Functional Studies on the Role of Reactive Oxygen Intermediates in the Resistance of Barley against Powdery Mildew

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Abstract

The role of reactive oxygen intermediate (ROI) accumulation in resistance and susceptibility of plants to parasitic fungi is still little understood. We examined the spatial and temporal occurrence of different ROIs in barley after inoculation with the biotrophic fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*, barley powdery mildew fungus). Using histochemical analyses, we collected correlative data indicating that H_2O_2 and $O_2^{\bullet-}$ play different roles in background penetration resistance to *Bgh*. To study the role of $O_2^{\bullet-}$ in detail, we isolated barley cDNAs encoding a NADPH oxidase GP91PHOX homologue and a RACB homologue, which may be involved in NADPH oxidase activation. Interestingly, transient silencing of RACB or GP91PHOX via sequence-specific RNA interference enhanced penetration resistance of barley to *Bgh*. Together, data reveal rather a negative than a positive role of superoxide generation in background resistance of barley to *Bgh*.

Keywords: barley; background resistance; *Blumeria graminis* f.sp. *hordei*; *Hordeum vulgare*; papilla; gene silencing; RNA interference

INTRODUCTION

Reactive oxygen intermediates (ROI) play multiple roles in plant pathogen interactions. $O_2^{\bullet-}$ or H_2O_2 are involved in cell wall cross-linking and they induce defense mechanisms including defense-related gene expression and the hypersensitive reaction (HR). On the other hand, ROI induce also the production of antioxidants and restrict cell death (LEVINE *et al*. 1994; TENHAKEN *et al*. 1995; JABS *et al*. 1996).

Basal background resistance of barley to the biotrophic, fungal pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*) is expressed mainly as penetration resistance that is characterized by formation of cell wall appositions at sites of fungal attack. In barley, $O_2^{\bullet-}$ production takes place during attack by *Bgh* at sites of successful penetration of epidermal cells, but not at sites where fungal penetration is prevented (HÜCKELHOVEN & KOGEL 1998). In contrast, H_2O_2 accumulates subcellularly in barley at sites were penetration by *Bgh* is successfully prevented as well as in entire cells that undergo HR. Together, accumulation patterns of $O_2^{\bullet-}$ and H_2O_2 differ temporally and spatially in barley during attack by *Bgh* (THORDAL-CHRISTENSEN *et al*. 1997; HÜCKELHOVEN & KOGEL 1998; KOGEL & HÜCKELHOVEN 1999; HÜCKELHOVEN *et al*. 1999, 2000).

Interaction of plant RAC homologues with an NADPH oxidase appears to regulate activity of NADPH oxidase that produces $O_2^{\bullet-}$ in response to pathogen attack (HASSANAIN *et al*. 2000; ONO *et al*. 2001). Therefore, functional analyses of barley RAC proteins and the core NADPH oxidase subunit GP91PHOX in the interaction with *Bgh* should provide a deeper insight into the role of ROI in the interaction of host and a biotrophic pathogen.

MATERIALS AND METHODS

The barley (*Hordeum vulgare* L.) lines Ingrid or Pallas were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen,

Denmark). Plants were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h (240 μmol/m^{2}s photon flux density). The barley powdery mildew fungus, *Blumeria graminis* (DC) Speer f.sp. *hordei* Em. Marchal, race A6 was inoculated onto barley primary leaves to give a density of 100 conidia per mm^2. *Bgh* was maintained on barley cultivar Siri under the same conditions. Reactive oxygen intermediates were detected *in situ* by infiltration 1mg/ml of 3,3-diaminobenzidine to stain H_2O_2 or nitroblue tetrazolium to stain $O_2^{\bullet-}$ according to THORDAL-CHRISTENSEN *et al*. (1997) or HÜCKELHOVEN and KOGEL (1998), respectively. We isolated cDNA fragments by the use of an Onestep RT-PCR kit and cloned them into pGEM-T. Primers were designed using GenBank or EST data base information (HÜCKELHOVEN *et al*. 2001). For probe generation, PCR-products of cDNA plasmid inserts were used for *in vitro* transcription with digoxygenin or fluoroescein labeled nucleotides. The OneStep RT-PCR kit was also used for semi-quantitative reverse transcription polymerase chain reaction. A transient transformation protocol was used to induce RNA interference via biolistic delivery of dsRNA into epidermal cells of barley leaf segments as described by SCHWEIZER *et al*. (2000). Principally, 312 μg of 1.1 μm tungsten particles were coated with dsRNA (2 μg) together with pGFP (1 μg, GFP under control of CaMV 35S promoter) as a transformation marker for each shot. Double stranded RNA was obtained by annealing of sense and antisense RNA synthesized *in vitro* (SCHWEIZER *et al*. 2000). Leaf segments were bombarded with coated particles 4 hours before inoculation with *Bgh*, race A6. Interaction outcome was judged subsequently by fluorescence and light microscopy. Attacked transformed cells contained either an easily visible haustorium and supported fungal growth or did neither contain a haustorium nor supported fungal growth.

RESULTS AND DISCUSSION

By histochemical staining, we found that H_2O_2 (visualized by 3,3-diaminobenzidine, DAB, according to THORDAL-CHRISTENSEN *et al*. 1997) accumulates especially in resistant barley (*mlo5*-mediated broad-spectrum resistance) as a response to *Bgh* attack. Detailed microscopic analysis revealed that formation of cell wall appositions preventing fungal penetration was associated with the accumulation of hydrogen peroxide (HÜCKELHOVEN *et al*. 1999, 2000). In contrast to H_2O_2, $O_2^{\bullet-}$ (visualized by nitroblue tetrazolium, NBT) accumulated at sites of successful fungal haustorium establishment (HÜCKELHOVEN & KOGEL 1998; KOGEL & HÜCKELHOVEN 1999).

By a candidate PCR approach, we isolated barley cDNAs encoding GP91PHOX subunits and a putative activator protein RACB (small GTP-binging protein of the ROP family) of the $O_2^{\bullet-}$ generating plasma membrane NADPH oxidase. Corresponding genes were constitutively expressed in barley epidermal cells that interact directly with *Bgh* (HÜCKELHOVEN *et al*. 2001). *Bgh* attack induced only slightly enhanced expression of *RacB* and *gp91phox*. Interestingly, transient knock-out (gene silencing) of RACB via sequence-specific RNA interference, triggered by dsRNA according to SCHWEIZER *et al*. (2000), enhanced penetration resistance of barley to *Bgh,* whereas silencing of two *Bgh*-induced genes (encoding cytosolic ascorbate peroxidase and papain cystein protease) had no or opposite effects, respectively. We demonstrated specificity of RNA interference by silencing the transient expression of fusion proteins with the green fluorescing reporter protein (SCHULTHEISS *et al*. 2002).We conclude that unexpectedly host RACB and NADPH oxidase activity might be involved in processes that are required for entry of *Bgh* into epidermal cells of barley.

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