Characterization of the Arabidopsis Lysine-Rich Arabinogalactan-Protein \textit{AtAGP17} Mutant (\textit{rat1}) That Results in a Decreased Efficiency of Agrobacterium Transformation\textsuperscript{1}[w]

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Arabinogalactan-proteins (AGPs) are a family of complex proteoglycans widely distributed in plants. The Arabidopsis \textit{rat1} mutant, previously characterized as resistant to \textit{Agrobacterium tumefaciens} root transformation, is due to a mutation in the gene for the Lys-rich AGP, \textit{AtAGP17}. We show that the phenotype of \textit{rat1} correlates with down-regulation of \textit{AGP17} in the root as a result of a T-DNA insertion into the promoter of \textit{AGP17}. Complementation of \textit{rat1} plants by a floral dip method with either the wild-type \textit{AGP17} gene or cDNA can restore the plant to a wild-type phenotype in several independent transformants. Based on changes in \textit{PRI} gene expression and a decrease in free salicylic acid levels upon Agrobacterium infection, we suggest mechanisms by which AGP17 allows Agrobacterium rapidly to reduce the systemic acquired resistance response during the infection process.

Arabinogalactan-proteins (AGPs) are a family of complex proteoglycans widely distributed in plants. They are found in the extracellular matrix associated with the plasma membrane and cell wall (Knox, 1995; Du et al., 1996). Although the precise function(s) that AGPs perform is unknown, they have been implicated in diverse developmental roles, including differentiation, cell-cell recognition, and embryogenesis (Knox, 1996; Schultz et al., 1998; Majewski-Sawka and Nothnagel, 2000).

Most studies investigating AGP expression and function have used an AGP-binding dye, \(\beta\)-D-glucosyl (\(\beta\)-D-Glc) Yariv reagent, and/or antibodies that recognize the carbohydrate epitopes of AGPs (for review, see Gaspar et al., 2001). These previous studies support a role for AGPs in plant cell growth and development; however, they do not inform us of the function of individual AGPs. The identification of AGP genes from Arabidopsis provides us with a wide range of tools to determine the function(s) of individual AGPs.

To date, almost 50 genes encoding putative AGP protein backbones (hereafter referred to as AGP genes) have been identified in Arabidopsis (Schultz et al., 2002). These include the classical AGPs, those with Lys-rich domains, the arabinogalactan (AG)-peptides with short protein backbones, and the fasciclin-like AGPs (Gaspar et al., 2001). Fasciclin-like AGPs are a class of chimeric AGPs that, in addition to AGP motifs, have fasciclin-like domains (Gaspar et al., 2001; Johnson et al., 2003a). In addition, another approximately 50 glycosylphosphatidylinositol (GPI)-anchored proteins are likely to contain AG chains as part of larger proteins based on the presence of short Pro-, Ser-, Thr-, and Ala-rich regions containing noncontiguous Pro residues (Borner et al., 2002, 2003). Pro-, Ser-, Thr-, and Ala-rich regions contain noncontiguous Pro residues are referred to as AG-glycomodules because there is increasing evidence that these motifs direct the O-glycosylation of Hyp with type II AG chains (Goodrum et al., 2000; Zhao et al., 2002; Tan et al., 2003).

Only a few AGP mutants have been identified to date. The haploinsufficient mutant, \textit{rat1} (resistant to Agrobacterium transformation), is resistant to transient and stable transformation of root segments by tumorigenic and nontumorigenic Agrobacterium strains (Nam et al., 1999; Zhu et al., 2003a). This

\textsuperscript{1}This work was supported by the Australian Research Council (grant nos. A10020017 and DP0343454 to A.B. and C.J.S.) and by the National Science Foundation (Plant Genome grant no. 99–75715 to S.B.G.). Y.M.G. was supported by a Melbourne Research Scholar grant (grant nos. A10020017 and DP0343454 to A.B. and C.J.S.) and by the

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\textsuperscript{[w]}The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.045542.
mutant has a T-DNA insertion upstream of the start codon of AGP17 (Nam et al., 1999; Gaspar et al., 2001). A root-specific nonclassical (chimeric) AGP from Arabidopsis, ATAGP30, has been implicated in root regeneration and seed germination (van Hengel and Roberts, 2003). The other AGP mutant, sos5/fla4, displays a salt overly sensitive phenotype with increased cell expansion under high salt conditions (Shi et al., 2003).

The rat1/agp17 mutant belongs to the Lys-rich AGP subclass in Arabidopsis, which consists of three genes, ATAGP17, ATAGP18 (Gilson et al., 2001), and ATAGP19 (Schultz et al., 2002). The predicted proteins all contain a short (approximately 12 amino acid) basic Lys-rich region. Both AGP17 and AGP18 are predicted to be GPI-anchored based on the big PI plant predictor (Eisenhaber et al., 2003). These AGPs share sequence similarity to NaAGP4, an AGP isolated from styles of Nicotiana alata (Gilson et al., 2001). Expression of NaAGP4 is reduced by wounding and pathogen infection (Gilson et al., 2001) and in this respect is similar to another AGP with a Lys-rich region, LeAGP-1 from Lycopersicon esculentum (Pogson and Davies, 1995; Li and Showalter, 1996; Zhao et al., 2002).

In this article, we characterize the rat1 mutant and show that the reduced binding of Agrobacterium correlates with down-regulation of AGP17 in the root and does not result from an inhibition of cellulose synthesis by the bacterium. The rat1 phenotype could be complemented with the wild-type AGP17 gene. By comparing levels of salicylic acid (SA) in Agrobacterium-infected and uninfected Arabidopsis plants, we provide evidence that wild-type control of AGP17 gene expression in the roots is necessary to allow Agrobacterium rapidly to reduce the systemic acquired resistance (SAR) response during infection.

RESULTS

The Rat Phenotype of rat1 Results from Reduced AGP17 Expression in Roots

The plant line CS12955 corresponding to rat1 (Nam et al., 1999) was independently identified from the Feldmann T-DNA collection using a reverse-genetic PCR based approach with AGP17 specific primers (McKinney et al., 1995). By sequencing the PCR product, we determined that the T-DNA tag is 1,097 bp upstream of the start codon of the AGP17 gene. The positions of oligonucleotide PCR primers used in this study are indicated. Not drawn to scale. B, RNA gel-blot analysis of the steady-state levels of RNAs encoding two Lys-rich AGPs, AGP17 and AGP18, in various Arabidopsis tissues. R, root; U, unopened flower (0.5–1.5 mm in length); O, open flower (1.5–2 mm in length); C, cauline leaf (0.5–1.5 cm in length); R, rosette leaf (1.5–3 cm in length); Y, young inflorescence stem first internode from the growing tip greater than 1 cm in length); M, mature inflorescence stem first internode from the base); S, young siliques (0.5–1 cm in length). To illustrate the loading of RNA, the ethidium bromide-stained formaldehyde gel is shown.

We analyzed expression of AGP17 and the most closely related Lys-rich AGP, AGP18, in wild-type plants using RNA gel-blot analysis (Fig. 1B). Expression of AGP17 was detected only in flowers (Fig. 1B). Surprisingly, we could not detect expression of AGP17 in roots even after a 2-week exposure of the blot. RNA-gel blot analysis of AGP18 showed high expression in flowers, but expression was also detected in other plant parts (Fig. 1B).

To compare levels of mRNA in the roots of wild-type and rat1 plants, we used a semiquantitative reverse transcription (RT)-PCR method (Lasserre et al., 1996; Zeggzoufi et al., 1999). We compared the expression of AGP17 in hydroponically grown wild-type and rat1 plants that were either uninfected or infected with Agrobacterium (Fig. 2) and sampled at different times up to 48 h. In wild-type roots, AGP17 cDNA was detected after 40 PCR cycles with or without Agrobacterium inoculation. No expression of AGP17 was detected in rat1 roots after 40 PCR cycles. However, the AGP17 transcript could be detected after 50 or more

![Figure 1. A, Position of the T-DNA insertion within the AGP17 gene (At2g3130) of the Arabidopsis T-DNA insertion line CS12955. The domain structure of the AGP17 protein and the position of the gene At2g3130, upstream of AGP17, are shown. The T-DNA insertion is 1,097 bp upstream of the start codon of the AGP17 gene. The positions of oligonucleotide PCR primers used in this study are indicated. Not drawn to scale. B, RNA gel-blot analysis of the steady-state levels of RNAs encoding two Lys-rich AGPs, AGP17 and AGP18, in various Arabidopsis tissues. R, root; U, unopened flower (0.5–1.5 mm in length); O, open flower (1.5–2 mm in length); C, cauline leaf (0.5–1.5 cm in length); R, rosette leaf (1.5–3 cm in length); Y, young inflorescence stem first internode from the growing tip greater than 1 cm in length); M, mature inflorescence stem first internode from the base); S, young siliques (0.5–1 cm in length). To illustrate the loading of RNA, the ethidium bromide-stained formaldehyde gel is shown.]
cycles (data not shown). Therefore, the T-DNA insertion results specifically in a reduction of root expression but not leaf expression in rat1 plants. The expression of AGP17 in wild-type roots and leaves and rat1 leaves was constant over the 0 to 48 h postinfection sampling period, indicating that Agrobacterium infection did not alter the level of AGP17 transcripts. We also examined the expression of AGP18 in rat1 mutants at these time points and found that there was no change compared to the wild type (data not shown).

The Expression of the Upstream Gene Flanking AGP17 Is Not Affected by the T-DNA Insertion in rat1

To rule out the possibility that other genes near the T-DNA insertion site were affected in rat1, the integrity of the upstream neighboring gene was determined by PCR and sequencing. The oligonucleotides used are indicated in Figure 1A. These experiments showed that there were no rearrangements or small insertions and deletions in the genomic region examined upstream or downstream of the T-DNA insertion site (data not shown). Additional experiments showed that two or more copies of the T-DNA in a head-to-head configuration are present, with the T-DNA left borders flanking the genomic DNA on both sides of the insertion (Fig. 1A). Examination of sequence around the T-DNA insertion site revealed the presence of an expressed gene of unknown function (GenBank accession no. A2g23120) approximately 1 kb upstream of the T-DNA. The expression level of the upstream gene, as determined by RT-PCR, was unaltered in the rat1 mutant when compared to wild-type plants (data not shown). Therefore, we conclude that the T-DNA insertion affects AGP17 and causes the rat1 phenotype by reducing the expression of AGP17 in Arabidopsis roots.

Complementation of the rat1 Mutant

To provide further evidence that loss of AGP17 expression in the roots of rat1 plants is responsible for the rat phenotype, we performed a genetic complementation analysis. The rat1 mutant was transformed with either a genomic fragment containing the AGP17 gene (RAT1::gRAT1) or with an AGP17 cDNA under the control of a cauliflower mosaic virus (CaMV) 3S promoter (35S::cRAT1). A floral dip method (Clough and Bent, 1998) was used to obtain multiple independent transformants because this method has worked successfully for other rat mutants (Mysore et al., 2000). We tested 27 independent transformants (T1) containing the AGP17 gene and 25 transformants containing the AGP17 cDNA using a root transformation assay (Nam et al., 1999). Analysis of the T1 was possible because the rat phenotype is haploinsufficient (semidominant; Nam et al., 1999). Figure 3 shows examples of successful complementation of rat1 by AGP17 genomic and cDNA clones. However, transformation was not always successful. The extent of tumor formation on root segments of wild-type plants, the homozygous rat1 mutant, and the individual complemented transformants is shown in Supplemental Figure 1 (available at www.plantphysiol.org). We initially speculated that the lack of full phenotypic complementation might result from inappropriate expression from the 3S5 promoter. However, this explanation is likely to be not valid because we saw a similar proportion of complementation using RAT1 genomic constructions. Therefore, the most likely explanation for the lack of full phenotypic complementation in many lines is that the expression of the complementing transgene was not sufficient, or was inappropriately controlled, due to the position of the insertion of the complementation construct into the genome. The haploinsufficient nature of the rat1 mutant (Nam et al., 1999) is consistent with the suggestion that different levels of AGP17 transcript have different effects on transformation efficiency.

Agrobacterium Can Still Synthesize Cellulose on rat1 Roots

To extend the results of Nam et al. (1999), we used scanning electron microscopy to look at Agrobacterium binding to rat1 roots (Fig. 4). In rat1 plants, Agrobacterium binding is reduced, with only occasional binding on the root surface (Fig. 4A) and at the root hair tips (Fig. 4B). Where bacteria were present on rat1 roots, cellulose microfibrils were evident (Fig. 4B, arrows). This suggests that the rat1 phenotype results from a defect in the loose or initial binding step in Agrobacterium infection and not in the second step involving the synthesis of cellulose by the bacterium.
that promotes close adhesion to the root surface (Matthysse, 1994; Matthysse et al., 1995; Matthysse and McMahan, 1998).

**β-D-Glucosyl Yariv Reagent Inhibits Transformation of Arabidopsis Roots**

Because roots of the agp17/rat1 mutant are resistant to Agrobacterium transformation, we were interested in determining the effect of an AGP-binding reagent on transformation. AGPs bind to the synthetic dye, β-D-Glc Yariv reagent, but not to the β-D-mannosyl (β-D-Man) Yariv derivative (Yariv et al., 1967). We investigated the effect of these two Yariv reagents on Agrobacterium transformation of Arabidopsis root segments (Fig. 5). To visualize transformation, we used the Agrobacterium strain At849. This nontumorigenic strain contains the T-DNA binary vector pBISN1 (Narasimhulu et al., 1996) encoding a *gusA*-intron gene under the control of a strong promoter (Ni et al., 1995). When wild-type root segments were infected with *A. tumefaciens* At849 either in the absence of β-D-Glc Yariv reagent or in the presence of β-D-Man Yariv reagent (which does not bind to AGPs), expression of β-glucuronidase (GUS) activity could be seen at the cut ends of the root segments (Fig. 5B). However, when the wild-type roots were pretreated with β-D-Glc Yariv reagent prior to infection, transformation occurred at only a very low level as indicated by reduced GUS activity (<10% that of control plants; Fig. 5). When observed by light microscopy, β-D-Glc Yariv reagent inhibited binding of Agrobacterium to whole wild-type roots, whereas binding of Agrobacterium to *rat1* roots under the same conditions remained low, i.e. unchanged (data not shown). Controls indicated that at the concentrations used, Yariv reagents did not inhibit the growth of Agrobacterium or reduce the ability of Arabidopsis roots to form callus on callus-inducing medium (data not shown). The Yariv effects upon transient GUS expression support the idea that AGPs are important in Agrobacterium-mediated transformation of roots.

**Down-Regulation of PRI Gene Expression during Agrobacterium Infection in Wild-Type Plants But Not rat1**

Infection of plant cells with transformation-competent Agrobacterium strains alters the pattern of plant gene expression (Ditt et al., 2001; Veena et al., 2003). Recently it has been shown that defense-related genes, such as β-1,3-glucanase, are repressed in tobacco...
BY-2 cells after inoculation with Agrobacterium (Veena et al., 2003). There are two major defense response pathways in plants, the SA-dependent, SAR pathway that is characterized by the induction of pathogenesis-related (PR) genes, such as PR1 and PR5 (for review, see Shah, 2003) and an SA-independent resistance pathway that is characterized by the induction of the plant defensin1.2 (PDF1.2) gene by jasmonate and ethylene (Gu et al., 2002). It is not known if either (or both) of these pathways is activated during the first few hours of Agrobacterium infection when root binding is known to occur (Matthysse, 1986).

Defense genes are generally expressed at low levels in unwounded plants (Uknes et al., 1992), so we used semiquantitative RT-PCR to investigate the expression of the PR1, PR5, and PDF1.2 genes in rat1 and wild-type roots, with or without inoculation with Agrobacterium (Fig. 6). Root samples were collected at 0, 1, 2, 3, and 4 h postinfection. These early time points were chosen because rat1 affects the binding of Agrobacterium, which occurs in the first few hours of Agrobacterium infection (Sykes and Matthysse, 1986). Both PR1 and PR5 mRNAs were detected in untreated hydroponically grown wild-type and rat1 roots (Fig. 6A), although it took 35 cycles to obtain an observable PCR product, suggesting that expression levels are low. When roots were inoculated with Agrobacterium, the level of PR gene expression remained relatively stable in the rat1 mutant. In wild-type roots, PR1 expression was reduced (approximately 4-fold) within 1 h postinfection. PR5 expression was also reduced during this time and was constitutively higher (approximately 4-fold) in rat1 plants than in wild-type plants. PDF1.2 cDNA was not detected in any of the samples. A PCR product of the correct size was amplified from genomic DNA (data not shown), indicating that the PCR primers would have amplified a PDF1.2 cDNA if the gene were expressed (Fig. 6A).

Figure 6. A, Semiquantitative RT-PCR showing expression in roots of the PR genes PR1 and PR5 and the jasmonic acid/ethylene-regulated gene PDF1.2. Hydroponically grown wild-type and rat1 Arabidopsis roots were incubated without or with Agrobacterium for 1 h. 18S rRNA transcript levels were used to normalize the samples. The 18S rRNA control panel is representative of the above experiments. Numbers at right indicate the number of PCR cycles used. B and C, Endogenous levels of SA in hydroponically grown Arabidopsis roots inoculated with A. tumefaciens. Concentrations of free SA (B; ng/g FW) and glucosyl SA (C; released by β-glucosidase treatment; ng/g FW) after the addition of Agrobacterium for 1 h. Bar is the mean of three replicated extracts (± se).
Expression trends of \textit{PR1}, \textit{PR5}, and \textit{PDF1.2} continued in tissues 2, 3, and 4 h postinfection with \textit{Agrobacterium} (data not shown).

\textbf{Decrease of SA Levels Is an Early Response to \textit{Agrobacterium} Infection}

The reduction in \textit{PR} gene expression observed in wild-type plants 1 h postinfection suggests that \textit{Agrobacterium} may affect the SAR pathway. To investigate this further, we determined the levels of SA in roots of both wild-type and \textit{rat1} mutant plants 1 h postinfection by \textit{Agrobacterium}. To quantify SA, the levels of total and free SA were determined using published methods with substantial modification (Dewdney et al., 2000; “Materials and Methods”). Total SA includes SA and the sugar conjugate of SA, \(\beta\)-glucosyl SA (G-SA; Hennig et al., 1993). The levels of both were determined, even though G-SA is not thought to be an active component because G-SA can be converted to biologically active SA in vivo (Hennig et al., 1993). To determine total SA levels from root extracts, G-SA was converted to SA in vitro using a \(\beta\)-glucosidase (see “Materials and Methods”). Mass spectrometry (MS) was used to confirm the identity of SA in the root fraction that eluted at the same time as an SA standard using reverse phase (RP)-HPLC. MS showed the fraction included SA and other unidentified components (data not shown). To obtain accurate quantification of SA, it was necessary to rechromatograph the SA containing fraction from the first separation using a step gradient (see “Materials and Methods”). This resulted in four major peaks with the SA-containing fraction representing approximately 25% of the original peak (data not shown).

In uninfected roots, the level of free SA in wild-type roots is almost double the level observed in \textit{rat1} roots (Fig. 6B), whereas the levels of G-SA in wild-type and \textit{rat1} roots are similar (Fig. 6C). When wild-type plants are challenged with \textit{Agrobacterium}, the levels of free SA decreased by about 40% (Fig. 6B). The difference between the treated and untreated roots is significant \((P < 0.05)\) based on ANOVA, as is the difference between the wild type and \textit{rat1}. The level of G-SA does not change significantly after \textit{Agrobacterium} infection in \textit{rat1}, although there is an observable decrease in wild-type plants.

\textbf{DISCUSSION}

\textbf{The Rat Phenotype of \textit{rat1} Is Caused by Down-Regulation of \textit{AGP17} in the Root}

The presence of a T-DNA insertion in the promoter of the Lys-rich AGP gene \textit{AGP17} results in reduced \textit{AGP17} expression in roots of \textit{rat1} Arabidopsis plants (Figs. 1 and 2). Several lines of evidence indicate that this is the basis of the resistance to \textit{Agrobacterium} transformation in the \textit{rat1} mutant. These include (1) cosegregation of kanamycin resistance with the \textit{rat} phenotype (Nam et al., 1999) and the presence of only one T-DNA locus in \textit{rat1}; (2) no DNA rearrangements near the insertion site affecting the expression of other genes (see “Results”); and (3) complementation of the rat phenotype in \textit{rat1} plants by introducing a wild-type \textit{AGP17} gene or cDNA (Fig. 3 and Supplemental Fig. 1).

\textbf{SA Levels Are Reduced in Wild-Type Plants during \textit{Agrobacterium} Infection}

It was surprising that SA was present, and \textit{PR} genes were expressed, at the zero time points in the hydroponics system used to grow Arabidopsis plants for this study. It is likely that the \textit{PR} gene expression is much lower than would be detected by RNA gel-blot analysis because it took 35 cycles for a modest amount of RT-PCR product to be produced. It is possible that these low levels of \textit{PR} gene expression are not relevant to SAR or that roots behave differently from leaves, and there is always a basal level of \textit{PR1} expression in uninfected roots. However, a more likely explanation is that the changes in \textit{PR1} gene expression are important, and the hydroponic system used to grow the plants is eliciting a wounding or stress response, leading to low levels of \textit{PR} gene expression and free SA accumulation. The sustained \textit{PR} gene expression in \textit{rat1} plants up to 4 h postinfection (data not shown) is consistent with the suggestion that the modest change in SA levels in \textit{rat1} plants is not enough to downregulate the SAR response and, hence, \textit{PR} gene expression remains unchanged. The higher levels of \textit{PR} gene expression but lower levels of SA in \textit{rat1} compared to the wild type may result from cross talk between different signaling pathways; ethylene has been suggested as an alternate activator of \textit{PR1} gene expression (Kunkel and Brooks, 2002).

Our analysis of wild-type and \textit{rat1} plants provides further support for the hypothesis that \textit{Agrobacterium} alters plant defense responses (Ditt et al., 2001; Veena et al., 2003). Furthermore, it suggests that plants may detect relative changes, not absolute levels of SA. This is based on the observation that uninfected \textit{rat1} plants have lower initial levels of SA but only a 25% change in SA levels, whereas wild-type plants have a 40% drop in free SA levels after \textit{Agrobacterium} inoculation (Fig. 6B). This suggests that it is the degree of change in SA concentration (disruption of homeostasis) that determines the level of response. Another observation from our data is that there is no corresponding increase in G-SA in wild-type plants, suggesting that plants can actively degrade SA as occurs in bacteria (Gaffney et al., 1993; Delaney et al., 1994).

These results are consistent with recent findings that the expression of hundreds of plant genes is modulated following \textit{Agrobacterium} infection in several plant systems, including \textit{Aggeratum conyzoides} (Ditt et al., 2001) and \textit{Nicotiana tabacum} BY-2 suspension culture cells (Veena et al., 2003). Among the tobacco genes affected by cocultivation with transfer-competent but not transfer-deficient \textit{Agrobacterium} strains are
defense genes, including PR genes, several classes of glutathione S-transferase genes, and other defense-related genes (Veena et al., 2003).

Mechanisms for the Possible Involvement of AGP17 in Agrobacterium-Mediated Transformation

We suggest two possible, nonexclusive mechanisms to explain the role of AGP17 in Agrobacterium-mediated transformation. In the first, AGP17 is required by Agrobacterium to bind directly to the root surface. Binding can be directly to AGP17, or AGP17 may mediate binding by altering the plant cell wall, thus presenting a different receptor. Because secreted AGPs are generally soluble, direct binding would probably only occur if AGP17 were cross-linked to other cell wall components, for example, cell wall polysaccharides (for review, see Serpe and Nothnagel, 1999). In Angelica roots, there is evidence that AGPs are cross-linked to pectic polysaccharides in the plant cell wall (Yamada et al., 1985; Kiyohara et al., 1997), and the Lys-rich region of the AGP17 protein backbone could provide suitable amino acid residues for cross-linking.

The Yariv binding experiments (Fig. 5) provide some support for the direct attachment model. The disruption of Agrobacterium attachment by β-Glc Yariv reagent suggests that receptor sites for binding are rendered inaccessible by this AGP-specific reagent (Fig. 5). An alternate explanation is that the cross-linking of many different AGPs by β-Glc Yariv at the cell surface provides a physical barrier that prevents binding of the bacterium and/or entry of the T-DNA into the cell.

For the second mechanism, Agrobacterium does not require AGP17 to bind to the root cell wall. Rather, AGP17 is involved in a signaling pathway(s) that consequently affects the ability of Agrobacterium to bind to the root surface. In this scenario, AGP17 is required to reduce the levels of SA, by an unknown mechanism, when Agrobacterium binds to the root. Assuming AGP17 is GPI anchored as predicted, it could form part of a signaling cascade in one of two ways. With its GPI anchor still attached, AGP17 would be bound to the outer surface of the plasma membrane and therefore could interact with receptor-like kinases such as the wall-associated kinases (Anderson et al., 2001). Receptor-like kinases have both an extracellular and cytoplasmic domain, and one of the wall-associated kinases is thought to interact with molecules containing AGP epitopes (Gens et al., 2000). Alternatively, AGP17 could become a soluble signaling molecule if released from the plasma membrane by GPI anchor-specific phospholipases (Schultz et al., 1998; Youl et al., 1998).

What Structural Features of AGP17 Are Important for Transformation?

In both the scenarios described above, the role of AGP17 is nonredundant because the rat phenotype would not be apparent if other AGPs were able to fulfill the role(s) of AGP17.

It is not known whether carbohydrate moieties or the Lys-rich domain of the protein backbone of AGP17 are important for the interaction of Agrobacterium with the plant surface. The basic Lys-rich domain, also found in AGP18 and AGP19, might directly interact with the acidic polysaccharide from Agrobacterium that was shown to be important for Agrobacterium binding to carrot cells (Reuhs et al., 1997). Alternatively, the carbohydrate moiety may be important because another rat mutant, rat3, is also deficient in Agrobacterium attachment (Nam et al., 1999). The RAT3 protein contains the amino acid sequence Ala-Pro-Ala-Pro-Thr-Thr-Ser (GenBank accession no. At5g63250) that is a motif, also known as a glycomodule, found in AGPs, and is likely to direct the addition of type II AG O-linked-polysaccharides (Goodrum et al., 2000; Borner et al., 2002, 2003; Johnson et al., 2003b; Tan et al., 2003). By selectively removing or modifying different domains within AGP17 and using these modified constructs in complementation experiments, we should be able to determine which regions of AGP17 are required for binding the bacterium and/or its elicitors.

CONCLUSION

The likelihood of multiple rounds of communication between Agrobacterium and plant cells to establish binding was first proposed by Matthysse (1994) based on experiments with Agrobacterium mutants. The availability of Arabidopsis rat mutants allows us to investigate the role of the plant genes involved in Agrobacterium binding. Of the more than 125 rat mutants identified, only a few of these (rat1, rat3, and rat4) have been shown to be defective in binding (Zhu et al., 2003a, 2003b). Our results suggest a signal transduction pathway between AGP17/RAT1 at the plant surface and intracellular changes in SA levels and gene expression of the key defense response gene PRI. Future characterization of rat1 and other rat mutants deficient in Agrobacterium attachment should enable the identification of other components of the SA-dependent PR pathway that interact with AGP17.

MATERIALS AND METHODS

Plant and Bacterial Growth Conditions

Seeds of Arabidopsis (ecotype Wassilevskija-2 [Ws-2]) were grown either in soil and maintained under standard greenhouse conditions (approximately 26°C, 16 h light), on agar plates (0.5X Murashige and Skoog medium, 3% [w/v] Suc, 1% [w/v] agar), in agitated liquid cultures (Gamborg's B-5 liquid; Invitrogen, Carlsbad, CA; 26°C, 16 h light, 50 rpm; Reiter et al., 1992) or hydroponically (0.5X Optimum Grow hydroponic nutrient solution, pH 6; Growth Technology, O'Connor, Western Australia; aerated, 26°C, 16 h light). For hydroponic growth, 2-week-old plants from agar plates were transferred to precut holes in styrofoam sheeting and floated on sterile hydroponic liquid medium. The medium was aerated with filtered air from a standard aquarium pump. Plants were acclimated for 1 week before sampling or inoculation with Agrobacterium.
For axenic growth, seeds were surface sterilized for 5 min with 12% (v/v) sodium hypochlorite containing a drop of 50% (v/v) Tween 20 per 5 ml of sodium hypochlorite, then washed several times with sterile distilled water. Plant tissue was collected and stored at −70 °C before use.

_Agrobacterium tumefaciens_ A208 (Sciany et al., 1978; used for root tumorigenesis assays), A849 (Nam et al., 1998; used for GUS assays), or GV3101 (Koncz and Schell, 1986; used for all other assays) were grown in _Agrobacterium_ broth minimal, yeast extract peptone, or Luria-Bertani-rich medium (Lichtenstein and Draper, 1986) at 30°C.

**Screening T-DNA Lines**

Oligonucleotides specific to the _AGP17_ gene (AGP17F, 5'-TGCGGATAATCTTCTTGACG-3'; AGP17R, 5'-GCCGAGACTGTAATGAGACG-3') and the borders of the T-DNA insert (right border, 5'-GATTGGTTGTCCCGCTCC-3'; left border, 5'-GATGGCACTGATCACGCAATTTTAGAC-3') were used to screen DNA from the T-DNA transformed lines (Feldmann, 1991) by PCR. Standard molecular biology techniques were used as described (Sambrook et al., 1989). An oligonucleotide specific to the region of genomic DNA upstream of the T-DNA insertion site (Fig. 1A; T-DNAF, 5'-TTAAGTGATATGTTGCTACGTG-3') was conjuncted in conjunction with oligonucleotide AGP17R (5'-GAGAAAGACATCGCTTGTGCCC-3') and the left-border and right-border T-DNA oligonucleotides to determine the arrangement of T-DNA insertions and to sequence the surrounding DNA. Oligonucleotides specific to the gene upstream of _AGP17_, A12q23120 (A12qF, 5'-AAGCGACACATCCTAGAAAGC-3'; A12qR, 5'-TTCTGCGAGATCCA-CAAAC-3'), were used for RT-PCR as described below.

**Microscopy of Root Segments Inoculated with Agrobacterium**

Wild-type and _rat1_ mutant Arabidopsis plants were grown for 2 weeks in agaritated liquid cultures as described previously (Reiter et al., 1992). _A. tumefaciens_ GV3101 was grown for 2 d in Luria-Bertani broth at 26°C until they reached an _A_900 nm = 1. Flasks containing Arabidopsis plants were inoculated with the bacterial culture (0.5 ml/100 ml) and were incubated at 26°C with agitation at 50 rpm. Seedlings were harvested 12 h postinoculation. Immediately following harvesting, roots were carefully washed free of growth media with distilled water and cut into segments (2 mm in length). Root segments were fixed overnight in 2.5% (v/v) glutaraldehyde in 0.05 M PIPES buffer, pH 7.2, under vacuum. Segments were washed in 0.05 M PIPES buffer and transferred to a nylon membrane (ZETA-PROBE; Bio-Rad, Cambridge, MA) coated using an Edwards S150B Sputter Coater (Sussex, UK). The roots were critical point dried in a Samdri PVT-3 (Tousimis Research, Rockville, MD) critical point dryer and were platinum coated using a graded ethanol series. Segments were critical point dried in a graded ethanol series. Segments were critical point dried in a graded ethanol series. Segments were critical point dried in a graded ethanol series. Segments were critical point dried in a graded ethanol series.

**RNA-Blot Analysis**

Total RNA was extracted from soil-grown Arabidopsis tissues as described previously (Wadsworth et al., 1988) using a guanidinium isothiocyanate-based extraction buffer. Tissues were used as root tips, young segments (0.5–1.5 mm in length), opened flowers (1.5–2 mm in length), cauline leaves (0.5–1.5 cm in length), rosette leaves (1.5–3 cm in length), young inflorescence stems (first internode from the base), and young siliques (0.5–1 cm in length). Total RNA was subjected to electrophoresis through 1.2% agarose gels containing formaldehyde (Sambrook et al., 1989) and transferred to a nylon membrane (ZETA-PROBE; Bio-Rad, Cambridge, MA) following the manufacturer’s instructions. Membranes were hybridized overnight (65°C) using a dextran sulfate-based hybridization solution as recommended by Bio-Rad for increased sensitivity. Probes were made by labeling _AGP17_ DNA fragments with [α-32P]dCTP using a random primer DNA labeling kit (MegaPrime; Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer’s instructions. _A. tumefaciens_ GV3101 was grown for 2 d in Luria-Bertani broth at 26°C until they reached an _A_900 nm = 1. Bacteria (5 ml) were added to the hydroponic liquid solution (1 L), and the seedlings were harvested at various times postinoculation. Roots were removed from the plants, and leaf and root tissues were frozen separately at −70°C until RNA extraction.

RNA treatment, first-strand cDNA synthesis, and semi-quantitative RT-PCR were performed as described previously (Lasserre et al., 1996; Zegzouti et al., 1999) with modifications. RNase-free DNase I (Roche Diagnostics, Indianapolis) at 0.25 unit per μg of total RNA with Ribonuclease Inhibitor (Promega, Madison, WI) was added to 4 units per μg of total RNA. Enzymes were removed by purification with a Qiagen RNeasy kit ( Valencia, CA) following the manufacturer’s instructions. PCR was performed directly using the RNA, prior to RT, to verify the absence of contaminating genomic DNA. If needed, the DNase I treatment was repeated. Random primers (1 μg; Promega) were annealed to 2 μg of total heat denatured RNA in a final volume of 10 μl. First-strand cDNA was synthesized at 42°C for 1 h using 200 units of Superscript II Reverse Transcriptase (Invitrogen) in the presence of Ribonuclease Inhibitor (40 units; Promega), 1 × PCR buffer (Invitrogen), 2.5 mM MgCl₂, 0.5 mM of each deoxynucleotide phosphate, and 10 mM dithiothreitol. The reverse transcribed samples were used as a template for the amplification of _AGP17_ using 15 pmol of gene specific oligonucleotides (AGP17F, AGP17R), and, as an internal control, a fragment of the 18S RNA was amplified concomitantly with the _AGP17_ cDNA by addition, after a predetermined number of cycles, of 15 pmol of 18S-specific oligonucleotides (18SF, 5'-CATACGCTGGTTGACATC-3'; 18SR, 5'-GACTCCTTCGCA-GGTCAAC-3'; Cho and Cosgrove, 2000). The PCR conditions were as follows: an initial 5-min denaturation at 96°C, n cycles of 94°C, 30 s; 55°C, 30 s; and 72°C, 1.5 min, and a final extension of 7 min at 70°C. The amount of cDNA used in each PCR was adjusted so that RT-PCR amplification of 18S rRNA resulted in a band of similar intensity to that of the target mRNA. To determine that the number of cycles needed for 18S and _AGP17_ amplification was within the exponential amplification phase, PCR reactions were set up, removed from a GeneAmp 9600 thermocycler (Applied Biosystems, Melbourne, Australia) at consecutive cycles and the product amounts assessed by electrophoresis. To maintain RT-PCR amplification within the exponential phase, the number of PCR cycles used for 18S rRNA was 18 or 21 cycles for leaf and root cDNA, respectively, and for the _AGP17_ transcript, 30 or 40 cycles for leaf and root cDNA, respectively.

cDNA was subjected to electrophoresis through 1.4% (w/v) agarose gels and transferred to a nylon membrane (Magna; Micron Separations, Westborough, MA) following the manufacturer’s instructions. The membranes were hybridized with an equal concentration of digoxigenin probes for _AGP17_ and 18S rRNA. The digoxigenin-labeled probes were synthesized by PCR following the manufacturer’s instructions (Roche Diagnostics). All RT-PCR reactions were performed in duplicate and in two independent experiments.

**Genetic Complementation of the _rat1_ Mutant**

We employed the floral dip method (Clough and Bent, 1998) to introduce either a wild-type _RAT1_ cDNA under the control of a CaMV 35S promoter or a wild-type _RAT1_ genomic clone into _rat1_ plants (Mysore et al., 2000). The genomic complementing clone consisted of a 6-kb EcoRI fragment containing the entire _AGP17_ gene, including upstream and downstream elements, cloned into the T-DNA binary pCPTY-HPT (Becker et al., 1992). The genomic fragment used in the complementation experiments also includes two upstream genes At2g23110 and At2g23120. These two genes are both related to late embryogenesis abundant genes and are unlikely to be involved in the _rat1_ phenotype. This interpretation is supported by the hybridization with the cDNA clone for _AGP17_. The cDNA complementing clone consisted of a wild-type _RAT1_ cDNA clone inserted into the SalI site of the T-DNA binary vector p55S-HPT, under the control of a CaMV 35S promoter. The genomic and cDNA constructions are referred to as _RAT1::gRAT1_ and 35S::cRAT1, respectively. The binary vectors were introduced into _A. tumefaciens_ GV3101, and the plants were infected using a flower-dip protocol (Clough and Bent, 1998). Hygromycin-resistant transformants were selected and tested, in the T1 generation, for tumorgenesis as described in Nam et al. (1999). A minimum of 50 root segments was tested for each plant.

**Analysis of Genes Involved in the Defense Response**

Analysis of gene expression of the _PRI1, PR5_ (Uknes et al., 1992), and _PDF1.2_ (Pennisinneks et al., 1996) genes was performed using the RT-PCR
Inhibition of Transformation by Yariv Reagents

Arabidopsis root segments (150) pooled from 10 to 20 wild-type plants were incubated with 50 μM β-Glc or β-Man Yariv reagent ( Biosciences Australia Pty, Melbourne, Australia) or no Yariv (control) for 1 h, and then inoculated with A. tumefaciens AT849. After 15 h of cocultivation, the root segments were moved to Murashige and Skoog medium containing 100 μg/mL timentin to kill the bacteria. The roots were assayed for GUS activity after 4 d (Jefferson et al., 1987). All assays were repeated in triplicate.

Quantification of SA

SA was extracted and analyzed by RP-HPLC using a modification of the methods described by Dewdney et al. (2000). For SA analysis, 1 g fresh weight (FW) of Arabidopsis root tissue was divided into three equal samples, frozen, and stored at −70°C until use. One sample from each test was ground in liquid nitrogen to a fine powder and 250 ng o-anisic acid (Sigma, St. Louis) was added to each sample as an internal standard before extraction in methanol.

To determine total SA (free SA and glucosyl SA), one-half the extract was treated by enzymic hydrolysis with β-glucosidase (60 units mL−1; Sigma), whereas for free SA, the extract was not hydrolyzed. Final extracts were dried and resuspended in dichloromethane. Samples were analyzed by static HPLC peaks were collected and dried under nitrogen prior to derivatization for 10 min, and then constant for 15 min at a flow rate of 0.2 mL/min. All SA results were adjusted for recovery using the internal standards (o-anisic acid), and extracts were independently prepared in triplicate for each sample. RP-HPLC peaks were collected and dried under nitrogen prior to derivatization for analysis by MS. Methanolation was performed by addition of methanolic HCl (1 N) and incubation for 4 h at 80°C. Once cooled, samples were carefully dried and resuspended in dichloromethane. Samples were analyzed by static nanospray MS using Econotip10 (New Objective, Woburn, MA) in negative ion mode on the QSTAR XL mass spectrometer (Applied Biosystems). The full-scan mass spectrum was obtained from 50 to 600 m/z.

Acknowledgments

We are grateful to the Arabidopsis Biological Research Center, Ohio State University, for providing the Feldmann T-DNA lines. We thank Kris Ferguson for help with MS analysis and Assoc. Prof. Ed Newbigin for critical comments on this manuscript.

Received April 29, 2004; returned for revision June 1, 2004; accepted June 2, 2004.

CITATION


ACKNOWLEDGMENTS

We are grateful to the Arabidopsis Biological Research Center, Ohio State University, for providing the Feldmann T-DNA lines. We thank Kris Jefferson, TA, Kavanagh, TA, and Bevan, MW for critical comments on this manuscript.
Gaspar et al.


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