

To determine the subcellular localization of the PaWRKY1 protein, the GFP gene was fused to PaWRKY1 under the control of the Cauliflower mosaic virus 35S promoter (Fig. 6A). In the transient expression assay, 35S-PaWRKY1-GFP and an empty-vector control were introduced into onion epidermal cells by particle bombardment. The cells were stained with DAPI to reveal the nuclei and then examined using a fluorescence microscope. GFP signals of 35S-PaWRKY1 were detected in the nuclei of onion cells (Fig. 6A), whereas the empty-vector controls were uniformly distributed throughout the epidermal cells (data not shown). These data thus indicate that PaWRKY1 is a nuclear protein that may act as a transcription factor to regulate the expression of downstream genes.

WRKY transcription factors are thought to function by binding their cognate TGTGAC/T W-box cis-elements in the promoter regions of target genes and activating or repressing their expression (Rushton et al. 2010). To examine the DNA-binding capacity of PaWRKY1 for binding to the W-box elements, a series of Y1H assays were performed. Two DNA fragments containing W-box sequences were generated; one had a triple tandem repeat of the wild-type W box (W box; TTGAC) and the other had the repeat of an mW box (TTGAA). In addition, two promoter-containing fragments (300 and 700 bps in length) derived from an orchid gene encoding a CDPK (PaCDPK) (Tsai et al. 2007) were included in the experiment. Detection of protein–DNA-binding activity by growth performance of the yeast cells showed that PaWRKY1 possessed specific DNA-binding ability to the promoter of wild-type W box, but not to those of mutated W box and PaCDPK (Fig. 6B). The result indicated that the PaWRKY1 was able to recognize and interact with the W-box elements.

Potential regulatory roles of the pathogen-induced PaWRKY1

To better characterize PaWRKY1-mediated gene regulatory networks, the transgenic Arabidopsis plants

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Fig. 5. Amino acid sequence alignment and conserved domains of PaWRKY1 and PaECR1. (A) The deduced PaWRKY1 (HM596075) polypeptide was aligned with five closely related group IIc WRKY proteins in plants, including Triticum aestivum TaWRKY (ABN43184), Solanum tuberosum StWRKY 1 (CAB97004), Fragaria ananassa FaWRKY1 (ACH88751), Arabidopsis thaliana AtWRKY75 (NP_196812) and Brassica napus BnWRKY75 (AC114409). The black boxes indicate identical residues and gray boxes indicate conservative substitutions. Hyphens indicate gaps introduced to optimize alignments. The numbers on the left indicate the amino acid residues in the PaWRKY1 protein sequence. The WRKYGQK peptide stretch is highlighted in red. Putative nuclear localization signals are indicated with black stars under the sequences. The two conserved cysteine and histidine residues making up the potential zinc finger motif are marked with green. The black triangles and circles indicate amino acids conserved within the motif A([K/R]EPRVAV[Q/K]T[K/V]SEVD[I/V]L) and motif 3 (KAKKxxQK) described for subgroup IIc of the WRKY family, respectively (Eulgem et al. 2000). The alignments were generated by the BOXSHADE website (http://www.ch.embnet.org/software/BOX_form.html). (B) Amino acid sequence alignment of PaECR1 (GenBank accession number HM596076) with orthologs from Gossypium hirsutum (ABV60089), A. thaliana (NP_191096), Oryza sativa (NP_001042030), Nicotiana benthamiana (AAY17262) and Vitis vinifera (XP_002283594). The ECR protein sequences with homology to steroid-5-α-reductase domain are underlined. Blue shades mark the residues identified as critical for function of fatty acid elongation (Paul et al. 2007). A putative NADPH-binding domain at the C-terminal region (Song et al. 2009) is denoted with squares. Physiol. Plant. 145, 2012
were generated by constitutive expression of the PaWRKY1 gene under the control of the 35S promoter. Subsequently, the transgenic Arabidopsis plants harboring the 35S-PaWRKY1 construct were characterized by analyzing overexpression of the PaWRKY1 transgene using semi-quantitative RT-PCR. The transcripts of PaWRKY1 accumulated to a significant level in the transgenic Arabidopsis plants when compared with the control plants transformed with the empty vector (Appendix S5). The PaWRKY1-overexpressing transgenic lines did not exhibit any apparent phenotypic abnormalities compared with vector control plants (data

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not shown). The PaWRKY1-overexpressing Arabidopsis plants were subjected to microarray analysis using the Arabidopsis GeneChip ATH1. The gene expression profiles of transgenic Arabidopsis plants expressing PaWRKY1 were compared with those of vector control plants.

As a result, the expression levels of 50 genes were upregulated more than twofold in the transgenic PaWRKY1 Arabidopsis when compared with those in vector control plants. In contrast, the gene expression levels of seven genes were downregulated less than 0.5-fold (Table 1). According to the gene ontology analysis by agrigo (Du et al. 2010), the largest functional categories involved in the biological processes were those in response to stimuli (Appendix S6). A gene encoding cell wall bound POX was significantly downregulated in orchids in response to stimuli (Fig. 7B). At 14-h post-inoculation with E. chrysanthemi, the pathogen-induced PaECR1 gene expression was predominately induced by E. chrysanthemi infection in the leaf tissues. To better characterize the function of PaECR1 in the response to E. chrysanthemi infection, the orchid leaf tissues with suppression of endogenous PaECR1 transcripts were generated by VIGS. The PaECR1 cDNA fragment containing the partial sequence of ORF was cloned into the CymMV-based VIGS vectors (Fig. 7A). The leaf tissues of the orchid plants were inoculated with the resultant CymMV-PaECR1 construct to trigger VIGS. The effect of VIGS on endogenous PaECR1 mRNA levels was examined by semi-quantitative RT-PCR using leaf RNA samples (Fig. 7B). At 14-h post-inoculation with E. chrysanthemi, the pathogen-induced PaECR1 gene expression was compromised in the PaECR1-silenced orchid lines in comparison with the empty-vector control plants. Interestingly, the leaf tissues from the PaECR1-silenced orchid lines were more susceptible to E. chrysanthemi infection than that of vector control plants according to the CFU (Fig. 7B). The progression of maceration symptoms caused by E. chrysanthemi was more rapidly in the PaECR1-silenced lines than that of control plants (data not shown). The data indicated that E. chrysanthemi was able to accumulate to higher level in the leaf tissues of PaECR1-silenced orchid in comparison with that of empty-vector control. The relatively high susceptibility to E. chrysanthemi in PaECR1-silenced leaf tissues were consistent and observed in other VIGS-PaECR1 orchid plant lines (Fig. 7B). The results demonstrated the functional significance of PaECR1 in the orchid plants when infected with E. chrysanthemi.

Discussion

Understanding the complex transcriptional changes occurring in orchids in response to soft rot Erwinia is important for efficient management of this pathogen. Transcriptomics is a powerful approach for the global analysis of plant–pathogen interactions (Fekete et al. 2009, Wang et al. 2009, Avrova et al. 2003, Sarowar et al. 2011). To gain further information on the molecular basis in orchids upon E. chrysanthemi infection, this study describes the first large-scale investigation into the pathogen-induced genes during the host–pathogen interaction. SSH strategy was conducted to establish an EST library enriched for genes expressed in the orchid plants at the early stage of infection. A total of 102 unique pathogen-induced ESTs were successfully generated and annotated to different functional categories, especially those associated with redox regulation, responses to pathogens, metabolic process and lipid synthesis. Expression profiles of the selected ESTs were consistent with the SSH-based analysis, further strengthening the reliability of the results. As anticipated, several of the pathogen-induced ESTs were related to general responses of plants to pathogen attack such as PR10c, CHS and cell wall-associated enzymes (Appendix S3). On the basis of the functional analysis of PaECR1 gene, it is tempting to speculate that derivatives of the very long chain fatty acid (VLCFA) biosynthesis pathway may participate in the regulation of the pathogenesis. Taken together, these data may reflect an intricate signaling system, involving a number of signal compounds and interacting signal pathways, regulating the expression of pathogen-responsive genes in the orchid plants.

Necrotrophic pathogens appear to stimulate ROS accumulation so as to trigger host cell death and allow the pathogens to access nutrients, contributing to survival and disease development. In pear leaves, inoculation with E. amylovora resulted in superoxide accumulation, lipid peroxidation and electrolyte leakage (Torres 2010). In accordance with the report, collapse and maceration of the orchid cells induced by E. chrysanthemi.
Table 1. List of selected genes changed transcriptionally in transgenic PaWRKY1 Arabidopsis plants. The gene expression profiles of transgenic 35S-PaWRKY1 Arabidopsis were compared with those of control plants transformed with the empty vector. On the basis of microarray analysis, genes with expression level that is more than twofold higher or less at an FDR below 0.05 are shown. Rank products analysis reveals that most significantly differential expressed genes display the lowest RP value. AGI, Arabidopsis genome initiative; FC, fold change; RP/Rsum, rank product; FDR, false discovery rate.

<table>
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<th>AGI number</th>
<th>Product encoded</th>
<th>FC</th>
<th>RP/Rsum</th>
<th>FDR</th>
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<td>AT5G48570</td>
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<td>2.16</td>
<td>183.78</td>
<td>0.0135</td>
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Table 1. Continued

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<th>Product encoded</th>
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<th>RP/Rsum</th>
<th>FDR</th>
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<tr>
<td>AT5G15960</td>
<td>KIN1 cold and ABA inducible protein</td>
<td>2.52</td>
<td>184.91</td>
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<td>AT4G24960</td>
<td>Eukaryote-specific ABA- and stress-inducible gene</td>
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<td>AT2G34810</td>
<td>FAD-binding domain-containing protein</td>
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<td>300.86</td>
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<td>AT1G73330</td>
<td>Drought-repressed 4</td>
<td>0.49</td>
<td>137.74</td>
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was preceded by accumulation of ROS (Figs 1 and 2). Alteration in POX activity has been reported in various plant–pathogen interactions. POX activity is expected to reduce the level of ROS by metabolizing hydrogen peroxides, but POX is also capable of various ‘oxidase’ reactions leading to ROS generation (Nanda et al. 2010). In this study, the necrotrophic bacterium, *E. chrysanthemi*, induced in orchids a rapid production of ROS and a concomitant alteration in antioxidative systems (Fig. 2). Accumulation of ROS was detected mainly at the site of inoculation during the early stage of pathogenesis. Especially, the build-up of ROS levels was in correlation with the decrease in POX activity during the early stages post-inoculation (Fig. 2). The results suggested that the initial effect of infection was the reduction of POX activity resulting in the increase in ROS production. Furthermore, a subset of pathogen-induced ESTs associated with the oxidation regulation was also represented in the library. Among them there are genes that encode antioxidant enzymes such as CAT, GST and GR (PE40, PE26 and PE89; See Appendix S3). The abundance of antioxidant enzymes in the library indicated that the redox status in the attacked orchid plants had been disturbed. One pathogen-induced EST encoding an enzyme with homology to lipoxygenase (LOX, PE163; Appendix S3) was also isolated, suggesting a role of lipid peroxidation in the orchid–*Erwinia* interaction (Porta and Rocha-Sosa 2002). Taken together, the ROS production, POX activity and antioxidant-related ESTs provide information on an alteration in redox status that might coordinate regulation of molecular responses during the orchid–*Erwinia* interaction.

Upon infection of host plants by pathogens, a complex network of signaling components and transcription factors relays the information in the plant cell to the nucleus, where specific transcriptional responses is triggered. Members of several transcription factor families (bZIP, WRKY, MYB, ERF and Whirly) have been shown to participate in the regulation of plant responses to pathogens (Eulgem 2005). Several lines of evidence have indicated the potential involvement of WRKY family transcription factors in plant–*Erwinia* interactions. For example, *Erwinia*- or CF-induced expression of WRKY genes was observed in various plant species such as potato, apple and *Arabidopsis* (Dellagi et al. 2000, Li et al. 2004, Baldo et al. 2010, Sarowar et al. 2011). Dual functionality has also been suggested for WRKY transcription factors. *Arabidopsis* plants overexpressing AtWRKY41 exhibited enhanced resistance toward virulent *Pseudomonas* but decreased resistance toward *E. carotovora* (Higashi et al. 2008). This study described a full-length orchid gene (PaWRKY1) encoding a protein with sequence homology to members of the plant WRKY family (Fig. 5). Functional analysis of PaWRKY1 in the orchid plants was performed to address its role in the pathogenesis. However, the pCymMV-PaWRKY1 recombinant viruses were unable to trigger VIGS in the orchid plants (data not shown). This newly characterized gene is the first WRKY factor described in orchids and belongs to group IIC of the WRKY family. Nuclear localization and a DNA-binding activity assay indicated that PaWRKY1 is a functional protein characteristic of WRKY transcription factors (Fig. 6). The closest sequence homologs of PaWRKY1, the potato StWRKY recombinant viruses were unable to trigger VIGS in the orchid plants (data not shown). These findings collectively suggest a regulatory role for group IIC proteins of the WRKY family in plant–*Erwinia* interactions.

VLCFAs (fatty acids with chain lengths ranging from 20 to 36 carbons) and their derivatives are essential biological components found in lipid, suberin and cuticular waxes in plants (Raffaele et al. 2009). The role of VLCFAs in plant responses to *Erwinia* infection has not been reported previously. In this study, analysis of gene expression demonstrated that a number of genes encoding proteins involved in the VLCFA biosynthesis pathway were induced in orchid plants during infection by *E. chrysanthemi*. Two genes coding for key enzymes...
that catalyze the intermediate steps of VLCFA biosynthesis, ACC and ECR (Roudier et al. 2010), were represented in the pathogen-induced EST library (PE7 and PE97; Appendix S3). In particular, the orchid ECR was found to be the most abundant transcripts in the library suggesting a role for VLCFAs in the host response to *Erwinia* infection. Gene expression validation of the orchid ACC and ECR confirmed the induction of these genes in response to *E. chrysanthemi* infection (Fig. 4; Appendix S4). Noticeably, functional analysis of *PaECR1* gene in the orchid plants clearly demonstrated its roles in defense responses against *E. chrysanthemi* (Fig. 7). Indeed, recent findings indicate that the VLCFA biosynthesis pathway has been associated with plant defense through different aspects (Raffaele et al. 2009). VLCFAs contribute to biosynthesis of the plant cuticle and the generation of sphingolipids. The plant cuticle is believed to provide an efficient barrier against the majority of pathogens that colonize the plant surface and it represents a passive defense (Reina-Pinto and Yephremov 2009). Sphingolipids are key components of the membrane and may also serve as signaling molecules during plant–pathogen interactions. Accumulating evidence implies that sphingolipids play a crucial role in plant innate immunity including triggers of programmed cell death and elicitor-induced defense mechanisms (Takahashi et al. 2009). Therefore, this study implies that the orchid ACC and ECR may lead to alteration in cuticle structure or sphingolipid synthesis, which in turn contributes to a defense response against *E. chrysanthemi*. ECR is required for the synthesis of the VLCFA-containing lipids that is in association with deception for pollinator attraction in the orchid family (Schlütter et al. 2011). The finding in this study also revealed the unique orchid–*Erwinia* interaction mediated by the pathogen-inducible *PaECR1*.

It has been demonstrated in *Nicotiana benthamiana* and *Arabidopsis* that disruption of the ECR genes affected the VLCFA-mediated cell expansion during plant morphogenesis (Park et al. 2005, Zheng et al. 2005). Analyses of the *Arabidopsis* ECR mutants also provide *in planta* evidence that the ECR is involved in VLCFA biosynthesis for the production of wax, seed storage triacylglycerols and sphingolipids. Accordingly, it was speculated that silencing of *PaECR1* gene in the orchid leaf tissues would lead to alteration of VLCFA-derived metabolites such as wax and sphingolipids. It is reasonable to assume that epicuticular wax composition was responsible for the altered interaction with the pathogens, but it is also possible that lipids derived from the VLCFA biosynthesis pathway may increase the susceptibility of the orchid plants to *E. chrysanthemi* infection.

In conclusion, the host transcriptional responses of the orchid plants were initially triggered either by *E. chrysanthemi* attack or by its corresponding elicitor, which contained cell wall-degrading enzymes. During the early stage of pathogenesis, the increase in ROS production occurred at the site of infection. Alteration in cellular redox homeostasis through the antioxidant enzyme systems (i.e. CAT, GR, GST and POX) might contribute to activation of signal transduction. The pathogen-induced signals may affect activity of
transcription factors, including bZIP, MYB and WRKY. The ECR-mediated VLCFA biosynthesis pathway is potentially involved in the defense responses and unique in the orchid–Erwinia interaction. In addition, several genes whose homologs were detected during this study have not been characterized for their possible roles in the pathogenesis. Such genes could account for differences in gene expression specific to the orchid plants and need to be examined further. The determination of the changes in VLCFA-derived metabolites is required to get more insight into the pathogenesis. The findings in this study provide valuable information not only in understanding overall gene expression in orchids during E. chrysanthemi infection, but also in identifying potential targets for development of disease control strategies.

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contributes to antibacterial resistance by repressing auxin signaling. Science 312: 436–439


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. List of primers and expected lengths of PCR products described in this study.

Appendix S2. Electrophoresis analysis of the subtracted cDNA fragments from the orchid leaves after infection with Erwinia chrysanthemi.

Appendix S3. List of early pathogen-induced transcripts in Phalaenopsis amabilis plants infected by Erwinia chrysanthemi.

Appendix S4. Validation of representative pathogen-induced ESTs by semi-quantitative RT-PCR analysis.

Appendix S5. Characterization of transgenic Arabidopsis plants overexpressing the orchid PaWRKY1 gene.

Appendix S6. Gene ontology categorization of the differentially expressed genes in transgenic Arabidopsis plants overexpressing the orchid PaWRKY1 gene.

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