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RESEARCH PAPER

Evidence for an operative glutamine translocator in chloroplasts from maritime pine (*Pinus pinaster* Ait.) cotyledons

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ABSTRACT

In higher plants, ammonium is assimilated into amino acids through the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle. This metabolic cycle is distributed in different cellular compartments in conifer seedlings: glutamine synthesis occurs in the cytosol and glutamate synthesis within the chloroplast. A method for preparing intact chloroplasts of pine cotyledons is presented with the aim of identifying a glutamine–glutamate translocator. Glutamine–glutamate exchange has been studied using the double silicone layer system, suggesting the existence of a translocator that imports glutamine into the chloroplast and exports glutamate to the cytoplasm. The translocator identified is specific for glutamine and glutamate, and the kinetic constants for both substrates indicate that it is unsaturated at intracellular concentrations. Thus, the experimental evidence obtained supports the model of the GS/GOGAT cycle in developing pine seedlings that accounts for the stoichiometric balance of metabolites. As a result, the efficient assimilation of free ammonia produced by photorespiration, nitrate reduction, storage protein mobilisation, phenylpropanoid pathway or *S*-adenosylmethionine synthesis is guaranteed.

INTRODUCTION

Nitrogen retention in forest ecosystems is based on net nitrification, net mineralisation, microbial assimilation of ammonium (NH_4^+) and plant N uptake. Most plant species prefer nitrate (NO_3^-) rather than NH_4^+ as N source (Kronzucker *et al.* 1997). However, rice, ericaceous species and many conifers occur naturally on soils enriched in NH_4^+ and organic N (Lavoie *et al.* 1992). It has been firmly established that angiosperms assimilate NH_4^+ into organic N within chloroplasts by the sequential action of the enzymes glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.7.1; 1.4.1.14); this is called the GS/GOGAT cycle (Miflin & Lea 1980). This organic N can be reused as a substrate for the biosynthesis of major N compounds of plant cells (Lam *et al.* 1996; Cánovas *et al.* 1998). Independent of the N source, the main role of the GS/GOGAT cycle is the assimilation of ammonia generated during photorespiration by the mitochondrial glycine decarboxylase reaction (Hirel & Lea 2001).

Conifers are reported to contain storage proteins that are extremely rich in amino acids with high nitrogen–carbon ratios, such as arginine (Allona *et al.* 1994). The megagametophyte, which houses most of the protein reserves, exports, without prior metabolic conversion, high quantities of arginine and glutamine (Feirer 1995; King & Gifford 1997) originating from the breakdown of storage proteins during seedling development. The increase of free arginine coincides with a marked increase of arginase and urease activity in cotyledons and epicotyls (King & Gifford 1997) that produce a high free NH_4^+ concentration within the cell. There are other physiological processes that also increase free NH_4^+ release, such as: (i) the phenylpropanoid pathway, in which free NH_4^+ is obtained from phenylalanine or thyrosine by means of phenyl ammonium lyase; and (ii) synthesis of *S*-adenosylmethionine as a part of C1 metabolism in plastids, in which serine hydroxy-methyl transferases also produce high free NH_4^+ concentrations (Cantón *et al.* 2005). Nitrate reduction and photorespiration are physiological processes that continually produce free NH_4^+ (Gebauer & Schulze 1997).

The GS/GOGAT cycle is the best candidate for efficient recycling of this N to avoid severe N deficiency that would compromise growth and development of woody plants (Cañas *et al.* 2007; Cánovas *et al.* 2007). Most plants have a GS/GOGAT cycle within the chloroplast. Since 2-oxoglutarate (2-OG), the precursor for ammonia assimilation, is biosynthesised in the cytosol and/or mitochondria, plastids depend on a known translocator, DiT1, that imports 2-OG into plastids in counter-exchange for a dicarboxylate, mainly malate (Weber *et al.* 1995; Taniguchi *et al.* 2002). It is well established by silicon-oil-filtration–centrifugation that DiT1 is coupled to the malate/glutamate translocator DiT2 to provide 2-OG import and glutamate export (the product of ammonia assimilation) without net malate transport (Woo *et al.* 1987; Flügge *et al.* 1988), such coupling being essential for survival in ambient air (Somerville & Ogren 1983). Both

DiT1 and DiT2 have been identified at the molecular level (Weber *et al.* 1995; Taniguchi *et al.* 2002; Renné *et al.* 2003), and DiT1/DiT2 coupling has been demonstrated using mutant and transgenic plants (Renné *et al.* 2003; Schneidereit *et al.* 2006). DiT1 accepts the dicarboxylates 2-OG, fumarate, succinate, glutarate and malate. DiT2, in addition to DiT1 substrates, accepts glutamate and aspartate, although neither DiT2 nor DiT1 accept glutamine (Renné *et al.* 2003).

Notably, pines distribute the GS/GOGAT cycle in different cellular compartments in photosynthetic and non-photosynthetic tissues: GS is present in the peripheral cytoplasm, whereas GOGAT is located in the chloroplast stroma (García-Gutiérrez *et al.* 1998; Suarez *et al.* 2002). As a consequence, it has been postulated that a single glutamine/glutamate translocator should be present in pine chloroplasts (Weber & Flugge 2002) to enable GS/GOGAT cycle function. This translocator should import glutamine to the chloroplast and export glutamate to the cytosol, otherwise N would be continuously drained from this essential cycle. The molecular and biochemical characterisation of DiT1 and DiT2 has eliminated the possibility of glutamine transport into chloroplasts by any dicarboxylate translocator. Evidence has been presented indicating that there is a different, glutamine-specific translocator in organisms that do not have a compartmentalised GS/GOGAT cycle in photosynthetic tissues, such as oats, spinach (Yu & Woo 1988) and *Arabidopsis* (Vernon 1960). This study presents a method to isolate chloroplasts from green cotyledons of pine seedlings to obtain biochemical evidence for specific glutamine/glutamate exchange in pine, whose operation is required to enable the GS/GOGAT cycle.

MATERIAL AND METHODS

Plant material

Maritime pine (*Pinus pinaster* Aiton) seeds were obtained from the Instituto Andaluz de Reforma Agraria de la Junta de Andalucía (Spain), and soaked for 1 day in sterile distilled water. They were then germinated in plastic trays containing vermiculite (Eurover, Europerlita S.A.) without any extra fertiliser, but were moistened with distilled water. Seedling germination and early growth took place under white light (Sylvania F48T12/CW/VHO fluorescent light, 110 μmol photons·m^{-2}·s^{-1}) at 22 °C and with constant humidification in a Phytotron (Koxka, Pamplona, Spain), employing a photoperiod of 16 h light and 8 h darkness.

Chloroplast isolation

Intact chloroplasts were isolated from the cotyledons of 1.5-cm long maritime pine seedlings, 13–15 days after soaking. Twenty grams of green tissue were cut into small pieces and blended in 60 ml of cold GR1 (0.33 M sorbitol, 0.05 M MOPS, pH 7.5, 2 mM EDTA, 1 mM $MgCl_2$ and 2 mM ascorbate) using two 2-s bursts on a Polytron homogeniser (Kinematica AG, Littau, Switzerland) at 60% line voltage. The homogenate was squeezed through two layers of cheesecloth, the filtrate was recovered in concave-bottom tubes, BSA was added at 0.15%, and the suspension was centrifuged in a swinging bucket rotor at 1500 *g* for 3 min. The material retained in the cheesecloth was recovered and complemented with 40 ml GR1 to repeat the blending, filtration and centrifugation steps. The process was repeated a third time using 20 ml GR1. Each chloroplast pellet was gently resuspended in 2 ml of cold MR1 (0.33 M sorbitol, 0.05 M MOPS, pH 7.5, 2 mM EDTA, 1 mM $MgCl_2$, 2 mM ascorbate, 0.15% BSA) using a micropipette with a cut cone. Each suspension was layered over a discontinuous Percoll (Sigma, St. Louis, MO, USA) gradient containing 0.75 ml of 80% Percoll and 2 ml of 40% Percoll, and centrifuged in a swinging bucket rotor at 4500 *g* for 10 min. The interphase between the two Percoll layers containing intact chloroplasts was carefully aspirated with a cut cone, placed in a new tube, and diluted from 5- to 10-fold in cold MR1. The final suspension was centrifuged for 1 min at 1500 *g* and the pellet resuspended in 0.5 ml cold MR1. All procedures were carried out at 4 °C.

Chlorophyll determination

Chlorophyll content was estimated by extraction of the MR1 chloroplast suspension in 80% neutralised acetone. The tube was energetically mixed, stored in the dark for 5 min, and centrifuged for 3 min at 13,000 *g* to remove solid material. The supernatant was measured in a spectrophotometer at 663.2, 646.8 and 730 nm, and the chlorophyll concentration in microgram per millilitre in the acetone dilution was assessed using the formula (Lichtenthaler 1987)

$$7.15 \times (A_{663.2} - A_{730}) + 18.71 \times (A_{646.8} - A_{730}).$$

Silicone oil centrifugation techniques

This technique is based on previously described methods (Heldt 1980; Howitz & McCarty 1985). The arrangement of aqueous and oil layers in Beckman 0.4-ml microcentrifuge tubes, from top to bottom (Fig. 1) was 100 μl of chloroplast suspension at 0.1 mg Chl·ml^{-1}, 80 μl of silicone oil AR200:AR20 (Extinte SL, Valencia, Spain) 6:1, 100 μl of 7% Percoll in GR1, 70 μl of silicone oil AR200, and 20 μl of a hypotonic solution. Tubes were centrifuged in a Hettich Mikro (Tuttlingen, Germany) for 30 s at 10,000 *g*, the silicone layers remaining separate at the end of centrifugation. After removing the upper phases, the pellet in the lower phase was recovered with a Hamilton syringe (1702 N). This phase con-

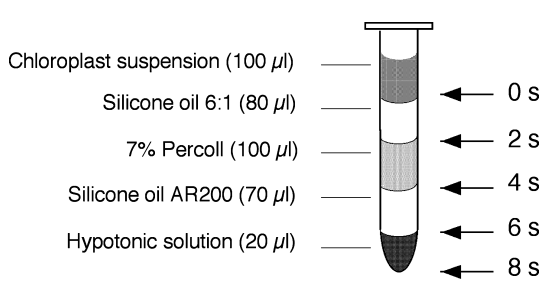

Fig. 1. Silicone oil centrifugation system in a 0.4-ml tube. Layer composition (left) and progress of the first chloroplasts through the centrifugation process (right) are shown.

tained Tris–HCl 1 M, pH 7.5, when the assay was performed to assess the percentage integrity, or AAH solution (6 N acetic acid, 0.135 M NaClO) when radioactive metabolite transport was measured.

Intactness and activity of chloroplasts

The intactness assay is based on the difference in density between intact and broken chloroplasts. From the chloroplast solution, the volume containing 10 μg of Chl was centrifuged through the silicone oil tubes (Fig. 1). Intact chloroplasts can migrate through the silicone oil layer, while broken chloroplasts are retained in the MR1–silicone oil interphase. The amount of pelleted chlorophyll confirmed an integrity ranging from 80% to 95%.

Since chloroplasts, even when intact, can be inactivated by incorrect treatment, the ferricyanide method (Walker 1980; Wang 1980; Walker *et al.* 1987) was used to assess the enzyme activity of chloroplasts only for preparations with more than 80% integrity. The amount of reduced ferricyanide in broken chloroplasts – using intact chloroplasts from the same preparation as control – was decisive in establishing that only preparations with reducing activity higher than 0.4 $nmol \cdot min^{-1} \cdot (\mu g\ Chl)^{-1}$ were suitable for use. Chloroplast enzymes remain 100% active for 24 h, slowly decreasing in activity to 50% after 12 days.

Measurements of metabolite transport

All metabolite transport, unless otherwise specified, was measured in chloroplasts preloaded with specific metabolites. Preloading of chloroplasts contained in 10 μg of chlorophyll was performed by incubation in MR1 in the presence of 15 mM specific metabolite for 30 min at 4 °C in the light (110 μmol $photons \cdot m^{-2} \cdot s^{-1}$), since chloroplast enzymes remain 100% active in these conditions and chloroplast metabolism remains competent (note that transport measurements in darkness are not significant in the GS/GOGAT cycle or any other related chloroplast metabolic pathway). Preloaded chloroplasts were diluted in MR1 to provide a concentration of 0.1 $mg \cdot ml^{-1}$chlorophyll – no wash was needed since metabolite contamination is negligible (Yu & Woo 1988). A total of 100 μl of this solution was pipetted into microfuge tubes containing the phases as shown in Fig. 1. The Percoll layer contained 1 mM [^{3}H]glutamine (Dupont) at 20 $Ci \cdot mol^{-1}$ and unlabelled competitors (when present). The tubes were incubated for 5 min at 22 °C in the light (110 μmol $photons \cdot m^{-2} \cdot s^{-1}$) and centrifuged as above. Uptake occurred during centrifugation as the plastids moved through the Percoll layer. The two silicone oil layers remained separate at the end of centrifugation. The uptake time determined was 4 s (see Results) and radioactivity in the pellet fraction was determined as described (Heldt 1980).

Glutamate export was measured by preloading chloroplasts with 7.5 mM [^{3}H]glutamate at 10 $Ci \cdot mol^{-1}$ for 30 min at 4 °C in the light. The tested metabolite counter-exchanged with glutamate was included in the 7% Percoll layer at 1 mM (Fig. 1). Tubes were incubated for 5 min in the light at 22 °C and centrifuged as above. Exchange occurred in the Percoll layer during centrifugation. This layer was taken off to measure the exported glutamate.

Statistical analyses

Data are presented with their standard error, and significant differences were determined with a confidence level of 95% in single or paired *t*-tests, or with non-parametric Mann–Whitney *U*-test (Hassard 1991). Regression equations were performed as described (Claros & Cánovas 1998), providing that the standard error of prediction as a measure of the discrepancy (residual error) between observed values and those predicted fitted (Motulsky 1995).

RESULTS

Chloroplast isolation for metabolite transport measurements

Transport studies on living chloroplasts require a large amount of intact, active material. A protocol for the isolation of chloroplasts from pine leaves has been published (Oku *et al.* 1971), but a high degree of purity was required to avoid unwanted artifacts. Several general-purpose protocols found in the literature (Reeves & Hall 1980; Joy & Mills 1987; Walker *et al.* 1987) were also tested in pine, but were unable to provide sufficient starting material (yields below 1 $\mu g\ Chl \cdot g^{-1}$ fresh weight). Several changes were performed to refine the protocol for isolation of chloroplasts from pine cotyledons as starting material, without contamination due to any other organelles and with sufficient yield. Germination stages, Polytron pulses, homogenisation cycles, centrifugation time and speed, and Percoll layers were found to be especially important for intact chloroplast yield (Fig. 2) with reduced contamination by dense organelles (nuclei and peroxysomes). Ordinary yields were around 15 $\mu g\ Chl \cdot g^{-1}$ fresh weight. The use of a stable double silicone layer system for chloroplast transport measurement (Fig. 1) instead of the single-layer method avoids excessive washing of chloroplasts and permits calculation of initial reaction rates. The incubation time was estimated as described previously (Howitz & McCarty 1985). The first entry of chloroplasts into the silicone oil (Fig. 1, right) occurred within 2 s, and after 4 s, the chloroplasts reached the 7% Percoll layer. After 6 s, 27% of chloroplasts reached the bottom of the tube, and after 12 s no more accumulation was observed, indicating that all the chloroplasts had been pulled down. Chloroplasts do not pass through the

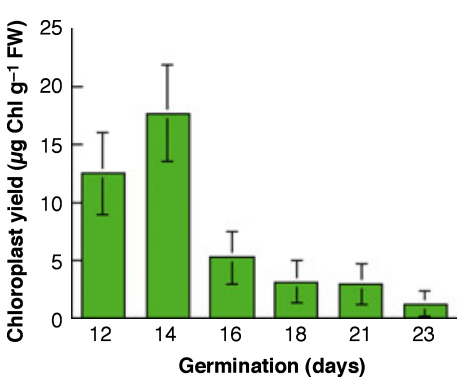

Fig. 2. Effect of harvesting at different germination stages on the yield of chloroplasts from *P. pinaster* cotyledons. Seedlings were harvested every 2 days once the cotyledons had reached 1.2 cm in length (14 days).

7% Percoll phase in a single, discrete period, but the asymmetrical distribution of centrifugation times (0% at 4 s, 27% at 6 s, 44% at 8 s, 3% at 10 s, 0% at 12 s) provided a reaction time of 4 s, which guarantees that the import results correspond to the linear range of the enzymatic reaction, *i.e.* the initial rates.

Glutamine uptake

To determine whether glutamine uptake was mediated by the antiport of another metabolite, preloading with different dicarboxylates was performed. Preloading is essential for maximal activity of metabolite transport, which occurs by counter-exchange, because chloroplasts generally lose a substantial part of their endogenous metabolite pool during isolation. Moreover, glutamine can interact non-specifically with other proteins of the outer chloroplast membrane, providing artifactual uptake activities (Molina *et al.* 1995; M. A. Medina, personal communication). This accounts for the fact that maximum activities were found without preloading chloroplasts (Fig. 3, dark bars): [^{3}H]glutamine uptake activity in pine cotyledon chloroplasts preloaded with glutamine, malate, 2-OG and aspartate were two- to fourfold lower than control (not preloaded) chloroplasts. Compared to these metabolites, [^{3}H]glutamine uptake was significantly increased when chloroplasts were preloaded with glutamate ($P < 0.05$). This result suggests that glutamine import occurred preferentially with the export of glutamate. This was confirmed by the reverse experiment, in which chloroplasts were preloaded with [^{3}H]glutamate and its efflux measured in the presence of glutamine and other metabolites. Here, the main export is observed when glutamine can be used as the counter-ion of glutamate (Fig. 3, clear bars).

The fact that glutamate is a good counter-exchange substrate for glutamine but not aspartate, prompted us to assess the substrate specificity of this translocator. Hence, [^{3}H]glutamine uptake into pine cotyledon chloroplasts was measured in the presence of different concentrations of dicarboxylates that are structurally related to glutamine, at 1 mM and 5 mM: aspartate, glutamate and 2-OG. Figure 4 shows that these dicarboxylates do not inhibit [^{3}H]glutamine uptake significantly (in fact, 2-OG increased uptake), strongly suggesting

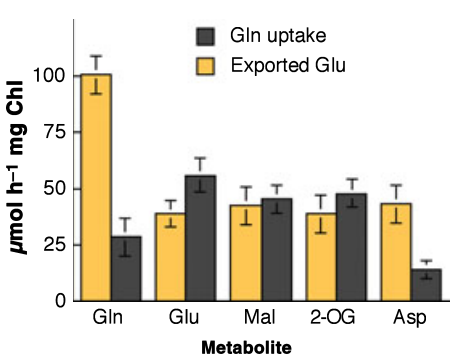

Fig. 3. Preferred substrates for the translocator, using the double silicone layer system with pine cotyledon chloroplasts at 22 °C. Dark bars show the effect of preloading with 15 mM metabolite on 1 mM [^{3}H]glutamine uptake. Clear bars represent the effect of adding different 1 mM external substrates when preloading with 7.5 mM [^{3}H]glutamate.

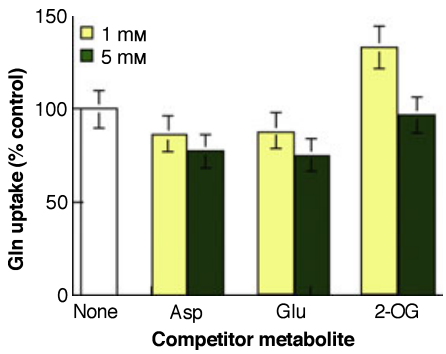

Fig. 4. Effect of dicarboxylates on 1 mM [^{3}H]glutamine transport activity in intact pine cotyledon chloroplasts at 22 °C in the double silicone layer system. Chloroplasts were preloaded with glutamate. Two concentrations (1 and 5 mM) were used.

that the glutamine/glutamate translocator is specific for these metabolites and is a separate event unrelated to dicarboxylate import.

In order to obtain basic kinetic data on glutamine uptake, the dependence of the transport rate on the external [^{3}H]glutamine concentration was determined in three independent experiments with chloroplasts preloaded with glutamate. The best estimates were obtained using direct linear plots (Johnson & Faunt 1992) since this provided a standard error of prediction of 23, whereas other methods provide one ranging from 33 to 63. The apparent K_m value obtained without sorbitol and water correction was 4.7 mM (Fig. 5). This non-normalised value is within the range of the normalised values described in the literature for glutamine and dicarboxylate translocators (Woo *et al.* 1987; Flügge *et al.* 1988; Yu & Woo 1988; Molina *et al.* 1995; Renné *et al.* 2003), which can guarantee that glutamine will not be drained from the cycle. The apparent V_{max} of 362 µmol·h^{-1}·(mg·Chl)$^{-1}$ is, however, an order of magnitude higher than that described, suggesting that the glutamine translocator is not easily saturable at the

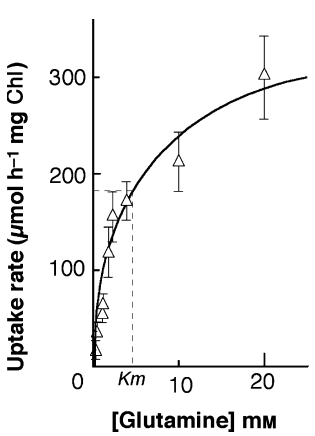

Fig. 5. Concentration dependence of glutamine transport into chloroplasts preloaded with glutamate using the stable double silicone layer system. A direct linear plot was used to determine the apparent K_m (4.7 mM) and the apparent V_{max} (362 µmol·h^{-1}·(mg Chl)$^{-1}$), with a standard error of prediction of 23. These values were estimated using Lines&Kinetics software (Claros & Cánovas 1998).

intracellular concentration of dicarboxylates (Gerhardt & Heldt 1984).

DISCUSSION

Isolation of chloroplasts from maritime pine cotyledons

In general, trees are not the most suitable subjects for research on molecular processes in plants. The first difficulty involves obtaining metabolically active organelles in large amounts. This problem was circumvented using pine seedlings as the chloroplast source. Developmental stage and tissue homogenisation have been shown to be the main variables that influence final yield (Fig. 2). These findings can be explained by the fact that mature cotyledons store more proteins and fatty acids, which substantially increase the fresh weight and damage chloroplasts during extraction (Papageorgiou 1980; Reeves & Hall 1980; Walker *et al.* 1987). This is why researchers usually use the ferricyanide method (Walker 1980) to assess the integrity of chloroplasts, assuming that they are active. In the present study, integrity and activity have been considered as different aspects, since integrity can be measured taking into account the different buoyant density of broken and intact chloroplast. Once integrity has been verified, activity can be measured with an assay that involves several enzymes, such as the reduction of ferricyanide. The ferricyanide reaction has commonly been used to calculate the integrity of chloroplasts, assuming that the lower the activity, the more intact the chloroplasts, without taking into account that low activity can be due to broken chloroplasts undergoing enzyme inactivation. To avoid this, and to know whether chloroplast enzymes are active, an aliquot of intact chloroplasts was broken and ferricyanide reduction measured (see Material and methods). Therefore, high levels of ferricyanide reduction indicate that enzymatic activities are still working in purified chloroplasts, making them suitable for transport analyses. The mean yield of our method was 15 μg Chl·g^{-1} fresh weight, and the mean percentage integrity was 90%. These results were confirmed by optical microscopy (results not shown).

Characterisation of the chloroplast glutamine translocator

Metabolite transport in isolated chloroplasts occurs primarily by counter-exchange (Lehner & Heldt 1978), and so metabolite transport activities of isolated chloroplasts would depend on the endogenous metabolite pools present in chloroplast preparations. The short reaction time used (4 s, Fig. 1) guarantees that metabolic conversion by GOGAT and other enzymes is negligible and no enzyme inhibitor is required. In the case of dicarboxylates, the use of inhibitors is mandatory since they can be transported in and out of chloroplasts by DiT1 and DiT2 translocators with overlapping specificities and because the measured activity is the sum of the activity of the different translocators involved (Woo *et al.* 1987). Although glutamine is not a substrate for these translocators (it is not a dicarboxylate), the best way to minimise counter-exchange with nonspecific internal metabolites is by preloading chloroplasts with a single substrate. Preloading is expected to block all nonspecific binding sites and to replace all exchangeable metabolites by the preloaded species inside the chloroplasts. This guarantees that [^{3}H]glutamine uptake would only occur by counter-exchange with the preloaded metabolite, and avoids nonspecific binding (Fig. 3, dark bars). Although many studies on metabolite transport use the single-layer silicone system, the double-layer system shown in Fig. 1 facilitates chloroplast preloading with specific metabolites without the need for a subsequent organelle wash and recentrifugation step, as occurs in single-layer systems. Additionally, the reaction time is short enough to ensure initial reaction rates (where the reaction kinetics are linear) in order to minimise interference from metabolic conversion. These conditions enable the study of glutamine transport across pine chloroplast envelope membranes during germination or wood synthesis, since at these stages, the internal levels of NH_4^+ are high (King & Gifford 1997; Cantón *et al.* 2005), and NH_4^+ should be metabolised by a compartmentalised GS/GOGAT cycle (García-Gutiérrez *et al.* 1998; Suarez *et al.* 2002). Our results suggest that glutamine uptake in isolated chloroplasts is different from that of dicarboxylates, and can account for a continuous exchange of substrates between cytosol and chloroplasts.

The transport activity measured in the chloroplasts was, as expected, affected by the metabolite species preloaded into the organelle (Fig. 3): glutamate was the preferred counter-ion for glutamine transport, importing glutamine and exporting glutamate. The specific activity of glutamine import is quite similar to that described for pea (Barber & Thurman 1978). High concentrations of aspartate or glutamate (two substrates of the dicarboxylate translocator) had only a small inhibitory effect on glutamine uptake in glutamate-preloaded chloroplasts (Fig. 4). These results may indicate a distinct site for glutamine transport in pine cotyledon chloroplasts. Since no dicarboxylate seemed to be able to inhibit glutamine uptake, only one system would be involved in such a process. However, the possibility that the small reduction in glutamine uptake compared to the control (Fig. 4) could reflect a certain amount of glutamine uptake by some other route cannot be ruled out. The fact that glutamate, being the counter-ion of glutamine, does not inhibit import of glutamine would suggest that the two substrates are recognised on different sides of the membrane, and that the translocator seems to be able to discriminate between the two substrates on either side of the membrane. This is also supported by the fact that glutamine can effectively use glutamate as counter-ion (Fig. 3). Such specificity prevents an exchange of glutamine and glutamate in the reverse order, or a fruitless exchange of glutamine or glutamate. The unexpected increase in glutamine uptake observed with 2-OG (Fig. 4) might be explained as a result of activation of the GOGAT enzyme by the 2-OG abundance, since: (i) chloroplasts are simultaneously importing 2-OG and Gln (both are GOGAT substrates); (ii) the 2-OG conversion to glutamate by GOGAT increases as more 2-OG is available (Schneidereit *et al.* 2006); and (iii) as a result of GOGAT activity with high availability of 2-OG, more glutamate is accumulated and the Gln concentration inside chloroplasts would decrease faster than in other import reactions that do no receive external 2-OG (Fig. 4). The increased 2-OG conversion into glutamate due to the continuous availability of its substrates would require more Gln import, and this would explain the apparent Gln uptake activation due to 2-OG. Although the hypothesis of

glutamine import activation by 2-OG is supported by the argument that 2-OG transport into chloroplasts is crucial for proper plant development (Schneidereit *et al.* 2006), more experimental evidence is required to demonstrate it unambiguously.

Dicarboxylates are highly concentrated (Gerhardt & Heldt 1984; Woo *et al.* 1987; Renné *et al.* 2003) in the chloroplast stroma (21 mM glutamate and 10 mM aspartate) as well as in the cytosol (4 mM both dicarboxylates). The high K_m and V_{max} values, in agreement with values for other metabolite translocators (Woo *et al.* 1987; Flügge *et al.* 1988; Molina *et al.* 1995; Renné *et al.* 2003), including the oat and spinach glutamine translocator (Yu & Woo 1988), suggest that substantial *in vivo* transport of glutamine would occur in pine chloroplasts (Gezelius & Nasholm 1993), ensuring that the glutamine would not be inappropriately retained in the cytosol, and similarly, no excess glutamate would accumulate within the chloroplast.

GS/GOGAT cycle in maritime pine

Ammonium in pine is produced through several pathways, such as photorespiration, nitrate reduction, lignin and *S*-adenosylmethionine synthesis, or storage protein mobilisation. Therefore, it must be rapidly incorporated into amino acids through the GS/GOGAT cycle (Cánovas *et al.* 2007). The previously proposed hypothesis that the glutamine translocator is involved in the photosynthetic cells of pine seedlings (Weber & Flugge 2002) has been validated by this study (Fig. 6). The glutamine formed in the cytosol is transported into the chloroplast by a specific translocator. It is then metabolised by GOGAT to form glutamate, which would subsequently become the counter-exchange ion for glutamine transport through the glutamine translocator, or further metabolised to synthesise nitrogen-containing compounds. The glutamine/glutamate translocator facilitates the stoichiometric balance of the GS/GOGAT cycle, ensuring that nitrogen is not drained from it. Furthermore, this could explain how, during seedling development (King & Gifford 1997) or lignin or *S*-adenosylmethionine synthesis (Cantón *et al.* 2005), the continuously produced NH_4^+ is efficiently incorporated into amino acids (Cánovas *et al.* 2007) by the specificity and high activity of the glutamine/glutamate exchange between chloroplasts and cytosol, provided that enough 2-OG is available (Fig. 4; Schneidereit *et al.* 2006).

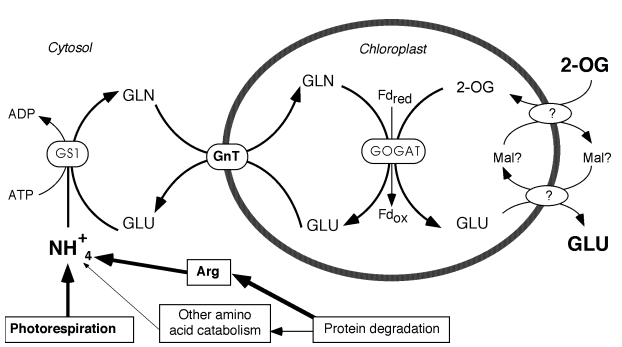

Fig. 6. Model for the compartmentalised GS/GOGAT cycle in photosynthetic cells of pine seedlings. The translocator identified in this study, named GnT in the Figure, facilitates the exchange of glutamate and glutamine to complete the cycle, ensuring that organic nitrogen is not drained from the cycle. Although the presence of dicarboxylate translocators for 2-OG and glutamate has not been demonstrated in pine cotyledons, they have been included to complete the flux of metabolites through the GS/GOGAT cycle since they appear in many other plant species.

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REFERENCES

Allona I., Collada C., Casado R., Aragoncillo C. (1994) 2s arginine-rich proteins from *Pinus pinaster* seeds. *Tree Physiololgy*, **14**, 211–218.

Barber D.J., Thurman D.A. (1978) Transport of glutamine into isolated pea chloroplasts. *Plant, Cell & Environment*, **1**, 297–303.

Cañas R.A., de la Torre F., Canovas F.M., Canton F.R. (2007) Coordination of *psas1* and *psaspg* expression controls timing of re-allocated N utilization in hypocotyls of pine seedlings. *Planta*, **225**, 1205–1219.

Cánovas F.M., Cantón F.R., García-Gutiérrez A., Crespillo R., Gallardo F. (1998) Molecular physiology of glutamine and glutamate biosynthesis in developing conifer seedlings. *Physiologia Plantarum*, **103**, 287–294.

Cánovas F.M., Ávila C., Cantón F.R., Cañas R.A., de la Torre F. (2007) Ammonium assimilation and amino acid metabolism in conifers. *Journal of Experimental Botany*, **58**, 2307–2318.

Cantón F.R., Suárez M.F., Cánovas F.M. (2005) Molecular aspects of nitrogen mobilization and recycling in trees. *Photosynthesis Research*, **83**, 265–278.

Claros M.G., Cánovas F.M. (1998) Lines&kinetics: a graphic tool to deal with linear regressions and enzyme kinetics. *Embnet News*, **5**, 5–7.

Feirer R.P. (1995) The biochemistry of conifer embryo development: amino acids, polyamines and storage proteins. In: Jain S., Gupta P., Newton R. (Eds), *Somatic embryogenesis in woody plants*. Kluwer Academic Publishers, Dordrecht, The Netherlands: pp. 317–336.

Flügge I.U., Woo K.C., Heldt H.W. (1988) Characteristics of 2-oxoglutarate and glutamate transport in spinach chloroplasts. *Planta*, **174**, 534–541.

García-Gutiérrez A., Dubois F., Cantón F.R., Gallardo F., Sangwan R.S., Cánovas F.M. (1998) Two different models of early development and nitrogen assimilation in gymmosperm seedlings. *Plant Journal*, **13**, 187–199.

Gebauer G., Schulze E.D. (1997) Nitrate nutrition of Central European forest trees. In: Rennenberg H., Eschirch W., Ziegler H. (Eds), *Trees – contributions to modern tree physiology*. Backhuys Publishers, Leiden, The Netherlands: pp. 273–291.

Gerhardt R., Heldt H.W. (1984) Measurement of subcellular metabolite levels in leaves by fractionation of freeze-stopped material in non-aqueous media. *Plant Physiology*, **75**, 542–547.

Gezelius K., Nasholm T. (1993) Free amino acids and protein in Scots pine seedlings cultivated at different nutrient availabilities. *Tree Physiology*, **13**, 71–86.

Hassard T.H. (1991) *Understanding biostatistics*. Mosby-Year Book, Inc, St Louis, MO, USA.

Heldt H.W. (1980) Measurement of metabolite movement across the envelope and of the pH in the stroma and the thylakoid space in intact chloroplasts. *Methods in Enzymology*, **69**, 604–613.

Hirel B., Lea P.J. (2001) Ammonia assimilation. In: Lea P.J., Morot-Gaudry J.F. (Eds), *Plant nitrogen*. Springer-Verlag, Berlin, Germany: pp. 79–99.

Howitz K.T., McCarty R.E. (1985) Kinetic characteristics of the chloroplast envelope glycolate transporter. *Biochemistry*, **24**, 2645–2652.

Johnson M.L., Faunt L.M. (1992) Parameter estimation by least-square methods. *Methods in Enzymology*, **210**, 1–37.

Joy K.W., Mills W.R. (1987) Purification of chloroplasts using silica sols. *Methods in Enzymology*, **148**, 179–188.

King J.E., Gifford D.J. (1997) Amino acid utilization in seeds of lobolly pine during germination and early seedling growth. *Plant Physiology*, **113**, 1125–1135.

Kronzucker H.J., Siddiqi M.Y., Glass A.D.M. (1997) Conifer roots discrimination against soil nitrate and the ecology of forest succession. *Nature*, **385**, 59–64.

Lam H.M., Coschigano I.C., Oliveira I.C., Melo-Oliveira R., Coruzzi G. (1996) The molecular-genetics of nitrogen assimilation into amino acids in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **47**, 569–593.

Lavoie N., Vézina L.-P., Margolis H. (1992) Adsorption and assimilation of nitrate and ammonium ions by jack pine seedlings. *Tree Physiology*, **11**, 171–183.

Lehner K., Heldt H.W. (1978) Dicarboxilate transport across the inner membrane of the chloroplast envelope. *Biochimica et Biophysica Acta*, **501**, 531–544.

Lichtenthaler H.K. (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology*, **148**, 350–382.

Miflin B.J., Lea P.J. (1980) Ammonia assimilation. In: Miflin B.J. (Ed.), *The biochemistry of plants*. Academic Press, New York, USA: pp. 169–202.

Molina M., Segura J.A., Aledo J.C., Medina M.A., Núñez de Castro I., Márquez J. (1995) Glutamine transport by vesicles isolated from tumour-cell mitochondrial inner membrane. *Biochemical Journal*, **308**, 629–633.

Motulsky H. (1995) *Intuitive biostatistics*. Oxford University Press, New York, USA.

Oku T., Kawara H., Tomita G. (1971) The Hill reaction and oxygen uptake in isolated pine chloroplasts. *Plant and Cell Physiology*, **12**, 559–566.

Papageorgiou G.C. (1980) Stabilization of chloroplasts and subchloroplast particles. *Methods in Enzymology*, **69**, 613–634.

Reeves S.G., Hall D.O. (1980) Higher plant chloroplasts and grana: general preparative procedures (excluding high carbon dioxide fixation ability chloroplast). *Methods in Enzymology*, **69**, 85–94.

Renné P., Dressen U., Hebbeker U., Hille D., Flügge U.I., Westhoff P., Weber A.P. (2003) The *Arabidopsis* mutant dct is deficient in the plastidic glutamate/malate translocator dit2. *Plant Journal*, **35**, 316–331.

Schneidereit J., Hausler R.E., Fiene G., Kaiser W.M., Weber A.P. (2006) Antisense repression reveals a crucial role of the plastidic 2-oxoglutarate/malate translocator dit1 at the interface between carbon and nitrogen metabolism. *Plant Journal*, **45**, 206–224.

Somerville S.C., Ogren W.L. (1983) An *Arabidopsis thaliana* mutant defective in chloroplast dicarboxylate transport. *Proceedings of the National Academy of Sciences USA*, **80**, 1290–1294.

Suarez M.F., Avila C., Gallardo F., Canton F.R., Garcia-Gutierrez A., Claros M.G., Canovas F.M. (2002) Molecular and enzymatic analysis of ammonium assimilation in woody plants. *Journal of Experimental Botany*, **53**, 891–904.

Taniguchi M., Taniguchi Y., Kawasaki M., Takeda S., Kato T., Sato S., Tabata S., Miyake H., Sugiyama T. (2002) Identifying and characterizing plastidic 2-oxoglutarate/malate and dicarboxylate transporters in *Arabidopsis thaliana*. *Plant Cell Physiology*, **43**, 706–717.

Vernon L.P. (1960) Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. *Analytical Chemistry*, **32**, 1144–1150.

Walker D.A. (1980) Preparation of higher plant chloroplasts. *Methods in Enzymology*, **69**, 94–104.

Walker D.A., Cerovic Z.G., Robinson S.P. (1987) Isolation of intact chloroplasts: general principles and criteria of integrity. *Methods in Enzymology*, **148**, 145–157.

Wang R.T. (1980) Amperometric hydrogen electrode. *Methods in Enzymology*, **69**, 409–434.

Weber A., Flugge U.I. (2002) Interaction of cytosolic and plastidic nitrogen metabolism in plants. *Journal of Experimental Botany*, **53**, 865–874.

Weber A., Menzlaff E., Arbinger B., Gutensohn M., Eckerskorn C., Flügge U.I. (1995) The 2-oxoglutarate/malate translocator of chloroplast envelope membranes: molecular cloning of a transporter containing a 12-helix motif and expression of the functional protein in yeast cells. *Biochemistry*, **34**, 2621–2627.

Woo K.C., Flügge U.I., Heldt H.W. (1987) A two translocator model for the transport of 2-oxoglutarate and glutamate in chloroplasts during ammonia assimilation in the light. *Plant Physiology*, **84**, 624–632.

Yu J., Woo K.C. (1988) Glutamine transport and the role of the glutamine translocator in chloroplasts. *Plant Physiology*, **88**, 1048–1054.