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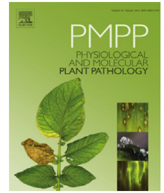

Overexpression of *StRbohA* in *Arabidopsis thaliana* enhances defence responses against *Verticillium dahliae*

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ABSTRACT

Plant NADPH oxidases are key regulators of plant–microbe interactions and reactive oxygen species (ROS) are essential to plant defences against pathogens. A significant part in the role played by ROS has been ascribed to plant respiratory burst oxidase homologs (RBOHs). In potato (*Solanum tuberosum*), where RBOHs were previously shown to be involved in wound-induced oxidative burst, we assessed their expression after inoculation with *Verticillium dahliae* Kleb. and showed that *StRbohA* was the only homolog to be differentially induced in potato in response to inoculation. In order to investigate the potential role of this gene in plant protection against wilt diseases, we used *Agrobacterium*-mediated transformation of *Arabidopsis* to assess the effects of its overexpression on plant responses to *V. dahliae*. After inoculation with this pathogen, the transformed Arabidopsis line overexpressing *StRbohA* showed lower disease severity (percent damaged leaf area and vascular discoloration) as compared to the wild type. It also had higher ROS production and more cell death caused by hydrogen peroxide (H_2O_2), compared to the wild type. Suberization of root cells was also more pronounced in the line overexpressing *StRbohA*, and supports a possible role for StRBOHA in plant resistance to *V. dahliae*. Together, these findings indicate that overexpressed *StRbohA* in *Arabidopsis* enhances the ROS-mediated defence mechanisms against *V. dahliae* and can be a potential tool to improve plant resistance to this and other soilborne pathogens that cause wilts in economically important crops.

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Introduction

Accumulation of reactive oxygen species (ROS), also termed “oxidative burst” [1], is one of the first defence events occurring in plants during their interactions with microbes [2]. ROS affect pathogens directly by damaging their cell components and indirectly through activation of host defence mechanisms such as lignin biosynthesis, *i.e.*, incompatible reaction of potato with *Phytophthora infestans* [3,4], lignification in bean tissues inoculated with *Pseudomonas syringae* pv. *phaseolicola* [5], formation of other defence barriers in barley against powdery mildew [6], production of pathogenesis-related proteins and phytoalexins, and a very complex signaling cross-talk [7]. However, many fungi have evolved counter-defence mechanisms to activate stress response regulators thereby tolerating the oxidative stress caused by ROS [8].

NADPH oxidases (NOXs), which are involved in the generation of the superoxide ion [9], are commonly known as RBOHs (respiratory burst oxidase homologues), and are similar to the mammalian NADPH oxidase [10]. Regulation of the activity of membrane-bound RBOH proteins by their functional domains has been reported in rice [11], potato [12] and Arabidopsis [13]. Similar to the human gp91^{phox}, potato StRBOH possesses six membrane domains and two N-terminal Ca^{2+}-binding EF hands [14]. The role of *Rboh* genes in ROS generation has been reported in rice [15], Arabidopsis [16], tomato [17], and tobacco [18,19]. However, only a few have been reported in ROS-mediated plant resistance or susceptibility to pathogens. In potato, *StRbohA* was shown to prevent infection in tubers through wound-healing induced by oxidative burst [20]. In *Arabidopsis*, homologues

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of mammalian gp91^{phox}, *AtRbohD* and *AtRbohF* induce reactive oxygen species (ROS)-mediated cell death during incompatible interactions with bacterial and oomycete pathogens [21]. In barley, while *HvRbohA* is involved in the induction of susceptibility to *Blumeria graminis* f. sp. *hordei* by enhancing the penetration into the host tissues [22], *HvRbohF2* was shown to be involved in resistance to *B. graminis* f. sp. *hordei* penetration and to leaf cell death [23]. These dual roles of the same gene had been explained in two ways: non-detectable single and non-cell autonomous function of *HvRbohF2* in response to *B. graminis* f. sp. *hordei* or loss of functions of *HvRbohF2* during plant development, which might have conditioned plants for higher susceptibility to *B. graminis* f. sp. *hordei*. Recently, *PvRbohB* was also shown to regulate bean root infection and nodulation by nodule-forming *Rhizobia* [24].

Verticillium wilt is an economically-important vascular wilt disease affecting a broad range of plant hosts and is most often caused by *Verticillium dahliae* Kleb., a soil-borne hemibiotropic pathogen [25]. *V. dahliae* produces microsclerotia for its long-term survival in the soil. After germination, its hyphae penetrate plant roots through wounds and/or root tips and results into wilt symptoms. Resistance against *Verticillium* spp. requires a coordinated activation of diverse plant responses which include ROS production, defence genes expression, synthesis of antimicrobial compounds and cell wall reinforcement [26–28].

Many of our recent studies that used proteomic [29], metabolomic [30,31] and transcriptomic approaches [32,33] to investigate the mechanisms underlying *V. dahliae* virulence, evoked the importance of ROS in host-*V. dahliae* interactions. Thus, better understanding of the role of ROS in such mechanisms is an important step towards developing sustainable solutions for verticillium and other wilts. At the transcriptional level, potato defence-related genes responded differentially to highly and weakly aggressive isolates of *V. dahliae*, including genes from both the salicylate [7] and jasmonate signaling pathways [27]. Along with prior findings on the involvement of *StRbohA* genes in wound healing, our results prompted us to investigate the use of the latter genes in building plant responses in anticipation of infection by soilborne pathogens such as *V. dahliae*. This is not meant to validate *Rboh* genes as contributors to plant defence, but rather to use them in developing anticipatory responses against soilborne pathogens. In the soil, microsclerotia are the starting point for *V. dahliae* inoculum and normally germinate in response to root exudates [32], thus initiating a faster infection in case of root wounding. Enabling the plant to establish a strong and rapid root tissue-healing before penetration by pathogens would be a novel and integral part of induced plant defence against soilborne pathogens such as *V. dahliae*. To test our hypothesis that strong and rapidly induced responses can restrict the infection caused by *V. dahliae* more efficiently, the aim of our study was to investigate the effects of *StRboh* overexpression in *Arabidopsis* using *Agrobacterium*-mediated transformation on verticillium disease development. This proof of concept using *Arabidopsis* is meant to build a strong case for future plant transformation that would reduce the loss incurred by Verticillium wilt in crop plants among the 400 species affected by this pathogen. Before transforming Arabidopsis, we investigated which potato *StRboh* gene homologs would express in response to this pathogen.

Materials and methods

Plant materials and growth conditions

Two potato cultivars viz. Kennebec (Susceptible) and Ranger Russet (moderately resistant) to *V. dahliae* [34], were used to assess the transcript levels of *StRboh* genes in response to inoculation with this pathogen. Four-week-old potato plants were used for inoculation as described previously [7]. Both wild type and transformed line L8 of *Arabidopsis thaliana* (Ecotype Columbia) overexpressing *StRbohA* were used in this study to analyze the effect of *StRboh* gene overexpression in *Arabidopsis* on its response to inoculation with *V. dahliae*. Arabidopsis plants were produced in plastic trays containing the same soil mix with NPK fertilizer (16:20:0) into a growth room with the conditions described earlier [35].

Fungal isolates and plants inoculation

For the assessment of the expression levels of *StRbohA*, *StRbohB*, and *StRbohC* in both potato leaves and roots, each cultivar was inoculated separately with two isolates of *V. dahliae*, Vs06-14 (weakly aggressive, WA), and Vd1396-9 (highly aggressive, HA) [36]. From over 60 *V. dahliae* isolates pre-screened in our laboratory [36], we selected five isolates (Vd1396-9, V104, Vs06-14, Vs1398-21 and Vs06-09) for the tests in the *Arabidopsis*-*V. dahliae* pathosystem. Pathogenic variability analyses of the tested isolates, using both susceptible and moderately resistant potato cultivars and sunflower hybrids had revealed three pathogenicity groups [36]. Isolates V104 and Vs06-09, from potato and sunflower, respectively, belonged to group 1, inducing only limited symptoms on both potato and sunflower, similar to non-inoculated wounded and non-wounded controls. Potato isolates Vd1398-21 and Vd1396-9 from group 2 were highly-aggressive on both potato and sunflower. Group 3 had all sunflower isolates including isolate Vs-06-14, which had weak aggressiveness on both potato and sunflower.

Inoculum preparation and potato inoculation was done according to Derksen et al. [7]. Briefly, conidial suspensions of 10^6 conidia/mL from *V. dahliae* single-spore cultures grown on potato dextrose agar (PDA) for 2 weeks at 20 °C (Fisher Scientific Incubator, Model 146E), were used to inoculate four-week-old potato plants via 'root dipping'. Briefly, the soil was gently washed from the uprooted potato plant roots with water followed by trimming the root tips with scissors. The roots were then immersed in the conidial suspensions for one minute before being transplanted. Wounded control plants had their root tips cut before immersion in sterile distilled water (SDW). The inoculation experiments were conducted with three replications. In Arabidopsis, inoculation was done with some modifications. Due to the frailty of *Arabidopsis* roots, these were inoculated 46 days after transplanting of seedlings by pouring 10 mL of inoculum for each isolate into four fresh cuts through the root zone in each pot and 10 mL of sterile water was used for wounded controls. Each treatment consisted of 3 pots with 5 plants each and the trays were kept in the growth room under the same conditions mentioned above.

Potato tissue harvesting and RNA extraction

For RNA extraction, potato leaf and root samples were collected at 0 h, 6 h, 24 h, 48 h, 1 week and 2 weeks post inoculation (wpi). The samples for time 0 h post inoculation (hpi) were collected from the plants immediately dipped in the conidial suspension and for the wounded control, from plants dipped in sterile distilled water (SDW). The samples were then kept at −80 °C until used and three replications were maintained for each treatment. RNA was extracted from both potato leaf and root tissues and from *Arabidopsis* leaf tissues using TRIzol reagent (Invitrogen) following the protocol reported by Derksen et al. [7].

Expression analysis of StRboh gene homologs, A, B and C using semi-quantitative RT-PCR

After the DNAse1 (Ambion) treatment of the total RNA, the cDNA was synthesized from 1 μg of the treated total RNA

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