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Production of cecropin A in transgenic rice plants has an impact on host gene expression

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Summary

Expression of the *cecropin A* gene in rice confers resistance to the rice blast fungus *Magnaporthe oryzae*. In this study, a polymerase chain reaction-based suppression subtractive hybridization approach was used to generate a cDNA macroarray from the elite japonica rice (*Oryza sativa* L.) cultivar 'Senia'. Gene expression studies revealed that the expression of components of the protein secretory and vesicular transport machinery is co-ordinately activated at the pre-invasive stage of infection of rice by the blast fungus. Comparisons of gene expression between wild-type and *cecropin A* plants revealed the over-expression of genes involved in protection against oxidative stress in transgenic plants in the absence of the pathogen, which correlated well with the tolerance of these plants to oxidative stress. A subcellular fractionation analysis suggested that cecropin A accumulates in the endoplasmic reticulum in *cecropin A* rice. Moreover, a large number of genes related to the processes of synthesis, folding and stabilization of proteins that enter into the secretory pathway are over-expressed in *cecropin A* rice, confirming that these plants constitutively express the unfolded protein response. Transgenic expression of *cecropin A* in rice has an effect on the transcriptional reprogramming that accompanies plant adaptation to fungal infection. Overall, this study provides evidence for transgene-induced changes in gene expression in *cecropin A* rice under both optimal growth conditions and stress conditions imposed by fungal infection. The data also indicate that resistance to blast in *cecropin A* rice may be the consequence of a combination of the antifungal activity of cecropin A and *cecropin A*-mediated over-expression of rice genes.

Keywords: blast, cecropin, endoplasmic reticulum, macroarray, *Oryza sativa*, oxidative stress, plant defence.

Introduction

Plant diseases caused by pathogens are responsible for huge annual losses in cultivated crops. Rice blast, caused by the fungus *Magnaporthe oryzae* (Hebert) Barr (anamorph *Pyricularia grisea* Saccardo), is one of the most devastating diseases of cultivated rice (*Oryza sativa*) worldwide (Ou, 1985). Rice blast infections are initiated by the spores of *M. oryzae*, which are spread by splash dispersal and adhere tightly to the rice leaf cuticle. The spores germinate quickly and, within 8 h, each can form a specialized infection structure, called an 'appressorium'. This fungus has been described as a hemibiotrophic pathogen that maintains an initial biotrophic relationship with its host (Talbot, 2003). The biotrophic stage of the rice–*M. oryzae* interaction is critical for the establishment of pathogenesis, and determines whether or not the pathogen will be successful in its colonization attempts. After the initial asymptomatic phase, which lasts for up to 3 days, *M. oryzae* causes large, necrotic disease lesions on rice leaves. In severe cases, the fungus can cause the whole seedling to die, whereas, in older plants, it can prevent grain filling or destroy the grain-bearing structures of the plant (Talbot, 2003). Breeding for durable resistance to this fungus seems to be difficult because of the

highly dynamic manner in which the blast pathogen population responds to a resistant rice cultivar. Fungicides are commonly used to control blast. However, the repeated use of hazardous agrochemicals for the control of this disease has several drawbacks, such as their lack of specificity, increased incidence of the development of resistance on prolonged application and the adverse impact on human health and the environment. The development of rice cultivars with durable resistance is one of the main objectives in rice breeding programmes. In this respect, plant genetic engineering provides an opportunity to introduce genes conferring resistance to the rice blast fungus into cultivated rice varieties.

Different genetic strategies have been used to generate disease-resistant plants, including the utilization of antimicrobial genes of both plant and non-plant origin (Broglie *et al*., 1991; Lorito *et al*., 1998; Datta *et al*., 1999). The outcome has varied, but the resistance obtained on introduction of a single plant antifungal gene, i.e. *chitinase* or *β-1,3-glucanase* gene, does not support the production of new disease-resistant varieties suitable for commercial agriculture. One of the main limitations is the relatively low level of resistance and the narrow spectrum of protection obtained with a single plant antimicrobial gene. In contrast, the expression of genes encoding antimicrobial peptides of animal, fungal or bacterial origin in transgenic plants has been proven to confer high levels of protection and a broad spectrum of resistance against pathogens (Lorito *et al*., 1998; Emani *et al*., 2003; Coca *et al*., 2004; de las Mercedes Dana *et al*., 2006). No negative effects on plant phenotype have been observed in many of these disease-resistant transgenic plants. However, the expression of foreign genes in transgenic plants may potentially lead to alterations in endogenous gene activities that are not detected during the phenotypic or agronomic evaluation of transgenic plants. Although the benefits of transgenic approaches in enhancing protection against pathogens have been repeatedly demonstrated, a deeper understanding of the potential changes in host gene expression that may be induced by transgenesis is still lacking. Equally, studies to determine whether the transgenic expression of an antimicrobial gene affects the natural inducible plant defence responses under infection conditions are needed. The information gained will be very important in adding to our understanding of 'substantial equivalence' between transgenic and wild-type plants.

Recently, the stable transformation of an elite japonica rice (*O. sativa* L. cv. 'Senia') with a plant codon-optimized synthetic *cecropin A* gene from the Cecropia moth, *Hyalophora cecropia*, has been reported (Coca *et al*., 2006). Constitutive expression of the *cecropin A* gene in rice confers high levels of protection against the rice blast fungus *M. oryzae*. The expression of the synthetic *cecropin A* gene is stable for at least four generations, and does not cause any effect on plant phenotype when grown under controlled glasshouse conditions. In this work, the possibility of *cecropin A*-induced alterations in host gene expression is explored. Our research objectives were twofold: (i) to determine whether transgenic expression of the *cecropin A* gene has an impact in the host plant, in terms of gene expression, under non-infection conditions; and (ii) to investigate whether transgenic expression of *cecropin A* has an effect on the pathogen-inducible rice defence response during interaction of the transgenic plant with the blast fungus. To accomplish these objectives, a polymerase chain reaction-based suppression subtractive hybridization (PCR-SSH) approach was used to construct a cDNA library from the rice cultivar Senia (cultivar used for the transgenic expression of the *cecropin A* gene; Coca *et al*., 2006). This cDNA library was employed to fabricate a rice macroarray containing 6144 rice expressed sequence tags (ESTs), which was then used in studies of gene expression in both transgenic and non-transgenic rice plants under optimal growth conditions and under stress conditions imposed by fungal infection. Evidence for transgene-induced changes in endogenous gene activities in *cecropin A* rice plants is reported.

Results

In order to investigate whether transgenic expression of the *cecropin A* gene in rice has an effect on host gene expression, the response of rice plants to *M. oryzae* infection was characterized. This study was carried out in the japonica rice cultivar Senia. Although many reports in the literature have described the induction of rice genes in response to infection by the rice blast fungus, most of these studies have been conducted at the colonization stage of the rice–*M. oryzae* interaction (necrotrophic lifestyle) (Kim *et al*., 2001; Rauyaree *et al*., 2001; Xiong *et al*., 2001; Lu *et al*., 2004). In this work, the rice response was investigated at the early stages of the rice–blast interaction, namely at the pre-invasion stage. To accomplish our objective, a PCR-SSH approach in combination with cDNA macroarray technology was used for the identification of genes that are up-regulated during the very early phases of the rice–blast interaction (3–48 h). The macroarray containing the subtracted cDNA library was then used in comparative analyses of gene expression between *cecropin A* and wild-type plants under normal growth conditions, as well as during the interaction of the transgenic plants with the rice blast fungus.

Construction of the rice subtracted library and macroarray analysis

The PCR-SSH Senia library was obtained by the subtraction of cDNA populations derived from *M. oryzae*-infected leaves from the cDNA representing uninfected leaves from Senia plants. Leaf tissues were collected at 3, 6, 9, 24 and 48 h after inoculation with *M. oryzae* spores. For each time point, leaf tissues from control, mock-inoculated leaves were also collected. Tester (samples of infected leaves) and driver (samples from control, non-infected leaves) cDNAs were prepared from a pool of at least three independent RNA preparations for each condition. Before using the RNA samples for the construction of the subtracted library, the expression of the *PR1a* gene, a widely used indicator for the induction of plant defence responses, was confirmed (results not shown). Two rounds of SSH were carried out to construct the fungal-induced, transcript-enriched library. Following PCR amplification of cDNA inserts, the PCR products were checked by agarose gel electrophoresis (results not shown). The average insert size was approximately 450 bp, ranging from 150 to 800 bp. A total of 6144 cDNA inserts, or ESTs, representing independent clones of the subtractive library and the internal controls of the macroarray, were spotted on to nylon membranes using robotic printing.

Differential gene expression was assessed by hybridizing the macroarray with ^{33}P-labelled cDNAs prepared from RNAs obtained from either non-infected or *M. oryzae*-infected rice leaves at 3 and 6 h after inoculation with fungal spores. Gene expression data at 48 h after infection were also obtained to assess the expression of marker genes or the rice response to blast infection. Thus, pair-wise comparisons were performed in which each fungal-infected condition (3, 6 or 48 h) was compared with each non-infected condition (3, 6 or 48 h). Two independent cDNA preparations and two replica membranes were made for each infection time. After scanning and global normalization, the signal intensity and expression ratio (infected vs. non-infected) were determined.

Identification of genes differentially expressed at the pre-invasive stage of rice infection with the rice blast fungus

Based on the differential screening of the 6144 ESTs arrayed in the rice macroarray, 590 ESTs showing an expression ratio equal to or greater than two (infected vs. non-infected) in one or more of the three comparisons [3, 6 or 48 h post-inoculation (pi)] were identified. None gave a positive signal on hybridization of the macroarray with a ^{33}P-labelled genomic

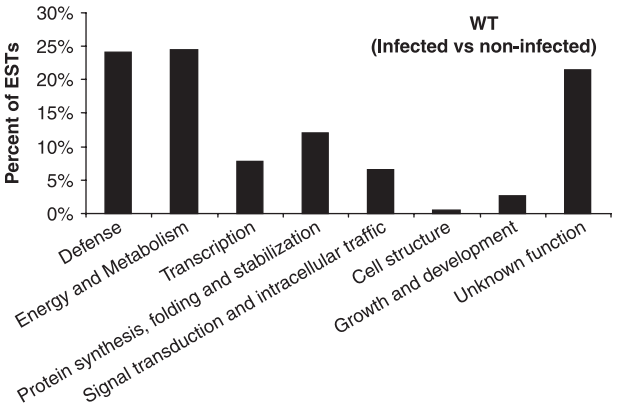

Figure 1 Functional classification of rice expressed sequence tags (ESTs) that are up-regulated in response to infection by the blast fungus *Magnaporthe oryzae* in wild-type rice plants (cv. Senia). The *y*-axis indicates the percentage of ESTs grouped in each category. Unigenes were categorized using the GenBank and KOME databases.

DNA from *M. oryzae* (results not shown). Nucleotide sequence analysis of 331 of the 590 ESTs showing differential expression generated 162 unique genes which were grouped into eight gene functional categories: (i) defence; (ii) energy and metabolism; (iii) transcription; (iv) protein synthesis, folding and stabilization; (v) signal transduction and intracellular traffic; (vi) cell structure; (vii) growth and development; and (viii) unclassified or unknown function (Figure 1; Table 1). The largest sets of ESTs were assigned to the categories of energy and metabolism, and disease/defence (24.5% and 24.2%, respectively). Some of the ESTs identified by differential screening were redundant, redundancy being defined as the presence of either several cDNA fragments corresponding to a particular gene, or several copies of a given cDNA fragment for that gene. Redundancy found in each functional category varied greatly, with the highest percentage of redundancy found in the defence and metabolism categories. Genes with most ESTs most probably represent genes that are abundantly expressed during *M. oryzae* infection and/or genes that are up-regulated at the various times of infection used for the construction of the subtracted library. Of the 162 unique genes showing differential expression, 142 unique genes were transcriptionally activated as early as 3–6 h after inoculation.

The defence gene category accounted for 24.2% of the differentially expressed ESTs encoding proteins that are considered to be part of the general plant defence response as they are induced in many different plant–pathogen systems (Table 1). Several pathogenesis-related (*PR*) genes, such as *PR1*, *β-1,3-glucanase, thaumatin-like protein, cysteine proteinase inhibitor, PR10* and *lipid transfer protein* genes,

Table 1 Rice genes up-regulated by *Magnaporthe oryzae* infection, sorted according to functional category

Description*	Accession number†	Number of ESTs‡	Expression ratio§
1. Disease/defence			
Ascorbate peroxidase (APX), thylakoid-bound	AB114856	4	2.7 (3 h)
β-1,3-glucanase	AK098943	1	3.0 (48 h)
Carbonic anhydrase 3	AF182806	4	4.1 (3 h), 2.0 (48 h)
Catalase isozyme A	X61626	1	3.2 (3 h)
Copper chaperone homologue	AP008214	1	2.4 (3 h), 2.7 (48 h)
Cysteine proteinase inhibitor	AK119511	1	2.2 (3 h)
Defender against apoptotic death 1 protein (DAD1)	D89727	1	4.4 (3 h)
Glycolate oxidase	AF022740	1	2.1 (3 h)
Glyoxalase I	AB017042	1	2.7 (3 h)
Glycine-rich protein (GRP 0.9)	X54449	30	3.1 (3 h), 2.1 (6 h), 2.6 (48 h)
Lipid transfer protein (LTP IV)	AK070414	1	2.3 (3 h)
Lipid transfer protein precursor (LTP2)	U31766	2	2.0 (3 h), 2.7 (6 h)
Lipid transfer protein, b1	X83434	6	2.3 (6 h)
Metallothionein-like type 1 (OsMT1)	U43529	2	2.0 (48 h)
Oryzacystatin (cysteine protease inhibitor)	S49967	6	2.5 (3 h), 2.4 (6 h), 2.2 (48 h)
Pathogenesis-related protein 1 (PR1)	AF306651	1	2.3 (48 h)
Pathogenesis-related protein class 1 (PRb1)	AY339373	1	2.4 (48 h)
Pathogenesis-related protein 10 (OsPR10), root specific	AB127580	1	6.3 (48 h)
Peroxidase (POX8.1)	AF014468	6	2.3 (6 h), 2.7 (48 h)
Peroxiredoxin	AK059845	1	2.3 (3 h)
Planthopper susceptibility protein Hd002A	AK066843	1	6.4 (3 h)
Protein, similar to CMV 1a interacting protein 1/tobacco	AK059127	1	2.1 (3 h)
Iron stress-related protein (RIS9), similar to a Citrus junos clone pBCR9	AK120252	1	2.8 (6 h)
Thaumatin-like protein	X68197	2	2.0 (3 h), 2.7 (6 h), 4.5 (48 h)
Thioredoxin f2 protein	AK101264	3	3.0 (3 h), 2.0 (6 h)
Water stress-induced protein (WSI724)	D26538	1	2.3 (6 h)
Wound-induced protein homologue IAI2	AB059238	1	3.4 (3 h), 2.0 (48 h)
2. Energy and metabolism			
23-kDa polypeptide of photosystem II	AF052203	2	2.1 (6 h), 2.6 (48 h)
33-kDa oxygen-evolving protein of photosystem II	AK119515	2	2.9 (3 h), 2.3 (6 h)
Adenylosuccinate synthetase	AK120407	1	2.3 (3 h)
Aspartate kinase-homoserine dehydrogenase	AK073790	1	2.7 (3 h)
ATP-dependent Clp protease ATP-binding subunit precursor (CLPD1)	AY166599	1	2.6 (48 h)
Chlorophyll a/b-binding protein (RCABP69)	AF058796	1	2.3 (3 h)
Chlorophyll a/b-binding protein precursor (Cab26)	AF094776	1	2.0 (6 h)
Cytidine or deoxycytidylate deaminase	AK100710	1	2.0 (6 h)
Cytochrome P450	AK104799	1	2.2 (48 h)
Farnesyl-pyrophosphate synthetase	AP008210	1	2.0 (48 h)
Ferredoxin-dependent glutamate synthase	Y12595	2	2.1 (3 h)
Fructose-bisphosphate aldolase class-I	AF017362	1	3.7 (3 h)
Glyceraldehyde-3-phosphate dehydrogenase	U31676	1	5.1 (3 h)
Hydrolase, α/β fold family protein	AK070827	1	2.2 (6 h)
Hydroxyanthranilate hydroxycinnamoyltransferase	AK119237	1	2.0 (6 h)
Magnesium-chelatase subunit H (Mg-protoporphyrin IX chelatase subunit H)	AK069545	2	2.0 (3 h)
Magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase, chloroplast precursor	AK069333	1	2.0 (6 h)
Malate dehydrogenase, cytoplasmic	AF353203	1	3.2 (48 h)
NADP-specific isocitrate dehydrogenase	AF155333	1	2.5 (3 h)
NOL1/NOP2/sun family protein, putative methyltransferase	AK073815	1	2.4 (6 h)
Phosphoglycerate kinase, chloroplast precursor	AK062214	3	2.0 (3 h), 2.2 (6 h)
Phosphoglycerate mutase family protein, putative	AK068167	1	2.5 (3 h)
Phosphoribulokinase precursor	AF529237	1	2.8 (6 h)

Table 1 (*Continued*)

Description*	Accession number†	Number of ESTs‡	Expression ratio§
Phosphoserine aminotransferase, chloroplast precursor	AK109385	1	2.3 (6 h)
Photosystem I reaction centre subunit IV	AP008213	2	2.2 (3 h), 2.1 (48 h)
Photosystem II 10-kDa polypeptide	AK121083	8	5.4 (3 h), 2.6 (48 h)
Photosystem I antenna protein	AK105002	1	2.3 (3 h)
Photosystem I antenna protein	AK119176	1	2.0 (48 h)
Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	AK070257	9	2.6 (3 h)
Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	AY445627	16	3.1 (3 h)
Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	D00643	1	7.1 (3 h)
Ribulose-5-phosphate-3-epimerase	AK066306	2	7.8 (3 h), 4.4 (6 h), 2.0 (48 h)
Ribulose-5-phosphate-3-epimerase	AF047444	1	4.0 (3 h)
S-Adenosyl methionine synthetase (pRSAM-1)	Z26867	1	2.9 (6 h)
Triose-phosphate isomerase	AK069488	1	2.9 (3 h)
Type I light-harvesting chlorophyll a/b binding protein of photosystem II (LHCPII)	D00641	5	2.3 (6 h), 2.9 (48 h)
Ubiquinol-cytochrome c reductase complex ubiquinone-binding protein	AK120341	1	2.0 (6 h)
UGP mRNA for UDP-glucose pyrophosphorylase	AB062606	1	2.8 (3 h)
3. Transcription			
AP2 domain-containing transcription factor, similar to DNA-binding protein RAV2	AK065008	1	3.2 (48 h)
Basic helix–loop–helix (bHLH) family protein	AK073385	5	3.3 (3 h), 2.1 (6 h), 2.1 (48 h)
Basic helix–loop–helix (bHLH) family protein	AP008207	4	3.0 (3 h), 2.0 (6 h)
Ethylene-responsive element binding protein (EREBP)-type transcription factor	AF364176	1	2.4 (6 h)
Glycine-rich RNA-binding protein (grp5 gene)	AJ302060	1	2.1 (48 h)
Glycine-rich RNA-binding protein	AF011331	1	7.6 (3 h)
Nuclear RNA-binding protein	AK121001	1	3.2 (3 h)
Plastid sigma factor 2B, OsSig2B	AB095095	1	2.6 (3 h)
RNA-binding protein homologue	AK060161	1	5.0 (3 h)
RNA helicase RH25	AK067570	1	2.0 (3 h)
RNA helicase	AK101403	1	2.1 (6 h)
RNA recognition motif (RRM)-containing protein, low similarity to RNA-binding protein RGP-3	AK070704	1	3.3 (3 h)
Splicing factor, putative strong similarity to splicing factor Prp8 (Homo sapiens)	AK099780	1	2.4 (6 h)
Transcription factor-like	AK059092	1	2.4 (6 h)
U2 snRNP auxiliary factor, small subunit 35a	Y18349	1	2.8 (6 h)
Zinc finger protein (Tranpr)	AY574990	2	3.4 (3 h)
Zinc finger transcription factor	AK100770	2	3.2 (3 h)
4. Protein synthesis, folding and stabilization			
Acidic ribosomal protein P2 gene	D29689	1	2.4 (6 h)
Aspartic proteinase Asp1 precursor (OsAsp1) (nucellin-like protein)	AK068664	1	2.4 (6 h)
β5 subunit of 20S proteasome (OsPBE1)	AB026568	1	2.0 (3 h)
Cyclophilin 2	L29469	1	2.0 (6 h)
Eukaryotic translation initiation factor 5A-2 (eIF5A-2)	AK099039	1	2.1 (6 h)
Eukaryotic translation initiation factor 5A (eIF5A)	AJ312906	1	2.0 (3 h), 2.7 (48 h)
Heat shock protein 90 (HSP90)	AB037681	2	2.0 (48 h)
Plastid-specific 30S ribosomal protein 1, chloroplast precursor	AK104733	5	2.4 (3 h), 2.1 (6 h)
Protein disulphide isomerase (PDI)	AY987391	1	2.4 (3 h)
Protein disulphide isomerase (PDI)	AY224470	1	2.0 (3 h)
Signal peptidase I-1	AK069581	1	3.1 (3 h)
40S ribosomal protein S15a	AK240912	1	3.3 (48 h)
40S ribosomal protein S3a (cyc07 protein)	D26060	1	2.1 (6 h)
40S ribosomal protein S8	AK068316	1	3.3 (3 h)

Table 1 (*Continued*)

Description*	Accession number†	Number of ESTs‡	Expression ratio§
40S ribosomal protein S21	D12633	2	2.0 (3 h)
40S ribosomal protein S23 (S12)	AK069399	1	2.7 (3 h)
60S ribosomal protein L35	AK119701	1	2.0 (6 h)
60S ribosomal protein L35a	AK102775	1	2.0 (6 h)
60S ribosomal protein L37a	AK059395	1	2.2 (6 h)
Ribosomal protein L17	AK120273	1	4.3 (3 h)
Ribosomal protein S13 (xd3 gene)	AJ417519	1	3.3 (3 h)
Ribosomal protein S27	AK059683	1	7.1 (3 h)
Translation elongation factor 1α (EF-1α)	D63580	2	3.3 (3 h)
Translation elongation factor 1α (EF-1α)	D63581	1	2.0 (6 h)
Translation elongation factor 1α (EF-1α)	D63583	1	2.3 (3 h), 3.0 (6 h)
Translation elongation factor 1β (EF-1β)	D23674	1	3.3 (3 h)
Threonyl-tRNA synthetase	Y14368	1	2.0 (3 h)
Threonyl-tRNA synthetase	AK058738	1	3.1 (3 h)
Ubiquitin/ribosomal protein S27a.1	AK061988	2	4.2 (3 h), 2.8 (6 h), 2.0 (48 h)
Ubiquitin-conjugating enzyme family protein	AK119659	1	2.0 (6 h)
Ubiquitin-like protein	AK058725	2	3.4 (3 h)
5. Signal transduction and intracellular traffic			
Aquaporin	AK119719	1	2.0 (6 h)
BRI1-kDa interacting protein 102 (Bip102)	AB117990	1	8.1 (6 h)
BRI1-kDa interacting protein 128 (Bip128)	AK066179	1	4.1 (3 h)
GTPase activating-like protein (GAP)	AK099843	1	2.1 (6 h)
Mitogen-activated protein kinase (MAPK) phosphatase	AK067768	1	2.4 (3 h)
Plasma membrane H^+-ATPase-like protein	AY224445	1	4.3 (3 h)
Profilin	AK121519	1	2.1 (48 h)
Protein kinase-like protein	AK065283	1	2.5 (3 h)
Ras-related GTP-binding protein (Rgp1)	X59276	1	2.0 (6 h)
Receptor-like protein kinase (DUF26-like)	AY847141	2	2.1 (6 h), 2.6 (48 h)
Remorin 1	AK106182	3	7.4 (3 h)
SIT4 phosphatase-associated family protein contains similarity to copper chaperone homologue	AK067338	1	2.0 (6 h), 2.0 (48 h)
Snf1-related protein kinase (OSK1)	D82039	1	4.2 (3 h)
Tonoplast intrinsic protein (OsTIP1)	AB114829	1	2.0 (6 h)
Transport protein particle (TRAPP) Bet3 component	AK111377	2	2.1 (3 h), 6.1 (48 h)
Vacuolar proton-translocating ATPase subunit E	AK071200	1	7.1 (3 h)
Vesicle transport v-SNARE	AK104463	1	2.9 (3 h), 2.2 (6 h)
Voltage-dependent anion channel (VDAC) porin	AK100231	1	2.7 (3 h), 2.0 (6 h)
6. Cell structure			
Chloroplast inner envelope protein	AK120578	1	2.0 (3 h)
Inner mitochondrial membrane protein	AK103851	1	2.3 (6 h)
7. Growth and division			
Auxin responsive protein, IAA1	AJ251791	1	4.0 (6 h)
DNA repair protein, RAD23-3	AK061556	1	2.5 (48 h)
Dormancy-associated protein, auxin repressed	AF467730	2	2.5 (6 h), 2.8 (48 h)
Light-induced protein	X68807	4	2.2 (3 h), 36.2 (48 h)
Senescence-associated protein, OSA15	AY037805	1	2.2 (6 h)
8. Unclassified or unknown function			
33-kDa secretory protein (DUF26 domain containing protein 2 precursor)	AK061425	4	2.1 (3 h), 2.0 (6 h), 2.4 (48 h)
Anther-specific proline-rich protein APG precursor	AK066367	1	2.5 (3 h), 2.6 (48 h)
Expressed protein contains 1 transmembrane domain	AK121047	1	2.0 (6 h)
Expressed protein	AK065040	1	3.0 (3 h)
Expressed protein	AK068443	1	13.4 (3 h)

Table 1 (*Continued*)

Description*	Accession number†	Number of ESTs‡	Expression ratio§
Expressed protein	AK068661	18	3.2 (3 h), 2.3 (6 h), 3.1 (48 h)
Expressed protein	AK121344	1	4.0 (3 h)
Expressed protein	XM_463125	2	2.8 (3 h), 2.0 (6 h), 3.1 (48 h)
Genomic sequence	AP008212	2	2.6 (3 h), 2.4 (6 h)
Genomic sequence	AP008213	22	2.9 (3 h), 2.2 (6 h), 2.6 (48 h)
Genomic sequence	AP008218	1	3.0 (6 h)
Hypothetical protein	AB110171	1	2.6 (3 h)
Hypothetical protein	AK105731	1	2.0 (6 h)
Pentatricopeptide (PPR) repeat-containing protein	AP008217	1	2.4 (3 h), 2.2 (6 h)
Reticulon family protein (RTNLB2)	AK073583	1	2.2 (3 h)
TMS membrane protein/tumour differentially expressed protein (TDE)	AK119191	1	3.8 (3 h)
Unknown protein	AK059554	1	2.0 (3 h)
Unknown protein	AK099155	1	2.2 (3 h), 2.0 (48 h)
Unknown protein	AK103517	1	2.5 (48 h)
Unknown protein	AK104019	1	2.2 (48 h)
Unknown protein	AK119691	1	3.2 (48 h)
Unknown protein	AK120355	1	2.5 (3 h)
Unknown protein	NM_186253	6	2.5 (3 h), 2.3 (6 h), 2.0 (48 h)

*Unigenes for which the transcript level was up-regulated are listed by functional category according to the Munich Information Centre for Protein Sequences (MIPS, http://mips.gsf.de). Only genes showing a ratio of ≥ 2.0 (infected vs. non-infected) are listed.

†Accession numbers for unigenes (GenBank and KOME databases).

‡Number of expressed sequence tag (EST) components in each gene is indicated.

§Expression ratio: signal intensity ratio of infected leaves over control, non-infected leaves of wild-type plants. The time of infection at which up-regulation was observed is shown in parentheses.

were up-regulated in rice leaves in response to *M. oryzae* infection. A carbonic anhydrase also showed fungal responsiveness in rice leaves. Genes encoding wound, water and iron stress-induced proteins were also up-regulated in fungal-infected rice leaves. In this respect, it is well known that a substantial number of genes display a common response to various types of stress, biotic and abiotic (Reymond *et al*., 2000). In addition, our macroarray data revealed that the expression of genes encoding enzymes involved in tolerance against oxidative stress and the detoxification of reactive oxygen species (ROS), such as catalase, peroxidase POX8.1, ascorbate peroxidase, peroxiredoxin, thioredoxin and metallothionein, was induced in the early response of rice plants to *M. oryzae* infection. The defence category also included the *DAD1* (*Defender against Apoptotic Death 1*) gene; this gene encodes an enzyme that performs the N-linked protein glycosylation required for protein transport from the endoplasmic reticulum (ER) to the target site of action. A suppressor activity in programmed cell death has been proposed for the *Arabidopsis DAD1* gene (Danon *et al*., 2004).

The functional category of energy and metabolism contained a large number of ESTs (24.5% of the ESTs). These included genes involved in photosynthesis and carbohydrate metabolism (fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase). This observation further supports a link between photosynthesis and carbohydrate metabolism and the plant defence response.

The functional category of protein synthesis, folding and stabilization comprised 12.1% of the ESTs (31 unique genes) showing pathogen responsiveness. Blast infection was accompanied by the up-regulation of components of the translational machinery, including the genes encoding translation initiation factor 5A (eIF5A, two genes), elongation factors 1α and 1β (EF-1α and EF-1β) and 13 ribosomal proteins, pointing to the occurrence of active protein synthesis during the pre-invasive defence response of rice. This study also revealed that the expression of genes encoding proteins involved in protein modification, folding and stabilization, such as signal peptidase I-1, protein disulphide isomerase (PDI), heat shock protein 90 (HSP90) and cyclophilin, was rapidly induced in response to *M. oryzae* infection.

Transcription and RNA modification (RNA-binding proteins, RNA helicases and the splicing factor Prp8, amongst others) accounted for 7.9% of the ESTs showing fungal responsiveness. In particular, macroarray analysis revealed the induction of transcription factors for which a role in

disease resistance has been described in other pathosystems, such as the ethylene-responsive factor [ethylene-responsive element binding protein (EREBP)-type] and the basic helix–loop–helix (bHLH)-type transcription factor (Berrocal-Lobo *et al*., 2002; Gutterson and Reuber, 2004).

Finally, 6.7% of the fungal-responsive ESTs identified in this work were categorized into the signal transduction and intracellular traffic category. Interestingly, genes that were up-regulated at the early stages of infection included several components of the vesicle-associated transport machinery, such as genes encoding the vesicle (v)-soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (v-SNARE) protein, the Ras-related guanosine triphosphate (GTP)-binding protein and the guanosine triphosphatase (GTPase)-activating protein or a transport protein particle (TRAPP). Additional genes encoding signalling components or regulatory proteins potentially involved in the early response of rice plants to *M. oryzae* were also expressed: genes encoding remorin, specific protein kinases and protein phosphatases, aquaporin, voltage-dependent anion channel (VDAC) porin, plasma membrane H^+-ATPase, vacuolar proton-translocating ATPase (subunit E), tonoplast intrinsic OsTIP1 protein, Bip102 and Bip128 (BRI1-kDa interacting protein 102 and 128, respectively).

Time course of transcript accumulation of selected *M. oryzae*-induced rice genes

To validate and extend the macroarray data, the time course of transcript accumulation for several randomly selected up-regulated genes was monitored further. Depending on the relative abundance of these transcripts, Northern blot, reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR (qRT-PCR) analyses were carried out (Figure 2). Most of the genes analysed here were induced at early time points of the infection period (4–6 h after inoculation). After this initial response, a decline in the expression of many of these genes was observed (Figure 2a). A second peak of fungal-induced expression occurred at later stages of the infection process (32–48 h pi), as observed for the *PR1a, β-1,3-glucanase, TLP* and *PR10* genes (Figure 2a). Changes in the level of expression of genes involved in the early response of rice plants to blast fungus (6 h, 9 h), such as *Ras-related GTP-binding protein* (*Rgp1*) and *GTPase-activating protein* (*GAP*) genes, and genes activated at a later stage of the infection process (24 h, 48 h), such as *TRAPP-Bet3* and *profilin* genes, were detected by RT-PCR (Figure 2b). Finally, changes in the level of expression of genes encoding EREBP, eIF5A and HSP90 were confirmed by qRT-PCR analysis (Figure 2c). Except for the classical *PR* genes, the expression studies of many of the selected up-regulated rice genes required the application of RT-PCR or qRT-PCR techniques, indicating that the PCR-SSH procedure successfully enriched low-abundance, fungal-responsive genes. Overall, these expression studies corroborated the results observed by macroarray analysis, and confirmed that the subtracted library reliably represents fungal-induced genes from rice.

Comparative analysis of gene expression in *cecropin A* and wild-type rice

The macroarray was used to investigate whether transgenic expression of the *cecropin A* gene induced changes in gene expression in the host plant when grown under optimal conditions (i.e. glasshouse-grown plants). The transgenic *cecropin A* rice lines analysed here express a codon-optimized synthetic *cecropin A* gene from *H. cecropia* under the control of the constitutive maize *ubiquitin 1* promoter (Coca *et al*., 2006). The *cecropin A* gene was designed for retention of cecropin A in the ER (Coca *et al*., 2006). For this, the signal peptide of the tobacco *PR1a* gene and the KDEL extension for retention in the ER were fused at the N- and C-terminus, respectively, of the mature *cecropin A*.

Macroarray hybridizations were conducted with cDNAs generated from leaves of 2-week-old *cecropin A* and wild-type plants. Leaves from three independent transgenic lines (R33-6, R33-7 and R33-24) and 10 individual plants per line were pooled. Two replica membranes were hybridized with each cDNA probe. One hundred and sixty-eight unique genes were identified as being over-expressed in *cecropin A* plants using an arbitrary cut-off of 2.5-fold, and were grouped into the different functional categories (Figure 3a). A description of the genes that were over-expressed in *cecropin A* plants, and the extent of variation of their expression compared with wild-type plants, is shown in Table 2. An important number of these genes were found to be expressed at high levels in *cecropin A* plants compared with wild-type plants in the absence of infection. Thus, 58 of the 168 unigenes showed fourfold expression in transgenic plants.

An important result of this study was the observation that genes encoding enzymes involved in protection against oxidative stress, such as peroxidases (three distinct genes), glutathione-*S*-transferase, iron superoxide dismutase and the metallothionein OsMT1, were highly expressed in *cecropin A* plants in the absence of *M. oryzae* infection. These genes represented up to 44.44% of the genes categorized in the defence category (Table 2). Comparison of the gene expression

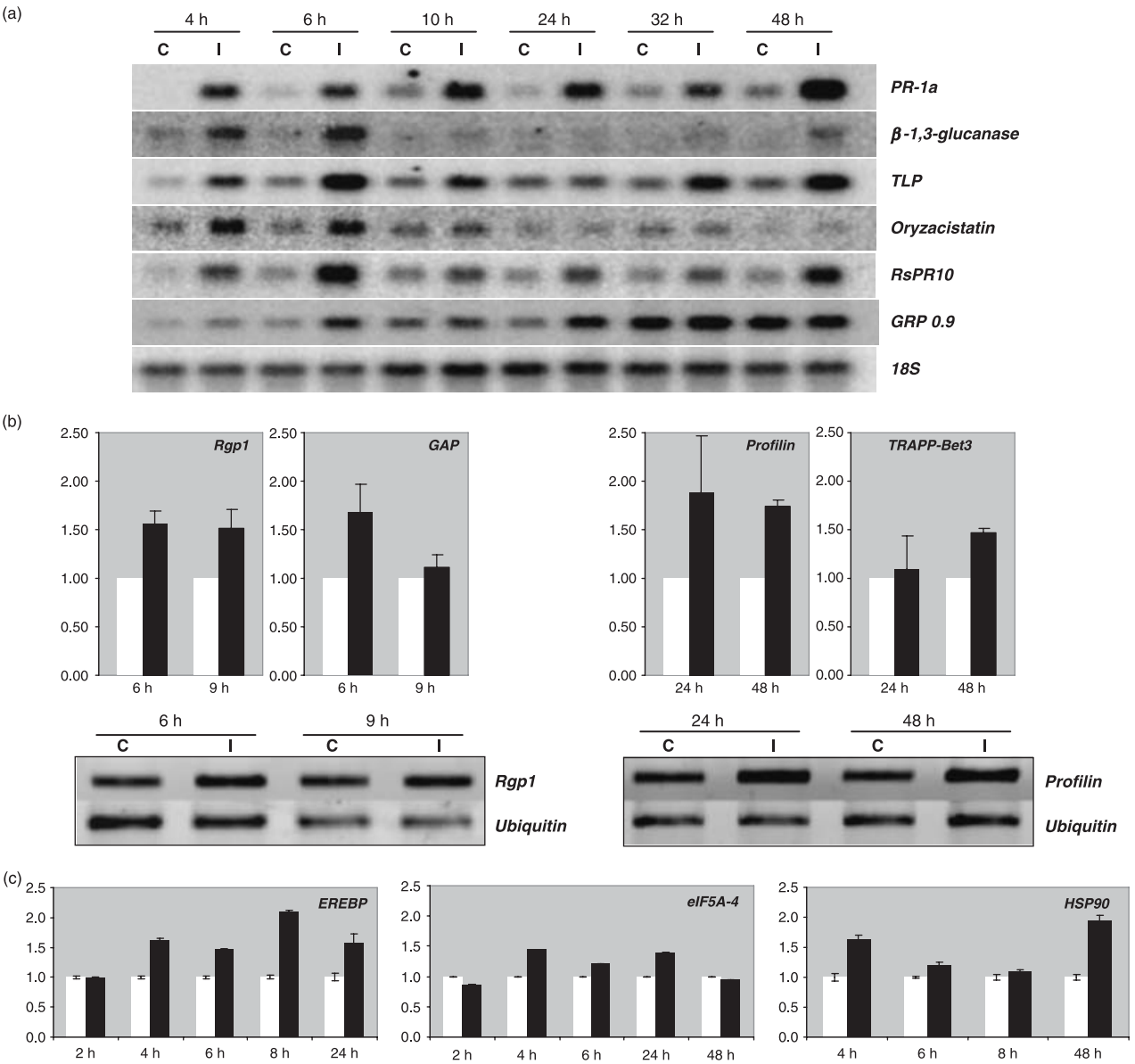

Figure 2 Validation of macroarray data. Expression patterns of genes selected by macroarray analysis during *Magnaporthe oryzae* infection. (a) Northern blot analysis with the indicated gene probes was performed on RNA samples obtained from rice leaves at different times after inoculation with fungal spores. Ten micrograms of total RNA from non-infected and infected rice leaves at the indicated times after inoculation were used in Northern blot analysis (C, control non-infected; I, *M. oryzae*-infected). RNA loading of each sample was verified by hybridization with the cDNA for the 18S ribosomal RNA. (b) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *M. oryzae*-infected (black bars) and non-infected (white bars) rice leaves. Top panels show the changes in transcript abundance of the indicated genes relative to the *ubiquitin* gene (loading control). The data shown are the mean values ± standard deviation of three independent experiments (error bars are indicated). Representative RT-PCR analyses for the *Rgp1* and *profilin* genes are presented in the bottom panels. (c) Changes in transcript abundance in rice leaves as determined by real-time quantitative RT-PCR (qRT-PCR) analysis. The relative expression of the *EREBP*, *eIF5A-4* and *HSP90* genes in fungal-infected (black bars) and non-infected (white bars) rice leaves is shown. To normalize the qRT-PCR data, each gene was compared with the actin transcript. Two biological replicates were used for these expression studies. See tables for gene abbreviations.

between transgenic and wild-type plants also revealed the over-expression of genes playing a role in protein synthesis, folding and stabilization (20.1% of the total number of ESTs) in *cecropin A* plants, particularly genes involved in the folding and stabilization of proteins that enter into the secretory pathway: calnexin (two distinct genes), calreticulin, HSP90 and HSP22, DNAJ heat shock protein (two distinct genes) and PDI. The expression of a random set of selected genes that were identified as being over-expressed in *cecropin A* plants in macroarray experiments was confirmed by Northern blot

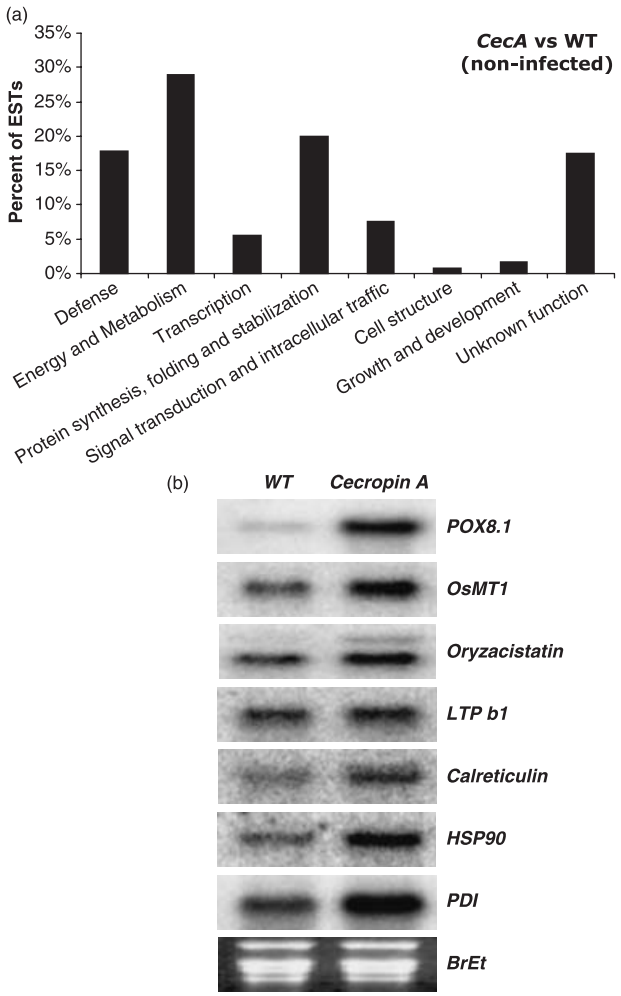

Figure 3 Gene expression data using the rice macroarray of *cecropin A* plants. (a) Distribution of expressed sequence tags (ESTs) with altered expression in *cecropin A* plants compared with wild-type plants amongst the different functional categories. CecA, cecropin A; WT, wild-type. (b) Northern blot analysis of genes showing differential expression in *cecropin A* plants compared with wild-type plants grown under normal growth conditions. See tables for gene abbreviations.

(Figure 3b). Overall, the results of RNA gel blot were consistent with the expression data obtained by macroarray analysis.

The transgenic *cecropin A* plants under study were produced by *Agrobacterium*-mediated transformation using the hygromycin resistance gene (*hptII*, hygromycin phosphotransferase) as the selectable marker. Therefore, there was the possibility that the expression of the selectable marker gene might influence the expression of rice genes. Accordingly, gene expression studies were performed with transgenic rice transformed with the empty vector (the pCAMBIA 1300 plasmid containing the *hptII* gene). None of the rice genes that were over-expressed in *cecropin A* plants showed differential expression in rice plants expressing the empty vector compared with wild-type plants (results not shown), indicating that the expression of the hygromycin resistance gene had little effect, if any, on the gene expression in rice. In other studies, the expression of the commonly used marker gene *nptII* (neomycin phosphotransferase), conferring kanamycin resistance, has not been reported to induce changes in the *Arabidopsis* transcriptome (El Ouakfaoui and Miki, 2005).

In summary, the comparative analysis of gene expression between transgenic and wild-type rice plants grown under optimal conditions revealed changes in the expression of an important number of rice genes. In particular, genes involved in protection against oxidative stress and genes related to processes of synthesis, folding and stabilization of proteins that enter into the secretory pathway were found to be over-expressed in *cecropin A* rice. Together, these findings indicate that the transgenic expression of *cecropin A* in rice has an impact on host gene expression.

Cecropin A accumulates in the ER in transgenic rice plants

Because an important number of rice genes that play a role in the processes of protein folding and stabilization in the ER are over-expressed in *cecropin A* plants, and because the *cecropin A* gene introduced into rice plants was engineered to retain cecropin A in the ER, this subcellular compartment was investigated for the accumulation of cecropin A. To this end, subcellular fractionation experiments were performed on both wild-type and transgenic rice plants, followed by Western blot analysis using a polyclonal antibody raised against cecropin A (Coca *et al*., 2006). After homogenization of rice leaves from wild-type and transgenic plants, the organelles were separated on a discontinuous sucrose gradient (20%–70% sucrose). Fractions were collected and separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting. Membranes were probed with anti-cecropin A or with antiserum raised against the BiP protein, an ER marker (luminal binding protein). Cecropin A was detected in the ER fraction (30%–50% sucrose) and co-fractionated with the BiP protein in transgenic plants, indicating that cecropin A and BiP share the same organellar compartment: the ER (Figure 4).

Enhanced tolerance to oxidative stress induced by hydrogen peroxide (H_2O_2) in *cecropin A* rice plants

The observation that genes encoding ROS-scavenging enzymes and, in particular, peroxidases (three distinct peroxidase rice

Table 2 Rice genes that are over-expressed by 2.5 fold or more in *cecropin A* plants compared with wild-type plants, classified according to functional category

Description*	Accession number†	Number of ESTs‡	Expression ratio§
1. Disease/defence			
Acidic endochitinase	AK065866	1	4.1
Band 7 family protein, strong similarity to hypersensitive-induced response protein	AK111965	1	4.2
Carbonic anhydrase 3	AF182806	1	6.6
Glutathione-S-transferase (GSTU6)	AK108376	1	7.0
Glycine-rich protein (GRP 0.9)	X54449	9	3.1
IAI2 mRNA for wound-induced protein homologue	AB059238	1	7.0
Iron stress-related protein from Citrus junos	AK120252	1	2.8
Iron superoxide dismutase (Fe)	AK111656	1	2.8
Lipid transfer protein, b1 (LTP)	X83434	2	2.9
Lipid transfer protein precursor (LTP2)	U31766	1	2.6
Metallothionein-like type 1 (OsMT1)	U43529	4	3.6
Oryzacystatin	S49967	6	3.2
Peroxidase (POX8.1)	AF014468	10	7.3
Peroxidase	AK058883	1	32.3
Peroxidase 22 class III (prx22)	BN000551	1	11.10
2. Energy and metabolism			
Adenosine kinase	AK101791	1	2.7
Aldehyde dehydrogenase	AK068297	1	3.8
Aldehyde dehydrogenase	AK120185	1	2.9
1-Aminocyclopropane-1-carboxylate oxidase	AK102472	1	2.6
Blue copper protein precursor	AK105053	1	4.8
β-Keto acyl reductase	AK064965	1	3.0
Branched-chain α-keto acid decarboxylase E1 β subunit (BCDH β1)	AK105761	1	3.6
Chlorophyll a/b-binding protein	U46159	1	3.0
Cinnamoyl-CoA reductase	AK062287	1	2.8
Cytidine or deoxycytidylate deaminase	AK100710	3	6.0
Cytochrome P450	AK069358	1	6.0
Δ-12 oleate desaturase-like	AK065239	1	3.0
3-Deoxy-D-arabino heptulosonate-7-phosphate synthase (DAHPS2 gene)	AB122058	1	3.8
Farnesyl-pyrophosphate synthetase fps2	XM_474182	1	7.4
Ferredoxin-dependent glutamate synthase	Y12595	1	2.7
Flavin-containing monooxygenase family protein	AK071801	1	20.1
Fructose-bisphosphate aldolase, precursor	AK073758	1	5.1
Glyceraldehyde-3-phosphate dehydrogenase	AK103777	1	2.5
3-Ketoacyl-CoA thiolase-like protein	AK061948	1	2.9
Lactoylglutathione lyase family protein	AK119669	1	5.6
Lysyl-tRNA synthetase	AK099302	1	3.0
5-Methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	AK099069	1	3.1
Magnesium-chelatase subunit H (Mg-protoporphyrin IX chelatase subunit H)	AK069545	2	2.5
Magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase	AK069333	2	4.9
Malate dehydrogenase (MDH)	AF444195	1	2.7
Mevalonate kinase	AP008207	1	2.7
NADP-dependent malic enzyme	AK121770	1	4.9
NADP-dependent malic enzyme	D16499	2	4.7
NADP-dependent malic enzyme (NADP-ME2)	AB053295	1	2.7
NAD-specific isocitrate dehydrogenase	AF155333	2	2.9
O-Sialoglycoprotein endopeptidase	AK099965	1	2.5
Phosphoglycerate dehydrogenase	AK120939	1	9.9
Phosphoglycerate kinase, chloroplast precursor	AK062214	1	2.6
Phosphoglycerate mutase family protein	AK068167	1	7.4
Phosphoserine aminotransferase	AK109385	1	4.8
Phosphoserine aminotransferase	AP008209	1	2.6

Table 2 (*Continued*)

Description*	Accession number†	Number of ESTs‡	Expression ratio§
Photosystem I reaction centre subunit IV	AP008213	1	4.1
Photosystem II 10-kDa polypeptide	AK121083	1	2.7
Phytoene synthase (psy gene)	AK070716	1	3.3
23-kDa polypeptide of photosystem II	AF052203	2	2.9
Pyridoxal kinase	AK066514	1	5.4
Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	AK070257	1	4.7
Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	AY445627	1	2.7
Ribulose-5-phosphate-3-epimerase	AF047444	1	4.4
RINO1 mRNA for myo-inositol phosphate synthase	AB012107	1	2.7
S-Adenosyl methionine synthetase (pRSAM-1)	Z26867	1	2.9
Sedoheptulose-1,7-bisphosphatase precursor	AY188797	1	3.8
Succinyl-CoA ligase (GDP-forming) β-chain, mitochondrial	AK103525	1	2.6
Tetraacyldisaccharide 4′-kinase (lipid A 4′-kinase)	AK070220	1	4.7
Transketolase-like protein	AK066830	2	3.8
Triosephosphate isomerase	AK069488	2	2.9
Type I light-harvesting chlorophyll a/b-binding protein of photosystem II (LHCPII)	D00641	6	2.9
Ubiquinol-cytochrome c reductase complex 8.0 kDa	AK062745	1	3.9
ζ-Carotene desaturase precursor (zds)	AF054629	1	2.6
3. Transcription			
Basic helix–loop–helix (bHLH) family protein	AK073385	2	2.8
Basic helix–loop–helix (bHLH) family protein	AP008207	1	2.9
Basic helix–loop–helix (bHLH) family protein	AP003683	1	7.5
DNA-binding protein	AP008213	1	2.8
Ethylene-responsive element binding protein, EREBP-type transcription factor	AF364176	1	11.1
High mobility group protein (HMG1)	AF541859	1	3.9
Leucine zipper-containing protein	AK101630	1	2.5
Myb family transcription factor	AK103973	1	3.8
RNA helicase	AK101403	1	3.4
RNA helicase RH25	AK067570	1	6.0
Transcriptional regulators of NagC/XylR (ROK)-like	AK101816	1	2.9
Zinc finger (C3HC4-type RING finger) family protein	AK067585	1	3.1
4. Protein synthesis, folding and stabilization			
β5 subunit of 20S proteasome (OsPBE1)	AB026568	1	4.9
Calnexin	AK069118	2	2.9
Calnexin	AK061185	1	2.8
Calreticulin-3 precursor	AK060834	1	3.7
Cytoplasmic ribosomal protein S13 (xd3 gene)	AJ417519	1	3.6
DNAJ heat shock N-terminal domain-containing protein	AK070019	1	3.6
DNAJ heat shock family protein similar to DnaJ homologue subfamily B member 11 precursor	AK062718	1	3.5
Eukaryotic translation initiation factor 5A (eIF5A)	AJ312906	1	2.5
Eukaryotic translation initiation factor 6 (eIF6)	AK074012	1	7.2
Heat shock protein 90 (HSP90)	AB037681	4	2.6
Heat shock protein precursor (HSP22), low molecular weight	AK105464	1	7.0
Mitochondrial 28S ribosomal protein S29-related	AK065624	1	3.4
Plastid-specific 30S ribosomal protein 1, chloroplast precursor	AK104733	1	8.0
Protein disulphide isomerase (PDI)	AY987391	2	4.6
26S protease regulatory subunit 4 homologue (TAT-binding protein homologue 2)	D17789	1	2.7
40S ribosomal protein S2	AK064984	1	3.5
40S ribosomal protein S8	AK068316	2	3.0
40S ribosomal protein S9	AK104660	1	3.3
40S ribosomal protein S11	AK120520	1	7.2
40S ribosomal protein S14	AK121223	1	3.0
40S ribosomal protein S15a	AK240912	1	2.6

Table 2 (*Continued*)

Description*	Accession number†	Number of ESTs‡	Expression ratio§
40S ribosomal protein S21	AP008209	1	2.6
40S ribosomal protein S21	D12633	1	2.5
40S ribosomal protein S23	AK241653	1	2.9
40S ribosomal protein S24	AK058248	1	7.4
40S ribosomal protein S28	AK242697	1	5.1
40S subunit ribosomal protein	D12633	1	3.7
60S ribosomal protein L17	AK120324	2	3.4
60S ribosomal protein L37a	AK119801	1	3.9
60S ribosomal protein L38	AK058262	1	3.1
Ribosomal protein L13a	AK103048	1	4.0
Signal peptidase I-1	AK069581	2	4.6
Translation elongation factor 1α (EF-1α)	D63581	2	3.0
Ubiquitin-conjugating enzyme E2-17 kDa	AK060954	1	5.5
Ubiquitin/ribosomal protein S27a	AK061988	2	2.6
Ubiquitin-like protein	AK058725	1	3.3
YK704 mRNA for acidic ribosomal protein P0	D21130	1	5.9
5. Signal transduction and Intracellular traffic			
BRI1-kDa interacting protein 102 (Bip 102)	AB117990	1	11.6
BRI1-kDa interacting protein 103 (proton pump interacting protein)	AB117987	1	3.1
Calmodulin	AK069620	1	5.1
Calmodulin-binding protein	AK072545	1	2.9
Rapid alkalinization factor (RALF) family protein similar			
Receptor-like protein kinase (DUF26-like protein)	AY847141	4	7.5
Profilin	AK121519	1	3.4
Protein kinase family protein	AK120660	1	2.6
Purine permease (putative)	AK072660	1	3.1
SEC14 cytosolic factor	AK064540	3	3.4
Stomatal cytokinesis-defective 1 (SCD1)	NM_193619	1	3.5
Sulphonylurea receptor-like protein	AK121451	1	3.3
Transport protein, similar to transport protein Sec23A	AK067849	1	2.9
6. Cell structure			
Actin-related protein 4	AK101582	1	10.8
Chloroplast inner envelope protein	AK120578	1	9.4
7. Growth and division			
Dormancy/auxin-associated family protein	AK064815	1	3.5
Dormancy-associated protein	AF467730	1	4.0
EL2 gene	D64038	1	2.8
Light-induced protein, CR9	X68807	1	8.7
8. Unclassified or unknown function			
21-kDa polypeptide; translationally controlled tumour protein homologue (TCTP)	AK105453	1	2.6
33-kDa secretory protein	AK061425	2	2.8
33-kDa secretory protein	AK240901	1	6.8
Agenet domain-containing protein	AK072110	1	3.5
Centromeric protein-related	AP008208	1	2.9
Crp1 protein	AK070212	1	4.0
Expressed protein	AK101256	1	13.8
Expressed protein	AK098951	1	3.4
Expressed protein	AK068661	3	2.6
Expressed protein	AK069538	1	2.6
Expressed protein	AK068925	1	2.7
Genomic sequence	AP008209	4	2.7
Genomic sequence	AP008210	1	2.6
Genomic sequence	AP008211	1	9.0

Table 2 (*Continued*)

Description*	Accession number†	Number of ESTs‡	Expression ratio§
Genomic sequence	AP008213	1	4.3
Genomic sequence	AP008214	1	3.0
Genomic sequence	AP008217	1	2.7
Hypothetical protein	AK240639	2	4.2
Hypothetical protein	AB110171	4	3.3
Hypothetical protein	AK073234	1	2.9
Hypothetical protein, expressed under carbonate stress	AB053296	1	2.5
Leucine-rich repeat protein	AK069710	1	4.6
LTR retrotransposon	AK242671	1	3.2
Protein gypsy/Ty3 element polyprotein	AK119906	1	3.7
Unknown protein	XM_463125	1	7.3
Unknown protein	AK068219	1	5.6
Unknown protein	AK099155	1	3.2
Unknown protein	AK071570	1	2.7
Unknown protein	AK240888	1	2.7
Unknown protein	AK243523	1	2.6
Unknown protein	AK059554	1	3.6

*Unigenes for which the transcript level was up-regulated are listed by functional category according to the Munich Information Centre for Protein Sequences (MIPS, http://mips.gsf.de). Only genes showing a ratio of ≥ 2.5 (*cecropin A* vs. wild-type) are listed.

†Accession numbers for unigenes (GenBank and KOME databases).

‡Number of expressed sequence tag (EST) components in each gene is indicated.

§Expression ratio: signal intensity ratio of leaves from *cecropin A* plants over leaves of wild-type plants.

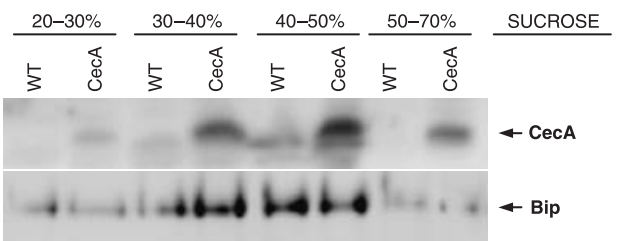

Figure 4 Co-fractionation of cecropin A and BiP proteins in transgenic rice plants. Total homogenates from wild-type (WT) and *cecropin A* (CecA) plants were subjected to centrifugation on a discontinuous sucrose gradient (20%–30%, 30%–40%, 40%–50% and 50%–70% sucrose). Each fraction was subjected to immunoblot analysis with anti-cecropin A (top panel) or anti-BiP (bottom panel).

genes) are expressed at very high levels in *cecropin A* plants compared with wild-type plants, in the absence of the pathogen, prompted us to investigate the response of transgenic plants to treatment with the ROS-generating agent H_2O_2, which is a substrate for a wide range of plant peroxidases. In this respect, external application of H_2O_2 has long been used to elicit oxidative stress in plants. Leaves of *cecropin A* and wild-type plants were treated with H_2O_2 and then stained with nitroblue tetrazolium (NBT). As shown in Figure 5 (top panels), intense staining was observed in areas randomly distributed on the leaves of wild-type plants, indicating that superoxide radicals (O_2^-) accumulate in these

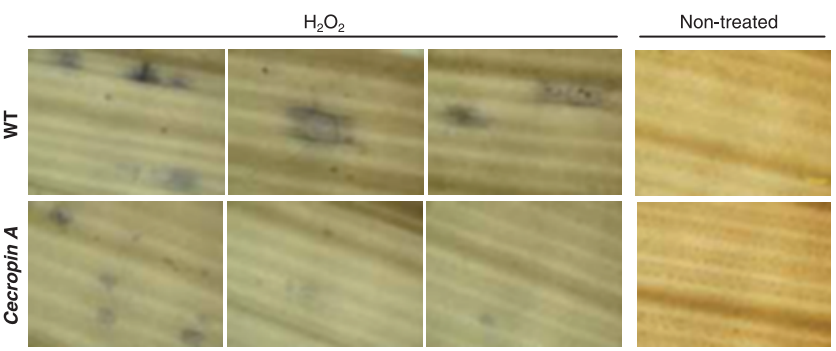

Figure 5 Effect of treatment with H_2O_2 in leaves of *cecropin A* (a) and wild-type (WT) (b) plants. Leaves of *cecropin A* and wild-type plants were treated with H_2O_2 for 8 h and then stained with nitroblue tetrazolium (NBT). After bleaching, leaves were examined by light microscopy. The reduction of NBT to formazan yields a bluish precipitate in the presence of superoxide radicals (O_2^-).

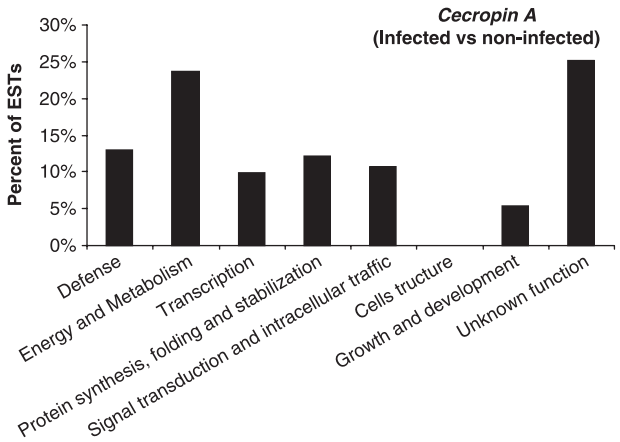

Figure 6 Functional classification of expressed sequence tags (ESTs) showing responsiveness to infection by the blast fungus *Magnaporthe oryzae* in *cecropin A* rice plants.

leaves after H_2O_2 treatment. In contrast, accumulation of O_2^- was barely detected in the leaves of *cecropin A* plants (Figure 5, bottom panels), confirming that transgenic plants are more tolerant than wild-type plants to the oxidative stress imposed by H_2O_2 treatment. This finding is in agreement with the results from macroarray analysis, which indicated that antioxidant systems are activated in transgenic plants in the absence of pathogen challenge.

Analysis of inducible rice defence responses in *cecropin A* rice under infection conditions

It was determined whether transgenic expression of the *cecropin A* gene has an influence on the defence mechanisms that are usually activated in rice plants on challenge with the blast fungus. To this end, the rice macroarray was used to compare gene expression profiles in non-infected and *M. oryzae*-infected *cecropin A* plants. Genes whose expression was up-regulated in transgenic plants during the early stage of infection, namely at 6 h pi, were identified. Changes in gene expression that might occur in *cecropin A* plants at later stages of the infection process were not investigated in this work.

Our study revealed that 94 unique genes were up-regulated in transgenic rice plants in response to *M. oryzae* infection, showing a difference equal to or greater than twofold. A description of the fungal-induced genes in *cecropin A* plants and their distribution in functional categories is shown in Table 3 and Figure 6, respectively. This study revealed that many of the genes that were up-regulated in response to *M. oryzae* infection in wild-type plants were also up-regulated in *cecropin A* plants in response to fungal infection (see Tables 1 and 3). The genes that were up-regulated in *M. oryzae*-infected *cecropin A* lines included those encoding lipid transfer protein (two genes), cysteine proteinase inhibitor, glycine-rich protein, ascorbate peroxidase, PDI, mitogen-activated protein kinase (MAPK), bHLH family protein, remorin and vacuolar proton-translocating ATPase (subunit E).

Discussion

In this work, the construction of a subtracted cDNA library containing rice genes that are induced during the early stage of the rice–blast interaction (3–48 h after inoculation) is reported. One hundred and sixty-two unique rice genes were identified as being up-regulated in response to infection by the rice blast fungus. Of these, 143 genes were found to be transcriptionally activated at 3 and 6 h after inoculation. From the different functional categories in which the *M. oryzae*-induced rice genes were placed, it appears that many different kinds of gene are involved in the early response of rice to the blast fungus. Clearly, the complexity of the plant response to pathogen infection indicates that the expression of plant genes belonging to different functional categories is reprogrammed. In this study, 331 of the 590 ESTs showing differential expression at one or more of the three time points studied (3, 6 and 48 h after inoculation) were characterized. This number represents a small fraction of the total number of ESTs contained in the subtracted cDNA library. It should be mentioned that the experimental design for preparation of the cDNA library comprised sampling of leaf tissues at five time points after inoculation (3, 6, 9, 24 and 48 h), and that, in this work, differential screening of the macroarray was carried out with RNA samples obtained at 3, 6 and 48 h after inoculation only. In addition, subtractive products may contain cDNAs that are induced at more than one time point after inoculation, which may also account for the large proportion of ESTs contained in the library. Although a large number of ESTs remain to be characterized, the results presented here provide a measure of the diversity of genes in the cDNA collection which show responsiveness to *M. oryzae* infection in rice plants.

Rice genes expressed during the early stages of infection with the rice blast fungus

Differential hybridization of the macroarray revealed the induction of expression of genes playing an important role in disease resistance, such as *PR* genes (van Loon *et al*., 2006) and genes involved in tolerance to oxidative stress and the detoxification of ROS. The production of ROS, including H_2O_2

Table 3 Genes up-regulated by *Magnaporthe oryzae* infection in *cecropin A* plants, sorted according to functional category

Description*	Accession number†	Number of ESTs‡	Expression ratio§
1. Disease/defence			
Ascorbate peroxidase, thylakoid-bound	AB114856	2	2.6
Carbonic anhydrase 3	AF182806	1	2.0
Cysteine proteinase inhibitor	AK119511	1	3.4
Defender against apoptotic death 1 protein (DAD1)	D89727	1	2.1
Glycolate oxidase	AF022740	1	2.2
Glyoxalase I	AB017042	2	2.3
Glycine-rich protein (GRP 0.9)	X54449	1	3.0
Lipid transfer protein (LPT IV)	AK070414	1	3.0
Lipid transfer protein, b1	X83434	1	2.0
Oryzacystatin	S49967	4	2.2
Protein, similar to CMV 1a interacting protein 1/tobacco	AK059127	1	2.2
Superoxide dismutase (Fe)	AK111656	1	2.1
2. Energy and metabolism			
23-kDa polypeptide of photosystem II	AF052203	3	2.0
33-kDa oxygen-evolving protein of photosystem II	AK119515	1	3.2
Adenylosuccinate synthetase	AK120407	1	3.4
Alanine aminotransferase	AK067732	1	2.0
Aspartate kinase-homoserine dehydrogenase	AK073790	1	2.6
ATP-NAD kinase family protein	AK073719	1	2.0
Chlorophyll a/b-binding protein (RCABP69)	AF058796	3	2.1
Chlorophyll a/b-binding protein precursor (Cab26)	AF094776	1	2.0
Dehydrogenase	AP008216	1	2.0
Ferredoxin-dependent glutamate synthase	Y12595	1	2.7
Hydrolase, α/β fold family protein	AK070827	1	2.0
Magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase, chloroplast precursor	AK069333	1	3.3
Non-cyanogenic β-glucosidase precursor	AK100820	1	2.1
Oxygen-evolving enhancer protein 3, chloroplast	AK099127	1	2.1
Oxygen-evolving protein	D21109	1	2.0
Phosphoenolpyruvate carboxykinase	AK102392	1	2.0
Phosphoribulokinase precursor	AF529237	1	2.8
Photosystem I reaction centre subunit VI, chloroplast	AP008213	1	4.0
Photosystem I reaction centre subunit VI, chloroplast	AF093635	1	2.0
Photosystem I reaction centre subunit PSI-N, chloroplast	AK059037	1	2.2
Photosystem I antenna protein	AK105002	1	2.2
Ribulose-bisphosphate carboxylase small chain C, chloroplast precursor	AY445627	4	2.9
Threonyl-tRNA synthetase	AK058738	1	4.1
UGP mRNA for UDP-glucose pyrophosphorylase	AB062606	1	5.4
3. Transcription			
29-kDa ribonucleoprotein A, chloroplast precursor	AK061072	1	4.1
Basic helix–loop–helix (bHLH) family protein	AK073385	3	2.6
Basic helix–loop–helix (bHLH) family protein	AP008207	2	2.9
DNA-binding protein	AP008213	1	2.7
HMG1 protein (HMG1)	AF541859	1	2.4
Nuclear RNA-binding protein (RGGA)	AK121001	1	2.3
Ethylene-responsive element binding protein, EREBP-type transcription factor	AF364176	2	2.0
RNA-binding protein homologue	AK060161	1	2.0
YT521-B-like family protein	AK119641	1	2.0
4. Protein synthesis, folding and stabilization			
Plastid-specific 30S ribosomal protein 1, chloroplast precursor	AK104733	3	2.5
Protein disulphide isomerase (PDI)	AY224470	1	2.0
Protein disulphide isomerase (PDI)	AY987391	1	2.7
40S ribosomal protein S21	D12633	1	2.0

Table 3 (*Continued*)

Description*	Accession number†	Number of ESTs‡	Expression ratio§
40S ribosomal protein S25	AK099010	2	3.7
60S ribosomal protein L37a	AK119801	1	2.1
Ribosomal protein L17	AK120273	1	2.7
Ribosomal protein S8	AK241816	1	2.2
Translation initiation factor (GOS2)	AF094774	2	2.0
Ubiquitin-conjugating enzyme	AK120251	1	2.0
Ubiquitin-like protein	AK058725	2	3.2
5. Signal transduction and intracellular traffic			
Aquaporin PIP2.8	AK109439	1	2.2
BRI1-kDa interacting protein, Bip 128	AK066179	1	4.7
Mitogen-activated protein kinase (MAPK) phosphatase	AK067768	1	2.7
Mitogen-activated protein kinase homologue	AK111579	1	3.3
Mitogen-activated protein kinase, wjumk1	AJ512643	1	2.1
Non-phototrophic hypocotyl 1a	AK066816	2	3.7
Phosphate transporter	AK065252	1	2.3
Receptor for activated C-kinase (RWD)	D38231	1	2.2
Remorin 1	AK106182	3	4.4
Transport protein particle (TRAPP) Bet3 component	AK111377	1	2.0
Vacuolar proton-translocating ATPase subunit E	AK071200	1	8.3
6. Cell structure			
7. Growth and division			
Auxin-responsive protein, IAA16	AK241477	1	2.1
Dormancy-associated protein	AF467730	3	2.2
Light-induced protein	X68807	3	2.4
8. Unclassified or unknown function			
33-kDa secretory protein	AK061425	2	2.4
Expressed protein	AK068443	1	4.4
Expressed protein	AK121361	2	2.0
Expressed protein	AK068625	1	2.1
Expressed protein	AK073300	1	2.1
Expressed protein	AK102573	1	2.0
Expressed protein	AK067965	1	2.0
Expressed protein	AK104214	1	2.0
Expressed protein	AK068661	3	2.0
Expressed protein	AK104595	1	2.0
Genomic sequence	AP008213	5	2.3
Hypothetical protein	AB110171	1	2.0
Maternal G10-like protein	D12628	1	2.4
Pentatricopeptide repeat-containing protein	AP008217	1	2.1
Reticulon family protein (RTNLB2)	AK073583	1	2.3
TMS membrane protein/tumour differentially expressed protein (TDE)	AK119191	1	3.4
Tobamovirus multiplication protein 3 (TOM3)	AK101318	1	2.6
Unknown protein	NM_186253	1	3.3
Unknown protein	AK059554	1	2.5
Unknown protein	AK120355	1	2.4
Unknown protein	AK242499	1	2.2
Unknown protein	AK240888	3	2.1
Unknown protein	AK241007	1	2.2

*Unigenes for which the transcript level was up-regulated are listed by functional category according to the Munich Information Centre for Protein Sequences (MIPS, http://mips.gsf.de). Only genes showing a ratio of ≥ 2.0 (infected vs. non-infected *cecropin A* plants) are listed. Those genes that are commonly induced in wild-type and *cecropin A* plants in response to fungal infection are indicated in bold.

†Accession numbers for unigenes (GenBank and KOME databases).

‡Number of expressed sequence tag (EST) components in each gene is indicated.

§Expression ratio: signal intensity ratio of infected leaves over non-infected leaves of *cecropin A* plants.

and O_2^-, the so-called oxidative burst, is one of the earliest events in the plant defence response against pathogen attack (Lamb and Dixon, 1997). At low concentrations, ROS have beneficial effects on plant cells responding to pathogen attack by reinforcing plant cell walls and acting as signalling molecules for the mediation of responses to pathogen infection. They can also act directly to kill pathogens. However, high concentrations of ROS can result in non-controlled oxidation of a variety of cellular components of the plant cell (DNA, proteins and membrane lipids), which may lead to cellular dysfunction, the appearance of necrotic lesions and, ultimately, to cell death. To control the steady-state level of ROS, plants possess a range of ROS-scavenging enzymes, namely peroxidases, catalases, ascorbate peroxidases, superoxide dismutases, peroxiredoxins, thioredoxins and glutathione peroxidases. Amongst the rice genes that were found to be up-regulated in response to *M. oryzae* infection were those encoding ascorbate peroxidase, catalase, peroxidase POX8.1, peroxiredoxin and thioredoxin. The expression of these genes was induced as early as 3–6 h after inoculation with fungal spores. The expression of a metallothionein gene (*OsMT1*), metallothioneins being essential for cellular processes of metal detoxification, was also induced in *M. oryzae*-infected rice.

The macroarray data also indicated that components of the machinery for protein synthesis, folding and modification were co-ordinately and rapidly up-regulated during infection of rice with the rice blast fungus *M. oryzae*. In particular, the induction of translation initiation factors (eIF5A) and elongation factors (EF-1α and EF-1β) was observed. The up-regulation of genes encoding eIF5A has also been described in the maize–*Fusarium verticillioides* pathosystem (Campo *et al*., 2004). It has been proposed that the different eIF5As might facilitate the translation of subsets of mRNAs required for specific physiological functions, such as cell division or cell death. Further studies are required to elucidate whether the *M. oryzae*-induced *eIF5A* rice gene facilitates translation of the set of mRNAs required for the rice defence response against fungal infection. With regard to the translation elongation factor EF-1, changes in the level of transcript accumulation have been reported to modulate the rate of apoptosis in mammalian cells (Duttaroy *et al*., 1998). In addition to their involvement in the detoxification of H_2O_2, thioredoxins and peroxiredoxins also participate in the thiol disulphide interconversion of proteins. Presumably, a co-ordinated up-regulation of components of the translational machinery will ensure the production of the newly requested defence proteins, these proteins being, in turn, part of the pre-invasive defence response of the rice plant.

An interesting observation arising from this study was that, in addition to inducing the expression of the genes involved in the translational machinery, fungal infection rapidly activated the expression of an important number of genes encoding components of the vesicle-associated transport machinery. The machinery for the transport of proteins between the organelles of the secretory pathway is highly conserved amongst eukaryotes and occurs by the budding of vesicles from a donor membrane and fusion with an acceptor membrane. Thus, the fusion of vesicles involves the interaction of v-SNAREs localized on the vesicle membrane and t-SNAREs localized on the target membrane (Jurgens, 2004). Additional proteins are also known to participate in the process of docking and fusion of transport vesicles to form protein complexes with v-SNAREs. Thus, v-SNARE interacts with a particular family of GTPases (Rab proteins) that participate in vesicle trafficking which, in turn, interact with GAP to hydrolyse GTP effectively. Of interest, *M. oryzae* infection rapidly induced the expression of components of the v-SNARE complex, namely the v-SNARE protein, Rgp1 and GAP. In *Arabidopsis*, pathogen-induced expression of a t-SNARE protein has been reported (Wick *et al*., 2003). Profilin, a protein that modulates actin polymerization and vesicular trafficking in plant cells (Aparicio-Fabre *et al*., 2006), and TRAPP (showing homology to yeast BET3), a protein involved in targeting and fusion of the ER to Golgi transport vesicles, were also up-regulated in the rice response to blast infection.

The macroarray data also revealed the fungal responsiveness of a rice gene encoding remorin. Remorin is a major component of plasma membrane lipid rafts (Mongrand *et al*., 2004). In *Arabidopsis*, the existence of lipid rafts, microdomains in the plasma membrane acting as 'signalling platforms' on which components of signal transduction cascades are locally condensed, is well documented (Bhat and Panstruga, 2005; Borner *et al*., 2005).

Taken together, a comparison of the gene expression profiles of *M. oryzae*-infected and non-infected Senia plants demonstrates that fungal infection results in the rapid activation of the protein secretory pathway and vesicle trafficking. All of these processes are co-ordinately up-regulated as part of the pre-invasive stage of the infection process. On perception of the pathogen, there is a massive request for defence proteins, and many of these proteins are targeted to the vacuole or the extracellular space. An increased level of components of the vesicular transport machinery will facilitate delivery of the defence-related proteins to the site of pathogen invasion. Increased vesicle fusion may also be required to repair the damage provoked by ROS to the

plasma membrane in infected tissues. Accordingly, these differentially expressed genes or early response regulators prepare the cell for an efficient defence response. The determination of the mechanisms by which these genes mediate the secretion of specific vesicles will not only contribute to our understanding of the early signalling events occurring during rice–blast interaction, but should also provide unique insights into the regulation of protein secretion in plants.

Transgenic expression of the *cecropin A* gene in rice has an impact on host gene expression

In the literature, many reports have illustrated the usefulness of antimicrobial genes for plant protection against pathogens. The production of these antifungal proteins aims to interfere with the growth of target pathogens in infected plant tissues, as it is generally accepted that pathogen resistance is solely a result of the effect of the transgene product on the pathogen. The results presented here, however, demonstrate that the introduction of a fungus resistance trait in a rice cultivar induces alterations in the expression of specific endogenous genes under normal growth conditions, i.e. in the absence of the pathogen. Thus, genes encoding several ROS-scavenging enzymes, which play a significant role in the basal resistance to pathogens, are over-expressed in *cecropin A* plants compared with wild-type plants. Most remarkable is the high level of expression of three different peroxidase genes in *cecropin A* plants. The observation that antioxidant systems are activated in transgenic plants in the absence of the pathogen is supported by the result that *cecropin A* plants are tolerant to oxidative stress imposed by treatment with H_2O_2. Although *cecropin A*-mediated changes in the expression of endogenous rice genes are observed, this phenomenon may be beneficial to the host plant, as illustrated by the observed tolerance of transgenic plants to oxidative stress.

It is well known that peroxidases play a significant role in conferring resistance to both fungal and bacterial pathogens in *Arabidopsis*. As an example, transgenic *Arabidopsis* plants with reduced levels of expression of a peroxidase gene were more susceptible than wild-type plants to fungal and bacterial pathogens (Bindschedler *et al*., 2006). In wheat, over-expression of a peroxidase gene resulted in enhanced resistance to the powdery mildew fungus *Blumeria graminis* f. sp. *tritici* (Altpeter *et al*., 2005). As previously reported by our group, *cecropin A* rice shows enhanced resistance to the rice blast fungus (Coca *et al*., 2006). In addition to this, *cecropin A* plants have been shown to exhibit enhanced resistance to the bacterial pathogen *Erwinia chrysanthemi*, the causal agent of foot rot disease of rice (results not shown). This observation indicates that transgenic expression of the *cecropin A* gene in rice confers protection against fungal and bacterial pathogens. The increased level of expression of rice peroxidase genes in *cecropin A* plants relative to that in wild-type plants may enhance their resistance to pathogen infection, thus reinforcing the antimicrobial activity of *cecropin A*. If so, the resistance of *cecropin A* plants to pathogens may be the consequence of a combination of both the activity of cecropin A and the activation of expression of endogenous defence genes.

Moreover, expression studies revealed that several ER-resident chaperones taking part in the process of the folding and modification of polypeptides targeted for secretion were expressed at significantly higher levels in *cecropin A* plants than in wild-type plants. This was the case for the genes encoding calnexin, calreticulin, HSP90, DNAJ heat shock protein and PDI. In this respect, it is well known that plant cells monitor protein misfolding by triggering the synthesis and accumulation of stress-specific chaperones that bind temporarily to proteins (Federoff, 2006). Otherwise, the accumulation of misfolded proteins might become catastrophic, leading to cell death. Calreticulin/calnexin protein complexes act as molecular chaperones in the ER (Crofts and Denecke, 1998). The PDI protein not only has a chaperone function in the secretory pathway of proteins, but also participates in thiol disulphide interconversion in the ER. The rice HSP90 identified in our macroarray experiments shows a high degree of amino acid identity with two HSP90 proteins from barley and Madagascar periwinkle, for which an ER localization has been demonstrated (although the majority of cellular HSP90s are located in the cytoplasm, distinct organelle forms are found in the ER, plastid and mitochondria). Other genes that are over-expressed in *cecropin A* plants compared with wild-type plants are those encoding DNAJ heat shock proteins, which participate in the process of protein translocation into the ER and folding (Miernyk, 1999). It should be stressed that the transgenic *cecropin A* rice lines used in this study express a *cecropin A* gene engineered for retention of the cecropin A polypeptide in the ER (Coca *et al*., 2006). The results presented here on the subcellular fractionation of transgenic rice strongly suggest that cecropin A is indeed located in the ER. It is therefore reasonable to postulate that differences in gene expression between wild-type and *cecropin A* plants may be attributed to the site of accumulation of the transgene product: the ER. The high level of expression observed for genes involved in the folding and stabilization of proteins that enter into the secretory pathway in transgenic plants may reflect the need for an adjustment of chaperone levels in the ER, a response that

resembles the so-called unfolded protein response for ER-resident proteins. Therefore, the basal activity of the protein folding and stabilization machinery may not be sufficient to accommodate the level of cecropin A protein synthesis in transgenic rice plants, and these plants may need to maintain the machinery for the synthesis and folding of proteins in an activated state. This study provides strong evidence that the transgenic expression of an antimicrobial gene, the *cecropin A* gene, in rice has an impact on host gene expression.

Evidence was also provided that transgenic and wild-type plants share common responses to infection, as demonstrated by the type of genes induced in response to *M. oryzae* in both *cecropin A* and wild-type plants. A closer examination of the number and distribution of the fungal-induced genes in the functional categories, however, revealed some differences between *cecropin A* and wild-type plants. Firstly, the number of fungal-responsive genes was significantly lower in *cecropin A* plants than in wild-type plants when using the same arbitrary twofold cut-off and at the same time after inoculation with fungal spores (94 and 162 unique genes were up-regulated by fungal infection in transgenic and wild-type plants, respectively). Secondly, the contribution of ESTs encoding defence genes in the overall response of transgenic plants to *M. oryzae* infection was lower than that in wild-type plants (13.0% and 24.2% of the ESTs were grouped in the defence category in transgenic and wild-type plants, respectively; see Figures 1 and 6). The high level of expression of certain defence genes in *cecropin A* plants under normal growth conditions, namely antioxidant systems, may account for the lower contribution of genes categorized in the defence group during the response of transgenic plants to infection. Together, these results indicate that, although expression of the *cecropin A* gene in transgenic rice does not impede the endogenous defence response, it exerts an effect on the transcriptional reprogramming that accompanies adaptation of the rice plant to infection conditions.

From the results obtained with *cecropin A* rice plants, it cannot be concluded that all transgenic plants must be considered to be 'not substantially equivalent' to wild-type plants at the level of gene expression. In other studies, transgenic wheat expressing a fungal phytase gene, or a high-molecular-weight glutelin subunit, exhibited only small differences in gene expression compared with untransformed plants (Gregersen *et al*., 2005; Baudo *et al*., 2006). Collectively, these data illustrate the need for the use of transcriptomics to explore the impact of transgene expression in plants, whilst establishing the need to approach these studies on a case-by-case basis. The availability of the rice defence gene collection obtained here provides an important tool for the analysis of gene expression in cultivated rice plants expressing other types of antimicrobial gene. A thorough understanding of the molecular mechanisms that operate in transgenic rice expressing antimicrobial genes, either during normal growth conditions or during interaction with the rice blast fungus, will undoubtedly aid in the evaluation of future strategies for the engineering of resistant rice cultivars using foreign antimicrobial genes.

Experimental procedures

Plant material and infection conditions

Rice (*O. sativa* L. cv. Senia) plants were soil grown at 27 ± 2 °C using a 18-h/6-h light/dark photoperiod. *M. oryzae* (PR9 isolate, CIRAD collection, Montpellier, France) was maintained on rice flour medium (20 g/L rice flour, 15 g/L agar and 2.5 g/L yeast extract) until the mycelium covered the surface of the plate. Spores were collected by adding sterile water to the surface of the mycelium. After filtration, the spores were adjusted to the appropriate concentration with sterile water using a Bürker counting chamber. Infection of rice leaves with *M. oryzae* spores was performed using a detached leaf assay, as described previously (Coca *et al*., 2004). Briefly, the second leaf of 2-week-old soil-grown rice plants was placed into plate dishes with 1% w/v water agar containing 2 μg/L kinetine. Whatman filter paper saturated with an *M. oryzae* spore suspension at a concentration of 10^6 spores/mL was placed on to the upper face of the leaf. The inoculated leaves were maintained in a chamber under humid conditions at 28 °C using a 16-h/8-h light/dark cycle for the required period of time.

RNA isolation and Northern blot analysis

Plant material was ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted from rice leaves using TRIzol reagent (Invitrogen, Life Technologies, Basle, Switzerland). For Northern blot analysis, total RNAs (10 μg) were subjected to 1.2% formaldehyde-containing agarose gel electrophoresis and transferred to nylon membranes (Hybond-N, Amersham, Little Chalfont, Buckinghamshire, UK). Hybridizations were carried out in 0.125 M Na_2HPO_4 pH 7.2, 7% SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), at 65 °C. Membranes were washed three times, 30 min each wash, in 0.02 M Na_2HPO_4 pH 7.2, 1 mM EDTA and 1% SDS at 65 °C. All cDNA probes were gel purified and radioactively labelled by random priming.

SSH and cDNA library construction

The principle of PCR-selected cDNA subtraction (referred to as PCR-SSH) is to subtract, by hybridization, sequences that are shared between a control (or 'driver') sample and the sample of interest (or 'tester'), followed by the selective PCR amplification of the differentially expressed cDNAs. Thus, the SSH approach originally developed by Diatchenko *et al*. (1996), and subsequently improved by the ligation of generic linkers to cDNAs followed by PCR amplification and

cloning of the differentially expressed cDNAs (PCR-SSH), allows a library to be normalized, thus minimizing clone redundancy. Thus, the cDNA subtraction method allows a cDNA library to be obtained which is enriched in sequences differentially represented between the two samples, making it easier to detect low-abundance transcripts than in the theoretically more optimal 'whole transcriptome' array. Moreover, the probes used for microarray preparation in global profiling studies are usually designed to interact with the 3′-regions of the targeted transcripts. Thus, it is possible that transcripts could be missed in global transcription profiling studies with the Senia variety using commercial rice arrays because of the poor hybridization between the probes and the labelled cDNA targets. Together, these aspects make the PCR-SSH technique, in combination with cDNA macroarray technology, especially suited for gene expression studies on the cultivated rice variety used in this investigation.

In this work, the PCR-SSH method was used to obtain a cDNA library representing different time points during the early response of rice to blast infection. Equal amounts (100 μg) of total RNA from each infection time (3, 6, 9, 24 and 48 h after inoculation with fungal spores) were combined prior to mRNA isolation. In each case, the starting biological material was a pool of leaves from at least six individual plants for each infection time. Equal quantities of total RNAs derived from control, non-infected leaves were also pooled. Poly(A)$^+$ RNAs were purified using biotinylated oligo(dT) and streptavidin-coupled paramagnetic particles (PolyA-Tract System, Promega, Madison, WI, USA). Double-stranded cDNA was produced from 3 μg of mRNA obtained from *M. oryzae*-infected (tester cDNA) or non-infected (driver cDNA) rice leaves. The SSH library was constructed following supplier instructions (Clontech, Palo Alto, CA, USA). The tester and driver cDNAs were digested with *Rsa*I. The *Rsa*I-digested tester cDNAs were ligated to different adapters (adapters 1 and 2). Two rounds of hybridization and PCR amplification were carried out to enrich the differentially expressed cDNAs. The SSH library enriched for differentially expressed cDNAs was constructed by ligating the subtracted cDNAs into the pGEM-Teasy vector (Promega) and transferring them into *Escherichia coli* DH5α. Individual recombinant bacterial colonies were picked and grown on 96-well microtitre plates.

Amplification of cDNA inserts and cDNA macroarray preparation

The cDNA inserts were amplified from colonies by PCR using adapter primers provided in the PCR-select cDNA subtraction kit, which were complementary to sequences flanking both sides of the cDNA insert. The PCRs contained 1.25 μL of each primer (10 μM each), 0.5 μL deoxynucleoside triphosphate (dNTP) mix (10 mM each), 2.5 μL $MgCl_2$ (25 mM), 0.5 μL of Taq DNA polymerase (Roche, Mannheim, Germany) and 1 μL of bacterial culture in a total volume of 25 μL. PCRs were performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Ramsey, Minnesota, USA) as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 2 min, followed by a final extension step at 72 °C for 7 min. The PCR products were electrophoresed on a 0.8% agarose gel to confirm the amplification quality and quantity.

The PCR products were transferred to 384-well plates. The BioGrid Robotic System (BioRobotics, Cambridge, UK) with a 384 gridding tool (diameter, 0.4 mm) was used to spot cDNAs on to nylon membranes (Hybond N+, little Chalfont, Buckinghamshire, UK). Each clone was spotted 10 times on to the membrane (about 0.2 μL per spot) with a spot diameter of 0.6 mm. The membranes were kept humid by setting them on to three Hybond™ blotting paper sheets soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH). After printing, the membrane was neutralized with 1.5 M NaCl, 0.5 M Tris/HCl pH 7.2, 1 mM EDTA pH 8.0 for 1 min. No subsequent UV fixation was necessary, and the membranes were kept on filter paper until completely dry.

In addition to the cDNA rice inserts, the pGEM-T plasmid DNA was spotted on to each membrane as a negative control. Known rice defence genes, namely those encoding β-1,3-glucanase (accession number AB027432), two chitinases (accession numbers X56063 and AB016497), OsPR10 (accession number AF395880) and thionin (accession number AB072337), and *Eco*RI-digested rice genomic DNA, were also printed on the membrane to serve as internal positive controls. The rice genomic DNA was extracted from 4-week-old rice leaves according to the method of Murray and Thompson (1980), but using mixed alkyltrimethylammonium bromide (MATAB) as the extraction buffer (0.1 M Tris/HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% MATAB, 1% PEG6000, 0.5% sodium sulphite).

Macroarray hybridization, scanning and data analysis

As a control test for printing quality, hybridization with genomic DNA from *M. oryzae* was performed, which was obtained by growing the fungus on a rice flour liquid medium (500 mL) for 1 month, with the mycelium harvested from the liquid culture by filtration. *M. oryzae* genomic DNA was extracted as described above. The *M. oryzae* genomic DNA (1 μg) was digested with *Eco*RI, radioactively labelled by random priming (Roche) and used as hybridization probe.

Total RNAs were obtained from rice leaves inoculated with *M. oryzae* and control leaves. Rice cDNAs were obtained by reverse transcription from 40 μg of total RNA and labelled with ^{33}P-α-deoxycytidine triphosphate (dCTP) using SuperScript II Reverse Transcriptase (Invitrogen), as described previously (Alberola *et al.*, 2004). Total RNA was also isolated from leaves of 2-week-old *cecropin A* plants (lines R33-24.3, R33-33.7 and R33-6.3, with 10 plants per line), as well as from leaves from wild-type plants at the same developmental stage. The labelled cDNA probes were denatured at 100 °C for 5 min, followed by 5 min on ice, and then used for hybridization. Before hybridization, the membranes were washed in 0.5% SDS for 30 min at 80 °C. The membranes were pre-hybridized in 5 mL of 5 × standard saline citrate (SSC), 0.5% SDS, 5 × Denhart's solution at 65 °C for at least 1 h. Hybridization was carried out by adding the denatured probe to fresh hybridization solution (5×10^6 dpm/mL) for 40–64 h at 60 °C. After hybridization, the membranes were washed at 65 °C in 2 × SSC, 0.1% SDS for 20 min, and twice in 0.2 × SSC, 0.1% SDS for 30 min. Two replica membranes were hybridized with each ^{33}P-labelled cDNA population.

Hybridization signals were recorded by phosphorimaging (FujiFilm FLA3000 Phosphorimager, Berlin, Germany). The image data obtained were imported into the software program ArrayVision 7.0 (Imaging Research, St. Catharines, ON, Canada) for spot detection and quantification of hybridization signals. Backgrounds were subtracted using ArrayVision 7.0 to obtain raw signal intensities. To assess the reproducibility of the macroarray analysis, two independent hybridizations were conducted for each condition. To control against biological errors, hybridizations were performed with replica membranes for two independently labelled cDNAs from RNA samples obtained

from two independently pooled leaf samples. Data for the expression analysis were averages of the two independent experiments. The nucleotide sequences of the selected cDNAs were determined and compared with nucleotide and protein sequence databases (GenBank, http://www.ncbi.nlm.nih.gov; KOME, http://cdna01.dna.affrc.go.jp; MGOS, http://www.mgosdb.org).

RT-PCR and qRT-PCR analyses

For RT-PCR and qRT-PCR analyses of the rice defence genes, total RNA was extracted from leaves of rice plants at the three- to four-leaf stage at different times after fungal inoculation with fungal spores. For each time point, leaves were collected from 10 individual plants. Total RNA was treated with DNase I (Roche) to remove DNA contamination before cDNA synthesis. The cDNA was synthesized from 5 μg of total RNA using 500 ng of oligo(dT)$_{12–18}$ in a total volume of 20 μL. Aliquots (1 μL of a 1 : 10 dilution of the resulting RT reaction product) were used as template for RT-PCR analysis.

The primers used for RT-PCR analysis were as follows: *Rgp1* (forward, 5′-GAGTTAAGAGGCCATGCTGACAAGAA-3′; reverse, 5′-CAGTGTTGATATGGCAGGAGTAAACT-3′); *GAP* (forward, 5′-GCAACTGAACATGGTAGCCAGGATAA-3′; reverse, 5′-CACTACTC AATCCTACCGTGAAGGGT-3′); *profilin* (forward, 5′-GGAGATTAC TGGCATCATGAAGGACT-3′; reverse, 5′-GTACAGAACTCAGT GAATGGACAGCT-3′); *TRAPP-Bet3* (forward, 5′-GCTGACTCACTTG AGAAAGGAACAGA-3′; reverse, 5′-GTACACAAATCATGTGGAGTC AGCCA-3′). The control of the rice *ubiquitin 1* gene was performed using the primers: forward, 5′-CGCCTTCTCAAGATGCAGATCTT-3′; reverse, 5′-CTGGATGTTGTAGTCAGCAAGGGT-3′.

The forward and reverse primers for qRT-PCR were designed using the PrimerXpress program (Applied Biosystems, Foster City, CA, USA). They were as follows: *EREBP* (forward, 5′-TGTTGGAGACT-GATTCAAATAATGC-3′; reverse, 5′-CACATTACAGCACACACAT-GGC-3′); *eIF5A-4* (forward, 5′-CCCCTAAACCACCATCCCTAG-3′; reverse, 5′-AATGATATTTTCACCTTAAGCACACAAT-3′); *HSP90* (forward, 5′-GGAGCCGTCGTCGTATGATAA-3′; reverse, 5′-TTGGT-GGCGACACATCACTAC-3′). A set of primers for the rice *actin 1* (*Rac1*) gene was also designed for use as an endogenous control to normalize the data for differences in input RNA and efficiency of reverse transcription (forward, 5′-CCTCTTCCAGCCTTCCTTCATA-3′; reverse, 5′-GCAATGCCAGGGAACATAGTG-3′).

qRT-PCR analysis was performed in optical 96-well plates using SYBRGreen to monitor double-stranded DNA synthesis (Platinum SYBRGreen qPCR with ROX, Invitrogen). Each reaction contained 2 μL of a 1 : 100 dilution of cDNA and 300 nM of each of the two gene-specific primers in a final volume of 20 μL. PCR was performed on an AbiPrism 7000 instrument (Applied Biosystems). After the initial activation step of the DNA polymerase at 95 °C for 2 min, samples were subjected to 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing and extension together at 60 °C for 30 s). No RT controls were included in the PCR runs to ensure negligible contamination of total RNA samples with genomic DNA.

Quantification of target gene expression was performed using the comparative threshold cycle (C_T) method. The C_T value is defined as the PCR cycle number that crosses an arbitrarily chosen signal threshold in the logarithmic phase of the amplification curve. Primer validation experiments were performed using 2 μL of fivefold dilutions of cDNA. The average C_T values from triplicate PCRs were normalized to the average C_T values for the *actin* gene from the same cDNA preparations. For each gene, normalized transcript levels in *M. oryzae*-infected leaves were compared with those of the respective control leaves.

Subcellular fractionation

Leaves from wild-type and transgenic plants were pulverized and homogenized in PBP buffer (0.1 M Tris/HCl pH 8.0, 0.05 M KCl, 0.01 M $MgCl_2$, 2 mM EDTA, containing 10% sucrose; 0.025 g fresh weight per millilitre) for 15 min at 4 °C with slow stirring. The homogenate was filtered through two layers of miracloth to remove cellular debris. Three millilitres of the filtrate were loaded on to a discontinuous sucrose gradient (20%, 30%, 40%, 50% and 70% sucrose in PBP buffer, 2 mL each) and centrifuged at 24 000 ***g*** for 2 h at 4 °C using a Beckman SW40 rotor (Beckman, Muskegon, MI, USA). Then, 1-mL fractions were collected and subjected to immunoblot analysis. The fractionation profiles of cecropin A and the ER marker BiP protein were examined. For this, a polyclonal antibody raised in rabbit against cecropin A (Coca *et al*., 2006) and BiP (kindly provided by M.D. Ludevid and M. Torrent) were used. Blots were incubated for 2 h at room temperature with anti-cecropin A or the anti-BiP protein (diluted 1 : 500 and 1 : 2000, respectively), followed by incubation with a secondary antibody conjugated with horseradish peroxidase and detection by the ECL system (Amersham Bioscience, Braunschweig, Germany).

NBT staining for O_2^-

For histochemical detection of O_2^-, leaf sections of approximately 2 cm in length were excised from either transgenic or wild-type plants and stained with NBT, as described by Fitzgerald *et al*. (2004).

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