The two senescence-related markers, GS1 (cytosolic glutamine synthetase) and GDH (glutamate dehydrogenase), involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in *Nicotiana tabacum* L. leaves

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Abstract

To investigate the role of stress in nitrogen management in plants, the effect of pathogen attack, elicitors, and phytohormone application on the expression of the two senescence-related markers GS1 (cytosolic glutamine synthetase EC 6.3.1.2) and GDH (glutamate dehydrogenase, EC 1.4.1.2) involved in nitrogen mobilization in senescing leaves of tobacco (*Nicotiana tabacum* L.) plants, was studied. The expression of genes involved in primary nitrogen assimilation such as GS2 (chloroplastic glutamine synthetase) and Nia (nitrate reductase, EC 1.6.1.1) was also analysed. The *Glubas* gene, coding a β-1,3-glucanase, was used as a plant-defence gene control. As during natural senescence, the expression of GS2 and Nia was repressed under almost all stress conditions. By contrast, GS1 and GDH mRNA accumulation was increased. However, GS1 and GDH showed differential patterns of expression depending on the stress applied. The expression of GS1 appeared more selective than GDH. Results indicate that the GDH and GS1 genes involved in leaf senescence are also a component of the plant defence response during plant–pathogen interaction. The links between natural plant senescence and stress-induced senescence are discussed, as well as the potential role of GS1 and GDH in a metabolic safeguard process.

Key words: Glutamate dehydrogenase, glutamine synthetase, leaf senescence, nitrate reductase, nitrogen metabolism, plant–pathogen interaction, stress.

Introduction

Nitrogen nutrition has a significant impact on plant disease development. The size of lesion areas that develop when fungal or bacterial pathogens infect plants is changeable depending on plant nutrition (Hoffland *et al.*, 2000; Long *et al.*, 2000). Reduced availability of nitrogen often increases the susceptibility of plants to diseases. Nitrogen limitation also affects pathogenesis and the form of nitrogen available to plants and pathogens can also affect the severity of the disease (Solomon *et al.*, 2003).

Many reports have shown that nitrogen-limiting conditions can induce genes of pathogenic bacteria and fungi in the same way as during infection (Talbot *et al.*, 1997). In some plant–microbe interaction models, the pathogenicity (*Path*), virulence (*Vir*), and avirulence (*Avr*) genes that are highly expressed in planta were also induced when axenic cultures were subjected to nitrogen limitation (Snoeijers *et al.*, 2000). For example, the avirulence gene *AvrD* from *Pseudomonas syringae* pv. *tomato* was highly induced upon colonization of host tissues and also when bacteria were growing in media containing a low concentration of nitrogen (Shen and Keen, 1993). The avirulence gene *Avr9*...
from the biotrophic fungal pathogen Cladosporium fulvum (Van Den Ackerveken et al., 1994) was also induced in planta and during nitrogen starvation in vitro (Snoeijers et al., 1999). The mpg1 pathogenicity gene from the rice blast fungus Magnaporthe grisea was expressed during pathogenesis and in axenic culture during nitrogen or glucose limitation (Lau and Hamer, 1996).

When pathogens are entering into their host plant, they are confronted with new and changing nutritional environments that lead them to adopt a new trophic behaviour. In Colletotrichum gloeosporioides an elevated expression of glutamine synthetase (GS) occurred during disease development (Stephenson et al., 1997), particularly during the early stages after infection. The transcripts of the fungal GS were also induced by nitrogen starvation in axenic cultures. Many studies on plant–fungus interactions have also suggested that nutrient deprivation in axenic culture might mimic growth conditions during the early stages of the infection process (Stephenson et al., 1997). It has been proposed that the pathogen genes, which are induced by nitrogen starvation, may play an important role in pathogenicity.

The infection of plants by bacterial and fungal pathogens also alters the nitrogen metabolism of the host plant. The physiological processes induced during pathogen infection appear similar to natural senescence. For example, the infection of tomato leaves by P. syringae pv. tomato induced the accumulation of a novel cytosolic glutamine synthetase (GS1) isoform, very similar to the GS1 isoform induced in tobacco leaves during senescence (Pérez-García et al., 1995, 1998; Brugière et al., 2000; Masclaux et al., 2000). The tabtoxin produced by P. syringae irreversibly inhibited the GS activity of the host plant and resulted in chlorosis of the leaves and senescence-like symptoms (Turner and Debbage, 1982; Bender et al., 1999). Moreover, in most cases, the stress response pathways appeared to be activated after the onset of senescence. Links between the expression of Senescence Associated Genes (SAG) and the pathogen response were indicated by the discovery that pathogenesis-related (PR) genes are expressed during the senescence of healthy leaves (Quirino et al., 1999). Conversely, transcripts of genes identified as being senescence-enhanced were detected in leaves exposed to many different stresses such as pathogen infection (Butt et al., 1998; Pontier et al., 1999) and ozone response (Miller et al., 1999). Signalling molecules such as salicylic acid (SA), jasmonic acid (JA) or ethylene have also been implicated in complex interconnecting pathways that control gene expression in plant–pathogen and stress responses as well as during senescence (Buchanan-Wollaston, 1997).

The aim of this study was to investigate the effect of biotic and abiotic stresses on the expression of the senescence-enhanced GS1 and GDH genes of tobacco. It has previously been shown that leaf nitrogen metabolism is modified when plants senesce (Masclaux et al., 2000). Leaf senescence represents the final stage of development and is characterized by the transition from nutrient assimilation to nutrient remobilization (Masclaux et al., 2001; Hörtensteiner and Feller, 2002). The expression of enzymes involved in the primary assimilation of nitrogen [nitrate reductase (NR), nitrite reductase (Nir), glutamate synthase (GOGAT), and chloroplastic glutamine synthetase (GS2)] decreased with age, and, at the same time, glutamate dehydrogenase (GDH) and cytosolic glutamine synthetase (GS1) transcripts, proteins and activities increased (Masclaux et al., 2000). It was also shown that the rate of senescence and nitrogen remobilization is related to the nitrogen nutrition status of the plant and to source/sink relationship (Kamachi et al., 1991; Ono and Watanabe, 1997; Crafts-Brandner et al., 1998; Masclaux et al., 2000).

The effect of infection with viruses and bacteria was investigated. The effect of fungal elicitors (cryptogein and Onozuka R10) was also analysed. Signal molecules such as ethylene, salicylic acid and jasmonic acid, known to be implicated in both pathogen response and senescence pathways were tested. Since many studies also reported the involvement of oxidative stress in senescence and pathogen-related processes (Buchanan-Wollaston et al., 2003), hydrogen peroxide was used as a signal in this study. The physiological role of the nitrogen mobilization makers, GS1 and GDH, in response to stress-induced senescence is discussed.

Materials and methods

Plant material and growth

Tobacco plants (Nicotiana tabacum cv. Xanthi G28 and Nicotiana tabacum cv. Xanthi XHFD8; INRA, Versailles, France) were grown in a clay loam soil in a greenhouse under natural lighting (temperature ranging between 20°C and 30°C). Plants were watered every 2 d with N12 (10 mM NO3- and 2 mM NH4+) until flowering. At this stage, plants developed around 30 leaves. In all these experiments, mature leaves, ranging between the 15th and 17th leaf from the bottom were used (Masclaux et al., 2000).

Pathogen infection

In order to test the effect of pathogen infection on the expression of genes related to nitrogen metabolism, the following experiments were carried out. Two month-old N. tabacum cv. Xanthi G28 plants were inoculated with three viruses: CMV (Cucumber Mosaic Virus, Cucumovirus), TEV (Tobacco Etch Virus, Potyvirus), and PVY (Potato Virus Y, Potyvirus) (Beclin et al., 1998). Three weeks after inoculation, fully expanded leaves, located at similar positions along the main axis of infected and uninfected tobacco plants were collected and frozen in liquid nitrogen before storing at –80°C. Two independent biological experiments were performed.

Two strains of Pseudomonas syringae pv. syringae, CFBP3077 (compatible reaction) and CFBP3077 hrp mutant, and one strain of Pseudomonas syringae pv. tabaci, CFBP1503 (compatible reaction), kindly provided by C. Manceau, INRA Angers, France, were used for bacterial infections. Bacteria were grown in 100 ml of LB medium until OD reached a value of 0.7. After centrifugation, the pellet was resuspended in 25 ml of MgCl2 (10 mM) and 1 ml of bacterial suspension was used for the inoculation of...
the leaves (Dangl et al., 1992). Each bacterial strain was infiltrated sub-epidermally using a syringe into fully expanded leaves (similar leaf position) of four to six tobacco plants. Four control plants were infiltrated with H2O. Tobacco leaf tissues containing the inoculated spot plus a 2 cm surrounding area were collected from six tobacco plants at 0 h (non-infiltrated), 2 h, 5 h, 9 h, 21 h, and 5 d after inoculation. Tissues were frozen in liquid nitrogen before storing at −80°C. Two independent biological experiments were carried out.

**Elicitors and signal molecules**

Leaf disc tissues (1 cm²) were randomly collected from mature leaves of 12 tobacco plants, avoiding the veins, and pooled. Discs were then rinsed in distilled water and floated on 30 ml of buffer (10 mM MES pH 6.5, 40 mM KCl, and 10 mM CaCl₂) in Petri dishes in a growth chamber (16 h light, 350–400 μmol photons m⁻² s⁻¹, 26°C; 8 h dark, 18°C). Signal molecules were added or not (control) to buffer solutions. Floating assays began at 11:00 h.

In order to test the effect of elicitors on the expression of nitrogen metabolism genes, cryptogeen (kindly provided by J-C Pernollet, INRA Jouy-en-Josas, France) was dissolved in buffer to final concentrations of 50 μM and 500 μM (Ricci et al., 1989). Onozuka R10 (Yakult Biochemicals Co, Ltd) was also diluted in buffer to 0.05% and 0.1% (Moreau-Mihri et al., 1996). Since oxidative stress occurs frequently during pathogen infection, the effect of 1 mM and 5 mM of H2O₂ (Sigma) was analysed. The effect of signal molecules implicated in stress response pathways was also tested and leaf discs were floated for 5 h and 21 h on 50 μM, 100 μM, and 1 mM of sodium salicylate pH 7 (Sigma) or jasmonic acid (Sigma). Buffers without the signal molecules were used as controls.

In order to test the effect of ethylene, this was generated by using 1 M or 5 M ethephon (2-chloroethylphosphonic acid, XTR4, CFP) solutions. Ethephon was injected in the septum of 500 ml flasks containing the leaf discs floated on 50 ml buffer. After 5 h and 21 h incubation, 1 ml of the atmosphere from each flask was collected and analysed with a gas chromatograph (Carbo Erba 4100, Thermofinnigan, France) to estimate the quantity (ppm) of generated ethylene. Leaf discs were collected at the same time, frozen in liquid nitrogen and stored at −80°C.

**Protein extraction and enzymatic assays**

The GDH and GS enzymes were extracted and assayed as described previously (Masclaux-Daubresse et al., 2002).

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

Total RNA was extracted and northern blot hybridization were performed as described previously (Masclaux et al., 2000). The following 32P-labelled probes were used for mRNA detection: Nia from Nicotiana sylvestris (Vaucheret et al., 1989), GS2 from N. tabacum (Becker et al., 1992), GS1 from N. tabacum (Dubois et al., 1996), GDH from N. tabacum (Masclaux-Daubresse et al., 2002), 18S from pea (Deng et al., 1991), and Glubas, basic β-1,3 glucanase from Lycopersicon esculentum (Van Kan et al., 1992).

The relative amounts of mRNA were determined by densitometric scanning (Photoshop5.1 Microsoft office) and quantification (NIH image 1.2, public domain) of the signals detected on the autoradiograms.

**Results**

**Viral infection of tobacco leaves induces GDH and GS1 expression**

Tobacco plants were infected with three pathogenic viruses: CMV (Cucumber Mosaic Virus, Cucumovirus), TEV (Tobacco Etch Virus, Potyvirus), and PVY (Potato Virus Y, Potyvirus). Three weeks after inoculation, leaves from similar positions were harvested from infected and control plants. Two independent infection experiments were carried out and gave similar results.

The expression of Glubas isolated from tomato and encoding a β-1,3 glucanase was analysed as a plant-defence control. Glubas was differentially expressed depending on virus infection, showing differential virus-pathogenicity (Fig. 1A, B). The GS2 and Nia (data not shown) expression was not affected by virus infection, whereas GDH and GS1 expression was induced in infected plants in the same manner as Glubas (Fig. 1A, B). The highest expression of Glubas, GDH, and GS1 were obtained with the PVY infection, whereas the effect of the infection with CMV was the weakest.

The aminating and deaminating GDH activities were measured in vitro. The leaf GDH aminating-activity was increased (Fig. 1C), whereas deaminating-activity was not affected by infection with viruses (data not shown). The aminating reaction, resulting in glutamate formation, appeared to be favoured by virus infection, particularly for PVY and TEV. GS activity was not affected by the CMV infection in leaves, whereas inoculation of plants with TEV and PVY led to a decrease in the total GS activity in leaves (Fig. 1D).

**Bacterial infection of tobacco leaves induces GDH and GS1 expression**

In order to differentiate the senescence-like symptoms triggered by pathogen attack from a defence response, tobacco plants were infected with three different Pseudomonas syringae pv. syringae and pv. tabaci strains (see Materials and methods).

The CFBP3077 strain (harp⁺) is avirulent and triggers an incompatible reaction on tobacco plants, which is characterized by the development of a typical hypersensitive response (HR). The strain CFBPM88-1 is an harp⁻ mutant of CFBP3077. This avirulent strain triggers an incompatible reaction but no HR. The CFBP1503 strain is virulent and induces a compatible reaction resulting in disease symptom development. The leaves of the control plants were infiltrated with water. The treated leaves were collected at 0 h, 2 h, 5 h, 9 h, 21 h, and 5 d and the time-course of the expression of the different N-metabolism genes was analysed using northern blot. Two independent experiments were performed and gave similar results (data from one experiment are shown).

The expression of Nia (data not shown) and GS2 (Fig. 2A, B) genes was weakly or not affected by Pseudomonas syringae or water inoculations. The GS2 expression increased steadily with time (specially at time 5 d). This increase was observed with all the treatments as well as with the H₂O control and is certainly due to a change in the global physiological state of the leaves that does not depend on the kind of treatment. The expression of Glubas, GDH,
and GS1 was differentially induced in infected plants depending on the bacteria strain (Fig. 2A, B). The expression of Glubas was enhanced by the three strains from 9 h after inoculation and peaked at 21 h. However, the increase in Glubas mRNA level was higher in leaves inoculated with the two avirulent strains CFBP3077 and CFBPM88-1 (Fig. 2A, B). The GDH mRNA accumulated preferentially in plants inoculated with the avirulent CFBP3077 (hrp +) and the virulent CFBP1503 strains. The induction of GDH expression started between 2 h and 5 h after inoculation and peaked after 9 h before decreasing until 5 d (Fig. 2A, B). It has to be noted that the level of GDH mRNA detected in the water control at 9 h post-inoculation is due to the day/night rhythm previously described by Masclaux-Daubresse et al. (2002), since leaf discs were harvested at 17.30 h, when GDH gene showed the higher expression. No accumulation could be detected in leaves inoculated by the avirulent hrp /C255 CFBPM88-1 mutant. Induction of GDH expression by bacterial infection thus depended on the hrp /C255 genotype, but not on the virulence/avirulence genotype. This suggested that GDH expression was induced when either the HR reaction or disease symptoms promoted cell death.

By contrast, GS1 mRNA accumulated in plants inoculated with the avirulent CFBP3077 (hrp +) or CFBPM88-1 (hrp +) strains, but not in plants inoculated with the virulent CFBP1503 strain (Fig. 2A, B). GS1 induction therefore depended on the virulence/avirulence genotype of P. syringae. The increase of GS1 mRNA level was detected as soon as 2 h post-inoculation, peaked 9 h later, and remained high after 21 h.

Two fungal elicitors activate GDH and GS1 expression and repress GS2 and Nia expression

After showing that GDH and GS1 expression can be induced in tobacco leaves by viruses and bacteria (Figs. 1, 2), experiments were performed to investigate whether fungal elicitors can have a similar effect.

Leaf discs were floated on cryptogein (Ricci et al., 1989) or Onozuka R10 (Moreau-Mihri et al., 1996) solutions for 5 h and 21 h. Controls were obtained using leaf discs floated on the buffer without any elicitor. Two independent experiments were performed and gave similar results.

Cryptogein and Onozuka R10 triggered similar effects when applied to leaf discs for 5 h. Glubas and GDH expression was induced whereas GS2 and Nia expression was repressed (Fig. 3A, B). After 21 h of incubation, these genes showed the same pattern of expression (data not shown). By contrast, GS1 expression showed no change after 5 h in the presence of elicitors, but was higher after 21 h of treatment (Fig. 3A, B).

GDH activity was correlated with GDH expression and the GDH enzyme preferentially catalysed the aminating reaction, leading to glutamate formation (Fig. 3C).
Effect of stress-related plant hormones on N-metabolism gene expression

The effect of the signal molecules known to trigger plant defence gene expression was investigated on the expression of N-metabolism genes.

Leaf discs were incubated for 5 h and 21 h in the presence of two concentrations of ethylene, or floated for 5 h and 21 h on buffer containing three concentrations of jasmonic acid or salicylic acid (see Materials and methods). Controls were obtained using leaf discs floated on the buffer without any stress-related plant hormone. Two independent experiments were performed and gave similar results. The different concentrations applied to leaf discs gave similar effects (data not shown) and only the results of one experiment, obtained with one dose are presented.

It was observed that all signal molecules used induced an increase in the expression of Glubas and GDH after 5 h and 21 h (Fig. 4A, B). In addition, all these molecules except ethylene, repressed Nia expression, suggesting a special role of ethylene in the control of Nia expression. The Nia mRNA levels detected in the water control were low at 5 h compared to 21 h. The reason was that, for the 5 h time point, the leaf discs were harvested at 17.30 h when the Nia expression, which is subjected to circadian rhythm with a peak at the end of the night, is very low (Masclaux-Daubresse et al., 2002). By contrast, for the time point of 21 h, leaf discs were harvested at 09.00 h, when the Nia expression is still highly detectable.

Interestingly, signal molecules triggered differential changes in GS1 and GS2 expression. At 5 h, jasmonic...
acid repressed both GS2 and GS1 expression, while at 21 h, this molecule triggered GS2 expression and still repressed GS1 expression (Fig. 4A, B). Ethylene also had a reciprocal effect on GS1 and GS2 expression and induced GS1 expression whereas it repressed GS2 (Fig. 4A, B). At 21 h, salicylic acid induced the expression of both genes (Fig. 4A, B).

The total GS activity was (i) unchanged after 5 h and 21 h of jasmonic acid treatment, (ii) increased after 5 h and 21 h of salicylic acid treatment, and (iii) increased after 5 h of ethylene treatment but decreased to control values after 21 h (Fig. 4C). The change in GS2 mRNA levels observed under various treatments appeared to parallel the change in the GS total activity.

The aminating GDH activity was increased significantly for all the signal molecules at 21 h, except for salicylic acid, which did not give results statistically different compared to the control (Fig. 4D). Activity was well correlated with GDH mRNA accumulation and the aminating/deaminating ratio (data not shown) indicated that the aminating activity was favoured, leading to glutamate formation (Fig. 4D).

### Fig. 3. Changes in nitrogen metabolism markers in leaf discs treated with the cryptogein and Onozuka R10 elicitors. Controls are the same carrying solutions without the signalling molecules. Northern blot (A) analysis of Glubas, GDH, GS1, GS2, and Nia expression. An 18S rRNA probe was used as a loading control. Note that the exposure times were different for each membrane, thus explaining the signal intensity variability in lane ‘C’. Quantification (B) of the accumulation of transcripts; each value was corrected according to the 18S control and expressed as a percentage of the control (discs floated without elicitor, lane ‘C’). The change in GDH activity (C) was investigated and expressed as a percentage of the control. Values are means ±SE of three measurements. Standard errors are not shown when they are smaller than the symbol. Two to three independent experiments were performed and gave similar results, only the results of one are presented. Fisher test shows *P < 0.05, **P < 0.001, and ***P < 0.0001 when difference with the control is significant and ns P > 0.05 (non significant).
Fig. 4. Effect of ethylene, jasmonic acid, and salicylic acid on nitrogen metabolism markers. Leaf discs were floated on buffer solution containing 100 µM of jasmonic acid (JA), 100 µM of salicylic acid (SA), or equal volume of water as a control (C) for 5 h and 21 h. Ethylene treatment was performed using confined flasks containing 0 M (control, C) or 1 M (250 ppm) ethephon as the ethylene donor. Note that the exposure times were different for each membrane, thus explaining the signal intensity variability in lane ‘C’.

The expression of Glubas, GDH, GS1, GS2, and Nia genes, quantification of the transcripts, and changes in GDH (C) and GS (D) activities are expressed as in Fig. 3. Values are means ± SE of three measurements. Standard errors are not shown when they are smaller than the symbol. Two independent biological experiments were performed and showed similar results. Fisher test shows *P < 0.05, **P < 0.001 and ***P < 0.0001 when difference with the control is significant and ns P > 0.05 (non-significant).
stress participates in the plant defence response against pathogens. It was interesting to know whether oxidative stress also controls the expression of N-metabolism genes.

Leaf discs were floated for 5 h and 21 h on a buffer solution containing H$_2$O$_2$. The results obtained after 21 h of incubation are presented in Fig. 5. Similar results were observed after 5 h and 21 h of incubation for gene expression and enzyme activities.

Interestingly, the effect of H$_2$O$_2$ on the expression of N-metabolism genes was parallel to the effect of ethylene (Fig. 4A, B).

An increase in the aminating GDH activity correlating with gene expression was observed (Fig. 5C). The total GS activity was reduced to a similar extent as the GS2 mRNA level (Fig. 5D). GDH and GS activities were both affected by H$_2$O$_2$ in a dose-dependent manner (Fig. 5C, D).

**Discussion**

In this study the effects of pathogen attack, elicitors, and phytohormones on the regulation of the metabolic pathways that control nitrogen management in plants are presented. The control of the genes involved in primary nitrogen assimilation (GS2 and Nia) and in organic nitrogen remobilization (GS1 and GDH) was investigated. The aim of this work was to compare the differential regulation that controls these genes, depending on the signals applied, and to compare the natural senescence process with stress-induced senescence effects.

Since a lot of reports have emphasized the importance of nitrogen nutrition in both pathogen virulence and plant defence, the effects of pathogen attack on nitrogen mobilization in tobacco plants were investigated first (Solomon et al., 2003). The use of pathogens to study GS1 and GDH regulation was interesting for two reasons. (i) Pathogen attack is a stress for the plants that can mimic natural senescence symptoms and that leads to cell death through disease or through the active cell death process called HR (hypersensitive response). Many Senescence-Associated Genes (SAG) are indeed induced during plant–microbe interactions. Conversely, genes coding for plant defence factors such as PR proteins or chitinase can be induced in the late phase of leaf senescence (Buchanan-Wollaston, 1997, 2003). It was therefore interesting to investigate the regulation of the two senescence markers GS1 and GDH in infected plants. (ii) In plants, pathogens are rerouting the entire nutrients they need from their host. They can thus be considered as new sinks supported by plant metabolism. The major function of leaf senescence is to mobilize nutrients to the new and growing part of the plant (Feller and Fischer, 1994). The role of senescence-like symptoms, promoted by pathogens, in the trophic interaction between plants and micro-organisms was therefore investigated.

To test the effect of pathogen attack, tobacco plants infected by three different viruses (CMV, TEV, and PVY) were analysed. All three viruses triggered similar symptoms that were visible as slightly chlorotic spots. Chlorophyll contents showed, however, that photosynthesis was
differentially affected depending on virus strain, since PVY triggered a more severe decrease in total chlorophyll than TEV, CMV, and the controls (data not shown). The decrease in chlorophyll reflected the senescence-like symptoms occurring in the plants infected with PVY and TEV. Interestingly, a decrease in GS2 and Nia mRNA contents was observed in infected plants and paralleled the increase in GS1, GDH, and Glubas mRNA. Both GDH and GS activities were measured and appeared differentially affected by viruses. In a similar manner as during senescence, the total GS activity of infected leaf tissues decreased whereas GDH activity increased. All these results support the idea that viruses, by inducing senescence-like symptoms, also induce the effects of senescence on N-management markers as previously described (Masclaux et al., 2000). However, since the importance of the physiological disturbance depended on the virulence of the virus strain, the role of pathogenicity was also analysed during bacterial attack.

When tomato plants were infected with Pseudomonas syringae pv. tomato, Pérez-Garcia et al. (1995, 1998) showed that a novel GSI encoding-gene was induced. The sequence coding for this tomato GSI is highly similar to the GSI-3 mRNA detected in tobacco mesophyll when leaves are senescing (Dubois et al., 1996; Brugière et al., 2000). A GS1 protein was also immunolocalized to the mesophyll cells of infected leaves (Pérez-Garcia et al., 1995, 1998).

In the present study, tobacco plants were infected with three different strains of Pseudomonas syringae that trigger different symptoms depending on their avirulence and hrp (hypersensitive response and pathogenicity) alleles. The P. syringae pv. syringae CFBP3077 (hrp+) is an avirulent strain that triggers an incompatible reaction on resistant tobacco plants, and develops necrosis typical of the HR. The strain CFBP3077 (hrp+) is strain CFBP3077 mutated in its hrp locus. This strain is avirulent and triggers an incompatible reaction but failed in HR induction. Therefore, neither symptoms nor necrosis can be observed at the infection site. The strain CFBP1503 of P. syringae pv. tabaci is virulent and triggers disease on the leaves of host plants as soaked spots at the infection site. A time-course of infection was designed in order to analyse changes in GS and GDH gene expression and activity occurring in infected tissues. The expression of Glubas was observed as a plant defence control. The GSI gene was induced when an incompatible reaction was triggered. Moreover, this induction did not depend on HR induction. This suggested that GSI shows the same pattern of expression as an early plant-defence gene. Indeed, when compared to Glubas expression, GSI mRNA accumulated earlier, as soon as 2 h after inoculation. GDH was also induced by P. syringae but its induction appeared later and was not specific to the incompatible reaction. Expression of GDH was also observed earlier than Glubas.

To gain more insight into the role of nitrogen management in response to pathogen attack, elicitors were also used. Elicitors are molecules released from pathogens that can trigger cell death of plant tissues (Aziz et al., 2003; Hagihara et al., 2004). It was proposed that these molecules could enhance the plant–pathogen recognition process that leads to the development of the HR. When tobacco leaf tissues were treated for 5 h and 21 h with the two fungal elicitors Onozuka R10 and cryptogein, a concomitant increase in Glubas and GDH expression and a concomitant decrease in GS2 and Nia expression were observed. Whereas no change in GSI mRNA content could be detected after 5 h of treatment, 21 h of treatment led to GSI induction. These results strengthen the importance of GDH and GSI expression in the response to pathogens. They showed again that GSI and GDH are not co-regulated and this suggested that these enzymes might have different roles in nitrogen mobilization (Masclaux-Daubresse et al., 2002). As during natural senescence, pathogen infection probably affects chloroplasts and primary nitrogen assimilation. The decrease in the expression of GS2 might explain why the total GS activity decreased during infection. The induction of GSI might have a compensatory effect to avoid ammonia accumulation and to ensure glutamine synthesis. The increase in both GDH mRNA and activity also emphasized the similarities with natural senescence and the aminating GDH activity increased in a similar manner as during senescence.

Even though pathogen attack and elicitors induced both GSI and GDH expression, the reason why organic nitrogen remobilization was promoted under such conditions remains obscure. One hypothesis is that pathogens mimic a new sink organ and send signals to activate the nitrogen-mobilization mechanisms in order to reroute plant nutrients and to support its growth. A second hypothesis is that the plant mobilizes and exports its nutriments far away from the infection site, in a metabolic safeguard resistance. The fact that GDH and GSI are not controlled in the same way by the different strains of Pseudomonas is informative. It was observed that GSI was preferentially induced by the two avirulent strains (CBPF3077 and CBPFM88-1) that promoted incompatible reactions rather than by the virulent CBPF1503 that led to disease development. Therefore, the second hypothesis proposing an N-mobilization safeguard appears more appropriate for GSI, which could thus function as a metabolic defence-gene. It was also observed that the induction of GSI was very rapid and detectable as soon as 2 h after inoculation. GDH induction was less rapid and peaked around 5–9 h after inoculation. GDH expression was also higher in leaves infected with CBPF3077 or CBPF1503 promoting HR and disease, respectively. Thus, the level of expression of GDH appeared related to cell death, the final stage of the infection by both strains. Affymetrix data available in silico (https://www.genevestigator.ethz.ch/) showed that GDH genes were up-regulated by cell death in several species. The role of GDH could therefore be related to a cell survival process during the late phase
preceding cell death. The analysis of the natural senescence process has allowed GS1 to be characterized as an early SAG (Senescence Associated Gene), whereas GDH is similar to a late SAG (Masclaux-Daubresse et al., 2005). The results presented here emphasize this difference.

Few reports discuss the interconnection of the signalling pathways leading to plant defence against pathogen or senescence (Buchanan-Wollaston et al., 2003). Several trans regulators are expressed in senescing leaves and controlled by stress (Chen et al., 2002), some of them also respond to phytohormones. Therefore, it was decided to test the role of phytohormones and signalling molecules, for example, salicylic acid (SA), jasmonic acid (JA), and ethylene, that have been implicated in the complex interconnecting pathways that control gene expression in plant–pathogen responses and stress, as well as in leaf senescence (He and Gan, 2001; Buchanan-Wollaston et al., 2003). It was observed that all these molecules induced Glubas expression after 5 h or 21 h of treatment. Interestingly, the same molecules also induced GDH and the timing and the importance of the effects triggered by JA and SA on GDH and Glubas were very similar. Surprisingly, only ethylene showed an inducing effect on GS1 expression, which again emphasized the discrepancy between GDH and GS1 regulation. The effect of the molecules on the expression of GS2 and Nia was also unclear and differential, depending on the molecule and the time of the treatment. It is important to note that the total activity of GS paralleled the GS2 mRNA accumulation. Taken together, these results suggest that GDH behaves like a stress-inducible gene, whereas GS1 regulation is more complex and more selective. This suggests that the roles of GS1 and GDH, in nitrogen management in response to stress, are independent.

The increase in reactive oxygen species (ROS) is a common factor between different stress responses as well as during senescence (Navabpour et al., 2003). The treatment of tobacco leaf discs with increasing doses of hydrogen peroxide enhanced Glubas expression and, in a similar manner, the accumulation of GS1 and GDH mRNA. A parallel decrease in GS2 mRNA was observed and seems to result in decreased GS activity.

The major outcome of this work is that almost all stresses studied showed similar effects as natural senescence and resulted in (i) a decrease in the expression of the primary N-assimilation genes GS2 and Nia, (ii) a decrease in total GS activity, (iii) an increase in GDH and GS1 expression, and (iv) an increase in the aminating GDH activity. The time-course or the magnitude of the response of GDH and GS1 to stresses was, however, different depending on the individual stress, thus suggesting that GDH behaves like a non-specific stress-related gene whereas GS1 is more selective. The role of GS1 and GDH is therefore probably independent, as suggested previously (Masclaux-Daubresse et al., 2002).

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