The plant nitrogen mobilization promoted by *Colletotrichum lindemuthianum* in *Phaseolus* leaves depends on fungus pathogenicity

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Abstract

Nitrogen plays an essential role in the nutrient relationship between plants and pathogens. Some studies report that the nitrogen-mobilizing plant metabolism that occurs during abiotic and biotic stress could be a ‘slash-and-burn’ defence strategy. In order to study nitrogen recycling and mobilization in host plants during pathogen attack and invasion, the *Colletotrichum lindemuthianum/Phaseolus vulgaris* interaction was used as a model. *C. lindemuthianum* is a hemibiotroph that causes anthracnose disease on *P. vulgaris*. Non-pathogenic mutants and the pathogenic wild-type strain were used to compare their effects on plant metabolism. The deleterious effects of infection were monitored by measuring changes in chlorophyll, protein, and amino acid concentrations. It was shown that amino acid composition changed depending on the plant–fungus interaction and that glutamine accumulated mainly in the leaves infected by the pathogenic strain. Glutamine accumulation correlated with the accumulation of cytosolic glutamine synthetase (*GS1α*) mRNA. The most striking result was that the GS1α gene was induced in all the fungus-infected leaves, independent of the strain used for inoculation, and that GS1α expression paralleled the PAL3 and CHS defence gene expression. It is concluded that a role of GS1α in plant defence has to be considered.

Key words: Amino acids, glutamine synthetase, nitrogen remobilization, senescence.

Introduction

Fungi are the group of micro-organisms that cause many of the world’s most serious plant diseases. To cause disease in plants, fungal pathogens have developed a wide variety of infectious processes from strict biotrophy to true necrotrophy. Although the penetration of the plant tissue is always a crucial step for plant–fungus interactions, the success of colonization depends on the capability of the pathogen to retrieve the nutrients from the host. This challenge can be achieved when plants contain adequate and enough nutrients. However, *in planta*, nitrogen availability seems to be limiting (Divon *et al.*, 2006, and references therein), and several fungal pathogenicity genes appear to be controlled by nitrogen starvation and may depend on nitrogen response transcription factors (Thomma *et al.*, 2006).

Superabundant or insufficient nitrogen supply is reported to influence the development of several pathogens (Huber and Watson, 1974). However, the effect of nitrogen on disease severity is variable, due to multiplicity of pathogen strategies that involve different metabolic requirements and different ways to acquire nutrients (Snoeijers *et al.*, 2000). Nitrogen fertilization has two conflicting effects, as it enhances plant defence while increasing nitrogen compounds available for pathogens in host plants. This effect seems prevalent for biotrophic and hemibiotrophic pathogens that benefit from increased metabolite pools in the host cells (Jensen and Munk, 1997; Hoffland *et al.*, 2000). By contrast, necrotrophic...
pathogen responses to nitrogen are variable (Hoffland et al., 2000; Long et al., 2000), probably because necrotrophs are able to break down plant cell elements, which allow them to use a larger range of nitrogen sources (Solomon et al., 2003).

Since the 1990s, the pathways that enable fungi to respond to the host plant nitrogen state have been investigated and partly clarified (see Solomon et al., 2003, for a review). One well-known illustration is given by the study of the avirulence gene avr9 of the tomato pathogen Cladosporium fulvum (van Kan et al., 1992; Thomma et al., 2006). Avr9 expression is induced in the case of nitrogen limitation in vitro and is regulated by the global nitrogen response factor NRF1, which suggests that C. fulvum lacks nitrogen in planta (van den Ackerveken et al., 1994; Pérez-Garcia et al., 2001). In addition, the expression of many pathogenicity genes or virulence and avirulence responses is modified by the nitrogen status in the host plant (Snoeijers et al., 2000).

Due to the tight nutrient relationships between plant and pathogen, pathogen attack is also expected to modify plant nitrogen content and, as a result, to activate important metabolic changes in the whole plant. The nutrient diversion caused by the fungus leads to drastic changes in source–sink relationships, thereby mimicking the source–sink relationship during leaf senescence (Masclaux et al., 2000). Some reports show that the plant metabolic pathways activated during abiotic stress and pathogen infection are similar to those enhanced during the senescence process (see Buchanan-Wollaston et al., 2003, for a review; Pageau et al., 2006). It was shown that biotic stress can enhance the expression of several plant genes involved in nutrient recycling, proteolysis and sugar, amino acid, and sulphur transporters (AbuQamar et al., 2006). Genes involved in the nitrogen mobilization process, normally occurring during senescence (Masclaux et al., 2000), seem to be up-regulated by stress. Several glutamine synthetase (EC 6.1.1.3), glutamate dehydrogenase (EC 1.4.1.2), and asparagine synthetase (EC 6.3.5.4) genes have been induced in response to pathogen inoculations (Pérez-Garcia et al., 1995, 1998a, b; Olea et al., 2004; Pageau et al., 2006; AbuQamar et al., 2006). The nitrogen mobilization promoted by infection could be considered as part of a slash-and-burn strategy that deprives the pathogen of nutrients. Nutritional and metabolic changes might occur as a defense against pathogen development (Hammond-Kosack and Parker, 2003). During the co-evolution with plants, pathogenic fungi may have become adapted to the modifications in plant nitrogen content caused by biotic stress, ultimately turning metabolic changes to the benefit of the pathogen (Parniske, 2000; Hammond-Kosack and Parker, 2003). Nevertheless, the importance of metabolic pathways in plant defence remains poorly studied (Solomon et al., 2003).

Colletotrichum lindemuthianum is a hemibiotrophic fungus that causes anthracnose disease on common bean (Perfect et al., 1999). This interaction is assumed to fit the gene-for-gene interaction (Flor, 1971). During the biotrophic phase of infection that lasts three to five days and starts six days after spore germination, the fungus differentiates infection vesicles and primary hyphae, and probably utilizes apoplastic fluid nutrients to feed on. The transition to a necrotrophic phase at six days post-inoculation suggests that the fungus cannot find the nutrients needed to develop and then undergoes necrotrophic growth to make more nutrients available. C. lindemuthianum can utilize a wide range of nitrogen sources. Ammonia and glutamine are preferentially used, but when these primary sources are absent or present in low concentrations, other secondary sources, such as aspartate, asparagine, or alanine can be used (Pellier et al., 2003).

The aim of this work was to determine to what extent C. lindemuthianum infection is able to affect plant metabolism and to investigate if the plant infection impaired the nitrogen recycling and mobilization metabolism of the host, as was shown for other plant–microbe interactions (Pérez-Garcia et al., 1995, 1998a, b; Olea et al., 2004; Pageau et al., 2006). The availability of non-pathogenic C. lindemuthianum mutants blocked at different stages of the infection cycle offers a good opportunity to analyse the drastic changes enabling the pathogen to metabolize plant cell elements after the penetration and the transition from biotrophic to necrotrophic stages (Parniske, 2000). The non-pathogenic clk1 mutant is unable to penetrate the plant leaf cuticle (Dufresne et al., 1998). However, it was shown that the contact of mutant spores and appressoria on the cuticle of the plant leaves, was sufficient to induce plant cell defence genes (Veneault-Fourrey et al., 2005). The clt1 mutant is also unable to cause lesion formation because it is stopped at the switch between the biotrophic to the necrotrophic phase. It cannot develop secondary hyphae (Dufresne et al., 2000). In the present study, the clt1 and clk1 mutants have been used to investigate the metabolic disturbance specifically induced during the biotrophic phase of infection. The modification of the amino acid and glutamine synthetase contents, in Phaseolus vulgaris leaves when infected by pathogenic and non-pathogenic strains of C. lindemuthianum, suggests that N mobilization is promoted by infection through the induction of GSI α gene.

Materials and methods

Fungal strains and culture conditions

Inoculi of the wild-type strain UPS9 (Fabre et al., 1995), the clt1 R255 strain (Dufresne et al., 2000) and the clk1 mutant H290 strain (Dufresne et al., 1998) were prepared from 10-d-old 3N+ plate cultures of C. lindemuthianum as described by Dufresne et al. (1998). C. lindemuthianum mycelium was grown in Roux’s flasks.
containing 50 ml of liquid potato dextrose medium. After 72 h of culture, mycelium was rinsed with water, filtered, and stored at –80 °C for further experiments.

**Plant material and infection assays**

The common bean (P. vulgaris) cv. La Victoire (Tezier, Valence-sur-Rhone, France), which is very susceptible to anthracnose, was used in all assays. Plantlets of common bean were grown as described previously (Dufresne et al., 1998). Ten-day-old 3N+ plate cultures of C. lindemuthianum were used to prepare conidial suspensions at a concentration of 10⁶ conidia ml⁻¹ for spray inoculation of 8-d-old whole plantlets. At 8 d after sowing plantlets harvested only two cotyledons. Whole plantlets were incubated at 19 °C in growth chambers, as described by Dufresne et al. (1998). The plantlets were covered with a plastic cap in order to maintain high humidity. For metabolic and molecular analysis, the two sprayed cotyledons of three plantlets were harvested at 0, 1, 2, 3, 6, 8, and 12 d post-inoculation (DPI). Harvests were performed at 11.00 h. Harvested material was pooled and immediately frozen in liquid nitrogen, then stored at –80 °C. The infection assay was replicated three times.

**Extraction of protein, enzymatic assays, and western blot analysis**

All extractions were carried out at 4 °C from frozen material stored at –80 °C. Samples were ground in liquid nitrogen to a fine powder with 30 mg of PVP (polyvinylpyrrolidone), before the addition of the extraction buffer (5 ml g⁻¹ fresh material). The extraction buffer was a TRIS–HCl buffer (pH 7.6; 25 mM) with MgCl₂ (1 mM), EDTA (1 mM), β-mercaptoethanol (1.5 mM), and leupeptin (4 μM). Protein contents were monitored in the supernatant of the same extracts after centrifugation at 15 000 rev. min⁻¹ for 5 min. The total soluble protein content was measured using a commercially available kit (Coomassie Protein assay reagent, Bio-Rad, California, USA). The remaining supernatant was then used to measure enzyme activities.

The GS activities were measured according to Masclaux et al. (2000).

Soluble proteins were separated by SDS–PAGE (Laemmli, 1970). An equal amount (5 μg) of protein was loaded in each track. The percentage of polyacrylamide was 10%. GS2 and GS1 detection was performed according to Masclaux et al. (2000), using polyclonal antibodies raised against tobacco GS2 (Becker et al., 1992). The relative amounts of polypeptides or transcripts were determined by using densitometric scanning of membranes (Photoshop5.1) and an advanced quantification tool (NIH image 1.3, public domain, http://rsb.info.nih.gov).

**Chlorophyll and metabolite extraction and determination**

Total leaf chlorophyll was measured in aliquots of the crude protein extract (100 μl) and determined as described by Arnon (1949). Leaf aliquots were ground in liquid nitrogen before the extraction in a 2% solution of 5-sulphosalicylic acid (100 mg FW ml⁻¹) at 4 °C. After centrifugation (15 000 rev. min⁻¹, 2 °C for 5 min) ammonium content and total amino acid content were measured and the individual amino acid composition was determined using ion-exchange chromatography and ninhydrin detection (Rochat and Boutin, 1989) as previously described by Masclaux-Daubresse et al. (2002).

**Extraction of total RNA and northern blot analysis**

Total RNA was extracted from plant material stored at –80 °C and northern blot analysis was performed as described previously (Masclaux et al., 2000). Specific ³²P-labelled probes for GS1 (Gebhardt et al., 1986; Lightfoot et al., 1988; Bennett et al., 1989), PAL3, CHS, and EF1-α (Veneault-Fourrey et al., 2005) from P. vulgaris were used for mRNA detection. Hybridizations were performed under high stringency conditions at 65 °C. Filters were washed with 2 × SET (0.06 M TRIS–HCl pH 8, 0.3 M NaCl, 4 mM EDTA) at room temperature for 5 min and at 65 °C for 10 min. Additional washing was performed successively using 1 × SET and 0.5 × SET at 65 °C for 15 min before drying and exposure to X-ray film.

**Results**

Colletotrichum infection affects leaf chlorophyll, protein, and amino acid concentrations

As previously described (Dufresne et al., 1998, 2000; Pellier et al., 2003; Veneault-Fourrey et al., 2005), infection of whole plantlets of the common bean with the wild-type strain UPS9 of C. lindemuthianum induced the outbreak of brown lesions on the main vein between 4 d and 5 d after inoculation. Symptoms extended further to secondary veins and, finally, led to the complete maceration of the whole leaf within 6–7 DPI. Inoculation with the two non-pathogenic strains, R255 and H290, was unable to produce anthracnose symptoms even after a long incubation.

Chlorophyll, protein, total free amino acid, and sugar concentrations can be used as senescence biomarkers. In this study, the effect of infection was monitored in a time-course experiment and biomarker changes depend on both (i) leaf ageing and (ii) infection. The water-sprayed leaves represent controls (i) for leaf ageing related modifications and (ii) as non-infected leaves.

For all the treatments, total chlorophyll increased and reached a maximum two days after inoculation (Fig. 1A). Thereafter chlorophyll contents decreased progressively in a similar manner in control leaves and in R255- and H290-inoculated leaves. UPS9 infection led to a stronger chlorophyll decrease than infection with the other strains from 6 DPI, when lesion symptoms appeared.

Protein contents were similar until 6 DPI in all the leaves. From 6 DPI until 12 DPI a progressive and similar decrease was observed in the control leaves and in the R255- and H290-inoculated leaves (Fig. 1B). A decrease was also observed for leaves inoculated with UPS9 and it was more pronounced than in other leaves at 6 DPI and 8 DPI. At 12 DPI, a sudden and huge increase in soluble protein content was observed in the UPS9-infected leaves. The separation and staining of proteins on gels (data not shown) as well as western blotting showed that the 12 DPI protein accumulation was certainly due to fungus development since none of the well-known plant proteins, such as Rubisco, could be detected at this stage in the UPS9-infected leaf extracts.

Since protein content decreased with ageing but accumulated in UPS9 infected leaves at 12 DPI, ammonium...
and amino acid contents were measured. Free ammonium content in bean leaves was low (under 0.5 μmol g⁻¹ FW) and did not fluctuate during infection, except in UPS9-infected leaves tissue at 12 DPI where it reached 1.98 μmol g⁻¹ FW (data not shown). The total free amino acid contents changed in a similar manner whatever the treatment, except at 12 DPI for the UPS9-inoculated leaves, which contained 5-fold more amino acids than at previous DPI (Fig. 1C).

Amino acid contents in healthy and infected Phaseolus and in Colletotrichum mycelium and conidia

The content of individual amino acids was monitored and the major amino acids found in healthy bean leaves were glutamate (Glu, 30%), aspartate (Asp, 10%), histidine (His, 10%), serine (Ser, 8%), and alanine (Ala, 8%) (Table 1). Amino acids were also determined in the C. lindemuthianum mycelium grown in Petri dishes. An equal amount of alanine, glutamate, and glutamine (Gln) were measured (17% of the total free amino acid pool each). γ-amino butyric acid (GABA) and arginine (Arg) represented 10% and 8.5%, respectively, and asparagine (Asn) and serine accounted for 3%. In conidia, glutamate and γ-amino butyric acid were the most abundant amino acids (16%) with arginine and alanine accounting for 9% (Table 1).

During infection, the threonine, cysteine, tyrosine, valine, histidine, phenylalanine, γ-amino butyric acid, leucine, and isoleucine percentages were similar in the control leaves and in fungus-infected leaves (data not shown). The main differences between control and infected leaves were observed for glutamate, proline (Pro), aspartate, arginine, glutamine, phospho-serine (P-Ser), glycine (Gly), and alanine (Fig. 2).

Glutamate, which represents 30% of the total free amino acids in water-treated bean leaves at the beginning of the assay, rose to 40% at 3 DPI and then decreased and represented 20% at 12 DPI for all the treatments. The decrease was more pronounced in UPS9 infected leaves.

Proline biosynthesis directly depends on glutamate metabolism and proline content fluctuated in a similar way to glutamate content. The proportion of proline was similar in water and in H290- and R255-inoculated leaves. At first, proline was 2% of total amino acids, and its amount slightly rose to 4% at 3 DPI. Proline then remained constant until 12 DPI, except in UPS9 leaves, in which, like glutamate, the proportion of proline decreased at 8 DPI and 12 DPI.

Aspartate is a one-step product of glutamate through aspartate amino transferase. However, aspartate fluctuated independently of glutamate content. In the leaves of the control, the proportion of aspartate constantly decreased with ageing, from 8% to 2%. In leaves infected with H290, R255 or UPS9, the proportion of aspartate was higher than in the control and remained around 6% until 6 DPI. At 8 DPI, aspartate suddenly rose to 14% in the UPS9-infected leaves then suddenly decreased to 2% at 12 DPI. By contrast, the proportion of aspartate increased to 13% in leaves sprayed with the mutants.

Arginine was not very abundant in bean leaves and represented around 0.5% of total amino acids in healthy leaves. Arginine fluctuated throughout the time-course experiment in a similar way to the control, the R255- and the H290-inoculated leaves. By contrast, in the UPS9-inoculated leaves, the proportion of arginine was higher and increased continuously from 0 to 12 DPI to reach 5%.
Table 1. Proportion of major individual free amino acids detected in extracts from healthy *P. vulgaris* cotyledons and *C. lindemuthianum* mycelium and conidia

Values indicate individual amino acid contents as a percentage of the total amino acid pool. Amino acid composition was analysed in five cotyledons of *P. vulgaris* and the mean ±SE is presented. Analysis of *C. lindemuthianum* mycelium and conidia amino acid pools were performed on a mix of several mycelium and conidia preparations.

<table>
<thead>
<tr>
<th>Amino acids (g % total amino acids)</th>
<th><em>P. vulgaris</em> cotyledonary leaves</th>
<th><em>C. lindemuthianum</em> mycelium</th>
<th><em>C. lindemuthianum</em> conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala 8.7±2.0</td>
<td>17.5</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>Arg 0.4±0.13</td>
<td>8.7</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>Asn 4.1±2.6</td>
<td>3.8</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Asp 10.2±1.7</td>
<td>0.3</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>GABA 3.0±1.2</td>
<td>10.4</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>Gln 0.9±1.0</td>
<td>16.5</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Glu 30.1±2.2</td>
<td>17.4</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>Gly 1.2±0.6</td>
<td>1.9</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>His 14.1±3.9</td>
<td>0.5</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>Pro 1.8±0.4</td>
<td>–</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Ser 8.0±1.3</td>
<td>3.4</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>Thr 3.6±0.29</td>
<td>1.4</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Trp –</td>
<td>–</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Tyr 0.4±0.13</td>
<td>0.8</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Val 2.7±0.48</td>
<td>2.2</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Others 10.8</td>
<td>15.2</td>
<td>21.8</td>
<td></td>
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</table>

Glutamine was very low in bean leaves, representing only 1% of the total amino acids. However, at 6 DPI, glutamine increased to 8% of the total amino acids in the UPS9- and the R255-infected leaves. In the H290-infected leaves glutamine was higher than the control and represented 4%. At 8 DPI, glutamine was around 8% in all the fungus-infected leaves. At 12 DPI, the UPS9-treated leaves accumulated even more glutamine (33%), whereas glutamine content in the R255- and H290-treated leaves returned to similar levels as the control leaves (2%).

Phosphoserine is involved in a serine biosynthetic pathway that does not depend on photorespiration (Ho and Saito, 2001). Phosphoserine content was low (1.5%) and similar in control leaves and in the R255- and H290-infected leaves, whereas it continuously increased to 8% at 12 DPI in leaves inoculated with UPS9.

By contrast with glutamine, phosphoserine or alanine, the proportion of glycine only increased in the control leaves with ageing from 2% to 30% at 12 DPI. For all the fungus-infected leaves, the proportions of glycine remained under 10%. For all treatments, the fluctuation of the alanine content was surprising. Whereas alanine represented 9% of the total amino acids in healthy leaves and in infected leaves at 1 DPI, the levels decreased to 2–3% at 2 DPI in all leaves. Nevertheless, whereas alanine content continuously decreased to 1% in the control leaves at 12 DPI, alanine increased in all the fungus-infected leaves to 4–5% at 12 DPI.

**UPS9 plant infection triggered the decrease of GS total activity and the increase of GS1 protein content**

Enzymes suspected to play a role in nitrogen recycling during stress are cytosolic glutamine synthetase (GS1) and the glutamate dehydrogenase (GDH) (Pageau et al., 2006). The GS activity measured *in vitro* is the sum of the cytosolic GS1 and the chloroplastic GS2 activities. In the H2O-sprayed leaves, the total GS activity was maintained constant at 1000 nmol min⁻¹ g⁻¹ FW until 8 DPI, and decreased slightly to 800 nmol min⁻¹ g⁻¹ FW at 12 DPI (Fig. 3A). In the leaves of the plants inoculated with the R255 or the H290 strains, the GS activity was similar to the control at the first time points of the experiment, but decreased earlier than the control (at 6 DPI) and to a lower value than the control (700 nmol min⁻¹ g⁻¹ FW). In the UPS9-infected leaves, the GS activity decreased significantly and dramatically after 3 DPI and activity was almost undetectable at 12 DPI.

Polyclonal antibodies raised against the tobacco GS2 were used to detect GS2 and GS1 isoforms through western blot experiments (Fig. 3B). The GS2 and GS1 patterns observed were similar in the bean soluble protein extracts and in the tobacco protein extract used as the control (data not shown; Masclaux et al., 2000). In extracts from the control leaves and from R255- and H290-infected leaves, no obvious changes in the GS2 and GS1 contents were detected depending on leaf treatment or during leaf ageing. The main isoform occurring in leaves was GS2, and only a slight signal for GS1 was detected. By contrast, the signal for the GS1 isoform was much more clearly detectable in extracts from UPS9-infected leaves. In the UPS9-infected leaves, GS1 signal was increased throughout the time course and was noticeable at 6 DPI and 8 DPI. At 8 DPI the GS1 signal was as strong as the GS2 signal (Fig. 3C). By contrast with the GS1 increase, a GS2 decrease was observed from 6 DPI; this decrease was pronounced at 8 DPI. At 12 DPI, no GS1 or GS2 signal could be detected because the protein content in UPS9-infected leaves was very low.
a specific probe against GS1 α (Gebhardt et al., 1986) was used and the absence of cross hybridization with GS2 (gs-δ) or other GS1 (β and γ) was tested in our conditions through Southern blots (data not shown). The level of GS1 α mRNA was constant and low in control leaves throughout the time-course. In leaves infected with the UPS9 strain, GS1 α mRNA content increased at 1 DPI compared with 0 DPI and appeared slightly higher than in the water-sprayed control at 1 DPI. Afterwards and when the necrotrophic stage was reached (at 6 DPI and 8 DPI), a strong increase in GS1 α mRNA content was observed. Surprisingly, inoculation with the two non-pathogenic mutants R255 and H290 also increased GS1 α mRNA contents compared with control. This increase was not as pronounced as with UPS9 infection, but the difference between R255- and H290-infected leaves and the water control was clearly observable after 3 DPI, thus showing that both R255 and H290 inoculation led to a long-term GS1 α induction. Note that induction of GS1 α was detected earlier in the R255-infected leaves than in the H290-infected leaves (Fig. 4A).

To determine whether changes in the GS1 α mRNA content are related to plant defence, the expression of the defence markers PAL3 and CHS was monitored (Fig. 4). No expression of PAL3 or CHS was detected in the water-sprayed leaves. In contrast to the control, PAL3 and CHS were induced by fungus infection. Interestingly, the strain R255 seems to trigger an earlier accumulation of both GS1 α and CHS mRNA compared with the H290 strain.

Fig. 2. The amino acid composition of P. vulgaris leaves depends on Colletotrichum pathogenicity. Amino acids of bean leaves sprayed with water (control) or inoculated with C. lindemuthianum wild type (UPS9) or mutant (R255 and H290) strains were analyzed during the course of infection. Free amino acids were separated through chromatography and proportions of individual amino acid determined as a percentage of the total amino acid content. Results were obtained after pooling the three amino acid samples on the basis of equal total free amino acid concentrations. Two independent infection repeats were performed and gave similar results, only one repeat is presented.
**Discussion**

In a previous report (Pageau *et al.*, 2006), the effect of biotic and abiotic stresses on the expression of markers involved in primary nitrogen assimilation, and on markers involved in nitrogen recycling and mobilization was monitored. Pageau *et al.* (2006) used tobacco as a plant model and infections were performed using several *Pseudomonas syringae* and virus strains. Results showed that depending on compatible and incompatible interactions, the GDH and GS1 markers were differentially induced. Whereas GDH seemed to be induced when cell death was triggered by disease or hypersensitive reaction, it appeared that GS1 was preferentially induced when the plant–bacteria relationship was incompatible. Those results raised the question about a possible role of GS1 in the plant defence process.

In the present work, the metabolic changes in the plant were investigated in a well-characterized plant–pathogen interaction involving *Colletotrichum lindemuthianum* and its host *Phaseolus vulgaris*. Indeed, the development of a pathogen and especially of a fungus can be considered as an additive sink for the plant. This led us to consider if fungus development leads to the induction of some physiological process related to nitrogen remobilization via the induction of enzymes involved in amino acid catabolism and remobilization, such as GS1 and GDH. Moreover, our knowledge about the physiology of plant leaves during senescence and under biotic and abiotic stress (Masclaux *et al.*, 2000; Chaffei *et al.*, 2004; Pageau *et al.*, 2006) raised the question about changes in amino acid contents during infection. *C. lindemuthianum* induces the outbreak of brown lesions on the main vein between 4 d and 5 d after inoculation, thus enhancing the necrotrophic phase of the infection process. The *clk 1* and *clta 1* mutants that are unable to cause any lesions, because they are unable to penetrate the cuticule (H290 strain) or stopped at the switch between the biotrophic to the necrotrophic phase (R255 strain) respectively (Dufresne *et al.*, 1998, 2000), are however recognized by the plant and lead to plant defence induction. However, the senescence-like symptoms (protein and chlorophyll decrease) were only observed in the leaves infected with the wild-type UPS9 strain, thus suggesting that the degradation of cellular components was accelerated by disease.

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**Fig. 3.** UPS9 plant infection triggered the decrease of GS total activity and the increase of GS1 protein content. Bean leaves were sprayed with water (control) or inoculated with *C. lindemuthianum* wild type (UPS9) or mutant (R255 and H290) strains and total GS activity (A), GS1 protein content (B), and GS1 and GS2 protein densitometric quantification in UPS9 infected leaves (C) were measured. In the western blot experiment, equal amounts of protein were loaded in each lane. Results are the means ± SD of three samples. Two independent infection repeats were performed and gave similar results, only one repeat is presented.
Strong effects were observed on nitrogen content and quality depending on infection. Whereas the changes in the global amino acid and ammonium pools were not significant except at 12 DPI in UPS9-infected leaves, plant inoculation with the fungus led to changes in individual amino acid proportions.

The most striking results were that the wild-type UPS9 strain led to a dramatic decrease in glutamate and proline content while it dramatically increased both phosphoserine and arginine content. Since these effects appeared specifically during the UPS9-infection, they might depend on fungal metabolism. Indeed, mycelium analysis showed that mycelium contains as much as 8% of arginine when grown in vitro, and one can therefore propose that the increase in arginine might be a metabolic marker to monitor fungal biomass in plants. The glutamate and proline depletion might be due to fungus feeding as well as to amino acid interconversion through plant transamination.

The amounts of aspartate, glutamine, and alanine were increased by fungus infection independent of the strain used for inoculation. Surprisingly, the alanine content was 3-fold lower at 1 DPI compared with 0 DPI for all the treatments. The reason for such a decrease still remains unknown and several hypotheses can be proposed, related to the fact that plants were placed after inoculation in humid individual greenhouses to favour infection. Indeed, it is known that alanine can be modulated depending on the stress (hypoxia) (Miyashita et al., 2007) and photorespiratory conditions (Igarashi et al., 2006). Whereas the amount of glycine continuously increased in bean leaves with ageing, it remained significantly lower in fungus-infected leaves. These findings suggested that plant metabolism rather than fungus development was involved in those modifications. Changes in several pathways might have been involved. Changes in aspartate, alanine, and glycine contents suggested, for example, that plant infection with the fungus might have affected the leaf photorespiratory pathway. In addition, the transient increase in glutamine amount suggested that N-remobilization was enhanced leading to glutamine biosynthesis. Interestingly, the glutamine increase appeared at 6 DPI in the leaves that were infected with pathogenic and non-pathogenic fungi, thus suggesting that N-remobilization can be triggered even if the disease process was impaired. Moreover, as the 6 DPI time point is the checkpoint of the switch from the biotrophic to the necrotrophic phase, it is possible that there was a relationship between the increase in glutamine and the onset of the necrotrophic phase. A role of glutamine as a plant signal able to alert the fungus to nitrogen translocation can be suggested. It is quite surprising to observe that in other plant–pathogen interactions like Brassica napus/Leptosperma maculans, the necrotrophic phase also seems to start when the plant begins to mobilize nitrogen (Rossato et al., 2001).

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**Fig. 4.** The GS1α expression paralleled the infection-dependent PAL and CHS induction. Bean leaves were sprayed with water (control) or inoculated with C. lindemuthianum wild type (UPS9) or mutant (R255 and H290) strains. An equal amount of total RNA (10 μg) was loaded per lane for northern experiment and the GS1α expression (A) as well as the CHS and PAL3 gene defence expression (B) were followed. EF1-α probe was used as control of mRNA loading (B). Densitometric quantification of GS1α mRNA was expressed for each treatment as the percentage of the value measured at 0 DPI (A). Two independent infection repeats were performed and gave similar results, only one repeat is presented.
Enzymes suspected of controlling nitrogen remobilization are cytosolic glutamine synthetase and glutamate dehydrogenase (Masclaux et al., 2000). GS and GDH activities were measured and it was observed that, as in Pageau et al. (2006), the total GS activity decreased with the progress of plant disease, thus suggesting that primary nitrogen assimilation through the GS2/GOGAT pathway was affected by UPS9-infection and the GDH activity increased, thus suggesting that N-remobilization was favoured in UPS9-infected leaves (data not shown). In protein extracts from UPS9-infected leaves a large increase in the GS1 isoform was detected from an early stage of infection, thus confirming that nitrogen remobilization was certainly induced. A decrease in GS2 protein paralleled the increase in GS1 protein, thus explaining the decrease in the total GS activity.

Since the GS1 isoform appeared more abundant in UPS9-infected leaves, and since GS1α was shown previously to be induced by wounding (Watson and Cullimore, 1996), GS1α transcripts were monitored. The increase of bean GS1α mRNA in the UPS9-infected leaves was clearly observed at 6 DPI and 8 DPI and was consistent with the increase in GS1 protein. A more surprising result was the induction of GS1α mRNA in leaves infected with the non-pathogenic strains R255 and H290. Increase in GS1α mRNA was detected from 3 DPI in R255-infected leaves and from 6 DPI in H290-infected leaves. With regard to Watson and Cullimore (1996) and to Pageau et al. (2006), this result confirms that the plant GS1α is induced like defence genes. Indeed, like the PAL3 and CHS genes, GS1α expression was induced by the pathogenic fungus as well as by non-pathogenic mutants. Moreover, the timing of the expression of GS1α, PAL3, and CHS was similar and depended on the fungal strain. It is interesting to note that the accumulation of GS1α mRNA in UPS9-, R255-, and H290-infected leaves paralleled the accumulation of glutamine in bean tissues. This strongly suggested that bean leaves responded to pathogen recognition by inducing GS1α, and that this resulted in glutamine accumulation in infected leaf tissues. Another explanation for glutamine accumulation could be that after 6 DPI, UPS9 triggered lesions close to the main and secondary veins that may have affected the phloem loading of glutamine and thereby nitrogen export. In the case of R255- and H290-infection, glutamine may have increased only transiently because the phloem flux was not affected. However, the difference between the GS1α expression of H290- and R255-infected leaves remains to be elucidated. Glutamine accumulation occurred at 6 DPI, the time when necrotrophy is normally enhanced. Interestingly it was shown that the Clnr1Colletotrichum mutant, which is affected in a AREA/NIT2-like nitrogen assimilation regulator and impaired in glutamine assimilation, was unable to complete its infection cycle and was specially affected after 6 DPI (Pellier et al., 2003). Therefore, the process of N-remobilization seems to be correlated with the onset of necrotrophy and a role of glutamine in the cross-talk between plant and fungus has to be considered.

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