

Differential Regulation of Root Arginine Catabolism and Polyamine Metabolism in Clubroot-Susceptible and Partially Resistant Arabidopsis Genotypes

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The hypertrophy and hyperplasia of infected roots into clubs are the intrinsic characteristics of clubroot, one of the economically most important diseases in *Brassica* crops worldwide. Polyamines, arginine (Arg)-derived metabolites, have long been recognized as cell proliferation and differentiation regulators in plants and consequently are suitable candidates for potential gall development factors. Furthermore, Arg catabolism, through arginase, which is strongly connected to polyamine metabolism, would play an important role in response to wound trauma and pathogen infection. In this study, we exploited the Arabidopsis (*Arabidopsis thaliana*)-*Plasmodiophora brassicae* pathosystem to investigate the involvement of polyamine metabolism and Arg catabolism in host responses to the pathogen infection and in partial clubroot resistance mechanisms. We demonstrated at the transcriptional, enzymatic, and metabolic levels that polyamine metabolism and Arg catabolism are induced during the later stages of disease in compatible Arabidopsis-*P. brassicae* interactions. However, susceptible and partially resistant plants showed strikingly different Arg metabolism signatures. Susceptible plants were characterized by a transient agmatine production, a massive induction of arginase, and a strong accumulation of proline. The potential functions of this marked activation of the arginase pathway in the *P. brassicae* pathogenicity strategy are discussed. Partially resistant plants showed a continuous agmatine production and a weaker arginase pathway activity than the susceptible genotype. Results suggest that the symptom severity was strongly associated to the differential regulation of root polyamine metabolism and Arg catabolism. Further work using arginase transgenic plants will provide insight into the physiological function of the arginase pathway in partial clubroot resistance.

Clubroot, caused by the obligate biotrophic protist *Plasmodiophora brassicae* Woron., is one of the economically most important diseases of *Brassica* crops in the world. The life cycle of this soil-borne pathogen can be divided into two phases: a primary phase in which events are confined to the root hairs, and a secondary phase that occurs in the cortex and the stele of the hypocotyl and roots of the infected plants. During the second phase, multinucleate plasmodia cause the hypertrophy (abnormal cell enlargement) and hyperplasia (uncontrolled cell division) of infected roots into characteristic clubs (Ingram and Tommerup, 1972). These symptoms obstruct nutrient and water transport, stunt the growth of the plant, and consequently reduce crop yield and quality. Since the pathogen survives as resting spores for a long period (up to 15 years) in the soil, control of the disease by agricultural practices and/or chemical treatments is difficult and/or expensive. Thus, the development of resistant cul-

tivars is currently the most efficient way to control clubroot among *Brassica* crops. Both qualitative and quantitative clubroot resistances have been identified and analyzed in different cultivated *Brassicaceae* species (Manzaneres-Dauleux et al., 2000a; Suwabe et al., 2003; Hirai et al., 2004; Piao et al., 2004; Rocherieux et al., 2004; Hirai, 2006; Saito et al., 2006; Suwabe et al., 2006). Monogenic or oligogenic conferred clubroot resistance introduced into commercial cultivars leads to complete resistance (incompatible interaction) but is rapidly overcome. Partial resistance (compatible interaction), controlled by multiple genes, would be overcome by the pathogen more slowly and is thus an alternative for developing durable host-plant resistance. However, mechanisms associated with partial quantitative resistance remain poorly understood.

Arabidopsis (*Arabidopsis thaliana*), a wild *Brassicaceae*, also is a host species for clubroot (Koch et al., 1991). Natural variation in the responses to clubroot occurs in the model plant (Fuchs and Sacristán, 1996; Siemens et al., 2002; Alix et al., 2007) and our group discovered that the Burren-0 (Bur-0) accession is partially resistant to the eH *P. brassicae* isolate (Alix et al., 2007). The resistance harbored by this accession is under polygenic control (M. Jubault, C. Lariagon, M. Simon, R. Delourme, and M.J. Manzaneres-Dauleux, unpublished data) and is characterized by a reduction

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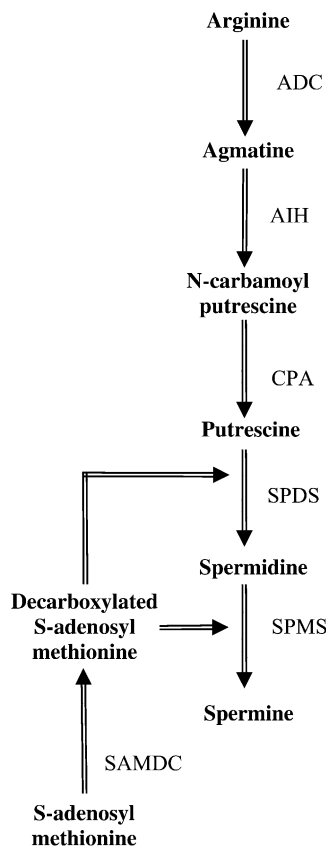


Figure 1. Polyamine biosynthetic pathway in Arabidopsis. ADC, Arg decarboxylase; AIH, agmatine iminohydrolase; CPA, *N*-carbamoylputrescine amidohydrolase; SPDS, spermidine synthase; SPMS, spermine synthase; SAMDC, *S*-adenosyl-Met decarboxylase.

of symptom severity. Compared to a susceptible genotype (main and secondary root systems are replaced by a big club), the partially resistant plants usually show only tiny clubs confined to the secondary root system. These findings make it possible to exploit the model plant and its panel of available biological and genomic resources to gain insight into clubroot pathogenesis and the mechanisms controlling partial resistance. Furthermore, *P. brassicae* does not show host specificity in *Brassicaceae* (i.e. the same isolate can infect different species). Consequently, knowledge acquired on Arabidopsis could then be rapidly integrated and transferred to cultivated crops. To date, research on clubroot using Arabidopsis as a model host system was mainly focused on the role of hormonal regulation by auxin (Ludwig-Muller et al., 1999; Grsic-Rausch et al., 2000; Neuhaus et al., 2000; Ando et al., 2006; Devos et al., 2006; Schuller and Ludwig-Muller, 2006) and/or cytokinins (Devos et al., 2006; Siemens et al., 2006) in the development of clubroot symptoms. However, other molecules, such as polyamines, could also be involved in cell proliferation and differentiation. These metabolites are of interest regarding the development of clubroot symptoms and

were previously proposed to participate in club formation in infected roots of susceptible *Brassica* (Walters and Shuttleton, 1985; Cao et al., 2008).

Polyamines are ubiquitous aliphatic cations that are produced by almost all living organisms, including plants, animals, fungi, and bacteria. Their biosynthesis and catabolism pathways have been fully characterized for many organisms (mammals, fungi, plants). In Arabidopsis, the amino acid Arg is the starting point for polyamine biosynthesis. Arg is decarboxylated by Arg decarboxylase to yield agmatine (Fig. 1), which then serves as the substrate for the biosynthesis of putrescine through the activities of two enzymes, agmatine iminohydrolase and *N*-carbamoylputrescine amidohydrolase. In higher plants, putrescine is also produced by an alternative pathway, from Orn, as the result of the action of Orn decarboxylase. However, several plant species, including the model plant Arabidopsis, show reduced or absent Orn decarboxylase activity, so that polyamine biosynthesis must be mostly dependant on the basic amino acid Arg (Hanfrey et al., 2001). Putrescine is then converted into the polyamines spermidine and spermine by addition of amino-propyl residues from decarboxylated *S*-adenosyl-Met, which results from decarboxylation of *S*-adenosyl-Met by *S*-adenosyl-Met decarboxylase. These reactions are sequentially catalyzed by two closely related but distinct enzymes, spermidine synthase and spermine synthase. In higher plants, in addition to their implication in fundamental cellular processes, such as chromatin organization, cell proliferation, differentiation, and programmed cell death (Thomas and Thomas, 2001; Bais and Ravishankar, 2002), polyamines were also reported to be involved in adaptive responses to abiotic (for review, see Bouchereau et al., 1999; Urano et al., 2003; Kuznetsov et al., 2006) and biotic stresses (for review, see Walters, 2003). First, they are essential components of the plant cell wall, a crucial physical barrier against pathogen invasion (Berta et al., 1997). In addition, polyamine catabolism may also contribute to defense responses through two reaction products (for review, see Cona et al., 2006): γ -aminobutyric acid, an important metabolite largely and rapidly produced in response to biotic and abiotic stresses (Bouche and Fromm, 2004), and the reactive oxygen species H_2O_2 , which has long been recognized to play a key role in defense (Gechev et al., 2006). Last, polyamine conjugation with phenolic acids was also linked with plant defense to pathogen infection (Walters, 2000).

Two major metabolic pathways are closely connected to polyamine metabolism. Arg is also the precursor for the biosynthesis of nitric oxide (NO), Orn, and urea (Fig. 2). The step catalyzed by NO synthase (NOS), which allows the two-step oxidation of Arg to NO and citrulline, is intensively studied during abiotic stresses and plant-pathogen interactions (Mur et al., 2006; Arasimowicz and Floryszak-Wieczorek, 2007). The second Arg catabolism pathway is catalyzed by arginase, which hydrolyzes Arg to Orn and urea. Whereas Orn contributes to the biosynthesis of Pro and Glu,

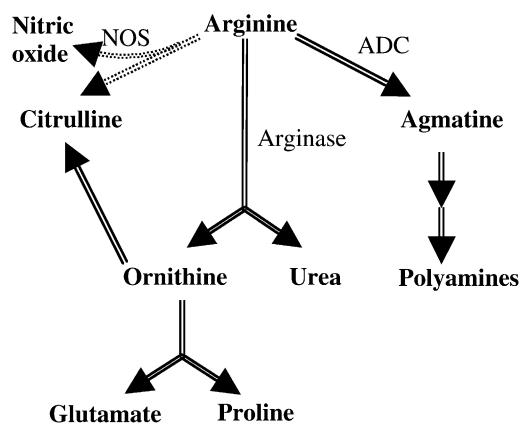


Figure 2. Arg catabolism in Arabidopsis. ADC, Arg decarboxylase.

urea is further catabolized by urease to carbon dioxide and ammonium. Although research on plant arginase has mainly focused on its role in mobilizing Arg as a nitrogen source during postgermination growth (Kolloffel and van Dijke, 1975; Zonia et al., 1995; Palmieri et al., 2006), plant arginase was also reported to be involved in stress responses. Arginase activity was induced in tomato (*Lycopersicon esculentum*) leaves in response to wounding; treatment with jasmonic acid, a potent signal for plant defense responses; and infection with a virulent strain of *Pseudomonas syringae* pv *tomato* (Chen et al., 2004). Potential roles in protection against herbivores and in pathogen virulence were consequently proposed. However, although they are strongly interconnected because they compete for a common substrate, these three Arg catabolic pathways, i.e. biosynthesis of polyamines, biosynthesis of NO, and the urea cycle, are frequently considered independently in higher plants. In contrast to animal systems, where a switch between the different branches of Arg metabolism has long been recognized to be involved in response to wound trauma and pathogen infection (Vincendeau et al., 2003; Duleu et al., 2004; Pfaff et al., 2005), the potential role of Arg metabolism regulation in the plant-pathogen relationship is still unclear.

Consequently, Arg metabolism appears to be an exciting metabolic pathway potentially involved in *Brassicaceae-P. brassicae* interactions due to, on one hand, its central role in plant defense-responses and, on the other hand, the role of polyamines in cell proliferation and differentiation regulation. The present work aims to determine, first, whether polyamine metabolism and Arg catabolism through arginase are implicated in host responses to *P. brassicae* infection, and, second, whether these metabolic pathways might be involved in partial clubroot resistance mechanisms. Thus, we examined the temporal responses of polyamines and arginase to clubroot in roots of both the susceptible Columbia-0 (Col-0) accession and the partially resistant Bur-0 accession. We analyzed the ex-

pression levels of genes involved in polyamine biosynthesis and encoding arginase, and quantified arginase activity, Arg-related amino acids, and free polyamine levels. Our results show that the expression of genes involved in Arg catabolism and polyamine metabolism is induced upon inoculation with *P. brassicae* in both susceptible and partially resistant accessions. However, free polyamine production and Arg utilization is clearly regulated differently in partially resistant plants compared to susceptible ones.

RESULTS

Clubroot Resistance Tests

In each test, the Arabidopsis accessions Bur-0 and Col-0 were evaluated at 21 d postinoculation (dpi) for clubroot symptoms. A set of differential hosts, including susceptible and resistant genotypes of different *Brassica* species, was also evaluated at 49 dpi to characterize the isolate's pathogenicity. This confirmed that the selection isolate eH (Fähling et al., 2003) used in this study belongs to the most virulent *P. brassicae* pathotype P1 (Somé et al., 1996). ANOVA revealed no significant difference between tests but a significant phenotypic variation between genotypes ($P < 0.001$). The Col-0 accession, with a mean disease index (DI) of 86, was classified as significantly more susceptible than the Bur-0 accession ($P < 0.05$), which showed an intermediate behavior with a mean DI of 64 as previously reported (Alix et al., 2007).

Transcriptional Profiling of Genes Involved in Polyamine Metabolism and Arg Catabolism

We used quantitative real-time reverse transcription (RT)-PCR to examine the expression levels of polyamine biosynthesis and arginase-encoding genes in control and infected roots of the partially resistant Bur-0 accession and the susceptible Col-0 accession. Four independent experiments were carried out at four time points (2, 7, 14, and 21 dpi) to relate specific host responses to the life cycle of the pathogen. The first time point corresponds to the primary phase of *P. brassicae* infection, i.e. the first contact between primary zoospores and root hairs and development of primary plasmodia. Seven, 14, and 21 dpi correspond in a susceptible genotype to the early events of cortical cells colonization and club formation, respectively, during the secondary phase of infection (Fuchs and Sacristán, 1996). For each time point, an ANOVA was performed to evaluate inoculation and genotype effects on gene expression.

First, we could not detect any significant differences between the transcriptional profiles of genes involved in polyamine biosynthesis and Arg catabolism in control roots of the two Arabidopsis genotypes. Similarly, no significant differences were observed between control and *P. brassicae*-infected roots for either gene set or accession at the first two time points (data

not shown). In contrast, at 14 and 21 dpi, the ANOVA showed that expression of most genes involved in polyamine biosynthesis and Arg catabolism had been significantly affected by the inoculation with transcripts accumulating in response to *P. brassicae* infection in both susceptible and partially resistant roots.

Close examination of specific gene expression profiles showed that expression of genes encoding Arg decarboxylase (*ADC1*), agmatine iminohydrolase (*AIH*), *N*-carbamoylputrescine amidohydrolase (*CPA*), spermidine synthase (*SPDS1*, *SPDS2*), and *S*-adenosyl-Met decarboxylase (*SAMDC2*) was significantly higher in *P. brassicae* inoculated roots than in the control at 14 and 21 dpi ($P < 0.05$ to $P < 0.001$; Fig. 3, A and B). Transcription of *SAMDC1*, a second gene encoding *S*-adenosyl-Met decarboxylase, and *SPMS*, encoding spermine synthase, was also induced by *P. brassicae* infection ($P < 0.05$ and $P < 0.01$ respectively), but only transiently at 14 dpi. In contrast, mRNA levels of *ADC2*, a second gene encoding Arg decarboxylase, did not change in response to infection, and expression of *ACL5*, a second gene encoding spermine synthase, decreased in infected plants at 14 and 21 dpi ($P < 0.05$ and $P < 0.001$). None of the genes showed significant different expression patterns between infected roots of the susceptible and partially resistant accessions.

The expression of the two genes encoding arginase, *ARGAH1* and *ARGAH2*, was also monitored throughout *P. brassicae* infection. *ARGAH1* mRNA levels increased significantly at 14 and 21 dpi in both accessions compared to control roots ($P < 0.05$; Fig. 4A), but there was no significant differences in response level between the two genotypes. *ARGAH2* mRNA levels were also higher in Col-0 and Bur-0 inoculated roots than in the control at 14 and 21 dpi. Interestingly, however, *ARGAH2* mRNA levels were drastically induced in susceptible infected roots compared to partially resistant infected roots (Fig. 4B). This observation proved to be statistically significant with a clear interaction between genotype and inoculation factors at 14 and 21 dpi ($P < 0.05$ and $P < 0.01$). Duncan's multiple-range test ($\alpha = 0.05$) performed on the four genotype \times inoculation treatments also showed that *ARGAH2* was expressed at significantly higher levels in infected Col-0 roots at 14 and 21 dpi. For example, at 21 dpi, the *ARGAH2* expression was 25-fold higher in inoculated roots than control Col-0 roots but only 3-fold higher for the partially resistant genotype Bur-0.

Arginase Activity

To validate our results showing induced arginase expression at the transcriptional level, arginase activity was measured in control and infected Col-0 and Bur-0 roots at 21 dpi (Fig. 5). A striking increase in arginase activity was observed in susceptible Col-0 roots in response to *P. brassicae* infection. Indeed, arginase activity was 10-fold higher in infected roots than in control roots. In contrast, arginase activity in

infected roots of the partially resistant Bur-0 accession only increased slightly, as was observed at the transcriptional level.

Arg-Related Amino Acids

Next, we measured the amino acid content of roots at 21 dpi, specifically looking at Arg and related amino acids, i.e. Orn, Glu, and Pro contents (Fig. 6). In non-infected roots, there was approximately 3 times more Arg in Bur-0 than Col-0. No significant change was observed for Arg contents in response to inoculation. Orn levels remained relatively low ($< 0.1 \mu\text{mol g}^{-1}$ dry weight [DW]) regardless of conditions or genotypes (data not shown). In contrast, infections had a strong impact on Pro accumulation, which reached high levels in the infected Col-0 roots. Pro also accumulated in Bur-0 roots, but in a much lower proportion. ANOVA revealed a significant interaction between genotype and inoculation factors ($P < 0.05$). A Duncan's multiple-range test ($\alpha = 0.05$) performed on the four genotype \times inoculation treatments also showed a significant higher accumulation of Pro in the infected Col-0 roots. In comparison, apparently higher levels of Glu in infected roots were very modest. ANOVA did not reveal significant changes in Glu contents in response to inoculation.

Free Polyamine Levels

To further investigate the role of polyamine metabolism following on from the above results obtained at the transcriptional level, we quantified the levels of the precursor diamines agmatine and putrescine and the levels of polyamines spermine and spermidine at 2, 7, 14, and 21 dpi (Fig. 7; Table I). These measures were performed on the four independent experiments previously used for the transcript profiling.

Metabolic profiling of control Arabidopsis roots showed that agmatine and spermidine are the most abundant polyamines. An ANOVA was performed to evaluate time-point and genotype effects on each metabolite level. No significant differences in putrescine and spermine content were detected between the two genotypes. However, whereas spermine content did not change along the time course, the putrescine level decreased significantly at 21 dpi in both Col-0 and Bur-0 roots ($P < 0.05$). Significant increases in agmatine and spermidine were observed at 14 dpi in Col-0 roots ($P < 0.05$).

For each time point, ANOVA was then performed to evaluate inoculation and genotype effects on metabolite level. At the two first time points, there was no significant difference in agmatine levels in noninfected and *P. brassicae*-infected roots (Fig. 7). At 14 dpi, however, the agmatine level significantly increased in response to infection in both susceptible and partially resistant roots. At 21 dpi, the effect of the interaction between accession and inoculation factors was

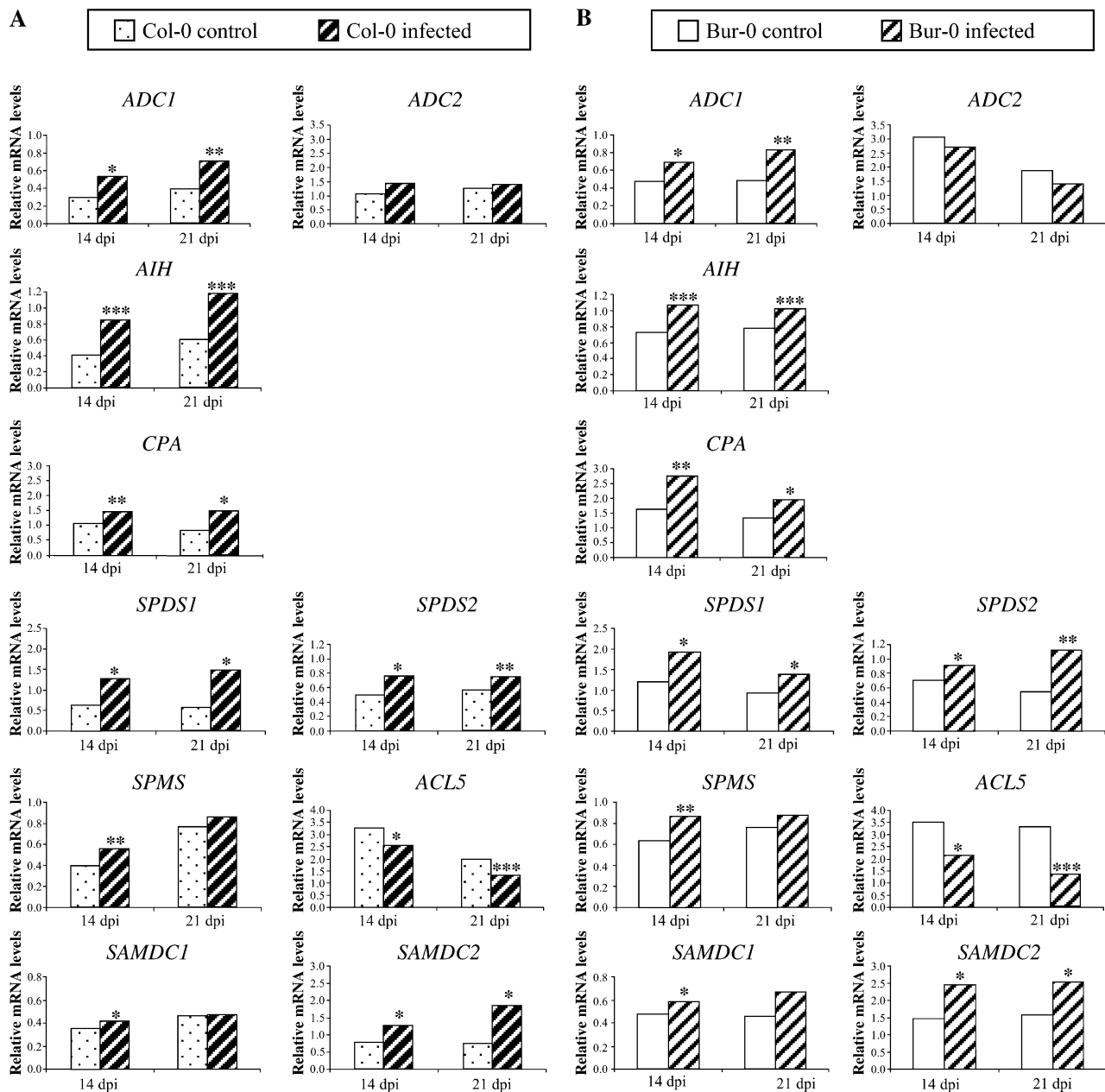


Figure 3. Relative transcript levels of polyamine biosynthetic genes encoding Arg decarboxylase (*ADC1*, *ADC2*), agmatine iminohydrolase (*AIH*), *N*-carbamoylputrescine amidohydrolase (*CPA*), spermidine synthase (*SPDS1*, *SPDS2*), spermine synthase (*SPMS*, *ACL5*), and *S*-adenosyl-Met decarboxylase (*SAMDC1*, *SAMDC2*) in noninfected (control) and infected roots of the susceptible accession Col-0 (A) and the partially resistant accession Bur-0 (B) at 14 and 21 dpi. Values were obtained by real-time quantitative RT-PCR and are normalized to the host *Actin8* gene. Samples of control and infected roots were analyzed in duplicate in four independent experiments. Significant differences from controls are shown at *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

significant ($P < 0.01$). Indeed, whereas agmatine level continued to rise at 21 dpi in the partially resistant roots, it stopped in the susceptible roots. A Duncan's multiple-range test ($\alpha = 0.05$) performed on the four accession \times inoculation treatments confirmed that there was a significantly higher level of agmatine in the Bur-0 infected roots (Fig. 7).

The level of putrescine did not change in response to clubroot infection either in susceptible or partially resistant roots (Table I). As opposed to Bur-0 roots, variations in spermidine and spermine levels were detected at 7 and 14 dpi in Col-0 roots. Upon *P. brassicae* infection, spermidine and spermine levels in susceptible roots tended to increase at 7 dpi and then

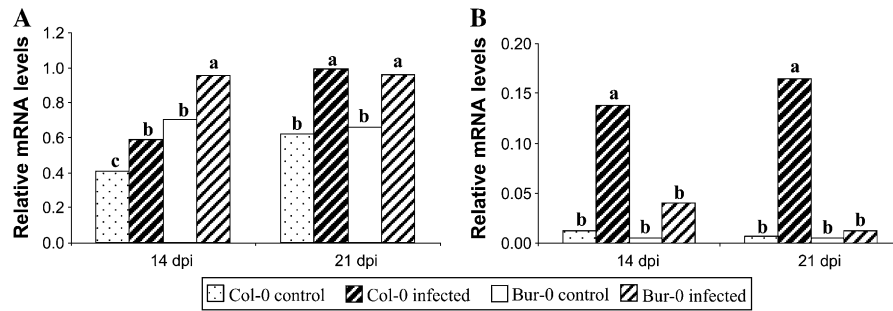


Figure 4. Relative transcript levels of the genes *ARGAH1* (A) and *ARGAH2* (B) encoding arginase in noninfected (control) and infected roots of the susceptible accession Col-0 and the partially resistant accession Bur-0 at 14 and 21 dpi. Values were obtained by real-time quantitative RT-PCR and are normalized to the host *Actin8* gene. Samples of control and infected roots were analyzed in duplicate in four independent experiments. For each time point, same letters indicate nonsignificant difference at $P = 0.05$.

to decrease at 14 dpi; however, these variations were not statistically significant.

DISCUSSION

This study reports the involvement of Arg metabolism in the Arabidopsis-*P. brassicae* interaction. Consistent results obtained at the transcriptional, enzymatic, and metabolic levels demonstrated that polyamine metabolism and Arg catabolism are induced in compatible Arabidopsis-*P. brassicae* interactions. Furthermore, we demonstrated that upon *P. brassicae* infection, susceptible and partially resistant plants exhibit striking differences in the regulation of Arg metabolism. In susceptible plants (Col-0), arginase activity was massively induced at 14 dpi and 21 dpi. This was associated with no change in Orn content but with a large accumulation of Pro. Furthermore, polyamine biosynthesis was also up-regulated with an accumulation of agmatine at 14 dpi. Partially resistant plants (Bur-0), on the other hand, exhibited a slight arginase induction and a moderate accumulation of Pro. In addition, as in susceptible plants, polyamine biosynthesis was also induced; however, agmatine accumulation, observed from 14 dpi, continued to increase at 21 dpi.

Transcriptional and Enzymatic Analyses of Arg Metabolism

We determined the transcriptional profile of genes involved in Arg metabolism in response to *P. brassicae* infection. In this study, we were particularly interested in the arginase and polyamine pathways. The NOS pathway was not included in this study because the nature of its coding gene remains elusive and controversial (Guo et al., 2003; Zemojtel et al., 2006). In Arabidopsis, most of the enzymes involved in Orn and polyamine production are encoded by duplicated genes. However, although they encode the same enzyme, duplicated genes encoding Arg decarboxylase

(*ADC1* and *ADC2*), spermine synthase (*SPMS* and *ACL5*), and arginase (*ARGAH1* and *ARGAH2*) were expressed differentially following *P. brassicae* infection. Whereas *ADC1* and *SPMS* transcripts accumulated at 14 and 21 dpi, like other polyamine biosynthetic genes, the expression of *ADC2* and *ACL5* was surprisingly opposite. While *ARGAH1* transcripts accumulated in a similar fashion in both susceptible and partially resistant accessions, *ARGAH2* overexpression was significantly higher in the susceptible accession than in the partially resistant accession at 14 and 21 dpi. This differential regulation of gene responsiveness was previously observed in biotic and abiotic stress. Indeed, *ADC2* was found to be specifically involved in hyperosmotic stress (Soyka and Heyer, 1999), in water stress (Alcazar et al., 2006), and in response to jasmonic acid and abscisic acid applications (Perez-Amador et al., 2002; Urano et al., 2003). Exogenous abscisic acid also up-regulated *SPMS* expression (Hanzawa et al., 2002; Urano et al., 2003) but not *ACL5*, which was specifically induced upon indolacetic acid application (Hanzawa et al., 2000). Even if two genes (*LeARG1* and *LeARG2*) encoding arginase were identified in tomato,

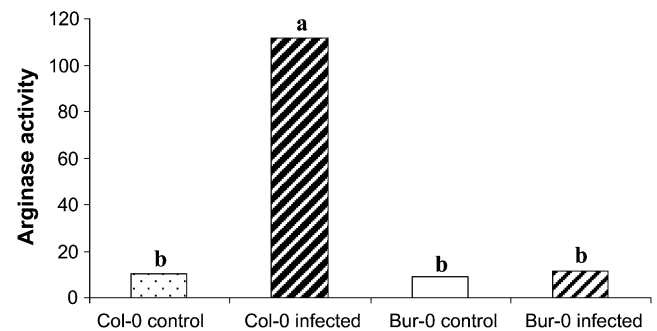


Figure 5. Levels of arginase activity in noninfected (control) and infected roots of the susceptible accession Col-0 and the partially resistant accession Bur-0 at 21 dpi. Arginase activity was expressed as nanomoles of Orn released per minute per milligram of protein. Values represent means of two replicates. Same letters indicate nonsignificant difference at $P = 0.05$.

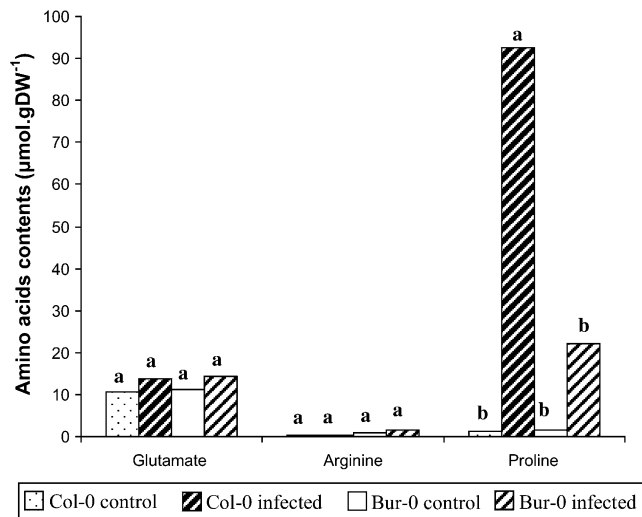


Figure 6. Effect of *P. brassicae* inoculation on Arg-related amino acid contents in roots of the susceptible accession Col-0 and the partially resistant accession Bur-0 at 21 dpi. Amino acid contents were expressed as micromoles per gram of DW. Values represent means of two replicates. For each amino acid, same letters indicate nonsignificant difference at $P = 0.05$.

specific induction of *LeARG2* was observed in response to wounding, jasmonic acid treatment, and infection with a virulent strain of *P. syringae* pv *tomato* (Chen et al., 2004).

Arginase activity measurements do not appear to exactly reflect the expression of both *ARGAH1* and *ARGAH2*, the two genes encoding arginase. *ARGAH1* showed higher basal and *P. brassicae*-induced expression levels than *ARGAH2*, but the strong enhancement of arginase activity in susceptible infected roots appears to be more consistent with the massive increase in *ARGAH2* expression in susceptible plants than with the overall higher *ARGAH1* expression. Taken together, these results suggest that *ARGAH2* is the predominant *P. brassicae*-inducible isoform in *Arabidopsis* roots and that the two arginase isoforms have

contrasting biochemical properties or differing post-transcriptional regulations.

Polyamine Metabolism and Arginase Are Induced in Compatible *Arabidopsis*-*P. brassicae* Interactions

Arg metabolism was induced in response to club-root infection in both susceptible and partially resistant plants. In the literature, similar induction was previously reported in biotic stress, both through arginase and polyamine pathways. Chen et al. (2004) reported an increase in arginase activity in tomato plants infected with a virulent strain of *P. syringae* pv *tomato*. Furthermore, in mammalian systems, arginase induction has long been associated with various parasite infections (Vincendeau et al., 2003; Duleu et al., 2004). The polyamine biosynthesis pathway was induced in both compatible and incompatible host-pathogen interactions (for review, see Walters, 2000, 2003). Furthermore, consistent with our results, Cao et al. (2008) also reported, at the proteome level, the up-regulation of the polyamine biosynthetic enzyme spermidine synthase in *Brassica napus* in response to *P. brassicae* infection. Previous reports on polyamine contents generally focused on the diamine putrescine and/or the polyamines spermidine and spermine; however, no information is currently available on the regulation of agmatine, the precursor to putrescine, in response to pathogen infection. The present study is thus the first report of agmatine involvement in biotic stress. Surprisingly, whereas agmatine accumulated in response to infection in Col-0 and Bur-0 roots, no significant variations in putrescine, spermidine, and spermine levels were reported. Burtin and Michael (1997) also reported that *ADC* overexpression in transgenic tobacco plants induced agmatine accumulation but did not affect putrescine, spermine, and spermidine levels. These results suggest that other polyamine-regulating mechanisms are involved, such as polyamine catabolism (for review, see Cona et al., 2006), conjugation, and transport (for review, see Martin-Tanguy, 2001). Hydroxycinnamic acid amide

Figure 7. Contents of agmatine in noninfected (control) and infected roots of the susceptible accession Col-0 and the partially resistant accession Bur-0 at 2, 7, 14, and 21 dpi. Agmatine contents were expressed as micromoles per gram of DW. Samples of control and infected roots were analyzed in four independent experiments. For each time point, same letters indicate nonsignificant difference at $P = 0.05$.

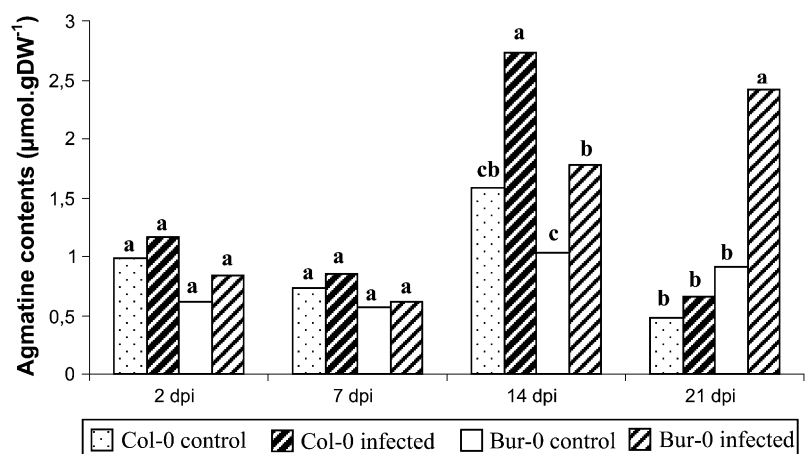


Table 1. Effect of *P. brassicae* inoculation on free polyamine concentrations in roots of the susceptible accession Col-0 and the partially resistant accession Bur-0 at 2, 7, 14, and 21 dpiValues represent means of four independent experiments \pm SE.

Polyamine	Genotype	Inoculation	Polyamine Content			
			2 dpi	7 dpi	14 dpi	21 dpi
$\mu\text{mol g}^{-1} \text{DW}$						
Putrescine	Col-0	Noninoculated	0.37 \pm 0.08	0.6 \pm 0.16	0.54 \pm 0.43	0.17 \pm 0.04
		Inoculated	0.32 \pm 0.11	0.58 \pm 0.3	0.44 \pm 0.14	0.26 \pm 0.15
	Bur-0	Noninoculated	0.45 \pm 0.17	0.46 \pm 0.18	0.25 \pm 0.03	0.22 \pm 0.10
		Inoculated	0.38 \pm 0.24	0.45 \pm 0.17	0.35 \pm 0.10	0.29 \pm 0.08
Spermidine	Col-0	Noninoculated	0.3 \pm 0.29	0.79 \pm 0.13	1.8 \pm 0.79	0.61 \pm 0.82
		Inoculated	0.18 \pm 0.06	2.02 \pm 1.10	0.44 \pm 0.29	0.87 \pm 0.84
	Bur-0	Noninoculated	0.18 \pm 0.1	0.42 \pm 0.39	0.33 \pm 0.39	0.63 \pm 0.64
		Inoculated	0.33 \pm 0.33	0.28 \pm 0.1	0.18 \pm 0.11	0.69 \pm 0.72
Spermine	Col-0	Noninoculated	0.22 \pm 0.17	0.35 \pm 0.26	0.46 \pm 0.55	0.06 \pm 0.03
		Inoculated	0.18 \pm 0.06	0.49 \pm 0.45	0.08 \pm 0.01	0.05 \pm 0.02
	Bur-0	Noninoculated	0.14 \pm 0.1	0.09 \pm 0.04	0.07 \pm 0.03	0.06 \pm 0.01
		Inoculated	0.19 \pm 0.13	0.14 \pm 0.05	0.06 \pm 0.02	0.09 \pm 0.04

conjugates were proposed to play a role in defense mechanisms against biotic and abiotic stress, by acting as radical scavengers, antifungal agents, or in strengthening the plant cell wall against microbial degradation (Bors et al., 1989; Walters, 2000; Von Roepenack-Lahaye et al., 2003). Walters and Shuttleton (1985) measured the free polyamine levels in turnip (*Brassica rapa*) roots infected by *P. brassicae* and showed that putrescine, spermidine, and spermine concentrations were higher in “clubbed” regions of the infected turnip roots than in noninfected roots, while the concentrations were lower in regions of infected roots not exhibiting symptoms of clubroot development. These results suggest that in infected roots, homeostatic regulation, involving transport of polyamines from regions not exhibiting symptoms to “clubbed” regions, may be taking place. Because we looked at whole roots, any localized variations in polyamine levels due to this type of transport mechanism between the different parts of infected roots were not detected. Last, we cannot exclude that some of the polyamines we measured were contributed by *P. brassicae*. Indeed, due to its exclusive intracellular life cycle, it is difficult to distinguish between metabolites of plant or pathogen origin.

Susceptible and Partially Resistant Plants Showed Differences in Arg Catabolism Regulation following *P. brassicae* Infection

Arginase catabolism was strongly induced in the susceptible plants. *ARGAH1* and particularly *ARGAH2* expression and arginase activity markedly increased upon *P. brassicae* infection. Induction of arginase may represent a pathogenicity strategy by *P. brassicae*. Indeed, because arginase competes with NOS for a common substrate, its induction could play an important role in pathogenesis by attenuating the production of NO-mediated host defenses. This hypothesis is

supported by increasing evidence from mammalian systems (Vincendeau et al., 2003). For example, trypanosomes can evade host defenses by stimulating the expression of macrophage arginase, which effectively inhibits NO production and NO-mediated trypanosome killing (Duleu et al., 2004). Arginase induction could also be a way of diverting nitrogen metabolism in favor of the pathogen. Induction occurred at 14 d and 21 dpi, corresponding to the second phase of the *P. brassicae* life cycle (Fuchs and Sacristán, 1996). During this phase, multinucleate plasmodia grow by mitotic division and consequently cause the hypertrophy and hyperplasia of host cells. By analogy to the proposed role of arginase in nitrogen metabolism during postgerminative growth (Kolloffel and van Dijke, 1975; Zonia et al., 1995), *P. brassicae*-induced degradation of Arg to ammonium and Orn may provide a mechanism to divert plant nitrogen into the production of amino acids indispensable for pathogen multiplication. Plasmodia would thus redirect host nutrients to their own benefits, thereby acting as a metabolic sink. *P. brassicae* was previously proposed to interfere in host carbon metabolism following observations that carbohydrates accumulate in infected tissues (Evans and Scholes, 1995). We also observed an acute accumulation of Pro in Col-0 infected roots, which strengthens the idea that susceptibility is associated with an enhancement of the metabolic flux from Arg to Pro. Free Pro biosynthesis and accumulation at high levels is very common in plants subjected to osmotic, drought, or saline strains (Delauney and Verma, 1993). Of note, although reasonable, the amounts of Pro detected in infected roots in this experiment were of surprising magnitude (92 $\mu\text{mol g DW}^{-1}$), and this may warrant further attention. Pro, γ -aminobutyric acid, and α -amino adipic acid were recently observed to strongly accumulate in T-DNA-induced Arabidopsis tumors cells and were viewed by the authors as stress metabolites (Deeken et al., 2006).

Our findings with this pathosystem, however, raise several issues. Was all the neosynthesized Pro pool actually derived from Orn through an Orn aminotransferase activity or was a more classically described stress-induced Glu to Pro biosynthetic pathway involved? In which cellular and subcellular compartments, including intracellular pathogen, do Pro biosynthesis and accumulation really take place? Without answers to these questions, it is difficult to conclude if, on one hand, Pro accumulated inside the pathogen favoring its growth through, for instance, osmotic or oxidative protection (Chen and Dickman, 2005), or if, on the other hand, Pro accumulation in the plant tissue simply reflects attempts by plant metabolism to manage the cellular stress caused by pathogen growth. In the partially resistant accession, agmatine accumulation occurred at 14 and 21 dpi and slight arginase induction and Pro accumulation were observed upon inoculation. This weaker response in partially resistant plants may suggest that the pathogen influence on host-metabolism was attenuated or delayed compared to the situation in susceptible plants. However, as yet we cannot conclude whether this regulation of Arg metabolism is the cause or the result of the partial clubroot resistance.

Further investigations using various sets of mutants and overexpressors are planned in our laboratory to test some of these hypotheses. For instance, genetic manipulation of arginase expression in Arabidopsis transgenic plants or quantification of arginase activity in a range of *Brassicaceae*, showing extreme and intermediate levels of resistance to clubroot, will provide insights into the physiological function of arginase in host-pathogen interactions and in partial clubroot resistance. Moreover, mutant genotypes for genes encoding enzymes involved in polyamine metabolism will be assessed for responsiveness to *P. brassicae*. However that may be, the coordination of the multiple routes from Arg directing metabolites toward nitrogen and carbon trophic channels, defense systems, growth regulators, or signaling compounds remains undoubtedly a major challenge for stressed plants and may be considered as a target of prime interest within the scope of quantitative resistance to *P. brassicae*.

MATERIALS AND METHODS

Pathogen

The selection isolate eH (Fähling et al., 2003), used in this study, belongs to the most virulent *Plasmodiophora brassicae* pathotype P1, according to the host differential set established by Somé et al. (1996). It was kindly provided by J. Siemens (University of Dresden, Germany).

Plant Materials

Seeds of the Arabidopsis (*Arabidopsis thaliana*) accessions Bur-0 (172AV) and Col-0 (186AV) were obtained from the Versailles Resource Center. These accessions are partially resistant and susceptible, respectively, to the *P. brassicae* isolate eH (Alix et al., 2007). *Brassica napus* subsp. *oleifera* 'Nevin' (ECD6), *B. napus* subsp. *rapifera* 'Wilhelmsburger' (ECD10), and *B. napus*

subsp. *oleifera* (Brutor), which constitute the host differential set established by Somé et al. (1996), and the highly clubroot susceptible *Brassica rapa* subsp. *pekinensis* 'Granaat' (ECD5) were included as controls in each clubroot test.

Growth Conditions and Inoculation Procedure

Five independent experiments were performed using a two-block design. Arabidopsis seeds were placed on wet blotting paper in petri dishes at 4°C for 3 d to synchronize germination. Seeds were then individually sown in 4-cm-diameter pots containing a [2/3 compost, 1/3 vermiculite] mix sterilized by autoclaving. Arabidopsis plants were grown under controlled environmental conditions (16 h light at 22°C and 8 h dark at 19°C) and inoculated 7 d after germination (stage 1.04; Boyes et al., 2001). The inoculum was prepared according to Manzanares-Dauleux et al. (2000a), and inoculation was performed by applying 1 mL of resting spore suspension (10^7 spores mL⁻¹) to the crown of each seedling. The resting spore suspension was replaced by distilled water for control plants. To relate specific host responses to the life cycle of the pathogen, infected and control plants were then harvested at four time points: 2, 7, 14, and 21 dpi (respectively, stages 1.04, 1.08, 1.12, and 3.90–6.50; Boyes et al., 2001). In the last experiment, infected and control plants were only harvested at 21 dpi (stages 3.90–6.50; Boyes et al., 2001). Depending on the kinetic point, between 84 and 600 individual plants were harvested per analysis point. Roots of the whole plants were thoroughly rinsed in different baths of distilled water. Roots and leaves dissected from whole plants were frozen in liquid nitrogen and stored at –80°C. To check that the inoculation was successful, in each test, six to 12 plants of each Arabidopsis accession were evaluated for clubroot resistance at 21 dpi and symptoms were recorded using the scale previously described for *Brassica oleracea* (Manzanares-Dauleux et al., 2000b): 0, no visible swelling; 1, very slight swelling usually confined to lateral roots; 2, moderate swelling on lateral roots and taproot; 2+, severe clubs on all roots but some roots remain; and 3, no root left, only one big gall. DI was calculated: $DI = (n_1 \times 25 + n_2 \times 50 + n_2^+ \times 75 + n_3 \times 100) / N$, where "ni" is the number of plants in the symptom class "i" and N is the total number of plants tested; an accession displaying a DI of zero is completely resistant and develops no clubroot symptoms while an accession with a DI of 100 is highly susceptible (Manzanares-Dauleux et al., 2000b).

Real-Time RT-PCR

Total RNA was extracted from approximately 30 mg of frozen root samples using the SV Total RNA Isolation kit (Promega). Any remaining genomic DNA was removed by digestion with DNase I (DNA-free; Ambion) according to the manufacturer's protocol. To check for genomic DNA contamination, a PCR reaction was carried out on the RNA samples using *Actin8* primers. The total RNA was quantified with a spectrophotometer and electrophoresed on a 2% agarose gel to check the concentration and integrity. First-strand complementary DNA (cDNA) synthesis was performed in a 20- μ L total reaction volume using 250 ng of DNase-digested total RNA, 1 μ M oligo(dT) primer, 1 mM dNTPs, 1 \times first-strand buffer (Invitrogen), 20 mM dithiothreitol (Invitrogen), 40 units RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), and 200 units SuperScript II reverse transcriptase (Invitrogen) by incubating for 2 h at 42°C. The reaction was terminated by incubation for 15 min at 70°C. The cDNAs were diluted by 1/40 and their quality was confirmed by conventional RT-PCR with *Actin8* primers (Table II).

For each gene, primers for real-time RT-PCR were designed on gene sequence tags (Hilson et al., 2004) with Primer Express v1.5 software (Applied Biosystems) and synthesized by Eurogentec. The genes, as well as the sequence of their specific oligonucleotides, are presented in Table I. Three microliters of cDNA was used for PCR amplification using the SYBR Green PCR Master kit containing a Hot Start *Taq* polymerase (Applied Biosystems) and 0.9 μ M of each specific primer, in the ABI PRISM 7700 sequence detection system (Applied Biosystems). Ten-microliter reactions were run in duplicate in a 96-well thin-wall PCR plate (MicroAmp Optical 96-wells reaction plate; Applied Biosystems). The PCR amplification protocol consisted of a denaturation step at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The SYBR Green I fluorescence signal was measured during the 60°C annealing step. To check the annealing specificity of each oligonucleotide, melting-curve analysis (55°C–94°C) was carried out at the end of amplification. For calculations, a standard curve was determined for each gene using different dilutions of cDNA products. Expression levels for each target gene were then quantified following normalization to *Actin8*, the endogenous reference.

Arginase Assays

A known weight of frozen powdered root tissues (usually 0.5 g) was ground and homogenized in 1 mL of extraction buffer for 3 min. The extraction medium consisted of 100 mM Tris-HCl, pH 7.5, 1% (v/v) 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride (Chen et al., 2004). Homogenates were centrifuged at 10,000g for 15 min at 4°C and the supernatants were used as the enzyme source. Protein concentrations were determined by the Bradford method (Bradford, 1976). Before the arginase activity was assayed, the enzyme extract was made up to 5 mM in MnCl₂ and left for 15 min at room temperature to activate arginase (Goldraj and Polacco, 1999). A standard reaction mixture contained 450 μL of 125 mM Tris-HCl, pH 9.5, 50 μL of 550 mM L-Arg (adjusted to pH 9.5), 25 μL of 44 mM MnCl₂, and 25 μL of enzyme source. Control assays were concurrently performed by removing L-Arg or replacing native enzyme extract by boiled enzyme extract or increasing enzyme dose in the assay. The reaction mixture was incubated with continuous agitation (500 rpm) for 45 min at 30°C, and the reaction was stopped at 0, 15, 30, and 45 min by adding 500 μL of 10% (v/v) perchloric acid. Precipitated proteins were removed by centrifugation at 12,000g for 15 min, and Orn released in the medium was spectrophotometrically measured by the Chinard method (Chinard, 1952) modified according to Roubelakis and Kliewer (1978). Absorbances were read at 515 nm, and standard L-Orn solutions (0–250 μM) were used for calibration. To verify the results obtained by this colorimetric method, the amino acid composition of the reaction mixture was determined by UPLC with the Waters AccQ-Tag amino acid analysis system as described below. Arginase activity was expressed as the average of triplicates assays as nanomoles of Orn released per minute per milligram of protein.

Arg-Related Amino Acid Contents

Samples were ground in liquid nitrogen and freeze-dried. Methanol-chloroform-water-based extractions were made on 10 mg of the resulting dry powder using the following procedure: the powder was suspended in 400 μL of a 100 μM DL-3-aminobutyric acid solution in methanol, followed by 15 min of agitation at room temperature. Two hundred microliters of chloroform was then added, followed by a 5-min agitation step. Finally, 400 μL of water was added, and samples were then vortexed vigorously to induce phase separa-

tion and centrifuged at 13,000g for 5 min. The upper phase, which contains amino acids, was transferred to a clean vial and dried under vacuum. Dry residues were resuspended in 50 μL of ultra-pure water and 10 μL was used for the derivatization using the AccQ-Tag Ultra derivitization kit (Waters). Derivatized amino acids were analyzed using an Acquity UPLC system (Waters). One microliter of the reaction mixture was injected onto an Acquity UPLC BEH C18 1.7-μm 2.1- × 100-mm column heated at 55°C. Amino acids were eluted with a mixture of 10-fold diluted AccQ-Tag Eluent A (Waters) and pure acetonitrile at a flow rate of 0.7 mL min⁻¹ according to the following gradient: initial, 99.9% A; 0.54 min, 99.9% A; 6.50 min, 90.9% A, curve 7; 8.50 min, 78.8% A, curve 6; 8.90 min, 40.4% A, curve 6; 9.50 min, 40.4% A, curve 6; 9.60 min, 99.9% A, curve 6; and 10.10 min, 99.9% A. Derivatized amino acids were detected at 260 nm using a photo diode array detector. Amino acids were characterized by cochromatography of individual standards and quantified by comparison of individual external calibration curves.

Isolation of Free Polyamines

Extraction

DWs were determined after freeze-drying. Approximately 5 to 30 mg of powdered root samples were thoroughly mixed with 800 μL of 1 M HCl supplemented with 10 μM heptanediamine, as internal standard, on a magnetic stirring plate (2,000 rpm) for 1 h at 4°C. The homogenates were then centrifuged for 15 min at 10,000g at 4°C and the supernatants collected. The pellets were further extracted twice with 600 μL of 1 M HCl and 10 μM heptanediamine and stirred for 30 min at 4°C. The homogenates were centrifuged for 15 min at 10,000g at 4°C. The combined supernatants were used as the crude extract for characterization and determination of free polyamines.

Chromatographic Analysis

Extracted polyamines were derivatized with dansyl chloride (5-dimethyl-amino-1-naphthalenesulfonyl chloride) according to the method of Smith and Davies (1985). Two hundred microliters of supernatant was added to 100 mg of sodium carbonate and 600 μL of dansyl chloride in acetone (10 mg mL⁻¹).

Table II. Genes and oligonucleotides used in the real-time RT-PCR experiments

Genes	Loci	Encoded Proteins	Primers (5'-3')
<i>Actin8</i>	At1g49240	Actin	TTACCCGACGGACAAGTGATC ATGATGGCTGGAAAAGGACTTC
<i>ADC1</i>	At2g16500	Arg decarboxylase	CCAAGGTGTGTATCCTGTGAAAT AGCTTCTAAACCGAATCGAAAAC
<i>ADC2</i>	At4g34710	Arg decarboxylase	GCGATGGACCACACAGCTTT AGAACATCCGCTGAGGACTGA
<i>AIH</i>	At5g08170	Agmatine iminohydrolase	TCGAGAATGCAAGAGAGATCGTT CATTTTCGGCGACGGAAGTA
<i>CPA</i>	At2g27450	N-carbamoylputrescine amidohydrolase	GATCAAGTCGAAAAGGCAAAGCT CCATCCATAGTAAGAAGCACCTTGT
<i>SPDS1</i>	At1g23820	Spermidine synthase	CTAATCTGGAGGATCGTTGAATTT ATAGTCGCTCTTGCAATTGTGTAC
<i>SPDS2</i>	At1g70310	Spermidine synthase	TTGCCCGTGAAGAGACCTAGA TCCACCGTTCTGTGTTCCAT
<i>SPMS</i>	At5g53120	Spermine synthase	TGGCTCCATACTCATCTTATTGAA CGCATAGTGAACACTTTTGAATG
<i>ACL5</i>	At5g19530	Spermine synthase	CCATCATTTGCGGACACATG GAGACGAAAGAAGGACCGTTTAGA
<i>SAMDC1</i>	At3g02470	S-adenosyl-Met decarboxylase	TCTTTGAGCCAAGCATCTTTCA GCAGCAGGTGAAGAATTCATCA
<i>SAMDC2</i>	At5g15950	S-adenosyl-Met decarboxylase	TCTCCGAGATCTACCTTGAATG GATTCCTATTCTCTCGTCCT
<i>ARGAH1</i>	At4g08900	Arginase/agmatinase	GTCGAGGATTATTGGTAGAAAAGG GAACGACGCAGAAATTTAGTCTATG
<i>ARGAH2</i>	At4g08870	Arginase/agmatinase	TGGTACTTTGGAGTTAATCGTTG TGTCTATGAGACCACACTTATTGC

The mixture was incubated overnight at room temperature. Three hundred microliters of aqueous Pro (150 mg mL⁻¹) was added to the mixture to remove the excess dansyl chloride. The dansylated polyamines were further extracted with ethyl acetate. The organic phase containing polyamines was dried under nitrogen stream and the residue solubilized with 800 μ L of methanol and stored at -20° C until analysis. Free polyamines were separated and quantified through HPLC after yield correction with the internal standard and calibration with external standards (Hayman et al., 1985; Duhaze et al., 2002). The mobile phase consisted of a solution of 17.5 mM potassium acetate (pH 7.17) as eluant A and acetonitrile as eluant B. The solvent gradient was modified according to Hayman et al. (1985) as follows: $t = 0$ min, 70% A; $t = 2$ min, 58% A; $t = 24$ min, 58% A; $t = 25$ min, 55% A; $t = 32$ min, 55% A; $t = 52$ min, 45% A; $t = 53$ min, 35% A; $t = 64$ min, 35% A; $t = 70$ min, 20% A; $t = 75$ min, 100% B; and $t = 80$ min, 70% A. The flow rate of the mobile phase was 1.5 mL min⁻¹. The column was then rinsed for 8 min with 70% A before the next injection. Fluorescence was measured with an excitation wavelength of 366 nm and an emission wavelength of 490 nm. The HPLC design consisted of an HP series 1050 system, an autosampler with a 20- μ L injection loop, a Shimadzu RF-10AXL fluorometer, and a Waters Spherisorb 5- μ m ODS2 column (4.6 \times 250 mm). Signals were computed and analyzed using AZUR software (Datalys).

Statistical Analysis

The data were statistically analyzed using a generalized linear model (PROC GLM of Statistical Analysis System software; SAS Institute Inc., 2000). Multiple comparisons of means were performed using Duncan's multiple-range test ($\alpha = 0.05$).

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