Ectopic Expression of Constitutively Activated RACB in Barley Enhances Susceptibility to Powdery Mildew and Abiotic Stress

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Small RAC/ROP-family G proteins regulate development and stress responses in plants. Transient overexpression and RNA interference experiments suggested that the barley (Hordeum vulgare) RAC/ROP protein RACB is involved in susceptibility to the powdery mildew fungus Blumeria graminis f. sp. hordei. We created transgenic barley plants expressing the constitutively activated RACB mutant racb-G15V under control of the maize (Zea mays) ubiquitin 1 promoter. Individuals of the T1 generation expressing racb-G15V were significantly more susceptible to B. graminis when compared to segregating individuals that did not express racb-G15V. Additionally, racb-G15V-expressing plants showed delayed shoot development from the third leaf stage on, downward rolled leaves, and stunted roots. Expression of racb-G15V decreased photosynthetic CO₂-assimilation rates and transpiration of nonstressed leaves. In contrast, racb-G15V-expressing barley leaves, when detached from water supply, showed increased water loss and enhanced transpiration. Water loss was associated with reduced responsiveness to abscisic acid in regard to transpiration when compared to segregants not expressing racb-G15V. Hence, RACB might be a common signaling element in response to both biotic and abiotic stress.

Early interaction of plants with the biotic and abiotic environment requires perception and transduction of extracellular signals. Communication between extracellular and intracellular compartments in eukaryotes is mediated by endocytotic pathways and/or plasma membrane receptor-mediated signaling. The processing of extracellular signals often involves GTP-binding proteins. The RHO-related subclass of plant small monomeric GTPases is called RAC or ROP (Rho of plants). RAC/ROPs act as molecular switches in cell polarity, hormone signaling, and plant defense. Activation of downstream effectors by RAC/ROP requires binding of GTP, and, in turn, GTP-mediated RAC/ROP activity is abolished by intrinsic or stimulated GTP-hydrolyzing activity. Hence, RAC/ROP proteins can be constitutively activated by mutation of the intrinsic GTPase function. In contrast, wild-type RAC/ROP activity is strictly regulated, in plants most likely by receptor-like kinases, GTPase-activating proteins, guanine nucleotide exchange factors, and guanine nucleotide dissociation inhibitors (Gu et al., 2004; Berken et al., 2005). Winge et al. (2000) subdivided the 11 Arabidopsis (Arabidopsis thaliana) RAC/ROP proteins into two major subgroups that can be distinguished by length due to an additional exon in group II. Cereals appear to express six to nine RAC/ROP genes (Christensen et al., 2003; Schultheiss et al., 2003). Based on the available barley (Hordeum vulgare) transcriptome data, six barley full-length RAC/ROP cDNAs have been isolated, and no further family members could be identified in more than 300,000 expressed sequence tags (Schultheiss et al., 2003). Similar to Arabidopsis RAC/ROPs, barley family members can be subdivided into group I and group II RAC/ROPs by length. Likewise, similar to Arabidopsis, barley RAC/ROPs can be further distinguished into a total of four clades (Schultheiss et al., 2003).

Plant susceptibility to biotrophic fungi is little understood (Schulze-Lefert and Panstruga, 2003; Hückelhoven, 2005). Successful pathogens target host proteins to bypass or to suppress basic defense mechanisms. Seemingly, such target proteins are essential to the host because resistant mutants often show pleiotropic growth or cell death phenotypes (Vogel and Somerville, 2000). Barley susceptibility to the biotrophic powdery mildew fungus Blumeria graminis f. sp. hordei (Bgh) is dependent on the host receptor-like MLO protein. MLO is believed to be used by the fungus for host

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defense suppression (for review, see Schulze-Lefert and Panstruga, 2003; Hucklehoven, 2005). Mutants that do not express functional MLO are completely resistant to penetration by Bgh. This type of resistance is in turn limited in ror1 and ror2 mutants, which are affected in independent loci required for mlo-mediated resistance (Freialdenhoven et al., 1996). ROR1 is not yet identified, but ROR2 is a plasma membrane syntaxin that locally accumulates at the site of attempted fungal penetration, where it appears to be involved in exocytosis of defense-related compounds (Collins et al., 2003; Assaad et al., 2004; Bhat et al., 2005).

Transient transformation-mediated overexpression and RNA interference are used as test systems to assess gene function in the interaction of cereals with powdery mildew fungi (Nielsen et al., 1999; Schweizer et al., 1999, 2000; Panstruga, 2004). Transient RNA interference-mediated knock down of the barley RAC/ROP protein RACB renders epidermal cells more resistant to penetration by Bgh. In contrast, transient overexpression of the constitutively activated RACB mutant racb-G15V but not of wild-type RACB supports fungal penetration (Schultheiss et al., 2002, 2003). Hence, abundance and activity of RACB appear to be crucial for single-cell accessibility to Bgh. The exact mechanism of RACB activity is not known. However, RACB effects on susceptibility are dependent on functional MLO and ROR1 (Schultheiss et al., 2002, 2003). RACB could thus be involved in MLO-dependent ROR1 antagonism. RACB is a group I RAC/ROP falling into clade IV with maize (Zea mays) ROP2, ROP4, and ROP9, rice (Oryza sativa) RACB and RACD, and Arabidopsis ROPs 1 to 6 (Christensen et al., 2003; Schultheiss et al., 2003). RACB is a plasma membrane-associated protein. This localization is mediated by a CAAX-box prenylation motif at the C terminus, which is required for RACB function in susceptibility to Bgh (Schultheiss et al., 2003). Additionally, single-cell RACB overactivation hampers actin-filament reorganization in Bgh-attacked cells, whereas RACB knock down promotes polarization (Opalski et al., 2005). Hence, RACB modulates actin reorganization during attack by Bgh. In contrast to barley RACB, rice RAC1 is functioning in the oxidative burst and defensive cell death in response to the hemibiotrophic fungus Magnaporthe grisea (Ono et al., 2001). Hence, small RAC/ROP G proteins appear to be active in resistance and susceptibility to plant pathogenic fungi.

In this study, we genetically transformed susceptible barley to constitutively express racb-G15V. Our results support the role of RACB in susceptibility to Bgh and suggest a potential involvement of RACB in plant development as well as in response to abiotic stress.

RESULTS

Generation of Transgenic racb-G15V Barley and Phenotypes

Transient transformation of barley epidermal cells is widely used to assess gene function in the interaction of cereals and powdery mildew fungi (Nielsen et al., 1999; Schweizer et al., 1999, 2000). However, little direct evidence exists for transferability of results from transient single-cell expression to entire plants (Altpeter et al., 2005). To validate that transient transformation is a suitable tool to assess gene function in interaction with powdery mildew fungi and to assess further functions of barley RACB, we produced transgenic barley plants that express the constitutively activated RACB-G15V protein under control of the maize ubiquitin 1 promoter ZmUbi1 (Christensen and Quail, 1996). To this end, the barley cultivar Golden Promise was stably transformed with Agrobacterium tumefaciens strain AGL1 (Lazo et al., 1991) employing a method described earlier (Hensel and Kumlehn, 2004). Seventy-one independent primary transgenic lines were generated from 470 immature embryos infected. We observed that the T0 generation of candidate racb-G15V barley was reduced in growth as compared to nontransgenic donor plants of Golden Promise, and only 43 of these lines set seeds, indicating an impact of the transferred expression cassette on plant development.

To test individual plants of the T1 generation for transgene expression by reverse transcription (RT)-PCR, we designed oligo DNA primers that distinguish endogenous RACB mRNA from racb-G15V mRNA. In contrast to what was expected, the T1 generation of nine independent transgenic lines segregated about 1:1 (65:62) instead of 1:2:1 (3:1 for transgene expression; 22:1:2 for expression cassette; 5:5:5) (Christensen et al., 2003). Seventy-nine individuals of the T2 generation of line 17-1-23 (P = 0.78) showed a downward rolled phenotype and unfolded germination. The subsequently developing leaves unfolded about 1 week later when compared to segregants not expressing racb-G15V. In eight out of nine independent lines, we found one or two copies (data not shown). This might indicate male sterility of racb-G15V barley. The transgenic status of individuals was confirmed by genomic DNA blotting. Therefore, DNA analysis of individual T1 segregants consistently corroborated previous RT-PCR results (data not shown). Therefore, we considered individuals with negative results in the racb-G15V RT-PCR as T1 plants that lost racb-G15V by segregation (seggregants not expressing racb-G15V; synonym: racb-G15V-negative individuals). Out of seven lines tested in DNA-blot experiments, six contained single transgene copies (17-1-11, 17-1-23, 18-1-6, 18-1-10, 18-1-16, 18-1-19, and 18-1-21). A χ2 test confirmed a 1:1 segregation (χ2 for a 1:1 segregation: χ2 = 0.07; P = 0.79). One:one segregation was also found for 50 individuals of the T2 generation of line 17-1-23 (χ2 = 0.08; P = 0.78). This might indicate male sterility of racb-G15V barley.

To assess a potential RACB function during plant development, we judged plant habit over time. Individuals that expressed racb-G15V were macroscopically indistinguishable from segregants that lost racb-G15V until they reached the three-leaf stage 3 weeks after germination. The subsequently developing leaves showed a downward rolled phenotype and unfolded about 1 week later when compared to segregants not expressing racb-G15V in eight out of nine independent lines (Table 1). After leaf unfolding, leaves often remained twisted. This phenotype was associated with expression of racb-G15V in 100% of cases as checked by RT-PCR. From 3 to 5 weeks after germination onward,
A 224:231 segregation (x^2 test for a 1:1 segregation; \chi^2 = 0.11; P = 0.75), which underscored the possibility that male sterility of racb-G15V-expressing plants might be caused by strong expression of racb-G15V in pollen.

**RACB-G15V-Expressing Individuals Are Photosynthetically Less Active**

To further test whether photosynthesis is impaired in racb-G15V-expressing plants, we measured CO₂ assimilation rates under different light conditions. When we compared segregants from three independent lines, we observed that racb-G15V-expressing segregants had a reduced photosynthetic capacity under medium to high light conditions (shown for line 18-1-6 in Fig. 3). This effect was of similar strength in three lines tested (17-1-11, 18-1-6, and 18-1-10).

**RACB-G15V-Expressing Individuals Are More Susceptible to Bgh**

We analyzed cellular accessibility to Bgh and mildew symptom development after artificial inoculation of the T1 generation. We tested first leaves from 10-d-old plants because they had no obvious developmental phenotype. About 10 segregants of seven independent lines were analyzed for fungal penetration success and haustorium establishment on the microscopic level at 48 h after inoculation. In total, on racb-G15V segregants, 20% more fungal germlings succeeded in penetration when compared to segregants not expressing racb-G15V. In individual lines, racb-G15V enhanced penetration rates by 10% to 40%. Due to the high variability of penetration frequencies on individual leaves and the partially limited numbers of seeds, this effect was statistically significant for only five of the seven individual lines tested (Table I). However, racb-G15V-mediated susceptibility to penetration was statistically significant at P < 0.01 over all seven lines of the T1 generation (Student’s t test). The frequency of hypersensitive cell death reactions of single attacked cells was not affected by racb-G15V in any line (data not shown). We scored 200 interaction sites on each of the segregants and grouped them into categories of fungal penetration rates. This also indicated that the population of segregants expressing racb-G15V was significantly shifted toward higher accessibility to fungal penetration (Fig. 4A).

Five days after inoculation of third leaves, mildew colonies were counted on the same lines. Segregants expressing racb-G15V supported about 40% more

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**Table 1. Overview of transgenic lines expressing racb-G15V and corresponding phenotypes**

<table>
<thead>
<tr>
<th>racb-G15V-Expressing Line</th>
<th>Fungal Penetration (RT-PCR+/Total)</th>
<th>Colony Development (RT-PCR+/Total)</th>
<th>Rolled Leaves</th>
<th>Dwarfed</th>
<th>Water Loss</th>
<th>Response to ABA</th>
<th>Reduced Photosynthetic Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-1-23</td>
<td>n.d.</td>
<td>+ (20/39)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>18-1-6</td>
<td>– (3/7)</td>
<td>+ (3/10)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>18-1-10</td>
<td>+ (11/19)</td>
<td>+ (14/20)</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>+</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* + labels lines with a detectable phenotype; – labels lines without detectable phenotype; +? labels lines with an altered phenotype that was not statistically significant at P < 0.05. *1Numbers of segregants expressing racb-G15V of total n tested in the T1 generation; for line 17-1-23, the T2 generation was tested. *2Phenotypes could be observed from 3 weeks after germination onward. *3Line 17-1-3 expressed racb-G15V on a very low level (Fig. 5).
disease development than individuals not expressing *racb-G15V* (Fig. 4B). This effect was detected in five of the seven lines analyzed (Table I). Again, despite the fact that the individual susceptibility level of single leaves is generally variable, the *racb-G15V* effect was statistically significant over all lines at \( P < 0.01 \).

Line 17-1-3 showed neither a growth phenotype nor a susceptibility phenotype. Hence, we re-evaluated *racb-G15V* expression in line 17-1-3 by RNA gel-blot experiments. This revealed that this line expressed the transgene at a much lower level when compared to two lines showing strongly altered phenotypes (Fig. 5). Thus, the lack of a *racb-G15V*-mediated phenotype in 17-1-3 appeared to be due to low transgene expression.

It was speculated that susceptibility to one fungal pathogen might limit susceptibility to fungi with different lifestyles (Brown, 2002). Since *racb-G15V*-expressing T1 individuals were more susceptible to the biotrophic fungus *Bgh*, we tested *racb-G15V*-transgenic leaves for their ability to resist inoculation with the hemibiotrophic fungus *Bipolaris sorokiniana* and the necrotrophic fungus *Fusarium graminearum*. In both cases, segregants expressing *racb-G15V* showed as much necrotic symptoms as segregants not expressing *racb-G15V* (data not shown).

### Barley Expressing *racb-G15V* Shows Enhanced Water Loss under Stress Conditions

Arabidopsis RAC/ROP-like proteins are involved in abiotic stress signaling. Therefore, we tested *racb-G15V* T1 plants for their ability to maintain turgor under stress conditions. Excised leaves of segregants that expressed *racb-G15V* completely lost turgor overnight when kept at room temperature on the bench. In contrast, segregants not expressing *racb-G15V* partially retained turgor. Within 48 h *racb-G15V*-positive segregants dried out completely, whereas *racb-G15V*-negative individuals partially retained water (data not shown). To quantify water loss, we gravimetrically analyzed excised leaves of three independent lines during a period of 24 h at 20°C and 40% relative humidity. Segregants not expressing *racb-G15V* lost...
about 60% of their original weight, whereas racb-G15V-expressing individuals lost 80% of their original weight (shown for line 18-1-10 in Fig. 6A). Expression of racb-G15V did not influence leaf dry weight (data not shown).

To distinguish potential transgene-dependent cuticle defects (Chen et al., 2004) from altered stomatal conductivity, we measured chlorophyll release into 80% watery ethyl alcohol over time and transpiration rates, respectively. Transcuticular chlorophyll release was not different in segregants not expressing racb-G15V versus those expressing racb-G15V (data not shown). However, porometry indicated that transpiration rates remained on a high level in racb-G15V-positive individuals 16 h after leaf detachment (50%–80% of \( t_{50} \)), whereas racb-G15V-negative individuals were able to reduce transpiration to about 20% to 40% of what was measured at \( t_{50} \) (shown for line 18-1-10 in Fig. 6B). Hence, leaves expressing racb-G15V held up high transpiration rates when detached from water supply. This was in contrast to nonstressed leaves.

Since transpiration under stress condition is regulated by abscisic acid (ABA), we measured the influence of ABA on stomatal conductivity. After 4 min of adaptation to the measuring cell, excised leaves were placed in a 50 \( \mu \)M ABA solution. This strongly reduced transpiration in T1 plants not expressing racb-G15V over time to 4% of what was measured at \( t = 0 \) when ABA was applied. In contrast, following the same treatment, individuals expressing racb-G15V less effectively reduced transpiration to 21% of the value at \( t = 0 \) (Fig. 7). Similar results were obtained with 10 \( \mu \)M ABA, except that final transpiration rates were higher (14% of \( t = 0 \) in racb-G15V-negative versus 36% of \( t = 0 \) in racb-G15V-positive individuals). Absolute transpiration rates of racb-G15V barley plants remained at a higher level after ABA treatment when compared to wild-type segregants. In contrast, if racb-G15V-expressing segregants were treated with water instead of ABA, they showed up to 50% less transpiration than racb-G15V-negative segregants before, during, and at the end of the experiments. These results indicate that racb-G15V-expressing individuals were not generally more conductive for water but were less responsive to ABA, and, hence, they could not effectively reduce transpiration.
DISCUSSION

We have generated transgenic barley lines expressing the constitutively active mutant RAC/ROP protein RACB-G15V. Individuals expressing racb-G15V show multiple altered phenotypes when compared to individuals not expressing racb-G15V from the same generation. Among them, organ expansion defects, enhanced transpiration rates under stress conditions, and enhanced susceptibility to Bgh are most obvious. This suggests that small G proteins are involved in barley development and response to both biotic and abiotic stress.

Biolistic transformation of single cereal cells with subsequent challenge by B. graminis has been widely used to assess gene function in defense and susceptibility (Panstruga, 2004). However, direct evidence for gene function on entire plant level assessed by single-cell transformation is rare (Altpeter et al., 2005). Here, we report that a similar degree of enhanced susceptibility to penetration by Bgh was obtained when we compared racb-G15V barley plants with single-cell transformation (Fig. 4; Schultheiss et al., 2003). RACB-G15V enhanced susceptibility of cultivars Ingrid, Pallas, and Golden Promise in transient or stable overexpression experiments (Schultheiss et al., 2003; this study; H. Schultheiss, unpublished data). Together, this supports a function of active RACB in background susceptibility to Bgh and further validates the transient transformation assay as a suitable tool for gene function assessment in the interaction with B. graminis. Enhanced susceptibility to penetration by Bgh was observed on the first leaf, not having an obvious racb-G15V-dependent developmental phenotype. This, together with evidence from transient single-cell transformation in fully developed leaves and dependency of RACB effects on MLO and/or Ror-1, indicates that RACB activity may directly support entry by Bgh. T1 plants expressing racb-G15V showed enhanced susceptibility at the macroscopic level 5 d after inoculation (Fig. 4B). Bgh developed more colonies on racb-G15V-positive segregants than on racb-G15V-negative segregants. This supports that single-cell accessibility limits background resistance. The fact that early fungal colony growth is supported by RACB activity in third leaves is astonishing considering the observation that racb-G15V barley had limited photosynthetic capacity (Fig. 3) because Bgh, as an obligate biotroph, is dependent on host carbohydrate supply.

The function of RACB was linked to the function of MLO (Schultheiss et al., 2002, 2003), and Bgh-resistant barley mlo mutants show enhanced susceptibility to the hemibiotrophic fungus M. grisea and to the toxic culture filtrate of B. sorokiniana (Jarosch et al., 1999; Kumar et al., 2001). Additionally, RAC/ROP functions have been linked to effective defense responses in several plant pathogen interactions (Ono et al., 2001; Agrawal et al., 2003; Moeder et al., 2005). Therefore, we speculated that RACB might have a role in background resistance to other pathogens. However, when...
we inoculated racb-G15V-positive leaf segments with either F. graminearum or B. sorokiniana, we did not detect a significant difference in background resistance when compared to racb-G15V-negative segregants (data not shown). Although this does not exclude altered responses of racb-G15V barley to other pathogens or in other tissues, data indicate that there is no general link between barley susceptibility to Bgh and resistance to necrotizing pathogens.

Constitutive RACB activity in transgenic barley apparently provoked developmental phenotypes in roots and shoots (Fig. 1). To confirm that RACB-G15V might be responsible for these phenotypes, we analyzed racb-G15V expression by northern blots and by use of a reporter plant expressing GFP under control of the same promoter that was used to drive racb-G15V expression. Northern blots showed that strong leaf expression of racb-G15V is associated with leaf phenotypes in regard to leaf folding and susceptibility to Bgh. The GFP-expressing reporter lines showed GFP expression in all tissues analyzed, including those showing racb-G15V-mediated phenotypes. This is in accordance with observations in wheat (Triticum aestivum), in which the maize ubiquitin promoter drives ubiquitous reporter gene expression, in particular in young metabolically active tissue and pollen grains (Rooke et al., 2000). Hence, RACB-G15V might be directly responsible for shoot and root defects. Additionally, our analyses of expression of RACB from its endogenous promoter and data mining in public barley databases (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=barley; http://pgrc.ipk-gatersleben.de/b-est/; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) for expressed barley sequence tag abundance (e.g. Zhang et al., 2004) indicate that RACB is expressed in leaf epidermis, leaf mesophyll, earlets, male and female inflorescences, Caryopses, pericarps, embryos, coleoptiles, stems, and roots (Schultheiss et al., 2002; data not shown). Since we observed a 1:1 segregation in T1 and T2 populations, we speculate that racb-G15V may cause male sterility at high expression levels. RACB-like proteins have already been implicated in pollen tube growth and male gametophyte development in Arabidopsis, rice, and maize. Overexpression of constitutively activated RAC/ROPs induces loss of polarity in pollen tube growth (Kost et al., 1999; Arthur et al., 2003; Xu et al., 2004; Gu et al., 2005).

RACB-G15V provoked obvious organ expansion phenotypes in roots and shoots. Leaves showed a rolled downward phenotype rather than a leaf expansion phenotype. Heading was delayed or inhibited. Individuals that expressed racb-G15V and produced ears finally reached the height of individuals not expressing racb-G15V. Some of the racb-G15V-induced phenotypes are reminiscent of hormone signaling defects such as those observed in auxin or ABA mutants. Since RACB function was recently linked to cell polarization and racb-G15V inhibits actin reorganization in host defense (Opalski et al., 2005), racb-G15V barley may be decelerated in cell polarization required for organ expansion. Because double-stranded-RNA-mediated knock down of RAC induces resistance to Bgh, we also speculated that RACB participates in the polar membrane growth process that is involved in plasmalemma invagination during haustorium establishment (Schultheiss et al., 2002, 2003).

Besides constitutively activated RACB-G15V, barley RAC3-G17V similarly induces enhanced accessibility to Bgh in transiently transformed cells (Schultheiss et al., 2003). Arabidopsis RAC10, a relative of barley RAC3, was recently ectopically overexpressed in Arabidopsis to study potential protein functions (Bloch et al., 2005). This revealed a role for RAC10 in actin organization and endocytosis. Similar to racb-G15V barley, rac10-G15V Arabidopsis had downward rolled leaves. However, in contrast to racb-G15V barley but similar to other constitutively activated Arabidopsis RAC/ROP mutants (Molendijk et al., 2001; Jones et al., 2002; Fu et al., 2005), rac10-G15V Arabidopsis had an altered root hair phenotype. Together, one might speculate that constitutively activated RAC/ROPs partially cross activate downstream events of individual RAC/ROPs when ectopically expressed.

Constitutive RACB activity in transgenic barley limited leaf water retention capacity. Barley excised leaves expressing racb-G15V completely lost turgor within 24 h. This can be explained by a failure of racb-G15V barley to reduce transpiration when cut off from water supply (Fig. 6). The reduced ABA responsiveness of racb-G15V barley in regard to reduction of transpiration argues for a defect in ABA-responsive stomata closure. We observed neither a cuticula defect nor a morphological stomata defect in racb-G15V
barley. Hence, constitutive RACB activity appears to antagonize ABA function in regulation of leaf water conductivity. In contrast, transpiration was lower in racb-G15V-expressing T1 plants that were not treated with ABA than in segregants not expressing racb-G15V under high light conditions. Reduced transpiration rates under optimal light conditions might also indicate a reduced CO₂ exchange capacity, and this might explain limited photosynthesis. Arabidopsis ROP10, another relative of barley RAC3, is specifically involved in negative regulation of ABA effects (Zheng et al., 2002). The close RACB relative Arabidopsis RAC1 negatively regulates ABA-induced stomata closure and ABA-induced actin disassembly in stomata (Lemichez et al., 2001). We compared patterns of assembled filamentous actin in stomata of racb-G15V-positive and racb-G15V-negative segregants by confocal laser scanning microscopy after ABA treatment and phalloidin staining. We observed that individuals not expressing racb-G15V disassembled a part of the filamentous actin in stomata after ABA treatment and that barley expressing racb-G15V had a tendency to hold up a greater portion of actin in a filamentous form (data not shown). Transient expression of racb-G15V inhibits actin dynamics and polarization processes in the interaction with Bgh (Opalski et al., 2005). Therefore, the function of RACB in actin reorganization may not only explain enhanced susceptibility, but also reduced ABA responsiveness and delayed organ expansion of racb-G15V barley. The overlap of phenotypes induced in dicots and the monocot barley by activated RAC/ROP supports the view that small RHO-like plant GTPases regulate similar processes in many higher plants. Eventually, barley RACB appears to be a susceptibility factor in the interaction with Bgh and to have a role in plant development and adaptation to leaf water potentials. This further supports the idea that biotrophic fungi corrupt essential plant signaling pathways for reprogramming of the attacked host.

DNA Cloning

For constitutive expression of the racb-G15V gene in barley, the BamHI/Sall fragment from plasmid pGY1-RacB-V15 (Schultheiss et al., 2003) was subcloned into appropriate sites of the binary vector pSB181 (S. Broders, unpublished data). Plasmid pSB181 was designed by integration of the SfiI fragment of plasmid pUbi-AB (maize [Zea mays] ubiquitin 1 promoter [Zm-Ubi-1] containing the first intron in front of the start codon and nos terminator; DNA Cloning Service) into the plH6000 vector (GenBank accession no. AY223428; DNA Cloning Service). Vector plH6000 contains a T-DNA with the hygromycin-resistance gene hpt driven by the cauliflower mosaic virus 35S promoter and terminator, and a multiple cloning site for integration of additional expression cassettes.

For in planta expression, the GFP gene (GFP out of pGY1-GFP; Schweizer et al., 1999) was subcloned with restriction enzymes into pUbi-AB. The transfer of the complete expression cassette into the binary vector pH6000 was then performed as described above using SfiI sites. All binary plasmids were then introduced into the Arabidopsis strain AGL1 (Lazo et al., 1991) by electroporation (Gene Pulser; Bio-Rad) according to manufacturer’s instructions. Analysis of stably transformed material using the gfp reporter was performed with either a standard fluorescence microscope or a confocal laser scanning microscope TCS SP2 AOBS (excitation: laser line 488 nm; emission: 500 to 540 nm; Leica).

Measurement of Photosynthetic Capacities

Photosynthetic capacities were measured in each of nine to 13 T1 individuals of three transgenic lines. Segregants were exposed to increasing light intensities at 52% relative humidity. Net CO₂ uptake rates, transpiration rates, stomatal conductance, and intercellular CO₂ concentration were determined according to Hajirezaei et al. (2002), using a portable photosynthesis system LI-6400 (LI-COR). The CO₂ concentration of the air entering the leaf chamber and the leaf temperature were adjusted to 400 μmol mol⁻¹ and 20°C, respectively. Photon flux density was varied between 20 and 2000 μmol quanta m⁻² s⁻¹.

Water Loss Assay and Porometry

For water loss measurements, third leaves were cut and kept on filter paper at 20°C (40% relative humidity and 16-h-light period with 60 μmol s⁻¹ m⁻² photon flux). To quantify water loss, we gravimetrically analyzed the leaves during a period of 24 h. To determine the dry weight, leaves were kept for 48 h in an incubator at 70°C.

For transpiration measurements, third leaves were cut and incubated on 0.5% water-agar at 20°C, 60% relative humidity, and a light intensity of 60 μmol s⁻¹ m⁻² photon flux. Transpiration rates of the leaves were measured under the same conditions 16 h after detachment using a LICOR 1600 porometer.

For measurement of ABA effects, the excised third leaves were kept under 1,500 μmol s⁻¹ m⁻² photon flux at 52% relative humidity and 25°C until they reached maximal leaf conductivity. Measurement was started 4 min before ABA application. Subsequently, leaves were transferred into aqua destillata or ABA solutions, respectively, and water conductivity was measured over time for 21 min (system LI-6400; LI-COR).

MATERIALS AND METHODS

Plant and Pathogen Material

Transgenic plants and the parent cultivar Golden Promise were grown in a growth chamber at 22°C, 60% relative humidity, and a photoperiod of 16 h (150 μmol s⁻¹ m⁻² photon flux density) up to E.C. 30. From the fifth week after germination onward, plants were grown in a greenhouse at 20°C (16-h day; 60 μmol s⁻¹ m⁻² photon flux density) and 16°C (8-h night), each with 60% relative humidity.

The barley (Hordeum vulgare) powdery mildew fungus Blumeria graminis (DC) Speer f. sp. hordei Em. Marchal, race A6 (Bgh; Wilberg, 1974), was maintained on barley cultivar Sri under the same conditions.

For microscopic evaluation, Bgh was inoculated onto detached primary leaves of 10-d-old barley plants to give a density of 30 conidia mm⁻². The outcome of the interaction was evaluated after 48 h using light and UV microscopy. For macroscopical analysis, detached third leaves were inoculated with a density of 5 conidia mm⁻² and evaluated 5 to 7 d after inoculation.

Barley Transformation by Agrobacterium tumefaciens

Stable genetic transformation of barley with ZmUbi::racb-G15V was performed as described by Hensel and Kumlehn (2004). In brief, immature embryos of barley Golden Promise were excised 10 to 12 d after anthesis and subsequently infected with Agrobacterium tumefaciens strain AGL1 (Lazo et al., 1991) harboring the appropriate binary vector. After inoculation, callus development was induced on medium containing 50 mg/L hygromycin B (Roche) to ensure preferential growth of transformed plant cells. Established calli were then subcultured on regeneration medium supplemented with 25 mg/L hygromycin B until rooted plantlets could be transferred to soil.

Barley plants were grown and transformed with ZmUbi::GFP as described by Tingay et al. (1997) and Matthews et al. (2001). After surface sterilization for 3 min with 70% ethanol and 20 min with a sodium hypochlorite solution containing 3% active chlorine and rinsing three times with sterile distilled water, the infection of immature embryos with Agrobacterium was performed directly after isolation. We induced callus growth on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 50 mg/L hygromycin B (Roche) as a selective agent for transformed plant cells. Regeneration medium contained 50 mg/L hygromycin B and rooting medium 25 mg/L hygromycin B. Timentin (150 mg/L; Duchefa Biochemie) was applied as long as tests for the presence of bacteria were negative.
RNA-Gel Blots

Total RNA was extracted from three leaves (fourth and higher) of T1 individuals using RNA extraction buffer (PEQLAB) according to the manufacturer’s instructions. For RNA gel blot analysis, 15 μg of total RNA was separated on a 1.2% agarose gel and blotted on Hybond N+ nylon membranes (Amersham Biosciences Europe GmbH). The RNA content of the extracts was measured by UV photometry and was adjusted after checking in ethidium bromide-stained gels taking RNA bands as a standard.

\[ ^{32}P \] probe labeling of PCR-amplified RACB cDNA (GenBank accession no. AJ344223; primers, 5'-GGATCCTAGGCGGCGTCAGATT-3' and 5'-GTCGACCTGGCCTGTTTCGTTG-3') was carried out using the random primer HexaLabel DNA labeling kit (Fermentas GmbH) following the manufacturer’s instructions. The RNA-gel blots were hybridized overnight at 62°C in 0.5 M sodium phosphate buffer, pH 7.2, containing 0.5% (v/w) bovine serum albumin, 3.5% (v/w) SDS, and 0.5 mM sodium EDTA and stringently washed three times at 62°C in 0.1X SSC/0.1% SDS for 5 min each. Signals were detected with a Molecular Imager FX PhosphorImager (Bio-Rad).

RT-PCR Analysis

Total RNA was extracted from one sary leaf using RNA extraction buffer (PEQLAB) according to the manufacturer’s instructions. The OneStep RT-PCR kit (Qiagen) was used for semiquantitative RT-PCR following the manufacturer’s instructions. To demonstrate the expression of transformed racb-G15V, 500 ng of total RNA was used for RT-PCR (prePCR: 50°C 30 min, 95°C 15 min; PCR: 95°C 30 s, 55°C 30 s, 72°C 60 s, 35 cycles). Primers were designed in a way to allow amplification of expressed transgenic racb-G15V but not endogenous RACB (5'-AACCAGATCCTCCCAATC-3' and 5'-GTCGACCTGGCCTGTTTCGTTG-3'). Primers target a untranslated 5' part of the mRNA originating from maize ubiquitin and flank a 5' maize ubiquitin 1 intron. Hence, primers allowed us to test T1 segregating individuals for racb-G15V mRNA expression by one-step RT-PCR (data not shown). The ubiquitin coding gene (GenBank accession no. M60175; primers, 5'-ACCTCCTGGGAC-TACAACAT-3' and 5'-CATGATGGGCGCTGAAGTCTG-3') was used to verify quality of used RNA and as a measure for equal RNA amounts. PCR products were separated in agarose gels.

DNA Blotting

Genomic DNA prepared from leaves (Palotta et al., 2000) was digested with HindIII, separated on a 0.8% (w/v) agarose gel (30 μg per lane), and blotted onto a Hybond N+ membrane (Amersham Biosciences Europe GmbH) by capillary transfer under alkaline conditions as essentially described by Sambrook et al. (1989). The membrane was hybridized with the PCR-amplified lab probe using the primers 5'-GATCCGACGATGAGCGCGTCCAGGTT-3' and 5'-CATGATGGGCGCTGAAGTCTG-3'. The hybridization was performed at 65°C overnight. After hybridization, the blot was washed first with 6X SSC for 5 min followed by a washing step with 2X SSC, 0.1% (w/v) SDS for 30 min and twice with 0.1X SSC, 0.1% (w/v) SDS for 15 min. The radioactivity was detected using Bio-Imaging Analyzer BAS 2000 (Fuji Film).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owner of all or parts of the material. Obtaining any permission will be the responsibility of the requester. No restrictions or conditions will be placed on the use of any novel materials described in this paper that would limit their use in noncommercial research purposes.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ344223, M60175, and AY234328.

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