Arginase Induction Represses Gall Development During Clubroot Infection in Arabidopsis

Antoine Gravot1,2,*, Carole Deleu1,2, Geoffrey Wagner2,3, Christine Lariagon4, Raphael Lugan5, Christopher Todd6, David Wendehenne7,8, Régine Delourme4, Alain Bouchereau1,2 and Maria J. Manzanares-Dauleux2,3

1Université Rennes 1, UMR1349 IGEPP, F-35000 Rennes, France
2Université Européenne de Bretagne, France
3AGROCAMPUS OUEST, UMR1349 IGEPP, F-35000 Rennes, France
4INRA, UMR1349 IGEPP, F-35653 Le Rheu, France
5CNRS, UPR IBMP, Plate-forme métabolomique, F-67084 Strasbourg, France
6University of Saskatchewan, Department of Biology, Saskatoon, SK S7N 5E2 Canada
7Université de Bourgogne, UMR 1347 Agroécologie, BP 86510, F-21000 Dijon, France
8ERL CNRS 6300, BP 86510, F-21000 Dijon, France
*Corresponding author: E-mail, antoine.gravot@univ-rennes1.fr; Fax, +33-2-23-48-57-02

(Received January 7, 2012; Accepted March 8, 2012)

Arginase induction can play a defensive role through the reduction of arginine availability for phytophageous insects. Arginase activity is also induced during gall growth caused by Plasmodiophora brassicae infection in roots of Arabidopsis thaliana; however, its possible role in this context has been unclear. We report here that the mutation of the arginase-encoding gene ARGAH2 abrogates clubroot-induced arginase activity and results in enhanced gall size in infected roots, suggesting that arginase plays a defensive role. Induction of arginase activity in infected roots was impaired in the jar1 mutant, highlighting a link between the arginase response to clubroot and jasmonate signaling.

Clubroot-induced accumulation of the principal amino acids in galls was not affected by the argah2 mutation. Because ARGAH2 was previously reported to control auxin response, we investigated the role of ARGAH2 in callus induction. ARGAH2 was found to be highly induced in auxin/cytokinin-triggered aseptic plant calli, and callus development was enhanced in argah2 in the absence of the pathogen. We hypothesized that arginase contributes to a negative control over clubroot symptoms, by reducing hormone-triggered cellular proliferation.

Keywords: Arabidopsis thaliana • Arginase • Callogenesis • Clubroot • Jasmonate • Plasmodiophora brassicae.

Abbreviations: ARGAH2, ARGININE AMINOHYDROLASE 2; BAP, benzylaminopurine; CIM, callus-inducing medium; COOH-JA-Ile, carboxylated jasmonoyl-L-isoleucine; dpi, days post-inoculation; GABA, γ-aminobutyric acid; GUS, β-glucuronidase; JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; MS, mass spectrometry; OH-JA, hydroxyjasmonic acid; OPDA, 12-oxo-phytodienoic acid; UPLC, ultra performance liquid chromatography.

Introduction

Conversion of arginine to ornithine and urea is catalyzed by arginase (EC 3.5.3.1), an Mn-dependent enzyme that functions as an arginine aminohydrolase. In mammals, this reaction has been mostly studied as a key final step in the urea cycle, which allows potentially toxic ammonium to be detoxified into urea. In addition to this major role in nitrogen waste management, arginase catabolism can positively modulate several subsequent ornithine-dependent pathways, including proline-, glutamate- and ornithine-dependent polyamine biosynthesis (Li et al. 2001). Plants have not developed any significant mechanism for nitrogen excretion, and although all of the enzymes of the urea cycle are present in plants there is no report that it actually functions as a cycle. Instead, arginase activity has been studied in plants mostly for its involvement in nitrogen remobilization processes from protein degradation, especially during seed germination (de Ruiter and Kolloffel 1983, Cao et al. 2010). More recently, arginase induction was reported to be a jasmonate-driven defense mechanism against phytophageous insects in tomato. In this system, plant arginase activity acts as an anti-nutritional factor that reduces the assimilation of arginine in the insect digestive tract (Chen et al. 2005).

Arabidopsis thaliana synthesizes two functional arginas, each predicted to be targeted to the mitochondrion and encoded by two duplicated tandem genes, ARGAH1 (At4g08900) and ARGAH2 (At4g08870). In Arabidopsis, the
mitochondrial localization of arginase was demonstrated by immunolocalization (Flores et al. 2008), which confirmed findings from subcellular fractionation experiments previously conducted in other plant species (Goldraj and Polacco 2000). ARGAH1 is predominantly expressed in pollen grains, whereas ARGAH2 expression is induced by methyl-jasmonate treatment. The induction of arginase activity in the single mutant line argah2 is nearly abolished in germinating seedlings and in jasmonate-treated tissues, whereas argah1 displays strongly reduced arginase activity in pollen, supporting a distinct functional specialization for the two paralogs (Brownfield et al. 2008). ARGAH2 was also reported to exert a negative control over the auxin response, as argah2 displays both enhanced auxin-induced rhizogenesis and enhanced auxin-triggered induction of DRS, an auxin-responsive gene marker (Flores et al. 2008).

In a previous study on the Arabidopsis–Plasmodiophora brassicae pathosystem, we reported the induction of ARGAH2 and high arginase activity in clubroot-infected tissues of the susceptible accession Col-0 (Jubault et al. 2008a). This biochemical phenomenon reaches a maximum 3 weeks after inoculation, i.e. during the last stages of the infection process under our experimental conditions. At this time point, pathogen-induced deregulation of auxin and cytokinin has already led to an almost complete conversion of the plant root system into galls filled with P. brassicae spores (Siemens et al. 2006). This pathological root structure is no longer capable of providing water and nutrients to the plant, ultimately leading to the late, sudden and severe depression of plant shoots.

Arginase induction in clubroot is accompanied by modest regulation of the arginine-derived polyamine content (Jubault et al. 2008a). In contrast, clubroot is associated with pronounced changes to a few amino acids, including the accumulation of proline. High levels of arginase activity, in combination with ornithine aminotransferase activity, might potentially feed a biosynthetic pathway towards proline, which could play a significant osmoprotecting role in root galls. Alternatively, it was proposed that plant arginase could play a role in the regulation of other primary amino acids, thus controlling nitrogen availability for the pathogen (Jubault et al. 2008a). However, these hypotheses remained to be tested.

Within this context, arginase induction during clubroot infection and its consequences on primary metabolism and ultimately the pathogenic process remain to be clarified. To address these questions, we studied the susceptibility to clubroot and the regulation of arginase activity in the argah2 mutant line of Arabidopsis. We investigated the potential role of the jasmonate signaling pathway in the triggering of arginase induction and analyzed the potential impact of argah2 mutation on amino acid contents in infected roots. Because arginase had also been proposed to exert a negative control on auxin-triggered rhizogenesis, we also tested whether arginase could also be involved in cellular proliferation stimulated by exogenous auxin/cytokinin treatments.

### Results

**The argah2 mutant is impaired in the clubroot-triggered induction of arginase, and displays enhanced disease symptoms**

Arabidopsis plants were inoculated with the eH mono-spore isolate of P. brassicae 7 d after seed germination. Arginase activity was quantified in protein extracts from infected and uninfected roots, 21 days post-inoculation (21 dpi). In uninfected plants, basal levels of arginase activity were almost identical—below 100 μmol ornithine h⁻¹ g FW⁻¹—for each of the two genotypes (Fig. 1a). Clubroot infection-induced arginase activity reached up to approximately 500 μmol ornithine h⁻¹ g FW⁻¹ in the fully susceptible accession Col-0. In contrast, arginase activity was hardly stimulated by clubroot infection in the argah2 mutant lines (Col genetic background).

Gall symptoms were evaluated at 21 dpi using the Ga/La disease index described in Gravot et al. (2011), expressing the extent of galls per plant relative to the size of leaf rosette. Using this method, symptom severity was seen to be enhanced in the argah2 mutant compared with Col-0 (Fig. 1b, c).

**Arginase induction is abrogated in the jar1 mutant. OPDA, jasmonate and JA-Ile hydroxylated catabolites are accumulated in clubroot galls**

Clubroot infection resulted in only low induction of arginase activity in the roots of the jar1 mutant (Fig. 1a), at levels comparable with those obtained in argah2. The mutant jar1 was found to be highly susceptible to clubroot infection, with a Ga/La disease index of 75 ± 5 which is higher than in the wild-type Col-0 (27 ± 3) and also higher than in the argah2 mutant (41 ± 4) (Fig. 1b, c). Hormone profiling revealed that clubroot infection led to the accumulation at 21 dpi of both jasmonate and its biosynthetic precursor OPDA (12-oxo-phytodienoic acid), and its hydroxylated catabolite OH-JA, in infected roots of Col-0 (Table 1). Similar profiles were obtained with both mutant lines argah2 and jar1. The jasmonate-isoleucine (JA-Ile) content was not statistically different in inoculated or non-inoculated roots, but its hydroxylated catabolite COOH-JA-Ile was highly accumulated in infected roots of Col-0 and argah2. In contrast and as expected, levels of JA-Ile and its catabolite were strongly reduced in jar1.

**The argah2 mutation has a minor impact on the principal amino acids**

Metabolic profiling was performed on infected and non-infected roots of Col-0 and the argah2 mutant at 21 dpi (Fig. 2). First, it appeared that the free amine contents in roots of non-inoculated plants were very similar between the two genotypes. Secondly, gall development had several effects on the free soluble amine content that were shared by the two plant genotypes. This included an increase in total free amino acids in Col-0 (about +30%) and argah2 (about +50%). This phenomenon was essentially related to a significant increase in
Regulation and consequences of arginase induction during clubroot infection (a) Arginase activity in root tissues of inoculated (eH, dark colored bars) or non-inoculated (NI, light colored bars) plants at 21 dpi. Data are the means of four replicates, each one consisting of root tissues pooled from at least seven individual plants. Error bars represent the standard error (SE). (b, c) Clubroot symptoms in the wild type, argah2 and jar1. (b) Illustration of the symptoms at 21 dpi. (c) Quantitative evaluation of gall symptoms at 21 dpi using the Ga/La disease index described in Gravot et al. (2011), expressing the extent of galous roots relative to the size of leaf rosette as determined by image analysis (see Materials and Methods). Error bars represent the SE (n = 8, and each replicate consisted of at least seven plants). Asterisks represent statistically significant differences according to Student’s t-tests (P < 0.05).

Table 1 Clubroot-induced variations in the content of jasmonate-related compounds in roots of the accessions Col-0, argah2 and jar1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>OPDA</th>
<th>JA</th>
<th>OH-JA</th>
<th>JA-Ile</th>
<th>COOH-JA-Ile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>NI 21 dpi</td>
<td>7,112</td>
<td>126</td>
<td>D</td>
<td>2,914</td>
<td>279</td>
</tr>
<tr>
<td></td>
<td>eH 21 dpi</td>
<td>44,170*</td>
<td>349*</td>
<td>733*</td>
<td>1,540</td>
<td>1,358*</td>
</tr>
<tr>
<td>argah2</td>
<td>NI 21 dpi</td>
<td>8,868</td>
<td>159</td>
<td>D</td>
<td>1,926</td>
<td>329</td>
</tr>
<tr>
<td></td>
<td>eH 21 dpi</td>
<td>36,417*</td>
<td>398</td>
<td>748*</td>
<td>2,485</td>
<td>1,704*</td>
</tr>
<tr>
<td>jar1</td>
<td>NI 21 dpi</td>
<td>9,011</td>
<td>175</td>
<td>D</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>eH 21 dpi</td>
<td>37,528</td>
<td>427</td>
<td>977*</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

Hormone contents were analyzed in infected and non-infected roots at 14 or 21 dpi. Results are expressed in arbitrary units calculated from mass signals relative to sample biomass. Only peaks areas >10 times the chromatographic background noise were considered for quantitative measurements. Data are means of three replicates, each one consisting of at least 15 plants.

ND, not detected; D, detected (peak areas between three and 10 times the background noise).

Asterisks indicate significant differences between eH and NI treatments for one given genotype, inferred from Student’s t-tests (*P < 0.05).

OPDA, 12-oxo-phytodienoic acid; JA, jasmonic acid; OH-JA, hydroxyjasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; COOH-JA-Ile, carboxylated jasmonoyl-L-isoleucine.
the nitrogen-rich amines (C/N ≤ 3) α-alanine, glutamine and serine, and to a minor extent histidine and glycine. The arginine content in roots was not affected by the infection, either in the wild-type Col-0 or in the mutant. Proline content increased considerably in only one of the four infected gall samples of argah2 (reaching 79 μmol g DW⁻¹). This illustrates the versatility of proline accumulation in clubroot experiments, and most of all it impedes any statistically grounded conclusion for this metabolite. Although there are few differences between metabolic profiles in infected roots of Col-0 and argah2, none of the metabolic perturbations caused by clubroot in Col-0 seemed to be dramatically affected by the mutation of ARGAH2.

**ARGAH2 is induced in the epidermal cells of clubroot galls**

We investigated organ-specific expression of ARGHA2 in the clubroot context, using an Arabidopsis line expressing the β-glucuronidase (GUS)-encoding sequence under the control of the ARGAH2 promoter, described in Brownfield et al. (2008). At 21 dpi, uninfected control plants displayed heterogeneous GUS coloration in leaves and weak coloration was also observed in the first centimeter of the primary root (Fig. 3a). A section cut through the roots revealed a faint ARGAH2 expression in mature roots, restricted to a few cells surrounding vascular tissues (Fig. 3b, e). Contrasting with the low expression levels in control roots, in well-developed galls high intensity GUS coloration was observed (Fig. 3c). Sectioning through galls revealed that ARGAH2 expression was almost exclusively localized in the epidermal cell layers (Fig. 3d, f). For some of the sample infected galls, GUS coloration was also observed, in a small area in the center of the stele (Fig. 3d).

**ARGAH2 is induced in pathogen-free hormone-induced root-derived calli**

Considering (i) that the expression of ARGAH2 was found localized in clubroot gall peripheral layers, where auxin accumulation has been documented by others using the auxin-responsive DRS::GUS construction (Pasold et al. 2010); and (ii) that arginase has already been reported to be induced by auxin treatments and exerts an antagonism toward auxin-induced rhizogenesis (Flores et al. 2008), we speculated that arginase induction could be a response to hormone accumulation in either the presence or absence of *P. brassicae* and could play a role in reducing hormone-induced cellular proliferation. To test this, we induced callogenesis on aseptic root explants from the ARGAH2::GUS line and performed GUS
staining on the resulting developing tissues (Fig. 4). A combination of kinetin (0.2 mM) and 2,4-D (2.2 mM) in the B5 culture medium, i.e. a conventional callus-inducing medium (CIM) used for Arabidopsis (Atta et al. 2009), led to the development of calli displaying high levels of ARGAH2 expression as shown by the intensity of GUS coloration after 3 h of incubation with X-gluc (Fig. 4a). As a control, ARGAH2 induction was also evaluated in explants induced for rhizogenesis using a combination of benzylaminopurine (BAP, 0.2 mM) and IAA (2.2 mM). On those samples, following an identical incubation of 3 h with the substrate X-gluc, GUS coloration was of comparatively very low intensity and restricted to only a few spots (Fig. 4b).

The argah2 mutation enhances the development of pathogen-free hormone-induced root-derived calli

Col-0 and argah2 seeds were germinated on CIM, and calli development was observed (Fig. 5). At 7 dpi, root growth was severely reduced by hormone treatment in both genotypes, but the initiation of calli was clearly visible only in argah2. At 12 dpi, calli were initiated in both genotypes, but callus development was clearly more pronounced in argah2, especially at the collar level, leading to typical heart-shaped structures in most of the observed plantlets. This phenotype was inferred...
from the evaluation of >300 plantlets. A similar phenotype was observed when BAP replaced kinetin in the medium (data not shown).

Discussion

Induction of arginase activity during clubroot infection essentially depends on the induction of the plant gene ARGAH2

Previously we showed that arginase activity is induced in clubroot galls during the last steps of the infection (Jubault et al. 2008a). The induction of this enzyme paralleled clubroot-triggered transcriptional induction of the plant arginase-encoding gene ARGAH2. However, in such a heterogeneous biological system where the P. brassicae and A. thaliana gene products intimately co-exist, it was necessary to verify that induction of the plant ARGAH2 gene is entirely responsible for the enhancement of arginase activity. From the results presented here, based on the low levels of arginase activity in argah2 clubroot galls, we conclude that the clubroot-triggered increase in arginase activity is essentially due to up-regulation of the plant ARGAH2 gene. The slight enhancement of arginase activity which remains in clubroot galls on the argah2 mutant could be explained by either the pathogen’s enzymatic machinery or the protein encoded by ARGAH1, the second arginase-encoding gene copy in the Arabidopsis genome. This latter possibility supposes that arginase activity enhancement is related to post-transcriptional regulation since the ARGAH1 gene is not induced during clubroot development (Jubault et al. 2008a).
Arginase induction exerts a negative control on the development of gall growth

To address the question of the biological significance of arginase induction in clubroot, we put forward essentially three possible scenarios: (i) plant arginase activity is one of the plant’s metabolic functions targeted by pathogen effectors, among the pathogen’s global strategy for controlling plant metabolism; (ii) arginase is a plant defense response; or (iii) induction of plant arginase is a side effect of the plant–pathogen warfare, for example one among many stereotypical responses to jasmonate, without biological significance in the context of clubroot infection.

The fact that arginase is induced in infected Col-0 roots at higher levels than in the partially resistant accession Bur-0 was initially considered to support the hypothesis that arginase serves the infectious process (Jubault et al. 2008a). However, the present study gave support to the opposite second hypothesis: arginase is hardly induced in the argah2 mutant, resulting in more symptoms compared with the control Col-0, and arginase induction is then a defense response limiting clubroot development.

Arginase induction by clubroot infection depends on jasmonate signaling

ARGAH2 induction was reported to be under jasmonate signaling control in non-pathological conditions (Flores et al. 2008). JA accumulation during clubroot infection has been previously reported (Grsic et al. 1999). Jasmonate profiling presented in this work confirms this idea, and additionally show a significant accumulation of both a jasmonate precursor (OPDA) and a jasmonate-derived metabolite. We did not detect any significant regulation of JA-Ile, but the accumulation of its catabolite COO-JA-Ile suggests an induction of the activity of the JA-Ile conjugating enzyme JAR1 in infected roots before the sampling time point. In parallel, we showed that the jar1 mutation strongly reduces JA-Ile and JA-Ile derivative (Table 1), and almost abolishes arginase induction by clubroot infection, supporting the idea that arginase is regulated by JA-Ile. According to the proposed model, clubroot symptoms were enhanced in jar1 when compared with Col-0. However, symptoms were greater in the jar1 mutant compared with argah2, suggesting that—indeed of ARGAH2 induction—other jasmonate-driven mechanisms also contribute to reduce gall growth. Further work is now needed using additional mutants to gain a better understanding of the role of oxylipin signaling in the response to clubroot infection. Our phenotypic analysis of jar1 agrees with the results obtained by another group which previously described the increased susceptibility of jar1 to the e3 monosporic isolate of P. brassicae (Siemens et al. 2002). In contrast, Agarwal et al. (2011) reported an absence of effect of jar1 and coi1 mutations on clubroot symptoms when using an Australian field isolate. This discrepancy could be related either to differences between isolates or to differences in the methods used for the evaluation of clubroot symptoms.

Arginase activation apparently does not play any critical role in the homeostasis of arginine, nor does it regulate the availability of amino acids for the pathogen

An initially attractive hypothesis was to suppose that arginase activity impacts on the availability of soluble arginine—which is a nitrogen-rich amino acid—for pathogen nutrition. Thus, arginase induction could be interpreted in the context of a plant–pathogen struggle for nitrogen-containing substances, echoing another model described in tomato–insect interactions (Chen et al. 2005). However, metabolic profiling of galls showed that the argah2 mutation has no consequence on the free arginine content (Fig. 2). Based on GUS analysis of ARGAH2 induction, which was found to be highly specific to clubroot epidermal cells (Fig. 3), we could argue that our global metabolic profiling in galls would have certainly missed such a localized depletion of arginine. Nevertheless, knowing that the P. brassicae secondary growth phase occurs essentially in the cortex and not in epidermal cells, our results do not support a role for arginase in the down-regulation of arginine availability for the pathogen.

Alternatively, ornithine produced by arginase activity can be converted to PSC by ornithine aminotransferase, and then contribute to (i) proline biosynthesis, i.e. which carries out an osmoprotective function, and/or (ii) glutamate biosynthesis, i.e. to feed a master node of free amino acid biosynthesis with potential consequences on the trophic relationships between the plant and pathogen. In both cases, the hypothesis predicted that clubroot-induced amino acid accumulation should be reduced in the argah2 mutant. In the present work, we show, however, that clubroot-induced proline accumulation is not compromised by the mutation of ARGAH2. This supports the idea that, as suggested by Funck et al. (2008), the activation of arginase activity is not necessary for proline accumulation. A similarly negative conclusion can be drawn about the potential role of arginase in sustaining the clubroot-induced accumulation of glutamate and aspartate (Fig. 2). Furthermore, among the significant accumulation of amino acids caused by clubroot infection in Col-0, none was reduced by the argah2 mutations, with the exception of γ-aminobutyric acid (GABA). However, it is not clear whether small but statistically significant variations in GABA content in Col-0 could actually be biologically significant for pathogen nutrition.

Arginase drives negative feedback against both calli and gall development

In this work, we used in vitro culture techniques and exogenous synthetic cytokinin and auxin to induce artificially cellular proliferation starting from root explants, in the absence of pathogen infection. The role played by cytokinin and auxin signaling in the hyperplasy and hypertrophy of plant host cells during the development of clubroot has been well documented (Ludwig-Müller et al. 2009). Then, notwithstanding that many additional perturbations of host plant signaling are
very likely to be involved in clubroot development, we consider that in vitro callus culture can be regarded in our work as a useful simplified model, which allows dissection of a possible cross-talk between arginase activity and hormone-induced cellular proliferation.

The induction of ARGAH2 in pathogen-free in vitro cultured developing root calli, together with the effect of argah2 on callus development in vitro (Figs. 4, 5) support a view where arginase induction controls a negative feedback to hormone-triggered cell proliferation, and is thus a response to the symptoms rather than to the presence of the pathogen itself. Thus, arginase induction should be a common response to many other gall-inducing plant bioaggressors. Unfortunately, because of probe cross-hybridization between ARGAH1 and ARGAH2, the expression of these genes has not been faithfully reported by most of the available microarray data. It would be interesting now to examine the expression of ARGAH2 in other plant models where hormone-triggered cell proliferation also occurs.

Flores et al. (2008) suggested that an arginase defect allows higher levels of arginine-dependent nitric oxide biosynthesis that would mediate an enhanced response to auxin. Accordingly, preliminary work in our laboratory indicates an accumulation of nitric oxide in clubroot galls, which is enhanced by the mutation argah2 (data not shown). Additional work and complementary approaches are, however, necessary to underpin this aspect. Furthermore, a negative role exerted by the jasmonate-induced protein ARGAH2 on cellular proliferation should be put into perspective with previous reports of a negative effect exerted by jasmonate on cell division and proliferation (Swiatek et al. 2002) and with other studies dealing with JA/auxin cross-talk (Hoffmann et al. 2011, Sun et al. 2011). Grsic et al. (1999) proposed that jasmonate accumulation in clubroot galls could contribute to the induction of genes involved in the biosynthesis of auxin, thus possibly linking JA signaling positively to the infection process, which would contradict our results. Additional work now remains necessary to better understand how the possible positive effect of JA signaling on auxin biosynthesis could co-exist with the negative effect of JA signaling on cellular proliferation.

Conclusion

Our initial hypothesis when this work began was that arginase induction in clubroot, because it was higher in the susceptible accession Col-0 than in the partially resistant Bur-0, should contribute to the pathogenic process. Because of the major role of arginase in the delivery of nitrogen during germination, we then endeavored to test if in clubroot, the induction of plant arginase could affect the nitrogen nutritional needs of the pathogen P. brassicaceae. The data that we present here do not fit with those ideas, and have led us to propose a different hypothesis, where the induction of the Arabidopsis ARGAH2 gene, in epidermal gall cells, contributes to a jasmonate-driven limitation of clubroot symptom development. It now remains necessary to study further the involvement of jasmonate signaling in triggering plant defenses against clubroot development, and specifically the potential role of arginase in a negative cross-talk between JA and cellular proliferation.

Materials and Methods

Plant material

The T-DNA mutant argah2 (Col background) is defective for the arginase-encoding gene At4g08870, as described in Flores et al. (2008). The ethylmethane sulfonate (EMS)-generated jar1 mutant (Col background, TAIR accession CS8072) displays a substitution polymorphism in the gene At2g46370 that leads to a strong reduction in the biosynthesis of the biologically active JA-Ile conjugate (Staswick and Tiryaki 2004). Col-0 seeds were obtained from the Arabidopsis thaliana Resource Centre (accession 186AV). Histochemical characterization of ARGAH2 expression patterns was performed using a homogenous line expressing the GUS-coding sequence under the control of the ARGAH2 gene promoter (ARGAH2-B), described in Brownfield et al. (2008).

Clubroot tests

Susceptibility to clubroot was estimated by resistance tests at 21 dpi as described in Jubault et al. (2008b), using the isolate eH, or water for non-inoculated controls. eH is a selection isolate (Fähling et al. 2003) belonging to pathotype P1 as determined by Somé et al. (1996). Susceptibility to clubroot was quantified by gall area evaluation (Ga in mm²) using image analysis. The size of the gall was expressed relative to the leaf surface, roughly evaluated by the square of the longest leaf length (maximal leaf length² = leaf area index = La in cm²). The Ga/La ratio was multiplied by 5,000 to give the Ga/La disease index to obtain values within the range obtained with the classical disease index. For this purpose, every plant was photographed with a scale, and image analyses were performed using ImageJ software. For each replicate (from three to eight biological replicates depending on experiments), data were expressed as the mean of the evaluation of at least seven plants. The distribution of symptoms in individual infected plants in eight replicates of at least seven plants (≥56 plants) is given in Supplementary Fig. S1, giving an idea of the intragenotype variability of the clubroot response. For each replicate, 3 cm of roots below the leaf insertion were sampled for all plants and pooled, then frozen in liquid nitrogen and stored at −80°C before further biochemical analysis.

Aseptic callus induction

Seeds were aseptically germinated on a full B5 medium containing 0.8% agar, 2% sucrose. Seven days after germination, root explants were transplanted onto B5 plates supplemented with 0.2 μM BAP and 2.2 μM 2,4-D (alternatively IAA) for the callus (alternatively secondary root) induction and cultivated
for an additional 4 weeks before GUS or 4,5-diaminofluorescein diacetate (DAF-DIA) staining. Alternatively, plantlets were germinated directly on hormone-containing medium and observed after 7 d.

Amino acid profiling

Freeze-dried samples were ground to a fine powder using a ball mill. Free amino acids were extracted, derivatized through the AccQ-Tag Ultra method and analyzed using an UPLC-DAD (ultra performance liquid chromatography-diode array detector) system, as described in Jubault et al. (2008a). As small citrulline peaks suffered from poor resolution with high peaks of glutamate, we used in parallel, for the quantification of this specific amine, the following alternative elution gradient: start 0.1% B, 2 min 0.1% B (curve 6), 6 min 2% B (curve 7), 10 min 4% B (curve 7), 12 min 20% B (curve 6), 15 min 59.6% B (curve 6), 16 min 59.6% B (curve 6), 17 min 0.1% B (curve 6), 18 min 0.1% B (curve 6).

Jasmonate metabolite determination

Fresh root samples (a minimum of 15 plants for each biological replicate, three replicates) were ground to a fine powder in nitrogen using a mortar and pestle. Extractions were performed starting from about 300 mg of fresh powder. The first step of extraction was performed in 15 ml tubes, using 1 ml of a solvent mixture of water/propanol/chlorhydric acid (2:1:0.002) per 100 mg of fresh root powder, followed by an agitation step at 4°C for 30 min. Then, the extracts were subjected to partition phase by adding 2 ml of dichloromethane per 100 mg then agitation at 4°C for 30 min. Tubes were centrifugated for 5 min at 3,000 r.p.m. and the lower apolar phase was withdrawn with Pasteur pipets and transferred to new tubes, then dried under nitrogen flux. Pellets were solubilized in 150 μl of methanol before chromatographic analysis. The identification and characterization of phytohormones was performed with UPLC coupled to tandem mass spectrometry (UPLC-MS/MS), using MS transitions determined from pure standards (JA, 12-OH-JA, JA-Ile and OPDA) or published data (Glauser et al. 2008). The relative quantification in samples was achieved by reporting MS peak areas to the mass of biological material. All analyses were performed using a Waters Quattro Premier XE equipped with an electrospray ionization (ESI) source and coupled to an Acquity UPLC system (Waters). Chromatographic separation was achieved using an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters), coupled to an Acquity UPLC BEH C18 pre-column (2.1 × 5 mm, 1.7 μm; Waters). The mobile phase consisted of (A) water and (B) methanol, both containing 0.1% formic acid. The run started with 2 min of 95% A, then a linear gradient was applied to reach 100% B at 12 min, followed by an isocratic run using B for 2 min. Finally the return to initial conditions (95% A) was achieved in 3 min. The total run time was 17 min. The column was operated at 35°C with a flow-rate of 0.35 ml min⁻¹ (sample injection volume 3 μl). Nitrogen generated from pressurized air in a N2G nitrogen generator (Mistral) was used as the drying and nebulizing gas. The nebulizer gas flow was set to approximately 50 l h⁻¹, and the desolvation gas flow to 900 l h⁻¹. The interface temperature was set at 400°C and the source temperature at 135°C. The capillary voltage was set at 3.2 kV and the cone voltage at 25 V; the ionization was in positive or negative mode. Low mass and high mass resolution were 14 for the first mass analyzer and 13 for the second, ion energies 1 and 2 were 0.6 V, entrance and exit potentials were 2 V and detector (multiplier) gain was 650 V. Data acquisition and analysis were performed with the MassLynx software (ver. 4.1). The transitions and collision energy were, in negative mode: JA 209→59 (23 V); OH-JA-Ile 338→130 (20 V); COOH-JA-Ile 352→130 (20 V); OH-JA 225→59 (23 V); and in positive mode: OPDA 293→275 (15 V) and JA-Ile 324→151 (20 V). Only peaks areas >10 times the chromatographic background noise were considered for quantitative measurements. Peaks area between three and 10 times the chromatographic background noise in at least one repetition are reported as ‘detected’.

Arginase activity

Arginase assays were performed as follows: nitrogen-frozen powdered root tissues (usually 100–150 mg) were ground and homogenized in extraction buffer (400 μl 100 mg⁻¹ FW) for 2 min. The extraction buffer contained 100 mM Tris–HCl, pH 7.5, 1% (v/v) 2-mercaptoethanol. Homogenates were centrifuged at 10,000×g for 1 min at 4°C and the supernatants were directly used as the enzyme source. Protein concentrations were determined by the Bradford method. Before the arginase activity assay, the enzyme extract was mixed with 7.5 mM MnCl₂ and left for 10 min at 4°C to activate arginase (Goldraij and Polacco 1999). A reaction mixture containing 100 μl of 125 mM Tris–HCl, pH 9.5, and 30 μl of 250 mM l-arginine (reduced to pH 9.5) was added to 20 μl of activated enzyme. Control assays were concurrently performed by replacing native enzyme extract with boiled enzyme extract in the assay. The reaction mixture was incubated with continuous agitation (500 r.p.m.) for 20 min at 37°C, and stopped by heating at 95°C. Ornithine was spectrophotometrically measured by a colorimetric method as follows: 450 μl of 125 mM Tris–HCl, pH 9.5, and 1 ml of reagent [1% ninhydrin (w/v) in a mixture of 60% acetic acid (v/v) and 40% water] were added to 50 μl of the enzymatic reaction and heated at 95°C for 20 min. Absorbances were read at 515 nm, and standard l-ornithine solutions (0–100 μM) were used for calibration. Arginase activity was expressed as the average of triplicate assays as nmol of ornithine released per minute per milligram of protein.

GUS staining

Whole plants of the ARGAH2:GUS line, infected or not infected, were sampled at 21 dpi and briefly washed free of soil particles with tap water. Histochemical staining of GUS activity was then performed following the procedure described by Renault et al. (2010) with an incubation time of 3 or 6 h.
(for pathogen-free tissue cultures and clubroots, respectively) with the X-gluc-containing reaction mix, before subsequent washes. Stained root samples were cut with a razor blade and observed using a stereomicroscope. In addition, a few roots were fixed with 2% glutaraldehyde in phosphate buffer (0.1 mM, pH 7.2), then embedded in resin (Technovit 7100, Heraeus Kulzer) according to the supplier’s instructions. Slices of 4 µm thickness were obtained with a semi-automatic rotary microtome (Microm Microtech) and used for microscopic observations.

Supplementary data
Supplementary data are available at PCP online.

Funding
This work was supported by core funding of the Institut de Génétique Environnement et Protection des Plantes (IGEPP), provided by Agrocampus Ouest, Université de Rennes 1 and Institut National de la Recherche Agronomique (INRA).

Acknowledgments
Florian-Xavier Duval is greatly acknowledged for his technical support in phytohormone analysis.

References


