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Research article

Modulation of NADPH-oxidase gene expression in *rolB*-transformed calli of *Arabidopsis thaliana* and *Rubia cordifolia*

Galina Veremeichik [a], Victor Bulgakov [a, b], Yury Shkryl [a, b, *]

[a] Institute of Biology and Soil Science, Far East Branch of Russian Academy of Sciences, Vladivostok, 690022, Russia
[b] Far Eastern Federal University, Vladivostok, 690950, Russia

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ABSTRACT

Expression of *rol* genes from *Agrobacterium rhizogenes* induces reprogramming of transformed plant cells and provokes pleiotropic effects on primary and secondary metabolism. We have previously established that the *rolB* and *rolC* genes impair reactive oxygen species (ROS) generation in transformed cells of *Rubia cordifolia* and *Arabidopsis thaliana*. In the present investigation, we tested whether this effect is associated with changes in the expression levels of NADPH oxidases, which are considered to be the primary source of ROS during plant–microbe interactions. We identified two full-length NADPH oxidase genes from *R. cordifolia* and examined their expression in non-transformed and *rolB*-transformed calli. In addition, we examined the expression of their homologous genes from *A. thaliana* in non-transformed and *rolB*-expressing cells. The expression of *Rboh* isoforms was 3- to 7-fold higher in both *R. cordifolia* and *A. thaliana rolB*-transformed cells compared with non-transformed cells. Our results for the first time show that *Agrobacterium rolB* gene regulates particular NADPH oxidase isoforms.

1. Introduction

Following agrobacterial infection, the *rol* genes of the plant pathogen *Agrobacterium rhizogenes* are transferred into the plant genome, causing tumor formation and hairy root disease (Tarkowski and Vereecke, 2014). The expression of the *rol* genes and, most importantly, the *rolB* and *rolC* genes, is critical for hairy root production (Spena et al., 1987). The function of *rol* genes is not restricted to root formation: they cause pleiotropic effects on several cellular functions, including morphological changes of the transgenic plants (Piispanen et al., 2003; Zia et al., 2010), induction of PR-proteins (Kiselev et al., 2006), sucrose transport and metabolism (Nilsson and Olsson, 1997) and primary (Günter et al., 2015) and secondary metabolism (Bulgakov, 2008).

A new function of the *rol* genes in the plant-*Agrobacterium* interaction became apparent with the discovery that these genes affect reactive oxygen species (ROS) production in transformed plant cells. Thus, the *rolC*-transformed *Rubia cordifolia* callus line showed significantly decreased ROS levels compared with a control culture (Bulgakov et al., 2008). A similar effect was also observed in *rolA*- and *rolB*-expressing cells (Bulgakov et al., 2013). Interestingly, the combined action of all three genes (*rolABC* construct) caused a moderate decrease in ROS, thus indicating that they do not act synergistically (Bulgakov et al., 2012, 2013). Transformation with wild-type *A. rhizogenes* A4 also caused ROS suppression (Shkryl et al., 2010), and this effect was associated with activation of antioxidant gene expression (Bulgakov et al., 2013). Similarly, decreased levels of H_2O_2 and a rise in antioxidant enzyme activities were observed in hairy roots and regenerated plants of *Nicotiana tabacum* (Nikravesh et al., 2012). Therefore, it appears evident that *A. rhizogenes*, acting via transferred T-DNA genes, decreased the steady-state level of ROS in transformed plant cells. Contrary to these data, however, was the recent publication by Wang et al. (2014), showing that ROS-related genes were significantly suppressed in *rolB*- or *rolC*-deficient hairy roots. Because ROS function as cellular second messengers that modulate many different proteins, leading to various responses (Mori and Schroeder, 2004; Lehmann et al., 2015), we suggested that at least part of the *rol*-induced effects could be associated with alteration of the redox status of transformed cells.

NADPH oxidases, also referred to as respiratory burst oxidases (Rboh), catalyze the production of superoxides, a type of reactive oxygen species (ROS) (Sagi and Fluhr, 2006). Members of the Rboh family mediate the production of apoplastic ROS during the defense

* Corresponding author. Address: Institute of Biology and Soil Science, 159 Stoletija Str., Vladivostok, 690022, Russia.
E-mail address: yn80@mail.ru (Y. Shkryl).

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responses, as well as responses to abiotic, environmental and developmental cues (Torres and Dangl, 2005). There are 10 *Rboh* genes in *Arabidopsis thaliana—AtRbohA*, *AtRbohB*, *AtRbohC*, *AtRbohD*, *AtRbohE*, *AtRbohF*, *AtRbohG*, *AtRbohH*, *AtRbohI and AtRbohJ*. Among these genes, *AtRbohD* and *AtRbohF* play a pivotal role in producing the apoplastic oxidative burst in response to pathogens (Torres et al., 2002), guard cell closure (Kwak et al., 2003; Suhita et al., 2004; Desikan et al., 2006) and PAMP-induced ROS production (Zhang et al., 2007).

The aim of the present investigation was to study the effect of *rolB* gene on the expression of *Rboh* genes encoding ROS-producing enzymes. For this purpose, we used *R. cordifolia* and *A. thaliana* callus cultures expressing *rolB* under the control of a constitutive promoter. Two NADPH oxidase genes, namely *RcRboh1* and *RcRboh3*, were identified in *R. cordifolia*, and their expression was studied compared with the expression of their homologs in *A. thaliana*. In both models of transformed plants, *rolB* showed a significant effect on the expression of *Rboh* genes.

2. Materials and methods

2.1. Plant cell cultures

The control, non-transformed callus culture (R) and the transformed callus cultures of *Rubia cordifolia* were established and characterized previously (Shkryl et al., 2008). Transgenic callus lines with moderate and high levels expression of the *rolB* gene (RBM and RBH, respectively) were employed in this study. Calli were cultivated using "W" agarized medium (Bulgakov et al., 2011) supplemented with 0.5 mg/L 6-benzylaminopurine and 2.0 mg/L α-naphthaleneacetic acid.

The *Arabidopsis thaliana* non-transformed (At) as well as *rolB*-transgenic callus cultures were obtained as described (Bulgakov et al., 2012) from wild-type Columbia (Col-0) seedlings. For transformation, we used *Agrobacterium tumefaciens* strains GV3101 harboring the pPCV002-CaMVB construction (*rolB* and *rolC* under the control of 35S CaMV promoter, respectively) (Spena et al., 1987). Several *rol*-transgenic lines were selected from primary transformed calli and two of them showing the low and high levels of the *rolB* expression (AtBL and AtBH, respectively) were used in this study. These callus cultures were cultivated using "W" medium supplemented with 0.4 mg/L 2,4-dichlorophenoxyacetic acid.

R. cordifolia and *A. thaliana* cell cultures were cultivated in the dark at 24 °C with 30-day subculture intervals.

2.2. Isolation of cDNA clones corresponding to R. cordifolia Rboh genes

The isolation of total RNA and first-strand cDNA synthesis were carried out as described previously (Shkryl et al., 2010, 2011). To amplify sequences corresponding to genes encoding *Rboh* isoforms in *R. cordifolia*, degenerate primers Rboh-DegD and Rboh-DegR (Table 1) were designed using the amino acid sequences LIMEEL and GFNAFWY, which are conserved in Rbohs from different plant species. These conserved amino acid segments were chosen based on the alignment of plant Rboh proteins from *A. thaliana* (AtRbohA, AtRbohB AtRbohC, AtRbohD, AtRbohE and AtRbohF; GenBank accession numbers AAO41907, AAO64136, BAB08752, AAO11567, AEE29821 and AAB87789, respectively), *Oryza sativa* (OsRbohA, OsRbohB and OsRbohE; GenBank accession no. AAB87790, BAF04934 and BAD36441, respectively), *Solanum tuberosum* (StRbohA, StRbohB, StRbohC, StRbohD and StRbohF, GenBank accession no. BAB70750, BAB70751, BAE79344, BAE79345 and BAB84124, respectively), *Nicotiana tabacum* (NtRbohD and NtRbohF, GenBank accession no. ABN58915 and ABS85195, respectively).

Using RT-PCR with these primers and total RNA from *R. cordifolia* cells, cDNA fragments of predicted lengths were amplified. These fragments were isolated from gels with a Glass Milk Kit (Sileks M, Russia) and subcloned into a pTZ57R/T plasmid using the InsT/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania). The clones were amplified with M13 universal primers and sequenced as described earlier (Shkryl et al., 2008) at the Instrumental Centre for Biotechnology and Gene Engineering at the Institute of Biology and Soil Science using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City CA, USA).

2.3. Rapid amplification of cDNA ends

To obtain full-length sequences of *R. cordifolia Rboh* genes, we performed Rapid Amplification of cDNA Ends (RACE), using step-out PCR technology, according to Matz et al. (1999) with modifications (see below). cDNA was synthesized from total RNA isolated from the RBH callus culture and subjected to 25 cycles of amplification, using the SMART cDNA Amplification Kit, according to the manufacturer's instructions (Clontech, CA, USA). In our modified protocol of RACE, we used the degenerate primers Rboh-DegD and Rboh-DegR in the first round of PCR instead of gene-specific primers. The use of these primers allowed for the simultaneous amplification of all expressed isoforms of *RcRboh*. The gene-specific primers used for real-time PCR were employed in the second round of RACE to obtain individual amplicons for each isoform of *RcRboh*. The fragments obtained by RACE were cloned into the plasmid pTZ57R/T (Fermentas) and sequenced.

2.4. Real-time RT-PCR

A quantitative real-time PCR (qPCR) analysis was performed, using the Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories) with 2.5× SYBR green PCR master mix, containing ROX as a passive reference dye (Syntol, Moscow, Russia) as described (Veremeichik et al., 2014).

The gene-specific primer pairs used in the qPCR are listed in Table 1. Actin genes of *R. cordifolia* and *A. thaliana* were used as reference genes. Primer pairs for the actin genes span an intron in corresponding sequences that allowed for testing of the absence of DNA contamination in the samples. Two biological replicates, resulting from two different RNA extractions, were used for analysis, and three technical replicates were analyzed for each biological replicate. No-template controls and RNA-RT controls were included in the analysis to verify the absence of contamination. The absence of nonspecific products or primer-dimer artifacts in the samples was confirmed by melting curve analysis at the end of each run and by product visualization using electrophoresis. Primer efficiency of >95% was confirmed with a standard curve spanning seven orders of magnitude. Data were analyzed using CFX Manager Software (Version 1.5; Bio-Rad Laboratories).

2.5. Molecular phylogenetic analysis

Sequences of plant respiratory burst oxidases were retrieved from GenBank in a search carried out using the amino acid sequence of RcRbohs from *R. cordifolia* against the non-redundant protein database. GenBank sequences and sequences described in this work (in total 20) were aligned using the MUSCLE program (Edgar, 2004) with default settings and refined manually. Phylogenetic tree were generated using the maximum-likelihood (ML) method of tree construction. Prior to the analysis, the ProtTest program v.2.4 (Abascal et al., 2005) was used to determine the most appropriate amino acid substitutions that best fit the data. The best