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Two phenylalanine ammonia lyase isoforms are involved in the elicitor-induced response of rice to the fungal pathogen *Magnaporthe oryzae*

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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.

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; 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; 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; 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; 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; 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

Abbreviations: PAL, phenylalanine ammonia lyase.

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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; 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, 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