Knock-down of the Small G-Protein RACB Enhances Penetration-Resistance of Barley against the Powdery Mildew Fungus

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Abstract

Small G-proteins (RAC and RHO) are known to be involved in regulation of superoxide ($O_2^{\bullet-}$) production and the assembly of actin fibres. These processes are known to be crucial for accessibility and inaccessibility of barley cells to the biotrophic fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*). Using a candidate RT-PCR approach six *Rac*-related cDNA-clones were isolated from barley. The transient knock-down of *RacB* led to a remarkably lower penetration efficiency of *Bgh* into susceptible barley lines (*Mlo*/*Ror1*). Surprisingly the inhibition of *RacB* expression had no effect in the double mutant line A89 (*mlo5*/*ror1*). This led us to the assumption that the *RacB*-dsRNA effect is dependent on functional ROR1 (such as *mlo5*-mediated resistance). Vice versa, overexpression of constitutive active RACB-V15 in the susceptible line Pallas resulted in hypersusceptibility to *Bgh*. Thus, we conclude that RACB is a signal transduction protein functional in the accessibility of epidermal barley cells to *Bgh*.

Keywords: GTPase; *Hordeum vulgare*; papilla; plant-pathogen interaction; powdery mildew fungus

INTRODUCTION

The biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) attacks epidermal cells of barley *(Hordeum vulgare* L.). The first step of fungal invasion is the penetration of the cell wall and the invagination of the plasma membrane followed by the establishment of a haustorium. The interaction of barley and *Bgh* is accompanied by the remodelling of the cytoskeleton and the accumulation of reactive oxygen species (KOBAYASHI *et al*. 1997; THORDAL-CHRISTENSEN *et al*. 1997; HÜCKELHOVEN & KOGEL 1998).

Small G-proteins (RAC and RHO in animals, RAC and ROP in plants) are cytosolic signal transduction factors activated by extracellular signals (e.g. via G-protein-coupled receptors). RAC-proteins are known to be involved in the production of superoxide ($O_2^{\bullet-}$) and the assembly of actin fibres. As members of the GTPase superfamily RAC-proteins are active in their GTP-bound and inactive in their GDP-bound form (YANG 2002).

In this study we report about identification of six members of the small G-protein family in barley and show that RACB functions as a susceptibility factor in the interaction of barley and the powdery mildew fungus.

MATERIALS AND METHODS

A transient transformation protocol originally developed for wheat (*Triticum aestivum*) to assess gene function in the interaction with powdery mildew was used to induce RNA interference via biolistic delivery of dsRNA into epidermal cells of barley leaf segments (SCHWEIZER *et al*. 1999, 2000, compare also NIELSEN *et al*. 1999).

For knock-down experiments (gene silencing via RNAi) tungsten particles for biolistic transformation were coated with pGFP (GFP under control of cauliflower mosaic virus 35S promoter) and dsRNA of *RacB* or a heterologous dsRNA, respectively. Barley leaves were bombarded with the particles and inoculated densely four hours later with the powdery

mildew fungus (*Blumeria graminins* f.sp. *hordei*). After 40 h incubation interaction outcome was judged by fluorescence and light microscopy. Transformed GFP-expressing cells were identified under blue light excitation. Three different categories of transformed cells were distinguished: (a) penetrated cells, which contained an easily visible haustorium; (b) cells that were attacked by a fungal appressorium but did not contain a haustorium; (c) and cells that were not attacked by *Bgh*. Surface structures of *Bgh* were detected by light microscopy or by fluorescence staining of the fungus with 0.1% calcofluor (w/v in water).

Deviation of penetration efficiency referring to control penetration efficiency was used as a measure for susceptibility of cells that were bombarded with *RacB*-dsRNA compared with those bombarded with heterologous human dsRNA.

For overexpression experiments the particles were coated with pGFP and an expression vector containing the testgene or empty vector for control, respectively. Leaves were transformed as previously described but incubated for 24 h before inoculation with *Bgh*. Subsequently the same conditions were used as described for RNAi experiments.

RESULTS AND DISCUSSION

To analyse the role of RAC-proteins in the barley – powdery mildew fungus interaction, we isolated *Rac* cDNA clones by RT-PCR (HÜCKELHOVEN *et al*. 2001). We obtained six *Rac* homologues with high sequence similarities but different constitutive expression levels in different leaf tissues. In the epidermis *RacB, Rac1, RacD* and *Rop6* were expressed strongly whereas *Rop8* expression was weaker and *Rop4* expression was not detectable. The expression level of *Rop8* was similar in the mesophyll and in the epidermis and *Rop4* was weakly expressed in the mesophyll.

To elucidate the role of small GTP-binding proteins in basal resistance or cellular accessibility, we bombarded Pallas leaf segments with *RacB*-dsRNA together with a GFP-expression vector (pGFP) to trigger sequence-specific gene silencing by RNA interference (SCHWEIZER *et al*. 2000). The transient knock-down of RACB in susceptible barley lines (*Mlo/Ror1*) using dsRNA of *RacB* led to a up to 50% decreased penetration efficiency compared to leaf-segments bombarded with heterologous control dsRNA (Table 1) (SCHULTHEISS *et al*. 2002).

Having discovered that RACB is a susceptibility factor in barley – *Bgh* interaction, we speculated that RACB could be linked functionally to the barley MLO-protein, which is known to be a negative control element of *Bgh*-resistance (SCHULZE-LEFERT & VOGEL 2000). To test this hypothesis, we repeated the former experiment using the partially resistant double mutant line A89 (*mlo5/ror1*), in which *mlo*-resistance is weakened by a second mutation in *Ror1* (FREIALDENHOVEN *et al*. 1996). The knock-down of RACB had no effect in the *mlo5/ror1* barley line. This indicates resistance triggered by *RacB* silencing depends on the same prerequisites as *mlo*-mediated resistance. Therefore, we speculate that functions of RACB and MLO are linked in barley *Bgh*-susceptibility (SCHULTHEISS *et al*. 2002).

To further characterize the mechanism of RACB function, we performed transient overexpression assays with a constitutive active form of RACB (RACB-V15).

Table 1. Impact of different barley RAC transformation variants on penetration success of *Bgh* in epidermal cells

Transformation construct	Penetration efficiency of *Bgh*
dsRNA RacB[1]	strongly reduced
pJP-RacB(as)-RacB(s)[2]	reduced
pGY1-RacB-V15[3]	strongly enhanced
pGY1-RacB-V15-CSIL[4]	unchanged
pGY1-RacD-V15[5]	unchanged

Effects on penetration efficiency of *Blumeria graminis* f.sp. *hordei* in barley epidermal cells transient transformed with different constructs

[1]epidermal cells were transformed with double stranded RNA of *RacB* (knock-down by RNA interference)

[2]hairpin-construct leading to endogenous production of *RacB*-dsRNA

[3]overexpression construct leading to production of a constitutive active form of RACB

[4]overexpression construct leading to production of a constitutive active form of RACB without prenylation-site

[5]overexpression construct leading to production of a constitutive active form of RACD

As expected, overexpression of RACB-V15 in the susceptible line Pallas resulted in enhanced susceptibility. In contrast the expression of a *RacB*-hairpin construct for endogenous expression of *RacB*-dsRNA led to enhanced resistance (Table 1).

In mammals as well as in *Arabidopsis* it is shown that the first step in RAC activation includes membrane attachment via a prenyl-lipid membrane anchor at the C-terminus of the protein. By overexpressing a active RACB-mutant lacking the membrane anchor site (RACB-V15-CSIL) we could show that the function of RACB in susceptibility is dependent on the membrane attachment motif of RACB (Table 1). Therefore, we assume that linkage of a prenyl group to the C-terminus of RACB is a prerequisite for its function in susceptibility of barley epidermal cells.

To control whether the regulation of defence reactions is a feature of further RAC-family members, we performed overexpression studies with a constitutive active form of barley RACD(RACD-V15). As shown in Table 1 overexpression of RACD-V15 does not influence the penetration efficiency of *Bgh*. This indicates that negative regulation of penetration resistance to *Bgh* is restricted to one or a few RAC-proteins.

Taken together we conclude that the small G-protein RACB plays an important role as a molecular switch in barley accessibility of epidermal cells for invasion by *Bgh*. Presently RACB is used as a starting point to further investigate signalling in barley attacked by fungal pathogens.

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