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Comprehensive analysis of early response genes to two different microbial elicitors in tobacco cells

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Abstract

The key to understanding the molecular mechanism of the defense response triggered by recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) is the identification and comparison of a set of early response genes to different microbial elicitors. We performed comprehensive and detailed monitoring of gene expression over time after application of two different microbial elicitors, PiE (an elicitor from the cell walls of an oomycete, *Phytophthora infestans*) and TvX (a xylanase from a fungus, *Trichoderma viride*), in tobacco cultured cells using the suppression subtractive hybridization and cDNA macroarray techniques. We identified various kinds of genes that are up- or down-regulated at the early stages of response to the elicitors. The majority of up-regulated genes are predicted to have a role in the defense response as signaling components and transcription factors as well as the metabolism involved in the production of secondary signaling molecules and anti-microbial compounds. The overall results revealed that there is no substantial difference in the expression profiles between cells treated with two different microbial elicitors, at least during the early phase of the defense response.
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Keywords: cDNA macroarray; Elicitors; MAMPs; PAMPs; Suppression subtractive hybridization; Tobacco

1. Introduction

Plants resist most potential pathogenic microorganism through a basal defense mechanism, the so-called innate immune system [1,2]. The innate immune responses are triggered by recognition of the microbe-derived molecules, namely, pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), which have often been referred to as general elicitors [3–6]. These microbe-derived elicitors include proteins, peptides, carbohydrates, and lipids [3]. Recognition of elicitors and the resulting induced defense responses primarily occur at the single plant cell level. The induced defense responses include multifaceted molecular, biochemical, and morphological events, such as changes in ion fluxes, oxidative burst, accumulation of antimicrobial proteins, biosynthesis of low molecular-weight antimicrobial compounds, reinforcement of cell walls, and/or hypersensitive cell death [7]. It is well known that the up-regulated expression of a diverse array of plant genes is closely associated with the induction of defense responses [7]. It has been demonstrated that the defensive events also include the down-regulated expression of the several genes [8–11]. Therefore, identification and characterization of up- and down-regulated genes in response to elicitors are important and necessary to gain an understanding of the molecular mechanism of these defense responses. The recent

Abbreviations: CDPK, calcium-dependent protein kinase; CNGC, cyclic nucleotide-gated ion channel; GST, glutathione S-transferase; MAMPs, microbe-associated molecular patterns; MAP kinase, mitogen-activated protein kinase; OPPP, oxidative pentose phosphate pathway; PAMPs, pathogen-associated molecular patterns; PiE, elicitor from the cell walls of *Phytophthora infestans*; PCR, polymerase chain reaction; SSH, suppression subtractive hybridization; TvX, xylanase from *Trichoderma viride*

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development of DNA microarray technique has facilitated monitoring of global changes in gene expression and such analyses have revealed the defense-associated comprehensive transcriptional re-programming in *Arabidopsis* [12]. The microarray analysis demonstrated the transcriptional re-programming associated with the PAPM/MAMP-triggered immune responses in *Arabidopsis*. The treatment with a general elicitor, flg22, induced the expression of numerous defense-related genes concomitantly with resistance to pathogenic bacteria in *Arabidopsis* plants [13,14]. In addition, a noticeable overlap between the early transcriptional changes that occur in the flg22-triggered defense response in Arabidopsis and the Avr9-trggered defense response in tobacco was shown [14].

The suppression subtractive hybridization (SSH) method is an efficient method, widely used in the study of differential gene expression [15–17]. The SSH method includes a normalization step, which enriches for differentially expressed transcripts; thus it is expected to facilitate the identification of cDNAs with low to medium transcript abundance, such as early-inducible genes. Moreover, it yields cDNA fragments that can be used for the construction of cDNA arrays. cDNA macroarrays have proved to be a useful tool in gene expression profiling [16,18]. The combination of these techniques is effective for the comprehensive approaches in plant species, including cotton [16] and maize [19] as well as tobacco [20], which lack the complete genomic sequences.

We are currently investigating signaling and transcriptional events for defense responses in a tobacco cell culture (line XD6S), which have been shown to respond to two different microbial elicitors, PiE (an elicitor from the cell walls of an oomycete, *Phytophthora infestans*), and TvX (a xylanase from a fungus, *Trichoderma viride*). These two elicitors induce several typical defense responses in this cell culture, including the activation of p47 mitogen-activated protein (MAP) kinase, oxidative bursts, and alkalinization, as well as the expression of several defense genes [21]. Generally, these responses are more rapidly induced by PiE than TvX. In addition, TvX but not PiE elicits hypersensitive cell death in the XD6S cells [22]. Therefore, the experimental system is especially useful for investigating specific and common molecular events in the responses of plant cells to PAMPs/MAMPs.

In this study, we performed a comprehensive and detailed monitoring of gene expression over time after application of elicitors in the tobacco cells using the SSH and cDNA macroarray techniques to gain a better understanding of the molecular events in the elicitor-induced response in plant cells. We also discuss the results of a comparative analysis of the expression profiles in the tobacco cells treated with the two different microbial elicitors.

2. Materials and methods

2.1. Plant materials and elicitor treatments

The conditions for cell culture and the treatment of cells with microbial elicitors were described previously [22,23]. Briefly, suspension cultures of tobacco cells (*Nicotiana tabacum* line XD6S) were transferred at weekly intervals to fresh Murashige-Skoog medium (Wako Pure Chemical, Osaka, Japan), pH 5.8, that contained 3% sucrose and 5 μM 2,4-dichrolophenoxyacetic acid. A suspension of XD6S cells, after culture for 4 days, was treated with PiE extracted from the cell walls of *Phytophthora infestans* [23] or with TvX purchased from Sigma (St. Louis, MO, USA). 2-(*N*-Morpholino)ethanesulfonic acid (pH 5.8; final concentration, 25 mM), was also added to the suspension of cells to stabilize the pH of the culture medium.

2.2. Isolation of poly(A)+RNA

To synthesize cDNAs for SSH, poly(A)$^+$RNA was isolated from a 100 mL of cell suspension of XD6S, which had been treated with or without microbial elicitors, 1 μg/mL TvX or 100 μg/mL PiE, for 45 min, using the PolyATract System 1000 and PlyATract mRNA Isolation System (Promega, Tokyo, Japan). For the macroarray analysis and RNA gel blot analysis, total RNA was isolated from the XD6S cells by the method described previously [23]. Poly(A)+RNA was also isolated from the total RNAs using the PolyATract mRNA Isolation System (Promega) and subjected to macroarray analysis.

2.3. Generation of subtracted library by SSH

SSH was performed using the PCR-Select cDNA Subtraction kit (Clontech Laboratories, Palo Alto, CA, USA) as described by the manufacturer, with minor modification. Poly(A)$^+$RNA isolated from untreated control and PiE- or TvX-treated XD6S cells were used to make double-stranded, blunt-ended cDNA, which was then digested with RsaI. The combinations of tester and driver cDNAs are shown in Table 1. One-half of the digested tester cDNA was ligated with adapter 1 and the other half with adapter 2, whereas the driver cDNA was not subjected to adapter ligation. The tester cDNA ligated with adapter 1 or 2 was denatured and hybridized separately with an excess of denatured driver cDNA. Then the two hybridization reactions were mixed together with tester cDNAs along with freshly denatured driver cDNA. The hybridization product was subjected to primary polymerase chain reaction (PCR) amplification using the Advantage cDNA PCR Kit (Clontech). This PCR amplification was performed using the PCR primers 1 and 2 or 2R complementary to the adapters 1 and 2, respectively. The PCR reaction was then

Table 1
A list of the SSH-libraries

SSH libraries	Tester	Driver	Number of clones
C0	CON	0 h	23
E0	PiE	0 h	88
X0	TvX	0 h	187
XE	TvX	PiE	96
0X	0 h	TvX	192

0 h, no treatment. CON, incubated for 45 min without elicitor. PiE, incubated for 45 min with PiE. TvX, incubated for 45 min with TvX.

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