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Research article

Cloning and characterization of a novel LpWRKY1 transcription factor in tomato

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Abstract

The initiation of defence responses in plants is accompanied by fundamental changes in gene expression: the expression of pathogenesis-related genes is co-ordinately regulated with metabolic changes such as down regulation of photosynthesis and induction of sink metabolism. To identify candidate regulators of this co-ordinated regulatory mechanism, the role of WRKY transcription factors in the initiation of defence response was analysed in tomato. A WRKY-type transcription factor (LpWRKY1) from tomato was cloned by a reverse Northern approach. The corresponding mRNA is rapidly and transiently induced after challenging the cells with an elicitor-preparation derived from the wilt inducing fungus *Fusarium oxysporum lycopersici* (E-FOL) and the fungal elicitor chitosan, whereas the endogenous signals systemin and salicylic acid are inactive. Inhibition of protein biosynthesis by cycloheximide results in sustained induction of mRNA for LpWRKY1. In contrast, the transient induction of the gene encoding LpWRKY1 in response to elicitation by E-FOL is inhibited by the protein-kinase inhibitor staurosporine and may be mimicked by the phosphatase inhibitors endothall and cantharidine indicating the involvement of protein phosphorylation in the regulation of WRKY-type transcription factors. Direct proof of this postranslational modification of LpWRKY1 was obtained by demonstrating in-gel kinase assays using recombinant LpWRKY1 as substrate. A 44 kDa and a 67 kDa protein kinase were shown to be transiently activated to phosphorylate LpWRKY1 protein in response to elicitation with E-FOL.

Keywords: WRKY; Transcription factor; Phosphorylation; Tomato; Stress; Elicitor

1. Introduction

If a plant is challenged by a pathogen, several waves of gene activation or repression occur. Depending on the pathogen, appropriate defence-response genes are activated [4] so that the plant restricts the growth and spread of fungi or bacteria. The expression of defence-related genes is invoked by a ligand-receptor interaction [27] which leads to the expression of immediate early genes. The products of these genes are usually believed to be regulatory proteins that control subsequent cellular reactions. A class of transcription factors that is involved in defence responses and that shows the characteristics of immediate early genes has been discovered in plants [10,20]. After a strictly conserved amino acid motif, these proteins are called WRKY transcription factors which comprise a superfamily of 72 members in *Arabidopsis* [35]. The WRKY domain contains the WRKYGQK motif and a putative zinc finger domain of the C_2–H_2 type at its C-terminal end [29,35]. The WRKY domain occurs either once or twice in the known WRKY proteins, which separates them into class 1 WRKY proteins (two WRKY domains) and class 2 proteins (one WRKY domain). A third group of WRKY proteins carries one WRKY domain with a C_2–HC zinc finger [10,35]. All WRKY proteins characterised so far bind to the cis-acting

Abbreviations: E-FOL, elicitor preparation of *Fusarium oxysporum lycopersici*; PAL, phenylalanine ammonia lyase; MAPK, mitogen activated protein kinase.

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element (T)(T)TGAC(T/C) known as the W-box [5,17,29,35] which is found in the promoter regions of WRKY [11] and defence-related genes [30,35,40]. WRKY transcription factors are involved in several diverse pathway (reviewed in refs. [12,35]) including regulation of starch, anthocyanin and sesquiterpene anabolism. The involvement of WRKY factors in plant defense has widely been investigated in recent years [12,36]. Different members of WRKY factors have been shown to confer resistance toward bacteria and fungi [1,3,6,21–23,26,37,44]. Several WRKY factors are also known to act as negative regulator of the resistance [19,38,43]. They are also involved in the regulation of diverse processes along with defence such as hormone [23,39], metabolic regulation as well as trichome development [18] involve WRKY transcription factors. Adding up to this complexity are more than 500 ESTs encoding putative WRKY proteins present in the databases, which points towards a central regulatory role of these plant specific transcription factors.

Elicitation of photoautotrophic suspension culture cells of *Chenopodium rubrum* with either the stress related stimulus chitosan or the metabolic stimulus D-glucose results in the downregulation of photosynthesis, whereas sink metabolism is induced [18]. Simultaneously defence responsive genes like phenylalanine ammonia-lyase are upregulated. We have shown, that the signal transduction pathways leading to this co-ordinated regulation share at least in part common components, and that these pathways involve the rapid and transient activation of MAP-kinases [9]. To further dissect the signal transduction pathway leading to a co-ordinated regulation of the defence response and source/sink relations, we investigated the mechanism that initiates this concerted gene regulation in photoautotrophic suspension culture cells of tomato (*Lycopersicon peruvianum*). To get a detailed insight into the regulation of the defence response we studied the possible role of immediate-early genes as the final targets of this signal transduction pathway, that display candidate early regulators of those genes subject to regulation by pathogen infection. An elicitor preparation of the wilt inducing fungus *Fusarium oxysporum lycopersici* (E-FOL), a naturally occurring pathogen of tomato, was shown to result in various elicitor induced reactions. Rapid induction of changes in phenylpropanoid metabolism [2] are accompanied by effects on source/sink relations [32] supporting the co-ordinated regulatory mechanism demonstrated in *C. rubrum*.

In the present study, a WRKY transcription factor was cloned from tomato by a reverse-northern approach, which is rapidly induced by E-FOL and the fungal elicitor chitosan in autotrophic suspension culture cells. We demonstrate, that the expression of this transcription factor is dependent on protein phosphorylation and that the WRKY protein is phosphorylated by two transiently activated kinases.

2. Materials and methods

2.1. Growth of cell suspension culture cells

Photoautotrophic suspension culture cells of *L. peruvianum* L. were established by ref. [9] and are being subcultured every two weeks in MS-medium and incubated shaking under continuous light conditions with an atmosphere containing 2% CO_2. Cells were used for the experiments during the second week after subculturing. Stimuli and inhibitors were applied at the following concentrations: FOL, 150 μg dry hyphae/ml culture; chitosan, 0.1%; SA, 250 μM; systemin, 250 nM; cycloheximide, 5 μg/ml; staurosporine, 2 μM; endothall, 50 μM; cantharidine, 50 μM.

2.1.1. Preparation of an elicitor from *F. oxysporum lycopersici*

The pathogenic fungus *F. oxysporum* Schlecht.: Fr.f.sp. *lycopersici* (Sacc.) was obtained from the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands). The fungus was cultured in a medium containing 50 g/L glucose, 8 g/L Casamino acids, 0.5 g/L yeast extract, 0.2 g/L $MgSO_4$ and $FeSO_4$ each, 20 mg/L $CaCl_2$, 1.5 mg/L $MnSO_4$ and Na_2MoO_4 each, in 25 mM potassium phosphate at pH 7.5. After four days of shaking at 28 °C the culture was autoclaved, washed with water, and lyophyllised. For the induction of stress response, 100 mg/L of the dried hyphae were added to a tomato cell suspension culture.

2.2. Construction of the WRKY cDNA library and reverse-northern screening

A cDNA library was constructed from tomato cells that were previously treated with E-FOL for 30 min. Total RNA was used for reverse transcription using random hexamere primers and M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturers instructions. Subsequently, PCR was performed with the degenerate oligonucleotides Omh-7 (5′-GAY GGW TAY AAY TGG MGD AAR TAY GGW CAR AA-3′) and Omh-8 (5′-RTG RTY RTG YTK WCC YTC RTA WGT WGT-3′) which are directed against the conserved WRKY domains. The products of this PCR were then cloned into pUC57 using the T/A cloning kit (MBI, St. Leon-Rot, Germany). For reverse-northern, DNA was isolated from these clones and PCR was performed in 96 well plates to amplify the inserts of the individual clones, using Omh-7 and Omh-8 primers. The PCR reactions were evaluated by agarose gel electrophoresis and then diluted with 1.5 M NaCl, 0.5 M NaOH. One half of this dilution was then spotted on one nitrocellulose filter prewettet in the same solution using a 96 well vacuum manifold. After UV-crosslinking, the membrane was blocked in a modified church buffer (0.24 M Na_2HPO_4 pH 7.2, 7% SDS, 1 mM EDTA). For the detection of putatively regulated clones, total RNA from control and FOL treated cells was reverse-transcribed in the presence of γ^{32}PdCTP. This reaction mixture was then treated with 1.3 mM NaOH at 95 °C and was then added to the prehybridisation solution. After overnight incubation, the membranes were washed with 2 × SSC/0.1% SDS and 0.2 × SSC/0.1%S DS and then evaluated using a phosphorimager. Those clones showing a positive signal compared to the corresponding control membrane were then re-evaluated by northern-blot analysis.