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Abstract

Suspension cultured cells of a blast-resistant rice genotype (*Oryza sativa* L. cv. Gigante Vercelli) were treated with cell wall hydrolysates prepared from the fungal pathogen *Magnaporthe oryzae*. As a consequence, a complex pattern of phenylalanine ammonia lyase time course specific activity levels was evident. Ion-exchange chromatographic fractionation of crude extracts suggested that the early (6 h) and the late (48–72 h after elicitation) increase of activity relied upon the sequential induction of two different isoenzymes. The relative expression levels of 11 genes putatively coding for a phenylalanine ammonia lyase were measured by semi-quantitative capillary gel electrophoresis of RT-PCR products. Two genes were indeed found to be induced by treatments with the hydrolysate, and data were validated by real-time PCR. Conversely, only the early-responsive enzyme form was observed following elicitation in a blast-sensitive rice genotype (cv. Vialone nano). Therefore, the late-responsive isoform may represent a candidate gene to select for decreased sensitivity to blast.

Abbreviations

- PAL, phenylalanine ammonia lyase

Keywords

- Aromatic secondary metabolism;
- Blast fungus;
- *Magnaporthe oryzae*;
- Elicitors;
- Multiple enzyme forms;
- Gene expression

Introduction

Plants perceive biotic stimuli by recognizing a multitude of different signaling compounds originating from the interacting organisms. Some of these substances are pathogen-related

compounds, which act as general elicitors of defense reactions (Bent and Mackey, 2007 and Vasconsuelo and Boland, 2007). During the co-evolution of plants and microorganisms, pathogens gained the ability to synthesize and deliver effector proteins to suppress plant defenses. In response, plants evolved receptors to detect the presence of effector proteins (Berrocal-Lobo and Molina, 2008 and Kim et al., 2008). The plant cell wall represents an effective physical barrier to pathogens, and microbial hydrolases targeting the plant cell wall are well-known components of virulence (Cantu et al., 2008). On the other side, several plant defense proteins, such as β-1,3-glucanases, class I chitinases and some uncharacterized enzymes showing chitosanase activity, target the fungal cell wall (Ferreira et al., 2007). In addition, compounds derived from an incomplete hydrolysis of either the cell surface of pathogens (Yamaguchi et al., 2000) or the plant cell wall (Federici et al., 2006) can act as potent elicitors. Despite the large variety of active molecules, general schemes for elicitor signaling leading to plant resistance can be drawn, and include reversible protein phosphorylations, changes in the activity of plasma membrane proteins, variations in free Ca^{2+} concentrations in cytosol and nucleus, and production of reactive oxygen species and nitric oxide (Garcia-Brugger et al., 2006). An enhanced synthesis of aromatic secondary metabolites may contribute to disease resistance either directly, as phytoalexins, or through incorporation of phenolic material into structural barriers, such as phenol-conjugated or lignified cell walls (Treutter, 2006 and Menden et al., 2007).

The first committed step of the branched phenylpropanoid metabolism, the conversion of l-phenylalanine (Phe) into *trans*-cinnamate, is catalyzed by phenylalanine ammonia lyase (PAL, EC 4.3.1.5) (MacDonald and D'Cunha, 2007). A rapid increase of PAL activity levels represents an early response to attempted penetration by pathogens (Hahlbrock and Scheel, 1989), and a partial suppression of PAL gene expression may lead to increased fungal susceptibility (Maher et al., 1994 and Shadle et al., 2003). Growing experimental evidence suggests that the pathogen-induced increase of aromatic biosynthesis is accomplished by the expression of specific isoforms of several shikimate and phenylpropanoid pathway enzymes (McCue and Conn, 1989, Keith et al., 1991, Görlach et al., 1995, Kervinen et al., 1998 and Forlani, 2002).

Magnaporthe oryzae [T.T. Hebert] Yaegashi & Udagawa (teleomorph of *Pyricularia grisea* [Cooke] Sacc.) is a filamentous heterothallic ascomycete causing blast, a disease that affects many species of the grass family (Talbot, 2003). Rice blast is considered the main fungal disease of this crop because of a wide distribution and destructiveness under favorable conditions. The interaction between rice and *M. oryzae* has been the focus of extensive studies on plant disease resistance and fungal infection mechanisms (Ribot et al., 2008). Plant cell cultures have been widely employed to unravel fine details of host-pathogen interactions. Calli derived from plants of resistant cultivars usually retain tolerance (*e.g.* Aver'yanov et al., 2001). In most cases, the opposite is also true, allowing the selection of resistant clones at the undifferentiated tissue level (Saxena et al., 2008). The use of partially purified elicitors instead of pathogen infection may further simplify the experimental system. Models in which plants are substituted by cultured cells and pathogens by elicitors can adequately simulate *in vivo* infections (Lamb et al., 1989 and Keller et al., 1996). Such *in vitro* systems have provided reliable tools to identify some of the biochemical steps in the rice dynamic response to blast (Kim et al., 2000 and Kim et al., 2003).

In previous works, suspension cultured cells of various rice cultivars were treated with mycelial wall hydrolysates prepared from different *M. oryzae* isolates. Soon (1–3 h) after elicitor addition, cells produced significant amounts of superoxide anion, which was rapidly converted into diffusible peroxide. Interestingly, a significant correlation was found between basal and elicited levels of peroxide released and the overall tolerance of a given cultivar to the pathogen (Rożkowicz et al., 2003). Taking this approach one step further, the induction of phenylpropanoid metabolism in elicited cells was then considered. When PAL-specific activity was measured in rice suspension

cultured cells following treatment with cell wall hydrolysates, even low hydrolysate concentrations were able to induce a significant increase of enzyme levels 24 h after elicitor addition. However, rice genotypes showing differential sensitivity to blast did not react differently, nor did elicitors obtained from various pathotypes induce different reactions. At a later stage, higher hydrolysate concentrations were required to trigger maximal enzyme induction. In this case, on the contrary, highly significant differences were observed among plant genotypes, and a remarkable relationship was evident between the mean increase in PAL activity 72 h after elicitation and the overall resistance to blast at the plant level (Forlani, 2010). Here, we report on the molecular basis for increased PAL-specific activity levels in elicited rice cells.

Materials and methods

Plant material, growth conditions and elicitor treatment

Oryza sativa L. cell suspension cultures were grown in a liquid medium consisting of Murashige and Skoog salts and vitamins supplemented with 30 g L^{-1} sucrose and 2 mg L^{-1} 2.4D; 0.2% (v/v) Plant Preservative Mixture solution (Plant Cell Technology, Inc, Washington DC, USA) was routinely added to reduce the risk of microbial contamination. Cells were grown in 250 mL Erlenmeyer flasks containing 62 mL suspensions. Flasks were incubated under dim light (<50 μmol s^{-1} m^{-2}) on a rotary shaker (100 rpm) at 24 ± 1 °C. Subcultures were made every 14 days by transferring 12 mL aliquots to 50 mL of fresh medium. However, cultures to be used for experiments were maintained in continuous balanced growth by subculturing every 7 days. Mycelial wall hydrolysates, obtained by thermal hydrolysis from the purified mycelial cell wall of *Magnaporthe oryzae* as described previously (Rożkowicz et al., 2003), were added to the culture medium 3 days after the inoculum.

Enzyme extraction and assay

Rice cultured cells were harvested by vacuum filtration on nylon filters (50 μm mesh), resuspended in 2 mL g^{-1} of ice-cold extraction buffer (50 mM Tris–HCl buffer, pH 7.4, containing 0.5 mM DTT and 0.5 mM EDTA), and homogenized on ice in a 30 mL Teflon-in-glass Potter homogenizer by 20 strokes; 10 mg mL^{-1} insoluble polyvinylpolypyrrolydone was added to prevent oxidation of phenolics. All subsequent operations were carried out at 4 °C. The homogenate was centrifuged for 10 min at 14,000 × *g*, and solid ammonium sulphate was added to the supernatant to yield 70% saturation. Salted-out proteins were collected by centrifugation as above, resuspended in a small volume of extraction buffer and desalted by passage through a Bio-Gel P6DG column (Bio-Rad) equilibrated with the same buffer.

PAL activity was measured by following the production of cinnamic acid, as described previously (Forlani, 2002). The assay mixture contained 50 mM Tris–HCl buffer, pH 9.0, 5 mM l-Phe and a limiting amount of enzyme (up to 100 pkat) in a final volume of 1 mL. Samples were incubated at 35 °C for 10 min, monitoring every 20 s the absorbance at 290 nm against blanks in which the substrate had been either omitted or replaced with d-Phe. Activity was calculated utilizing the linear regression equation of cinnamate production over time, on the basis of an extinction coefficient estimated with an authentic standard. According to IUB recommendations, one unit of enzymatic activity (katal) was defined as the amount of enzyme that catalyzes the formation of 1 mol of cinnamate s^{-1} under the above assay conditions. Protein concentration was quantified by the method of Bradford (1976), using bovine serum albumin as the standard.

PAL isoform detection

Following ammonium sulphate precipitation, pelleted proteins were resuspended in extraction buffer supplemented with 100 mM NaCl, and column-desalted against the same buffer. Desalted extracts were loaded at 4 °C at a constant flow of 60 mL h^{-1} onto a DEAE-Sephacel column (20 mL bed-volume) previously equilibrated with the same buffer, while collecting 2.5 mL fractions. After extensive washing, retained proteins were eluted with buffer containing 200 mM NaCl.

Alternatively, desalted extracts were further centrifuged at 40,000 × *g* for 30 min, and 10 mL aliquots of the resulting supernatant were injected by means of a superloop (Pharmacia) onto a 5 mL HiTrap Q (Pharmacia) column that had been previously equilibrated with extraction buffer supplemented with 120 mM NaCl. Proteins were eluted at a flow rate of 1 mL min^{-1} using a computer-controlled (Data System 450; Kontron, Munchen, Germany) linear gradient from 120 to 240 mM NaCl (60 mL), for the collection of 1 mL fractions, while monitoring the eluate at 280 nm (HPLC Detector 432, Kontron).

RNA extraction, retrotranscription and PCR analysis

Total RNA was extracted from 100 mg of plant material by using the Plant RNA Isolation kit (Agilent Technologies), according to the manufacturer's instructions. Following spectrophotometric quantisation and assessment of RNA integrity, RNA (3 µg) was used for single strand cDNA synthesis by reverse transcription in a 20 µL reaction mixture using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene) with random primers. Specific primers for each putative PAL gene (Fig. S1 of supplementary material) were designed by the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). A 1 µL aliquot of cDNA from the reverse transcription reaction was used for PCR. The amplification was carried out in a Whatman Biometra T-Gradient Thermal cycler in a 25 µL reaction mixture containing 2.5 µL 10× PCR reaction buffer (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol forward and reverse primers and 1 U of *Taq* DNA polymerase (Fermentas). Cycling conditions consisted of an initial 4 min at 94 °C, followed by 30 s denaturing at 94 °C, 60 s annealing at 50 °C, and 120 s elongation at 72 °C repeated for 35 cycles, and with 10 min final extension at 72 °C. PCR products were first visualized by conventional agarose (2%) gel analysis, and then one microliter of the amplification reaction was analyzed by capillary gel electrophoresis using the 2100 Bioanalyzer and the DNA 1000 LabChip Kit (Agilent Technologies) according to the manufacturer's instructions. The kit provides sizing and quantification of dsDNA fragments ranging from 25 to 1000 bp. A semi-quantitative estimate of relative expression was obtained from the ratio between the concentration of amplification products for PALs and that for the housekeeping gene β-actin, followed by normalization with respect to untreated controls.

Quantitative real-time PCR analysis was performed by standard protocols using an Mx3000P Real-Time PCR System (Stratagene). The process was carried out with 25 µL of a mixture containing 12.5 µL of 2× Brilliant SYBR Green QPCR master mix (Stratagene), cDNA (1 µL from 20 µL of each reverse transcriptase product pool), 100 nM primers, and 30 mM ROX as a passive reference dye. Initial polymerase activation: 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 60 s at 55 °C, and 30 s at 72 °C. PCR conditions were determined by comparing threshold values in a dilution series of the RT product, followed by non-RT template control and nontemplate control for each primer pair. Relative expression values were computed according to Pfaffl (2001). All determinations were carried out in triplicate.

Statistical analysis

Unresolved PAL activity peaks in anion-exchange chromatograms were quantified by Gaussian deconvolution of activity profiles using Fityk® (version 0.8.2), a curve fitting and data analysis freeware (http://www.unipress.waw.pl/fityk/).

Results

PAL-specific activity levels in rice cells following contact with fungal hydrolysates

Suspension cultured cells of a rice genotype showing full tolerance to all *M. oryzae* pathotypes (Faivre-Rampant et al., 2011), Gigante Vercelli, were treated with hydrolysates prepared from purified fungal cell wall. A significant and rapid increase of PAL-specific activity levels was found soon after the addition (Fig. 1). At earlier times (4–8 h), no significant differences were found between low and high hydrolysate concentrations. Thereafter, different patterns were evident. With a low elicitor rate (2 mg L^{-1}) activity levels started to lower back to basal values after 24 h. By contrast, at a higher concentration (100 mg L^{-1}) specific activity kept increasing until a second plateau was reached, 72 h after the treatment (Fig. 1).

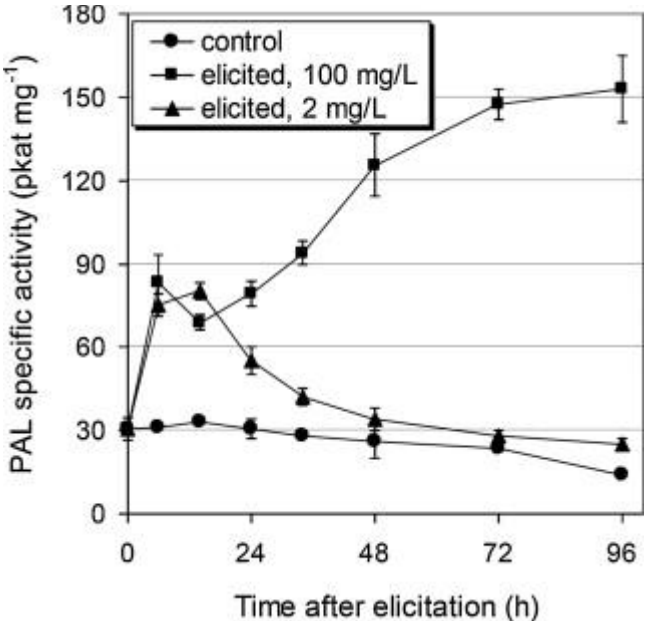

Fig. 1. PAL specific activity levels in suspension cultured cells of rice following the addition of crude elicitor from *Magnaporthe oryzae* mycelium. Actively-growing cultures of the blast-resistant cultivar Gigante Vercelli were added with 0, 2 or 100 mg L^{-1} of fungal cell wall hydrolysate. At increasing time after the addition cells were harvested and the specific activity of PAL was evaluated in cell-free extracts. Results are mean ± s.e. over three independent replications. The whole experiment was repeated twice, and very similar patterns were obtained.

Evidence for the occurrence of PAL isoforms and their expression in elicited cells

Experiments were then performed to understand whether the enzyme induced by the contact with fungal elicitor differs from that constitutively expressed in cultured cells. The shape of elution profiles obtained upon anion-exchange chromatographic fractionation of crude extracts suggested the occurrence of at least two different enzyme forms. Because conventional salt gradients did not resolve them, alternative protocols were set up by means of stepwise elution or anion-exchange FPLC (Fig. 2). These protocols allowed us to quantify the contribution of each putative isozyme to the overall activity. In extracts from untreated controls, low levels of both forms were detectable.

Soon after elicitor addition, the increase of activity was found to depend mainly upon the induction of isozyme I, whereas the level of isozyme II did not change significantly. The level of isozyme I remained quite constant thereafter. The further increase of PAL activity that took place 36–48 h after elicitation almost entirely relied upon an increased expression of isozyme II (Fig. 3).

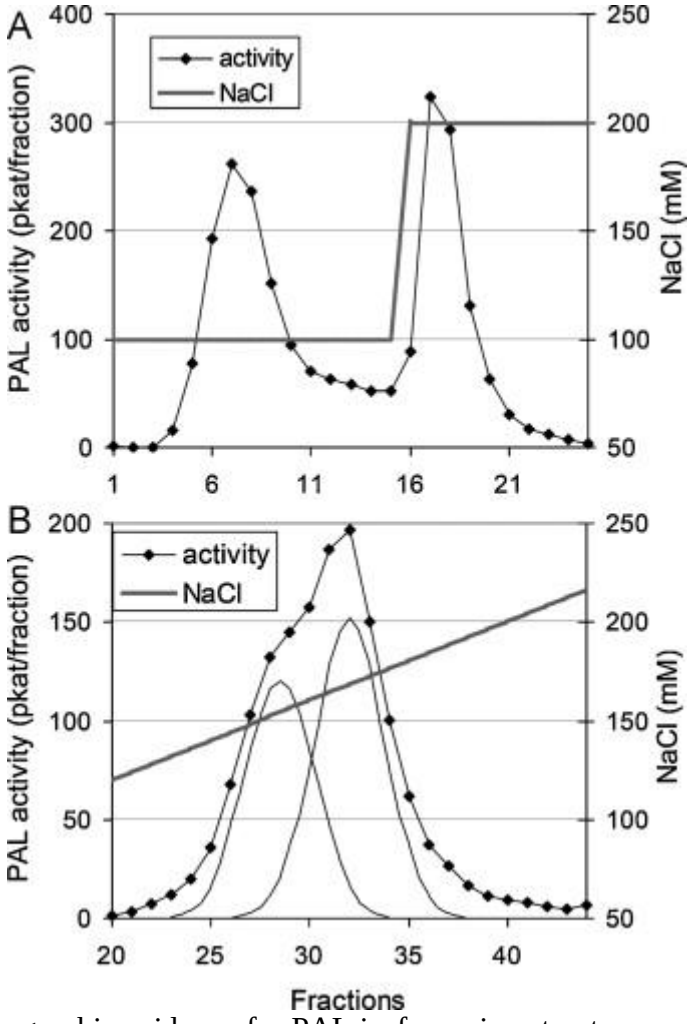

Fig. 2. Chromatographic evidence for PAL isoforms in extracts prepared from Gigante Vercelli cultured cells 72 h after the addition of fungal elicitor (100 mg L^{-1}) to the culture medium. Panel A. Stepwise fractionation by LC on a DEAE-Sephacel column. Panel B. Gradient fractionation by FPLC on a Hi-Trap Q column, where the opposite elution order was observed. The result of a Gaussian deconvolution analysis of the elution profile, performed with the curve fitting and data analysis freeware Fityk®, is also shown.

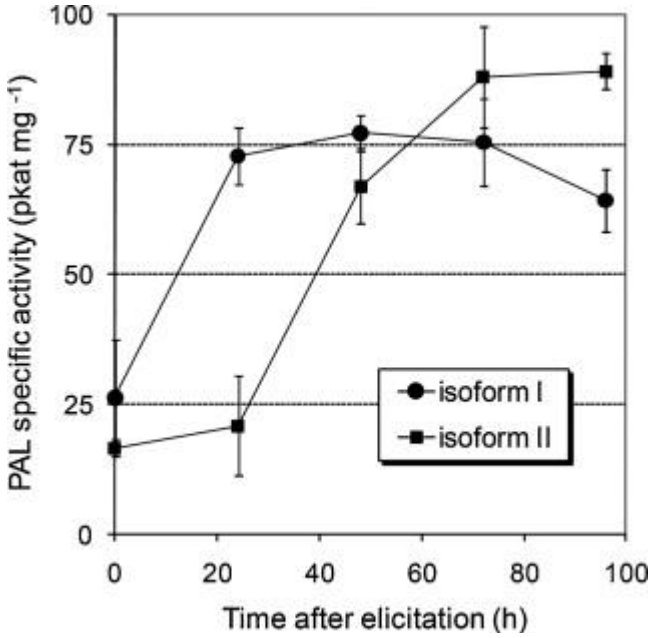

Fig. 3. Contribution of isozymes to the overall PAL specific activity in elicited cells. Activity levels, expressed as pkat mg^{-1} protein, were measured at increasing time after cell elicitation with *M. oryzae* cell wall hydrolysate (100 mg L^{-1}). Separation of enzyme forms was performed either by liquid chromatography on a DEAE-Sephacel column or by fast protein liquid chromatography on a HiTrap Q column. In the latter case, unresolved peaks were quantified by Gaussian deconvolution of activity profiles, as shown in Fig. 2. A good agreement was found between the two methods (SD never exceeding 20%). The putative isozymes were named according to their order of elution from the DEAE-Sephacel column. Results are mean ± SD over three independent replications.

Identification of PAL genes involved in the rice dynamic defense response

To further support the occurrence of true PAL isoforms, a molecular approach was chosen. In gene databanks, 19 sequences are annotated as putative rice PALs (Rice Genome Research Program, http://rgp.dna.affrc.go.jp/). An *in silico* analysis showed that some of them are redundant, but at least 11 different genes, herein named *PAL01* through *PAL11*, possibly code for this enzyme (Figs. S2 and S3 in the supplementary material). Specific primers were designed for each of these genes (Fig. S1), and reverse transcription PCR experiments were performed to identify those expressed in cultured cells. A detectable amplification product was obtained for 8 out of 11 genes. The analysis was then carried out with RNA extracted from elicited cells at increasing time after the treatment. A semi-quantitative estimate of gene expression was obtained by capillary electrophoresis of PCR products. Six genes showed quite uniform expression levels. The mRNA for the other two, *PAL07* and *PAL04*, was significantly more abundant in rice cells treated with fungal hydrolysate (Fig. 4). The time course of their expression was determined by quantitative real-time PCR. The patterns obtained (Fig. 5) were in reasonable agreement with those previously found at the biochemical level for isozyme I and II, respectively.

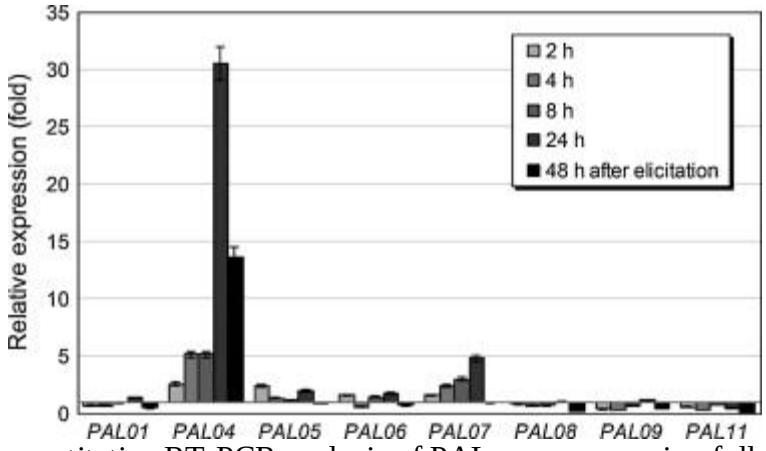

Fig. 4. Semi-quantitative RT-PCR analysis of PAL gene expression following elicitation. Suspension-cultured cells of rice, cv. Gigante Vercelli, were treated with fungal hydrolysates (100 mg L^{-1}). At increasing times after the addition cells were harvested, and total RNA was extracted. Expression levels for each of the 11 putative PAL genes were measured by semi-quantitative reverse transcription PCR by means of specific primers (Fig. S1 in the supplementary material). PCR products were visualized by conventional agarose gel analysis, and then by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Fig. S4 in the supplementary material). A semi-quantitative estimate of relative expression was obtained from the ratio between the concentration of amplification products for each PAL cDNA and that for the housekeeping gene β-actin, followed by normalization with respect to untreated controls.

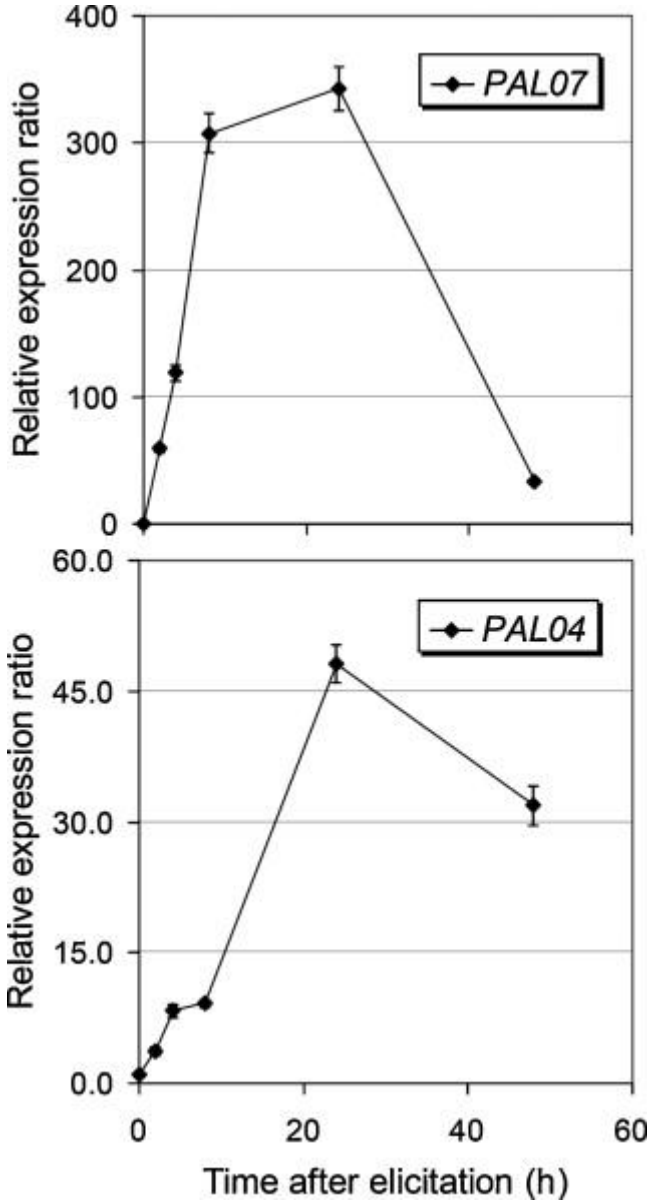

Fig. 5. Real-time PCR analysis of elicitor-induced expression of *PAL07* and *PAL04* genes. The same cDNA samples described in Fig. 4 were amplified by standard protocols using an Mx3000P Real-Time PCR System (Stratagene). Data were normalized using the β-actin mRNA level. Relative expression values were computed according to Pfaffl (2001). All determinations were carried out in triplicate.

PAL specific activity levels in a rice genotype with high susceptibility to blast

PAL activity was then measured in cultured cells of a rice cultivar that shows a high susceptibility to blast, namely Vialone nano (Faivre-Rampant et al., 2011). In this case also, a rapid increase of PAL levels was induced by the exposure to remarkably low concentrations of fungal hydrolysates. Different behavior was evident at a later stage. Whatever the initial elicitor level, the specific activity of the enzyme started to decrease back to the values present in controls (Fig. 6). The same pattern was obtained even if highest hydrolysate levels (up to 500 mg L^{-1}) were used. When isoforms were resolved, only isozyme I increased, and transiently, as a consequence of cell elicitation (data not shown).

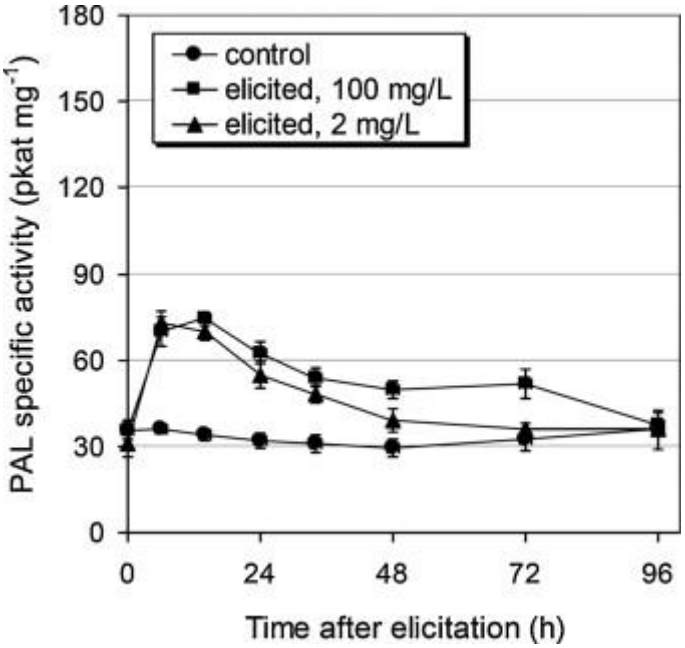

Fig. 6. PAL specific activity levels in suspension cultured cells of the blast-sensitive rice cultivar Vialone nano following elicitor addition. Actively-growing cultures were added with 0, 2 or 100 mg L^{-1} of fungal cell wall hydrolysate. At increasing time after the addition cells were harvested, and the specific activity of PAL was evaluated in cell-free extracts. Results, expressed as pkat mg^{-1} protein, are mean ± s.e. over three independent replications.

Discussion

Several PAL genes are usually expressed in higher plants in response to multiple biotic and abiotic stimuli (*e.g.* Kervinen et al., 1998, Sarma et al., 1998 and Cochrane et al., 2004). This small multigene family has been well studied in dicots, whereas relatively little is known to date about PAL expression and regulation in rice (Jwa et al., 2006). Because of the involvement in multiple responses, a reliable analysis of PAL induction at the plant level is often questionable. Even within the same tissue, cells may indeed show heterogeneous reactions following fungal attack (*e.g.* Kawamata et al., 1997). The use of cell suspension cultures in continuous balanced growth provided us with a system in which low PAL levels were maintained constant over quite a long period. In such a way, it was possible to accurately relate any variation of enzyme/mRNA level to the addition of fungal hydrolysate to the medium, and to evaluate such response on a homogeneous cell biomass.

Early after the contact with *M. oryzae* cell wall hydrolysates, a significant but not exceeding increase of PAL specific activity was found. In cells treated with low elicitor doses enzyme activity soon lowered back to control levels. On the contrary, in cultures treated with higher concentrations, similar to those that were able to induce the release of superoxide anion in the apoplast (Rożkowicz et al., 2003), a further increase was shown 48–72 h after elicitation. A dose-dependent biphasic profile of PAL induction might rely upon the presence of different elicitors in fungal hydrolysates. However, the results are consistent with previous findings showing the effect of purified *N*-acetylchitooligosaccharides on suspension-cultured rice cells. In that case, a biphasic profile of PAL induction was also found, with a first stage in the range from 0.01 to 1 mg L^{-1}, and a second one in the range from 3 to 300 mg L^{-1} elicitor (Inui et al., 1997). The involvement of multiple isozymes induced in response to acetylchitooligosaccharides by two distinct signaling mechanisms with a different dose-dependency was hypothesized, but no experimental evidence was shown (Inui et al., 1997). Data supporting the induction of at least two different PAL isozymes in elicited cells are on

the contrary reported here. Two peaks of activity with a different pattern with time were in fact partially resolved in extracts from elicited cells. Variation in elution profiles might depend on post-translational modifications of a single protein. However, molecular data supported the presence of early- and late-responsive PAL genes. Relative expression values for the two genes were not perfectly consistent with the corresponding activity ratio. However, such discrepancies could rely upon a different catalytic constant or a higher turn-over rate at either the messenger or the protein level.

To the best of our knowledge, this is the first study in which the expression of all non-redundant genes putatively coding in rice for a PAL has been measured as a function of cell elicitation. With only the exception of *PAL09*, all display an open reading frame compatible with the production of a catalytically active enzyme. Eight PAL genes out of eleven were transcribed in cultured cells at levels high enough to give rise to detectable RT-PCR products. Interestingly, the genes whose transcription was enhanced by the treatment with fungal elicitor (*PAL04* and *PAL07*) differed from those previously reported (*GP-1*, *GP-28* and *ZB8*, corresponding [Fig. S3] to *PAL01*, *PAL08* and *PAL06*, respectively; Minami et al., 1989, Minami and Tanaka, 1993 and Zhu et al., 1995), which are believed to be implicated in the plant defense reaction (Zhu et al., 1995, Blilou et al., 2000, Xu et al., 2003, Liu et al., 2005, Cho et al., 2007 and Takakura et al., 2008). The identification of new PAL genes specifically induced by contact with the *M. oryzae* elicitor is expected to provide us with useful information, and open new perspectives toward a better understanding of the rice response to blast.

To ascertain whether any reaction *in vitro* may be predictive of *in vivo* tolerance, blast-sensitive and blast-resistant cultivars were compared. No differences were found in PAL time course-specific activity levels early after elicitation. Irrespective of the genotype, a significant but mild increase of enzyme levels was evident following contact with even a low amount of fungal hydrolysate. On the contrary, significant variations were shown thereafter. In sensitive cultivars, PAL activity always lowered back to control levels. In tolerant genotypes, a further increase occurred 48–72 h after elicitation. A significant relationship had been shown between such a late response and the relative tolerance of a given cultivar at the whole plant level (Forlani, 2010). To date, most studies focused on early reactions, upon the assumption that the earlier the activation of defense pathway, the greater the chance of the plant to cope with the pathogen will be (*e.g.* Dixon and Paiva, 1995 and Kervinen et al., 1998). Even though the number of cultivars examined to date is limited, these results suggest that a capability to maintain active defenses over a longer time period may play a pivotal role in counteracting fungal attacks. The late-responsive PAL gene identified in the present work might therefore represent a useful marker for the assisted selection of increased blast tolerance. Work is currently in progress to further elucidate these aspects.

Acknowledgements

This work was supported by the Italian Ministero per l'Istruzione, l'Università e la Ricerca within the frame of the project PRIN2006 *Molecular aspects and systemic signals in plant responses to pathogen attack: the model Rice-Magnaporthe grisea*. The authors thank Dr. Sonia Casaro for skillful technical assistance.

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