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Identification of tobacco ESTs with a hypersensitive response (HR)-specific pattern of expression and likely involved in the induction of the HR and/or localized acquired resistance (LAR)[1]

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Abstract

Plant defense responses against pathogens often involve the restriction of the pathogen to its site of penetration. Restriction is achieved through the combined effects of the hypersensitive response (HR) and its tightly connected localized acquired resistance (LAR). As LAR is induced by unknown signals released by the cells undergoing the HR, LAR inducing/regulating genes must show a HR-specific pattern of expression. Here, we describe a differential display reverse-transcript polymerase chain reaction (DDRT-PCR) strategy to isolate tobacco expressed sequence tags (ESTs) characterized by such an expression profile, which also characterizes genes involved in the induction/execution of the HR. We compared the DDRT-PCR profile of tobacco cell suspensions treated with β-megaspermin inducing the HR with that of untreated cells and cells treated with α-megaspermin inducing a Defense No Death (DND) phenotype. The expression profile of the selected ESTs was analyzed in tobacco plants expressing a β-megaspermin-induced HR or a DND phenotype, including LAR, induced by three different elicitors. This comprehensive analysis allowed to identify 24 HR-specific ESTs, half of them shows no or non-significant homology with ESTs and genes in the databases. The other half exhibits homology with genes encoding a receptor-like kinase protein, proteins involved in the regulation of plasma membrane structure, proteins of the ubiquitin/26S proteasome proteolytic system, RNA binding proteins, and a protein hypothesized to be a true regulator of the HR.

Keywords: Differential display; Elicitin, expressed sequence tag (EST); Hypersensitive response (HR); Localized acquired resistance (LAR); *Nicotiana tabacum* L. cv. Samsun H

1. Introduction

Strategies developed by plants to defend themselves against pathogens involve restriction of the pathogen to its site of penetration. This can be achieved through the combined effects of the hypersensitive response (HR) and localized acquired resistance (LAR) [11,16]. The HR is a commonly activated resistance process characterized by the rapid induction of localized host cell death. Several reports describe strategies to clone genes involved in the regulation and execution of the HR, but only very few genes have been identified mostly because the screening methods selected also general defense-related genes such as genes encoding pathogenesis-related (PR) proteins or enzymes involved in the biosynthesis of phytoalexins. Furthermore the function of these few genes remains unknown in most cases [13,15,18,22,28,32,34,35]. LAR occurs in the narrow region of living cells surrounding a HR lesion in which a strong activation of defense responses occurs contributing to a local, highly inhospitable environment for the invading pathogen. LAR is most often described

Abbreviations: AFLP, amplified fragment length polymorphism; CCoAOMT, coumaroyl-CoA *O*-methyltransferase; DD, differential display; DDRT-PCR, differential display reverse-transcript polymerase chain reaction; DND, Defense No Death; EST, expressed sequence tag; HR, hypersensitive response; LAR, localized acquired resistance; PR, pathogenesis-related; STC, sesquiterpene cyclase.

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[1] The nucleotide sequences reported in this paper have been submitted to the NCBI database and the accession numbers are listed in Table 1.

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as the living component of the HR. As such, LAR exhibits a Defense No Death (DND) phenotype. Although LAR is tightly connected to the HR, it exhibits properties, which clearly distinguish it from the HR. The HR is induced by exogenous signals issuing from the invading pathogen [19], whereas LAR is induced by endogenous signals liberated by the cells undergoing the HR [11]. The chemical nature of the LAR signal is not known and no genes involved in the induction/regulation of LAR have been identified. LAR does not occur when a DND phenotype alone is induced [9]. For instance, in tobacco, there is no localized induction of defense responses in the vicinity of the tissue infiltrated with a DND dose of elicitin (i.e. 50 pM whereas the HR dose is 50 nM). This latter property indicates that genes involved in the induction of LAR must show a HR-specific pattern of expression. However, genes with such a pattern of expression could also be involved in the regulation/execution of the HR itself. Here, we describe a differential display reverse-transcript polymerase chain reaction (DDRT-PCR) strategy to isolate tobacco expressed sequence tags (ESTs) characterized by a HR-specific pattern of expression. The aim was to avoid to select general defense-related genes as defined above. Expression of the selected ESTs was further analyzed in planta under HR and various DND conditions to confirm the HR-specific pattern of expression and identify those potentially involved in LAR induction.

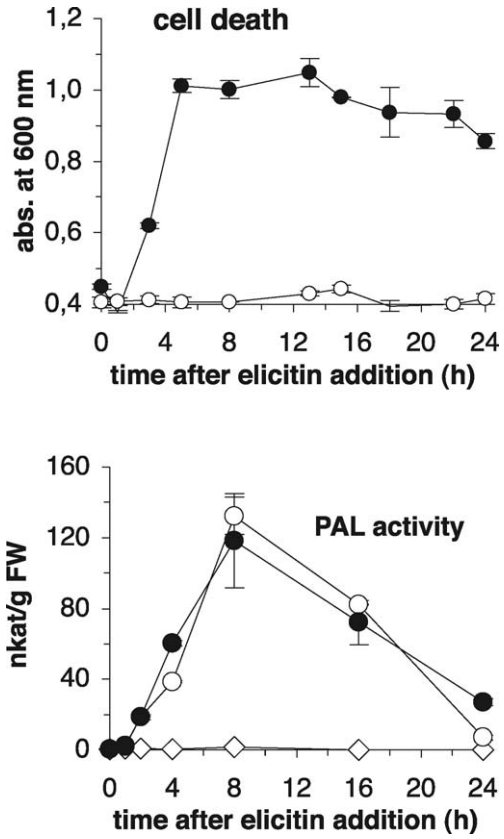

Fig. 1. Induction of cell death and of PAL activity after treatment of tobacco BY cells with α- or β-megaspermin. Cells were incubated in the presence of 250 nM α-megaspermin (open circles), 50 nM β-megaspermin (closed circles) or left untreated (open diamonds). The kinetics of cell death and of PAL activity were determined. For the cell death analysis, the results are expressed as values of elicitin-treated cells minus values of untreated cells.

2. Results

2.1. DDRT-PCR screening

Cell suspensions are the material of choice to perform DDRT-PCR as synchronous delivery of ligand to cells can be achieved more reproducibly in cultures than in leaves, making them ideal for biochemical, pharmacological and molecular studies. Furthermore, tobacco cell suspensions treated with β-elicitins release a diffusible signal activating defense responses [5,7]. This is consistent with the notion that, like cells in tissues, cell suspensions challenged with a pathogen-derived elicitor are able to release diffusible secondary signal molecules, which orchestrate the induction of complementary defense responses in neighboring cells, hence promoting LAR.

Typical defense-related genes are not the targets of our DDRT-PCR screen. Such genes encode PR proteins or enzymes involved in the biosynthesis of phytoalexins (in tobacco, phenylalanine ammonia lyase (PAL), is involved in the biosynthesis of the phytoalexin scopoletin). In tobacco, a sub-class of such genes has been shown induced early before the appearance of the cell death symptoms [11]. Moreover, these typical defense genes should represent the largest population of differentially expressed genes in elicitor-treated tobacco BY cell suspensions. Thus, to avoid screening for such genes we compared the DDRT-PCR pattern of HR-treated cells with that of DND-treated cells. Fig. 1 shows that a treatment of tobacco BY cells with β-megaspermin (50 nM) induces cell death and PAL activation, whereas α-megaspermin (250 nM) triggers a similar level of PAL activity without inducing cell death. α-Megaspermin proteins belong to the elicitin family [2] that induces the HR in *Nicotianae* plants in a gene-for-gene relationship at the species level [17]. However, when applied to our BY cells, α-megaspermin failed to induce the HR but induced a DND phenotype instead. This latter property of α-megaspermin, although unexpected, appeared particularly useful for the screening strategy.

The DDRT-PCR strategy to characterize HR-specific and early induced ESTs is depicted in Fig. 2. Tobacco cell suspensions were treated with 50 nM β-megaspermin (HR treatment), or 250 nM α-megaspermin (DND treatment) or left untreated as a control. Cells were collected 1 and 5 h after elicitin treatments, since a significant level of diffusible signal is already released by the cells 5 h after β-megaspermin treatment [7]. Total RNA was extracted and used to produce reverse trancripts (RT). PCRs with 21 pairs of arbitrary primers were performed using these RTs as the templates. About 5000 bands were generated and inspected, of which 56 showed a HR-specific profile, i.e. a PCR product was present in the HR lanes but absent in DND and control lanes. Interestingly, the intensity of these 56 bands was rather lower than that of