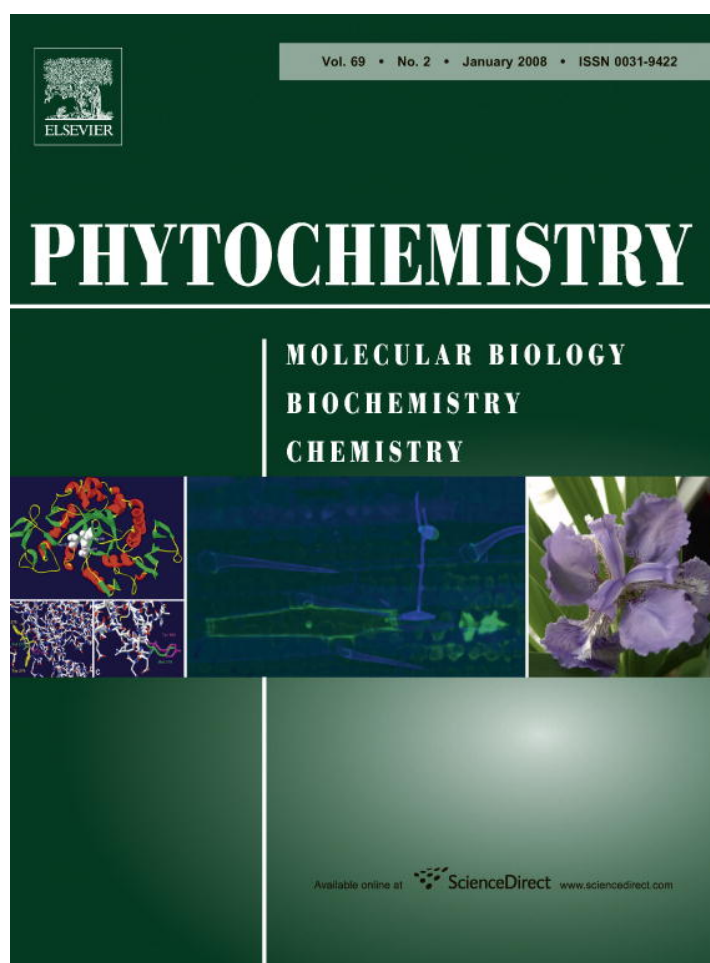


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## Response of transgenic poplar overexpressing cytosolic glutamine synthetase to phosphinothricin

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### Abstract

Glutamine synthetase (GS) is the main enzyme involved in ammonia assimilation in plants and is the target of phosphinothricin (PPT), an herbicide commonly used for weed control in agriculture. As a result of the inhibition of GS, PPT also blocks photorespiration, resulting in the depletion of leaf amino acid pools leading to the plant death. Hybrid transgenic poplar (*Populus tremula* × *P. alba* INRA clone 7171-B4) overexpressing cytosolic GS is characterized by enhanced vegetative growth [Gallardo, F., Fu, J., Cantón, F.R., García-Gutiérrez, A., Cánovas, F.M., Kirby, E.G., 1999. Expression of a conifer glutamine synthetase gene in transgenic poplar. *Planta* 210, 19–26; Fu, J., Sampalo, R., Gallardo, F., Cánovas, F.M., Kirby, E.G., 2003. Assembly of a cytosolic pine glutamine synthetase holoenzyme in leaves of transgenic poplar leads to enhanced vegetative growth in young plants. *Plant Cell Environ.* 26, 411–418; Jing, Z.P., Gallardo, F., Pascual, M.B., Sampalo, R., Romero, J., Torres de Navarra, A., Cánovas, F.M., 2004. Improved growth in a field trial of transgenic hybrid poplar overexpressing glutamine synthetase. *New Phytol.* 164, 137–145], increased photosynthetic and photorespiratory capacities [El-Khatib, R.T., Hamerlynck, E.P., Gallardo, F., Kirby, E.G., 2004. Transgenic poplar characterized by ectopic expression of a pine cytosolic glutamine synthetase gene exhibits enhanced tolerance to water stress. *Tree Physiol.* 24, 729–736], enhanced tolerance to water stress (El-Khatib et al., 2004), and enhanced nitrogen use efficiency [Man, H.-M., Boriel, R., El-Khatib, R.T., Kirby, E.G., 2005. Characterization of transgenic poplar with ectopic expression of pine cytosolic glutamine synthetase under conditions of varying nitrogen availability. *New Phytol.* 167, 31–39]. *In vitro* plantlets of GS transgenic poplar exhibited enhanced resistance to PPT when compared with non-transgenic controls. After 30 days exposure to PPT at an equivalent dose of 275 g ha<sup>-1</sup>, growth of GS transgenic poplar plantlets was 5-fold greater than controls. The response of young leaves to PPT treatment depends on physiological state as indicated by GS and Rubisco (LSU) levels. Young leaves from control plants, typically in a low differentiation state, respond to the herbicide showing up-regulation of GS and LSU. In contrast, young leaves from transgenic lines, with higher initial GS and LSU levels compared to control, display up-regulation of NADP<sup>+</sup>-isocitrate dehydrogenase. Differences between control and GS transgenics in their response to PPT are discussed in relation to their differences in photosynthetic and photorespiratory capacities (El-Khatib et al., 2004).

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**Keywords:** *Populus tremula* × *P. alba*; Salicaceae; Poplar; Phosphinothricin; Herbicide; Glutamine synthetase; Isocitrate dehydrogenase

### 1. Introduction

Trees, like herbaceous plants, respond negatively to both weed competition and limited nitrogen availability in the soil. The use of transgenic plants may offer new ways to enhance growth of agricultural crops and

**Abbreviations:** Fd-GOGAT, ferredoxin-dependent glutamate synthase; GS, glutamine synthetase; LSU, large subunit of Rubisco; NADP<sup>+</sup>-IDH, NADP<sup>+</sup>-linked isocitrate dehydrogenase.

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forest trees. Although a number of crops have been manipulated to achieve herbicide resistance, improving N uptake and assimilation efficiency is a more complex task since key enzymes involved in N assimilation and metabolism are often subject to regulation. The syntheses of glutamine and glutamate are crucial steps in the assimilation of inorganic nitrogen since all organic N-containing compounds required for plant growth are directly, or indirectly, derived from these two amino acids (Ireland and Lea, 1999).

The biosynthesis of glutamine is the target of one of most common herbicides used in agriculture, phosphinothricin or glufosinate (PPT). PPT is a component of a natural tripeptide first isolated from *Streptomyces hygroscopicus*. PPT is a structural analog of glutamate that binds to glutamine synthetase (GS, E.C. 6.3.1.2), the enzyme responsible for the synthesis of glutamine from ammonia into glutamate. The interaction between PPT and GS results in irreversible inactivation of the enzyme, impairing amino acid metabolism, thus leading to plant death (Lea and Ridley, 1989). GS expression is intimately associated with development of photosynthetic tissues, thus young leaves and the apical meristem are the main targets of PPT action.

Most plants have two GS isoforms: GS1, located in the cytosol of cells associated with vascular elements, and GS2 located in the chloroplasts of mesophyll cells. GS1 is mainly involved in the synthesis of glutamine for transport, whereas GS2 is involved in primary ammonia assimilation and recycling ammonium released during photorespiration (Ireland and Lea, 1999; Cren and Hirel, 1999). GS2 acts in coordination with chloroplastic ferredoxin-dependent glutamate synthase (Fd-GOGAT), resulting in net production of glutamate from 2-oxoglutarate and ammonia (GS/GOGAT cycle). GS/GOGAT reactions require photosynthetic energy, reducing power, and 2-oxoglutarate as carbon skeletons. The production of 2-oxoglutarate has been reported to be associated with photorespiration (Novitskaya et al., 2002) and it is produced primarily by cytosolic NADP<sup>+</sup>-isocitrate dehydrogenase (NADP<sup>+</sup>-IDH, Palomo et al., 1998; Hodges, 2002).

PPT-mediated inhibition of GS produces a rapid depletion of glutamate, the amino acid donor for the aminotransferase reactions in the C-2 photorespiratory pathway. This results in the accumulation of ammonia, leaf chlorosis and/or necrosis, and ultimately, plant death (Wendler et al., 1990; Lacuesta et al., 1992; Hoerlein, 1994). PPT-treated plants may experience an increase in GS expression (Ávila et al., 1998; Pérez-García et al., 1998), or amplification of GS genes; both may be viewed as mechanisms to overcome the inactivation of GS holoenzyme (Donn et al., 1984). In fact, *in vitro* resistance to PPT in vetiver cell suspensions was associated to increased GS activity (Prasertsongskun et al., 2002). According to these reports, enhanced GS expression in genetically engineered plants can improve nitrogen use efficiency, resulting in enhanced tolerance to PPT. Thus, resistance to PPT has

been recently reported in transgenic rice calli overexpressing GS (Hui et al., 2005).

In contrast to crop plants, there are minimal reports of PPT tolerance in forest trees. Hybrid poplar with enhanced tolerance to PPT has been achieved by the expression of a bacterial *bar* gene in transgenic lines (Confalonieri et al., 2000). In addition, in pine seedlings the effect of PPT on GS expression lead to the discovery of a new GS1 gene (Ávila et al., 1998).

Our work on poplar GS transgenics characterized by the ectopic expression of GS1 in photosynthetic cells has shown that transgenics display increased vegetative growth in both controlled growth conditions and in a field trial (Gallardo et al., 1999; Fu et al., 2003; Jing et al., 2004). In addition, GS transgenics exhibited increased photosynthetic and photorespiratory capacities (El-Khatib et al., 2004), enhanced tolerance to drought stress (El-Khatib et al., 2004), and enhanced nitrogen use efficiency (Man et al., 2005). In the present work we investigate the response of poplar GS transgenics to PPT.

## 2. Results

### 2.1. Tolerance of *in vitro* cultivated poplars to PPT

In order to determine the tolerance of both GS transgenic and control poplars to PPT, a total of 240 plantlets including control and four independent transgenic lines were cultured *in vitro* in the presence of 5, 25 or 100  $\mu$ M PPT and their survival was analyzed. Control plantlets respond to PPT by developing necrosis, which usually starts in the apical meristem, then spreads throughout the plantlets resulting in plantlet death (Fig. 1a). Low levels of PPT (5  $\mu$ M) had a limited effect on both control and transgenic lines, since about 80–100% of the plantlets remained viable after 20 days of culture (Fig. 1b). In contrast, significant differences in the survival rate of control and transgenics plantlets were observed with culture on 25 or 100  $\mu$ M PPT. Culture on 25  $\mu$ M PPT resulted in death of 75% of control plants after 20 days of culture, whereas 50–100% of transgenic plants remained viable during the same period (Fig. 1b). In the presence of 100  $\mu$ M PPT, the percentage of viable control plantlets decreased rapidly after 8 days of culture and all control plants were dead after 14 days of culture. Culture of transgenic lines on 100  $\mu$ M PPT resulted in 20–45% of transformed plantlets remaining viable after 14 days of culture (Fig. 1b).

The GS protein content was estimated using western blot analysis of proteins extracted from transgenic and control plantlets after 9 days of culture on 0, 5 and 25  $\mu$ M PPT (Fig. 1c). Two bands corresponding to chloroplastic (GS2) and cytosolic (GS1) GS isozymes were detected in the blots. In agreement with our previous work (Gallardo et al., 1999; Fu et al., 2003; Jing et al., 2004), *in vitro* cultivated transgenic plantlets showed total GS contents (GS1+GS2) 20–40% greater than that of non-transgenic

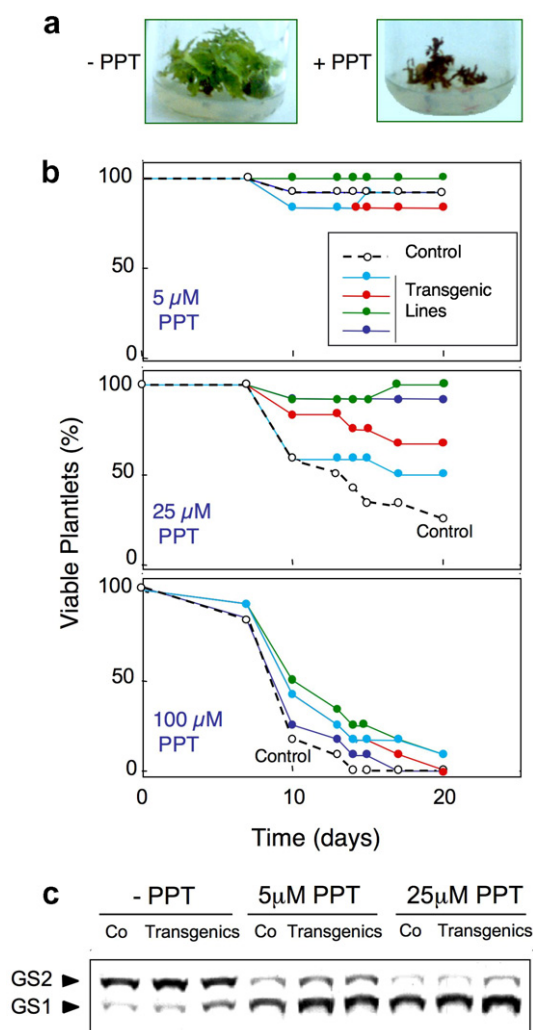


Fig. 1. *In vitro* tolerance of poplar seedlings to PPT. (a) Control poplar plantlets cultivated for 30 days in the presence of 25  $\mu\text{M}$  PPT develop extensive necrosis resulting in the death of the plant (+PPT). In the absence of herbicide the plantlets remains viable (–PPT). (b) Viability of control and GS transgenic plantlets in the presence of 5, 25 and 100  $\mu\text{M}$  PPT. Plantlets were considered not viable when the apex and 80% of the shoot presented necrosis. (c) Analysis of GS polypeptides. Control (Co) and transgenic plantlets were cultivated for 9 days in the absence of PPT (–PPT), or with 5 or 25  $\mu\text{M}$  PPT. Total soluble proteins were fractionated by SDS-PAGE and GS polypeptides detected by Western blot using a polyclonal GS antiserum (Cantón et al., 1996). Size of GS polypeptides is 45 kD for GS2, and 40–41 kD for GS1.

controls (Fig. 1c, signals of GS1+GS2 bands in absence of PPT). Changes in the relative contents of GS polypeptides were associated with the presence of PPT in the growth media. Plantlets cultivated on media without PPT contained higher GS2 than GS1 levels, while the reverse was observed in the presence of PPT, i.e. lower GS2 contents at higher PPT concentrations (Fig. 1c). Interestingly, GS2 levels were higher in transgenics than in control plants at 5 or 25  $\mu\text{M}$  PPT. Taken together, these results suggest that PPT initially affects the photosynthetic tissues and that enhanced viability of transgenics in the presence of PPT is associated with their higher GS contents.

## 2.2. Effect of PPT on protein expression in detached poplar leaves

To evaluate changes in protein expression associated with PPT treatment, young leaves from control and transgenic plantlets were detached, cut into two equal halves and painted with 1 mM PPT or water. Prior to PPT treatment, a disc was removed as control for the experiments (0 h, Fig. 2a). The PPT and water treated halves were then placed on MS media and analyzed after 4 and 22 h (Fig. 2a). SDS-PAGE showed that proteins from young leaves of control plantlets have low levels of the large subunit of Rubisco (LSU) in comparison to young leaves from transgenic lines (Fig. 2b, 0 h). Control leaves respond to culture by increasing their LSU steady-state levels. This response was more noticeable in leaves with lower LSU content and seems to be associated with the change in nutrient availability upon their transfer to MS media. In comparison to control leaves, extracts from GS transgenic lines showed higher initial levels of LSU, however increased time in culture and exposure to PPT did not result in further increases in levels of LSU (Fig. 2b). Western analysis of proteins extracted from leaves prior to exposure to PPT showed that levels of GS polypeptides were low in non-transgenic control leaves (Fig. 2c, control

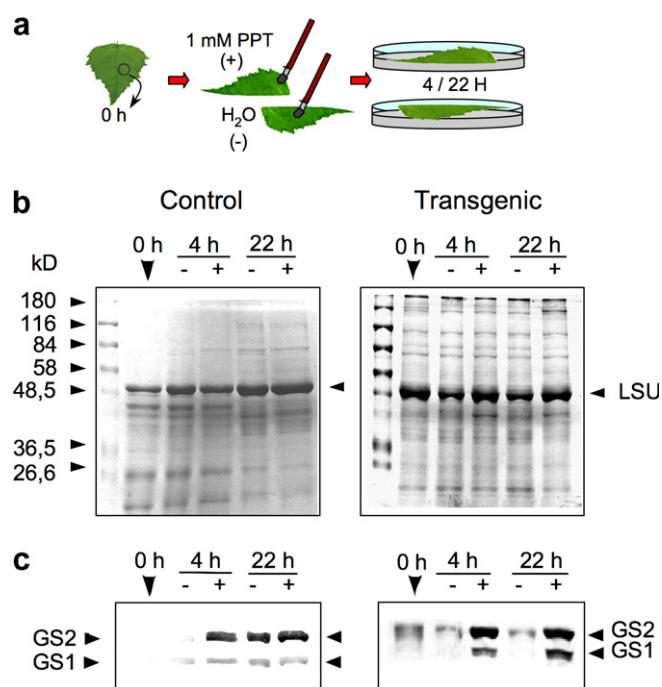


Fig. 2. Effect of PPT on the profile of polypeptides in detached poplar leaves. (a) Scheme of PPT treatment. Detached young leaves from control and GS transgenic plantlets were painted with 1 mM PPT (+) or with water (–) and cultivated for 4 or 22 h onto MS medium. Previous to PPT/water treatment, a leaf disc was obtained as a control for the experiment (0 h). (b) Representative protein patterns of detached poplar leaves. Leaf proteins (20  $\mu\text{g}$ ) were fractionated by SDS-PAGE and visualized by Coomassie-blue staining. (c) Representative GS polypeptide analyses by Western blot.

0 h), whereas the GS2 polypeptide was clearly detected in leaf extracts of transgenic plants (Fig. 2c, transgenic 0 h). The culture of leaves in the absence of PPT produced a slight increase in levels of GS polypeptides. This was more conspicuous in control leaves after 22 h of culture (Fig. 2c, 4 and 22 h without PPT). In contrast, a substantial increase in both GS1 and GS2 was observed 4 h after exposure to PPT. High GS polypeptide levels were still detected 22 h after herbicide exposure (Fig. 2c, 4 h, 22 h).

The experiment described above was repeated for 23 samples of control (9 samples) and transgenic (14 samples) plants. Levels of LSU and levels of total GS were determined prior to water or PPT treatment, and after 4 h in MS culture. Samples were grouped in three categories according to their LSU content prior to treatment (Fig. 3). Control leaves represented 57%, 38% and 0% of samples with initial LSU content of 2–5%, 8–15% and 23–32%, respectively. Transgenic leaves exhibited higher

LSU levels and they represented 45%, 62% and 100% of samples with LSU content of 2–5%, 8–15% and 23–32%, respectively. The results show that GS contents prior to treatments match well with initial LSU content in the samples (Fig. 3a). However, the increase in GS polypeptides 4 h after PPT treatment was higher in the samples with lower initial LSU contents (Fig. 3b). These data suggest that leaves with higher LSU and GS contents are less sensitive to PPT.

### 2.3. Effect of PPT on the development of control and transgenic poplar plantlets

Results described above demonstrate that GS transgenics display higher tolerance to PPT. Analyses of control and GS transgenics cultivated in a growth chamber were carried out using PPT treatments equivalent to doses used to control weeds in agriculture (125, 275 and 500 g ha<sup>-1</sup>). Three-week-old plants derived from tissue culture plantlets of control and GS transgenic lines were selected for this study. In previous work we observed that control and transgenic plants maintained similar growth rates during the first six weeks of culture (Fu et al., 2003).

Plants treated with PPT equivalent to 500 g ha<sup>-1</sup>, developed chlorosis and necrosis in leaves within the first 24 h after the application of the herbicide, and all plants died 72 h after the treatment (not shown). In contrast, all transgenic and control plants treated with PPT equivalent to 125 g ha<sup>-1</sup> PPT were not affected in their growth and only minor leaf areas with chlorosis were observed in young leaves. Thus, PPT equivalent to 275 g ha<sup>-1</sup> was selected for the following studies.

Growth of control and four GS transgenic lines was monitored before and after the application of PPT (equivalent to 275 g ha<sup>-1</sup>). Herbicide application quickly resulted in chlorosis and extensive necrosis was observed in leaves of both control and transgenic plants 3 days after the treatment. Eighteen days following treatment, the majority of leaves had abscised from all plants, although plants remained viable (Fig. 4a). Interestingly, the growth rate of control plants was substantially reduced after the treatment and plants exhibited a net growth of 2.4 cm 27 days after herbicide application (Fig. 4b, control). In contrast, transgenic plants showed only a slight reduction in growth rate and achieved a net growth of 12.3 cm 27 days after PPT application, a 5-fold increase over controls (Fig. 4b, transgenics).

Protein profiles of expanding leaves of control and transgenic plants treated with PPT for 4 h revealed that control leaves were in a less-differentiated state than transgenic leaves according to their LSU content (Fig. 4c, samples treated without PPT). The application of PPT increased the level of LSU in controls, while no effect on LSU was observed in transgenics (Fig. 4c). Low levels of GS polypeptides were observed in control leaves and they increased after PPT treatment (Fig. 4d, GS panel, control). The effect of PPT on GS expression in transgenics was very

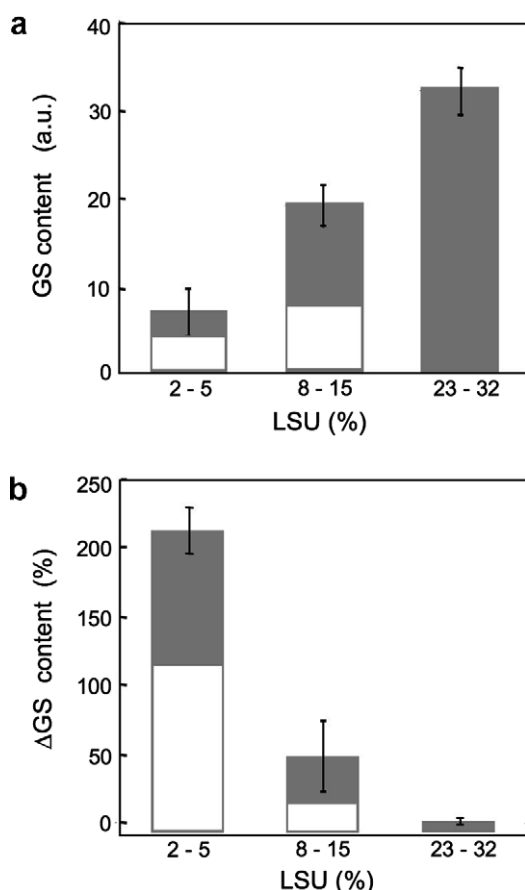


Fig. 3. The relationship between GS and LSU contents in response to PPT treatment. (a) GS content (arbitrary units; a.u.) in detached leaves as a function of LSU content prior to treatment with PPT. Values were determined from 23 pooled samples representing both non-transgenic and transgenic lines. LSU contents were grouped into 3 categories: 2–5%, 8–15%, and 23–32% of total protein. White columns represent the percentage of control samples in each group. (b) Increases in GS following PPT treatment as a function of LSU content. GS values (from arbitrary units, as above) for PPT-treated leaves are expressed as percent of water-treated controls and were determined after 4 h of herbicide treatment as indicated in Fig. 2. Data correspond to mean values  $\pm$  s.e.

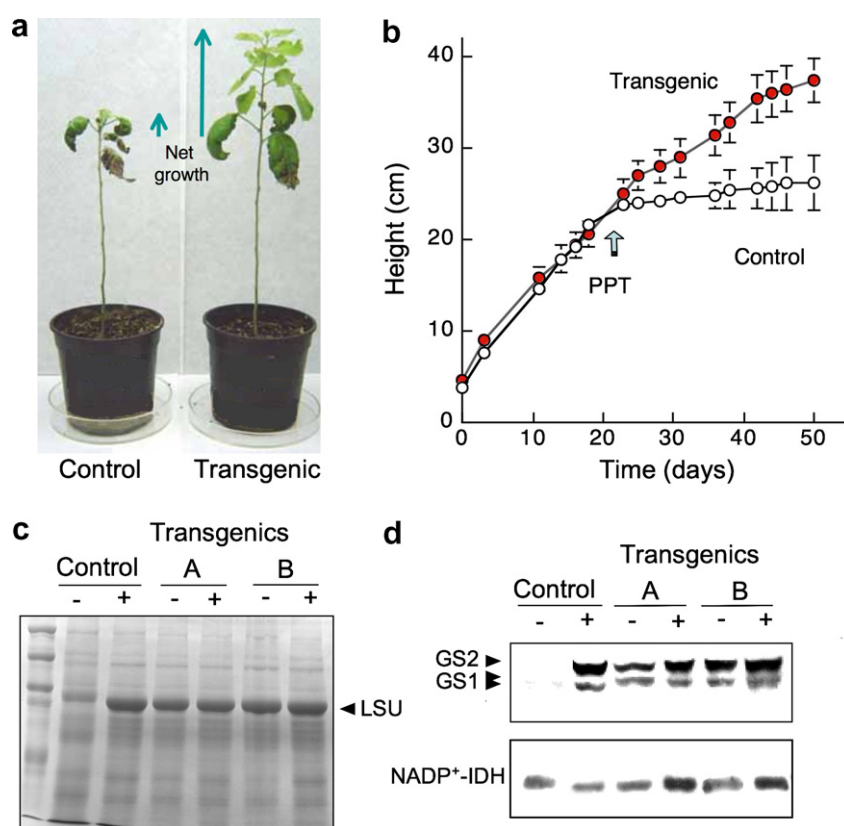


Fig. 4. Effect of PPT on growth of control and transgenic poplars. Three-week old plants were sprayed with PPT at an equivalent dose of  $275 \text{ g ha}^{-1}$  and growth was monitored until plants had the age of 50 days. (a) Representative control and GS transgenic plants at the end of the experiment. Poplar plantlets had lost most of their leaves, and net growth from PPT application is shown by a vertical arrow. (b) Growth curve of control and GS transgenic lines. The application of PPT-treatment is indicated by an arrow. Transgenic data are mean of four independent lines. Vertical bars correspond to the s.e. of the mean. There were significant differences in height between control and transgenics from day 25 to 50 ( $\alpha \leq 0.05$ ). (c) Protein profiles of young leaves after fractionation by SDS-PAGE and Coomassie-blue staining. Extracts were prepared 4 h after plant treatment with PPT (+) or water (-). LSU polypeptide is marked by an arrow. Twenty microgram of protein was loaded per lane. (d) GS and NADP<sup>+</sup>-IDH polypeptide analysis by western blot using polyclonal antibodies against pine proteins (Cantón et al., 1996; Palomo et al., 1998). Representative results from control and transgenics lines (A and B) are shown. Molecular masses of indicated proteins are as follows: LSU, 55 kD; GS2, 45 kD; GS1, 40 or 41 kD; NADP<sup>+</sup>-IDH, 48 kD. Differences in the mobility of GS1 bands are due to transgene expression (Gallardo et al., 1999).

limited and only a slight increase was observed (Fig. 4d, GS panel, transgenics). The expression of NADP<sup>+</sup>-IDH, a key enzyme in the production of carbon skeletons for amino acid synthesis, was also monitored by western blot analysis. A slight decrease in NADP<sup>+</sup>-IDH polypeptide levels was observed in control leaves after PPT treatment, however in transgenic leaves higher NADP<sup>+</sup>-IDH levels were detected after the application of the herbicide (Fig. 4d, NADP<sup>+</sup>-IDH panel).

### 3. Discussion

Nitrogen assimilation in plants is a key factor regulating growth that directly depends on photosynthesis. Reducing power, energy, and carbon skeletons generated through photosynthesis are necessary for the incorporation of ammonia into glutamine and glutamate through GS/GOGAT enzymes (Fig. 5). In addition, since glutamate also serves as the nitrogen donor to photorespiratory inter-

mediates through the aminotransferase reaction, nitrogen assimilation is intimately associated with photorespiration. Furthermore, GS is involved in the recovery of ammonia released in mitochondria by the photorespiratory conversion of glycine into serine (Tolbert, 1997; Fig. 5). Nitrogen released through photorespiration and subsequently re-assimilated through GS2 is the primary source of ammonia in photosynthetic tissues. Approximately ten-times more ammonia is assimilated through the GS/GOGAT pathway than is produced directly by nitrate reduction in the leaf (Keys et al., 1978). Although the role of the photorespiratory pathway in nitrogen recycling has been under considerable discussion for some time, its main biological function appears to be associated with protection of C3-photosynthesis (Kozaki and Takeba, 1996), and in supporting nitrate assimilation in the leaf (Rachmilevitch et al., 2004). As a result of its inhibitory effects on GS, the herbicide mechanism of PPT also resides in its ability to block photorespiration, resulting in the accumulation of ammonia, and the depletion of the amino acid pool

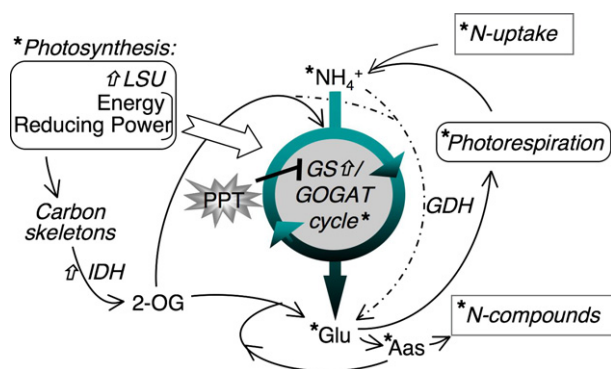


Fig. 5. Plant responses to glutamine synthetase (GS) inhibition by phosphinothricin (PPT), and features of transgenic poplars overexpressing GS. Glutamine synthetase/glutamate synthase (GS/GOGAT) cycle is involved in the assimilation of ammonia from N-uptake and photorespiration, and in the production of glutamate (Glu). A simplified balance of the GS/GOGAT cycle is shown: glutamate is produced from ammonia and 2-oxoglutarate (2-OG), and energy and reducing power are also required. PPT blocks glutamate production by inhibiting GS. Response to GS inhibition depends on the physiological state of the tissue. Increase ( $\uparrow$ ) in GS and LSU, or in NADP<sup>+</sup>-IDH (IDH) occurs in young leaves depending on their initial GS and LSU levels. Glutamate demand in photorespiratory active leaves under PPT effect could be transiently recovered from leaf amino acid pool (Aas) by aminotransferase reactions, or by glutamate dehydrogenase (GDH), both require 2-oxoglutarate (2-OG). Asterisks indicate plant processes, metabolites or enzyme expression altered, under normal growth conditions, that may be involved in the enhanced tolerance of GS transgenic poplar to PPT (Gallardo et al., 1999; Fu et al., 2003; Jing et al., 2004; El-Khatib et al., 2004; Man et al., 2005).

(Wendler and Wild, 1989; Downs et al., 1994). Thus, photosynthesis is rapidly inhibited by PPT when plants are cultivated under normal atmospheric conditions, but not under non-photorespiratory oxygen conditions (Sauer et al., 1987; Lacuesta et al., 1992). According to these findings, enhanced photorespiratory capacity may be a desirable trait for increasing natural plant tolerance to PPT.

Kozaki and Takeba (1996) reported that overexpression of GS2 in transgenic tobacco allowed a better recovery of photorespiratory ammonia resulting in enhanced protection of photosynthesis by reducing the photooxidative damage to the photosynthetic apparatus. Other reports of GS1 or GS2 overexpression in herbaceous plants indicated increased photosynthetic and photorespiratory capacities (Migge et al., 2000; Fuentes et al., 2001; Oliveira et al., 2002).

Transgenic poplar with ectopic GS1 expression in photosynthetic cells not only exhibited increased vegetative growth (Gallardo et al., 1999; Fu et al., 2003; Jing et al., 2004), but also enhanced photosynthetic and photorespiratory capacities, along with enhanced resistance to water stress (El-Khatib et al., 2004). In addition, results of the present work indicate that poplar GS transgenics exhibit enhanced tolerance to the herbicide PPT in both *in vitro* studies and in greenhouse experiments where plants were exposed to levels of PPT equivalent to those used for weed control in agriculture (Fig. 4). Although photosynthetic tissues are the main targets of PPT action, responses to PPT

seems to depend on the physiological state of the leaf. In fact, the effect of PPT on GS expression appears related to existing levels GS and LSU prior to exposure to PPT. Thus, PPT had a more marked effect on GS expression in leaves with lower initial GS and LSU contents, typically control leaves (Figs. 2–4).

Considering that photosynthesis, photorespiration, and nitrogen assimilation are simultaneously acquired during leaf development (Tobin et al., 1988; Yu and Woo, 1991), the differences observed in GS (Figs. 2–4) and LSU (Fig. 4) accumulation after PPT treatment may be associated with differences in the photosynthetic and metabolic competencies of young leaves. Moreover, higher photosynthetic and photorespiratory capacities of GS transgenic poplar lines were also associated with low free ammonia contents of transgenics and a greater leaf amino acid pool in comparison with control plants (El-Khatib et al., 2004; Man et al., 2005). Taken together these data suggest that the glutamate demand in photosynthetic cells could be different in young leaves of control and GS transgenic poplars.

Up-regulation of GS expression has been reported as a response to PPT application (Ávila et al., 1998; Pérez-García et al., 1998). Our results suggest that two different mechanisms could operate transiently to overcome PPT inhibition of GS in poplar leaves. In control plants, the modification of GS and LSU levels after PPT treatment (Figs. 2–4) could be considered as a coordinated mechanism to increase the flux through GS/GOGAT pathway in order for cells to recover from amino acid depletion and ammonia accumulation (Fig. 5). In contrast, GS transgenics experience up regulation of cytosolic NADP<sup>+</sup>-IDH after exposure to PPT with only slight effects on GS and LSU (Fig. 4). These findings suggest that a higher supply of 2-oxoglutarate is required to overcome the PPT effect in tissues with high GS and LSU contents (Fig. 5). In fact, the supply of 2-oxoglutarate has been considered a relevant factor supporting high photorespiratory fluxes (Novitskaya et al., 2002). In addition, the synthesis of 2-oxoglutarate has been considered to reflect the C/N status of the leaf, and a factor effecting carbon skeleton limitations resulting from photorespiration (Hodges, 2002; Schjoerring et al., 2000).

Furthermore, a similar mechanism seems to operate in pine cotyledons. After treatment with PPT, pine cotyledons show increased NADP<sup>+</sup>-IDH expression when steady-state levels of GS and LSU polypeptides are high (Pascual, 2007). Such results indicate that 2-oxoglutarate could be required to recycle reduced nitrogen components of young leaves by aminotransferase reactions, thus transiently overcoming PPT-inhibition of the GS/GOGAT cycle. Alternatively, glutamate dehydrogenase (GDH) may function to recover glutamate from photorespiratory ammonia and 2-oxoglutarate (Fig. 5). GDH is induced when the GS/GOGAT pathway is affected and ammonium accumulates in plant tissues (Tercé-Laforgue et al., 2004), or under abiotic stress (Skopelitis et al., 2006); and GDH overexpres-

sion in transgenic tobacco has also resulted in increased resistance to PPT (Nolte et al., 2004).

#### 4. Concluding remarks

This study has shown that transgenic poplar over-expressing GS exhibited higher tolerance to PPT than non-transgenic controls. Increases in GS and LSU or in NADP<sup>+</sup>-IDH seem to indicate that two different mechanisms operate in poplar leaves to confer PPT resistance. Since the herbicide levels used here are similar to those used in agricultural field applications, our results also suggest that herbicide resistance displayed by GS transgenic poplars could be advantageous in silviculture. Thus, the use of poplar GS transgenics in short rotation plantations could provide several significant benefits, namely, increased vegetative growth, enhanced tolerance to water stress, and higher tolerance to PPT.

#### 5. Experimental

##### 5.1. Plant material and herbicide treatment

Transformed poplar lines expressing the pine GS1 transgene (*Populus tremula* × *P. alba* INRA clone 7171-B4; Gallardo et al., 1999) were micropropagated *in vitro* on half-strength Murashige and Skoog medium (MS) as described previously (Gallardo et al., 1999). Unless otherwise noted, plantlets were maintained under standard conditions as described previously (Gallardo et al., 1999; Fu et al., 2003).

*In vitro* tolerance to phosphinothricin (glufosinate, PPT; Sigma–Aldrich Laborchemikalien GmbH D-30918 Seelze) was determined by culture of 3 cm plantlets on MS medium supplemented with 5, 25 or 100 μM PPT. A total of 240 plantlets corresponding to four independently transformed transgenic lines and controls were cultivated for 20 days, as described above. Plantlets were considered as non-viable when both the plant apex, and 80% of the plantlet were necrotic (Fig. 1).

The effect of PPT on young leaves of 3-week-old plantlets cultivated in a controlled grown chamber was also analyzed. Prior to treatment, leaflets were detached, disinfected and excised in two parts. One half of each leaf was painted with 1 mM PPT and the other half was treated with sterile water as control. All treated leaves were placed on MS medium and incubated under continuous illumination at 24 °C for 4 or 22 h (Fig. 2).

The effect of PPT on growth and development of control and transgenic poplar plants grown under greenhouse conditions was studied using 3-week-old plants. Assays of GS transgenic and control lines were carried out using treatments with PPT levels related to doses used to control weeds in the field, 125, 275 and 500 g ha<sup>-1</sup> (Pesticide Action Network UK, glufosinate ammonium fact sheet;

Martinson et al., 2002; Nelson et al., 2002; Brown et al., 2007). The herbicide was dissolved in sterile water and sprayed over the plants in a final volume of 60 mL. Leaf samples were taken 4 h after the treatment. Control and transgenic plants treated with sterile water alone served as controls. All experiments were repeated at least three times.

##### 5.2. Protein analysis

Total soluble proteins were extracted from poplar leaves as described earlier (Gallardo et al., 1999). Protein concentrations were estimated spectrophotometrically (Bradford, 1976) using bovine serum albumin as standard. Protein profiles were analyzed by SDS-PAGE according to the procedure of Laemmli (1970) followed by Coomassie blue staining. Leaf proteins were also fractionated using 10% polyacrylamide gels. Detection of proteins was conducted by SDS-PAGE followed by Western blot analysis using polyclonal antibodies raised against the recombinant pine GS protein (Cantón et al., 1996) and NADP<sup>+</sup>-IDH (Palomo et al., 1998). Protein transfers were conducted with a Multiphor II Novablot apparatus (Pharmacia Biotech, Uppsala, Sweden) by the application of 0.8 mA cm<sup>-2</sup> at constant current for 120 min. Immunocomplexes were identified with peroxidase-conjugated immunoglobulin with a peroxidase:immunoglobulin molar ratio of 3.3 (Vector Laboratories, Burlingame, CA, USA). GS and LSU quantification in blots and gels were conducted as described in Jing et al. (2004).

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#### References

- Ávila, C., García-Gutiérrez, A., Crespillo, R., Cánovas, F.M., 1998. Effects of phosphinothricin treatment on glutamine synthetase isoforms in Scots pine seedlings. *Plant Physiol. Biochem.* 36, 857–863.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Brown, L., Soltani, N., Shropshire, C., Spiser, H., Sikkema, P.H., 2007. Efficacy of four corn (*Zea mays* L.) herbicides when applied with flat fan and air induction nozzles. *Weed Biol. Manag.* 7, 55–61.
- Cantón, F.R., García-Gutiérrez, A., Crespillo, R., Cánovas, F.M., 1996. High-level expression of *Pinus sylvestris* glutamine synthetase in *Escherichia coli*. Production of polyclonal antibodies against the recombinant protein and expression in pine seedlings. *FEBS Lett.* 393, 205–210.
- Confalonieri, M., Belenghi, B., Balestrazzi, A., Negri, S., Faccioto, G., Schenone, G., Delledonne, M., 2000. Transformation of elite white poplar (*Populus alba* L.) cv. “Villafranca” and evaluation of herbicide resistance. *Plant Cell Rep.* 19, 978–982.



- Cren, M., Hirel, B., 1999. Glutamine synthetase in higher plants: regulation of gene and protein expression from the organ to the cell. *Plant Cell Physiol.* 40, 1187–1193.
- Donn, G., Tisher, E., Smith, J.A., Goodman, H., 1984. Herbicide resistant alfalfa cells: an example of gene amplification in plants. *J. Mol. Appl. Genet.* 2, 621–635.
- Downs, C.G., Christey, M.C., Maddocks, D., Seelye, J.F., Stevenson, D.G., 1994. Hairy roots of *Brassica napus*: I. Applied glutamine overcomes the effect of phosphinothricin treatment. *Plant Cell Rep.* 14, 37–40.
- El-Khatib, R.T., Hamerlynck, E.P., Gallardo, F., Kirby, E.G., 2004. Transgenic poplar characterized by ectopic expression of a pine cytosolic glutamine synthetase gene exhibits enhanced tolerance to water stress. *Tree Physiol.* 24, 729–736.
- Fu, J., Sampalo, R., Gallardo, F., Cánovas, F.M., Kirby, E.G., 2003. Assembly of a cytosolic pine glutamine synthetase holoenzyme in leaves of transgenic poplar leads to enhanced vegetative growth in young plants. *Plant Cell Environ.* 26, 411–418.
- Fuentes, S.I., Allen, D.J., Ortíz-López, A., Hernández, G., 2001. Overexpression of cytosolic glutamine synthetase increases photosynthesis and growth at low nitrogen concentrations. *J. Exp. Bot.* 52, 1071–1081.
- Gallardo, F., Fu, J., Cantón, F.R., García-Gutiérrez, A., Cánovas, F.M., Kirby, E.G., 1999. Expression of a conifer glutamine synthetase gene in transgenic poplar. *Planta* 210, 19–26.
- Hodges, M., 2002. Enzyme redundancy and the importance of 2-oxoglutarate in plant ammonium assimilation. *J. Exp. Bot.* 53, 905–916.
- Hoerlein, G., 1994. Glufosinate (phosphinothricin), a natural amino acid with unexpected herbicidal properties. *Rev. Environ. Contam. Toxicol.* 138, 73–145.
- Hui, S., Qiman, H., Jin, S., 2005. Overexpression of glutamine synthetase confers transgenic rice herbicide resistance. *High Tech. Lett.* 11, 75–79.
- Ireland, R.J., Lea, P.J., 1999. The enzymes of glutamine, glutamate, asparagines, and aspartate metabolism. In: Singh, B.K. (Ed.), *Plant Amino Acids. Biochemistry and Biotechnology*. Marcel Dekker, New York, pp. 49–109.
- Jing, Z.P., Gallardo, F., Pascual, M.B., Sampalo, R., Romero, J., Torres de Navarra, A., Cánovas, F.M., 2004. Improved growth in a field trial of transgenic hybrid poplar overexpressing glutamine synthetase. *New Phytol.* 164, 137–145.
- Keys, A.J., Bird, J.F., Cornelius, M.J., Lea, P.J., Wallsgrove, R.M., Miflin, B.J., 1978. Photorespiratory nitrogen cycle. *Nature* 275, 741–743.
- Kozaki, A., Takeba, G., 1996. Photorespiration protects C3 plants from photooxidation. *Nature* 384, 557–560.
- Lacuesta, M., Muñoz-Rueda, A., González-Murua, C., Sivak, M.N., 1992. Effect of phosphinothricin (glufosinate) on photosynthesis and chlorophyll fluorescence emission by barley leaves illuminated under photorespiratory and non-photorespiratory conditions. *J. Exp. Bot.* 43, 246–251.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lea, P.J., Ridley, S.M., 1989. Glutamine synthetase and its inhibition. In: Dodge, A.D. (Ed.), *Herbicides and plant metabolism*, Society for Experimental Biology Seminar Series, vol. 38. Cambridge University Press, Cambridge, pp. 137–170.
- Man, H.M., Boriel, R., El-Khatib, R.T., Kirby, E.G., 2005. Characterization of transgenic poplar with ectopic expression of pine cytosolic glutamine synthetase under conditions of varying nitrogen availability. *New Phytol.* 167, 31–39.
- Martinson, K.B., Sothorn, R.B., Koukari, W.L., Durgan, B.R., Gunsolus, J.L., 2002. Circadian response of annual weeds to glyphosate and glufosinate. *Chronobiol. Int.* 19, 405–422.
- Migge, A., Carrayol, E., Hirel, B., Becker, T.W., 2000. Leaf-specific overexpression of plastidic glutamine synthetase stimulates growth of transgenic tobacco seedlings. *Planta* 210, 252–260.
- Murashige, T., Skoog, K., 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497.
- Nelson, K.A., Renner, K.A., Penner, D., 2002. Yellow nutsedge (*Cyperus esculentus*) control and tuber yield with glyphosate and glufosinate. *Weed Technol.* 16, 360–365.
- Nolte, S.A., Young, B.G., Mungur, R., Lightfoot, D.A., 2004. The glutamate dehydrogenase gene *gdhA* increased the resistance of tobacco to glufosinate. *Weed Res.* 44, 335–339.
- Novitskaya, L., Trevanion, S.L., Driscoll, T.S., Foyer, C.H., Noctor, G., 2002. How does photorespiration modulate leaf amino acid contents? A dual approach through modelling and metabolite analysis. *Plant Cell Environ.* 25, 821–835.
- Oliveira, I.C., Brears, T., Knight, T.J., Clark, A., Coruzzi, G.M., 2002. Overexpression of cytosolic glutamine synthetase relation to nitrogen, light, and photorespiration. *Plant Physiol.* 129, 1170–1180.
- Palomo, J., Gallardo, F., Suárez, M.-F., Cánovas, F.M., 1998. Purification and characterization of NADP<sup>+</sup>-linked isocitrate dehydrogenase from Scots pine. Evidence for different physiological roles of the enzyme in primary development. *Plant Physiol.* 118, 617–626.
- Pascual, M.B., 2007. Expresión de glutamina sintetasa e isocitrato deshidrogenasa en árboles transgénicos: papel en el desarrollo. Ph.D. Thesis. Universidad de Málaga, Málaga.
- Pérez-García, A., de Vicente, A., Cantón, F.R., Cazorla, F.M., Codina, J.C., García-Gutiérrez, A., Cánovas, F.M., 1998. Light-dependent changes of tomato glutamine synthetase in response to *Pseudomonas syringae* infection or phosphinothricin treatment. *Physiol. Plant.* 102, 377–384.
- Prasertsongsun, S., Sangduen, N., Suwanwong, S., Santisopasri, V., Matsumoto, H., 2002. Increased activity and reduced sensitivity of glutamine synthetase in glufosinate-resistant vetiver (*Vetiveria zizanioides* Nas) cells. *Wood Biol. Manag.* 2, 171–176.
- Rachmilevitch, S., Cousins, A.B., Bloom, A.J., 2004. Nitrate assimilation in plant shoots depends on photorespiration. *Proc. Natl. Acad. Sci. USA* 101, 11506–11510.
- Sauer, H., Wild, A., Ruhle, W., 1987. The effect of phosphinothricin (glufosinate) on photosynthesis II. The causes of inhibition of photosynthesis. *Z. Naturforsch.* 42c, 270–278.
- Schjoerring, J.K., Husted, S., Mäck, G., Nielsen, K.H., Finnemann, J., Mattson, M., 2000. Physiological regulation of plant-atmosphere ammonia exchange. *Plant Soil* 221, 95–102.
- Skopelitis, D.S., Paranychianakis, N.V., Paschalidis, K.A., Pliakonis, E.D., Delis, I.D., Yakoumaki, D.I., Kouvarakis, A., Papadakis, A.K., Stephanou, E.G., Roubelakis-Angelakis, K.A., 2006. Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. *Plant Cell* 18, 12767–12781.
- Tercé-Laforgue, T., Dubois, F., Ferrario-Méry, S., Pou de Crezenzo, M.-A., Snagwan, R., Hirel, B., 2004. Glutamate dehydrogenase of tobacco is mainly induced in the cytosol of phloem companion cells when ammonia is provided either externally or released during photorespiration. *Plant Physiol.* 136, 4308–4317.
- Tobin, A.K., Sumar, N., Patel, M., Moore, A.L., Stewart, G.R., 1988. Development of photorespiration during chloroplast biogenesis in wheat leaves. *J. Exp. Bot.* 39, 833–843.
- Tolbert, N.E., 1997. The C2 oxidative photosynthetic carbon cycle. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 1–25.
- Wendler, C., Barniske, M., Wild, A., 1990. Effect of phosphinothricin (glufosinate) on photosynthesis and photorespiration of C3 and C4 plants. *Photosynth. Res.* 24, 55–61.
- Wendler, C., Wild, A., 1989. Effect of phosphinothricin (glufosinate) on photosynthesis and photorespiration. *Z. Naturforsch.* 45c, 535–537.
- Yu, J.W., Woo, K.C., 1991. Correlation between the development of photorespiration and the change in activities of NH<sub>3</sub> assimilation enzymes in greening oat leaves. *Aust. J. Plant. Physiol.* 18, 583–588.