Photorespiratory NH_4^+ Production in Leaves of Wild-Type and Glutamine Synthetase 2 Antisense Oilseed Rape[1]

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Exposure of oilseed rape (*Brassica napus*) plants to increasing leaf temperatures between 15°C and 25°C increased photorespiratory NH_4^+ production from 0.7 to 3.5 μmol m^{-2} s^{-1}. Despite the 5-fold increase in the rate of NH_4^+ production, the NH_4^+ concentration in root and leaf tissue water and xylem sap dropped significantly, whereas that in the leaf apoplastic fluid remained constant. The in vitro activity of glutamine synthetase (GS) in both leaves and roots also increased with temperature and in all cases substantially exceeded the observed rates of photorespiratory NH_4^+ production. The surplus of GS in oilseed rape plants was confirmed using GS2 antisense plants with 50% to 75% lower in vitro leaf GS activity than in the wild type. Despite the substantial reduction in GS activity, there was no tendency for antisense plants to have higher tissue NH_4^+ concentrations than wild-type plants and no overall correlation between GS activity and tissue NH_4^+ concentration was observed. Antisense plants exposed to leaf temperatures increasing from 14°C to 27°C or to a trifold increase in the O_2 to CO_2 ratio did not show any change in steady-state leaf tissue NH_4^+ concentration or in NH_3 emission to the atmosphere. The antisense plants also had similar leaf tissue concentrations of glutamine, glycine, and serine as the wild type, whereas glutamate increased by 38%. It is concluded that photorespiration does not control tissue or apoplastic levels of NH_4^+ in oilseed rape leaves and, as a consequence, that photorespiration does not exert a direct control on leaf atmosphere NH_3 fluxes.

Photorespiration is a complex network of biochemical processes taking place in the chloroplasts and peroxisomes, eventually leading to the evolution of stochiometric amounts of CO_2 and NH_3 in the mitochondria of C_3 plants. Photorespiration is light dependent and usually considered to be an energy-wasting process (Wingler et al., 2000), which may prevent photooxidation (Osmond et al., 1997) and ensure a steady flow of certain amino acids and keto acids used in other plant metabolic pathways, including the biosynthesis of antioxidants such as glutathione. Photorespiration is recognized as the quantitatively most important process generating NH_4^+ in plants during vegetative growth. The produced NH_4^+ is in equilibrium with NH_3, which easily penetrates membranes and may be lost in significant amounts to the atmosphere via the water film in the leaf apoplast embedding the mesophyll cells.

The chloroplastic isoform of Gln synthetase (GS2) is responsible for the assimilation of the large amounts of NH_4^+ produced in the mitochondria during photorespiration. This has been demonstrated by the use of barley (*Hordeum vulgare*) mutants lacking GS2 (Wallsgrove et al., 1987). These plants were not able to grow under photorespiratory conditions (21% [v/v] O_2) because NH_4^+ rapidly accumulated to toxic levels. Also, the use of the selective GS inhibitor DL-Met-DL-sulfoximine has shown that NH_4^+ accumulates within a few hours (Husted and Schjoerring, 1995; Mattsson et al., 1998). It is widely accepted that no compensatory and alternative pathways exist with sufficiently high assimilatory capacity for scavenging photorespiratory NH_4^+ (Martin et al., 1983; Lea, 1991). Thus, GS2 is responsible for keeping the symplastic NH_4^+ concentration at low levels and for preventing accumulation of toxic levels of NH_4^+ and NH_3.

Nevertheless, it has been shown that the system is far from being leak-proof and that substantial amounts of NH_3 may escape to the atmosphere via the leaf apoplast (Husted and Schjoerring, 1995, 1996; Husted et al., 1996). This NH_3 volatilization follows from the fact that the leaf apoplast is continuously supplied with NH_4^+ via efflux of NH_3/NH_4^+ from the cytoplasm (Husted and Schjoerring, 1995; Nielsen and Schjoerring, 1998). Despite the presence of effective NH_4^+ transport systems in the plasma membrane of mesophyll cells acting to re-absorb the lost NH_3/NH_4^+ (Gazzarini et al., 1999; Pearson et al., 2002), a certain NH_4^+ concentration exists in the leaf

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apoplastic solution and maintains a certain concentration of gaseous NH_3 in the leaf apoplastic air space. This concentration is referred to as the NH_3 compensation point and is usually in the nanomoles of NH_3 per mole air concentration range, i.e. of the same magnitude as the NH_3 concentration in the atmosphere surrounding the leaves. Increasing leaf temperatures favor NH_3 emission under controlled laboratory conditions (Husted and Schjoerring, 1996) as well as under field conditions (Husted et al., 2000) and may within a short timeframe cause a leaf to switch from being a sink for atmospheric NH_3 to becoming a source of atmospheric NH_3 (Husted and Schjoerring, 1996).

Photorespiration, and thereby photorespiratory NH_4^+ production, is known to increase with leaf temperature and ratio between O_2 and CO_2 (Jordan and Ogren, 1984; Brooks and Farquhar, 1985; Sharkey, 1988; Leegood, 1995). However, very limited information is available on how leaf temperature and O_2 to CO_2 ratio affect the steady-state NH_4^+ concentration in leaves and their NH_3 exchange with the atmosphere. The aim of this study was to test the hypothesis that photorespiration is important in controlling apoplastic and tissue levels of NH_4^+ in oilseed rape (*Brassica napus*) leaves and that as a consequence, photorespiration exerts a significant control on leaf atmosphere NH_3 fluxes. Both wild-type and transgenic oilseed rape plants with reduced GS2 activity were used in the experimental work.

RESULTS

The gas exchange parameters needed to estimate the rate of photorespiration and, thereby, the rate of NH_4^+ production during decarboxylation of Gly was estimated at leaf temperatures of 15°C, 20°C, and 25°C for wild-type oilseed rape cv Global (Table I). The essential parameters such as non-photorespiratory respiration in light (R_d), photosynthesis (A), transpiration (E), and the specificity factor (ϕ), describing the ratio between the rates of oxygenation and carboxylation, did all more than double when the leaf temperature increased from 15°C to 25°C at a photosynthetic photon flux density (PPFD) of 400 μmol m^{-2} s^{-1}. At the same time, photorespiration increased 5-fold, from 0.66 to 3.52 μmol m^{-2} s^{-1}, and the rate of photorespiration relative to that of photosynthesis increased from 13% to 30%.

The marked increase in photorespiration at increasing leaf temperatures was not followed by a concomitant increase in NH_4^+ concentration in any of the plant tissues analyzed, namely bulk leaf tissue water, bulk root tissue water, xylem sap, and apoplastic fluid. Instead, the NH_4^+ concentration decreased significantly in all these tissue types (Fig. 1, A–C), except for apoplastic NH_4^+, which was constant and very low, ranging between 50 and 85 μM (Fig. 1D). The most pronounced decrease in NH_4^+ concentration was observed in the bulk leaf tissue water, which dropped to one-third of the initial value, i.e. from 1.43 mM at 15°C to 0.51 mM at 25°C (Fig. 1A). This decrease was accompanied by an increase in total leaf in vitro GS activity, from 129 to 178 μmol g^{-1} fresh weight h^{-1} (Fig. 1A). Expressed on the basis of leaf area, the GS activity increased from 13 to 18 μmol m^{-2} s^{-1} (data not shown). Root GS activities were approximately 50% lower than the corresponding leaf GS activities (Fig. 1B). In contrast to the continuous increase in leaf GS activity with temperature, root GS activity did not change in the interval from 15°C to 20°C, but thereafter increased. Concomitantly, root NH_4^+ decreased from 0.90 to 0.43 mM.

The NH_4^+ concentration in the xylem sap was on average 2 to 3 times higher than that in the leaf and root tissues and decreased with increasing leaf temperature, as was the case in leaves and roots (Fig. 1C). However, due to a marked increase in transpiration rate when the leaf temperature increased from 15°C to 25°C, the NH_4^+ flux in the xylem increased from 43 to 61 nmol NH_4^+ m^{-2} leaf surface s^{-1} (Fig. 1C). Also, the NH_3 emission showed an increasing tendency ($P = 0.06$), going from 0.10 to 0.16 nmol NH_3 m^{-2} leaf surface s^{-1} at 15 and 25°C, respectively (Fig.

Table I. *Key parameters involved in the calculation of photorespiratory rates for wild-type oilseed rape measured at different leaf temperatures*

Parameters marked with an asterisk were obtained at a PPFD of 400 μmol m^{-2} s^{-1}. Errors represent ± SE. Values marked with the same letter are not significantly different ($P > 0.05$).

Parameter	Leaf Temperature		
	15°C	20°C	25°C
CO_2 compensation point when oxygenation equals carboxylation (Γ*, μmol mol^{-1})	28.3 ± 1.1[a]	41.1 ± 2.9[b]	40.8 ± 0.3[b]
Non-photorespiratory respiration (Rd, μmol m^{-2} s^{-1})	0.65 ± 0.12[a]	0.91 ± 0.17[b]	1.42 ± 0.28[c]
Photosynthesis (A, μmol m^{-2} s^{-1})*	5.26 ± 0.21[a]	8.08 ± 1.20[b]	11.84 ± 1.70[b]
Transpiration (E, mmol m^{-2} s^{-1})*	2.04 ± 0.34[a]	2.97 ± 0.12[b]	4.50 ± 0.10[c]
Leaf conductance (g, mmol m^{-2} s^{-1})*	78.4 ± 13.3[a]	70.2 ± 3.0[a]	79.3 ± 1.6[a]
Intercellular CO_2 concentration (C_i, μmol mol^{-1})*	283.1 ± 8.8[a]	232.7 ± 12.2[b]	195.4 ± 18.3[c]
Specificity factor (oxygenation/carboxylation; Φ, dimensionless)	0.20 ± 0.01[a]	0.31 ± 0.03[b]	0.42 ± 0.04[b]
Photorespiration* (v_o/2, μmol m^{-2} s^{-1})	0.66 ± 0.05[a]	1.65 ± 0.46[a]	3.52 ± 0.75[b]
Photorespiration relative to photosynthesis	12.5%	20.4%	29.7%

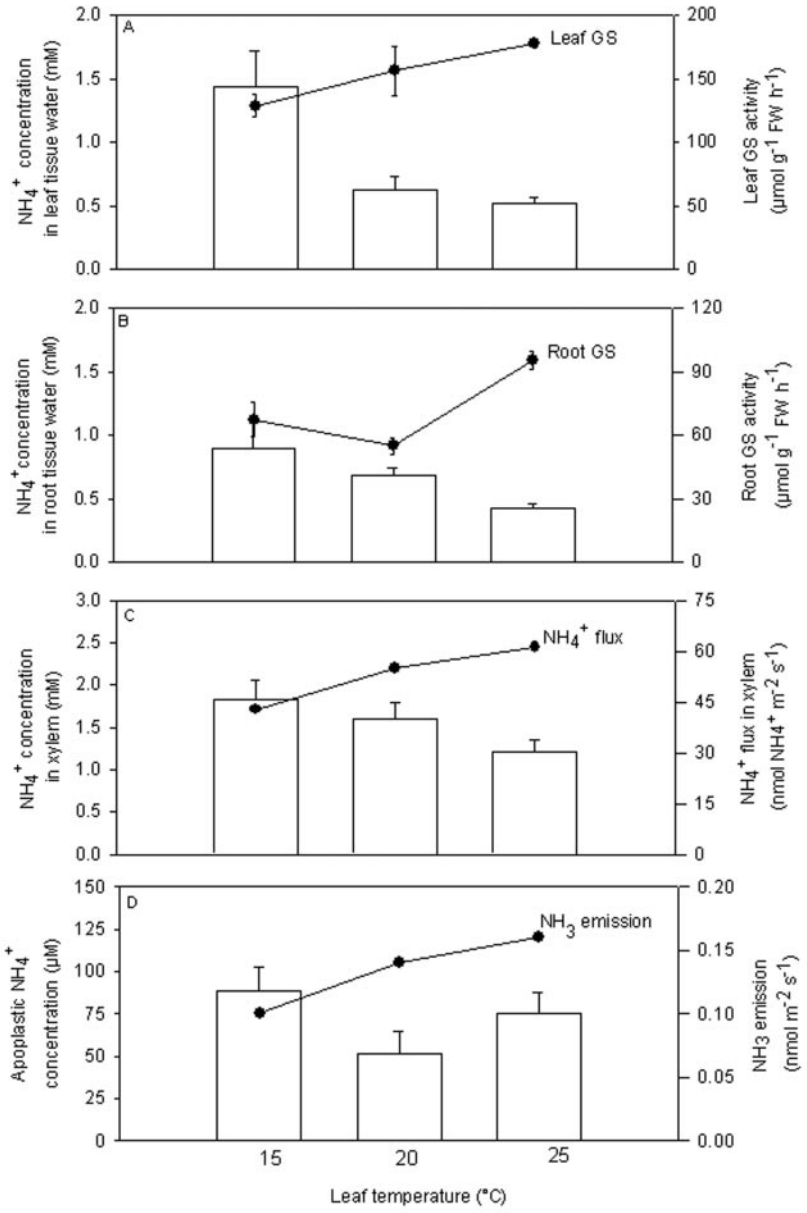

Figure 1. Effect of leaf temperature (15°C, 20°C, and 25°C) on NH_4^+ concentration in leaf tissue water (A), root tissue water (B), xylem sap (C), and apoplastic fluid (D) of oilseed rape. In addition, the total GS activity was measured in leaf (A) and root (B) tissue and the NH_4^+ flux in the xylem stream was calculated on the basis of NH_4^+concentrations and the transpiration rate (C). The effect of leaf temperature on the NH_3 flux on leaves was also determined (D). Plants were adjusted to the actual temperature for 24 h before the experiments were initiated. Values are means ± SE (n = 4).

1D). This increase could not be explained by an increasing apoplastic NH_4^+ concentration, which remained almost constant around 70 μM (Fig. 1D).

A GS2 antisense construct was expressed in oilseed rape cv Drakkar and compared with wild-type plants. The plants had the same phenotype, i.e. there was no difference in biomass production, leaf shape, size, and color (data not shown). The in vitro GS activity in leaves of 6-week-old antisense plants was only 24% of that in the wild type (Table II), whereas the corresponding amount of GS2 protein was 35% (Fig. 2). The amount of GS1 protein in 6-week-old plants was not affected by the antisense construct (Fig. 2). Immunogold labeling confirmed that GS was present in both the cytoplasm (GS1) and chloroplasts (GS2) of mesophyll cells in wild-type as well as antisense plants (Fig. 3). The close agreement between reduction in in vitro GS2 activity and protein in antisense plants indicates that only a minor part of the GS1 protein was active in the young leaves (predicted activation state around 0.15; see "Discussion"). Analyzed at the end of flowering (10-week-old plants), the reduction in total in vitro GS activity in antisense plants relative to wild type amounted to approximately 50% (Fig. 4). Over the following 3 weeks, up until the end of silique development (13-week-old plants), the in vitro GS activity declined 30% in leaves of both wild-type and antisense plants (Fig. 4), thereby maintaining approximately 50% lower total GS activity in antisense relative to wild-type leaves (Fig. 4). The GS activity in siliques was less affected by the antisense construct (25% reduc-

Table II. *Total in vitro GS activity and concentrations of NH_4^+ and selected amino acids in leaves of 6-week-old wild-type and GS2-antisense oilseed rape*

Values are means of four to six replicates ± SE. Total leaf *N* was 4.6% ± 0.3% and leaf nitrate was 0.75 ± 0.05 mM in tissue water of both wild-type and antisense plants.

Plant	Total GS Activity	Photorespiration	Tissue $[NH_4^+]$	Amino Acids			
				Glu	Gln	Gly	Ser
	μmol g^{-1} fresh wt h^{-1}	*μmol m^{-2} s^{-1}*	*μM*	*μM*			
Wild type	74.7 ± 13.4 (100%)	1.65 ± 0.46	530 ± 46	910 ± 59	528 ± 16	92 ± 16	283 ± 5
Antisense	17.9 ± 1.1 (24%)	1.98 ± 0.37	548 ± 47	1,253 ± 09	681 ± 94	92 ± 3	305 ± 6

tion compared with wild type in both 10- and 13-week-old plants; Fig. 4), in agreement with the fact that GS1 was the dominant isoform here (data not shown). Despite the substantial reduction in in vitro leaf GS activity (Table II; Fig. 4) and leaf GS2 protein level (Fig. 3), there was no tendency for antisense plants to have higher tissue NH_4^+ concentrations than wild-type plants (Table II; Fig. 4) and no overall correlation between GS activity and tissue NH_4^+ concentration was observed (Fig. 4).

Photorespiratory rates at 20°C in 6-week-old plants were similar in wild-type and antisense plants, being 1.65 ± 0.46 and 1.98 ± 0.37 μmol m^{-2} s^{-1}, respectively (Table II). To increase photorespiration, 6-week-old GS2 antisense plants were exposed to increasing O_2 concentrations ranging between 210 and 600 mmol mol^{-1} air, whereas atmospheric CO_2 concentration was maintained at 365 μmol CO_2 mol^{-1} at 20°C. Despite a 3-fold increase in the ratio between O_2 and CO_2 during this treatment and a decline in net photosynthesis from 5.2 to 2.9 μmol m^{-2} s^{-1}, no increase in foliar NH_3 emission was observed, but rather a decrease from 0.11 to 0.06 nmol m^{-2} s^{-1} (Fig. 5). Because NH_3 emission reflects the apoplastic and symplastic NH_4^+ concentrations (Husted and Schjoerring, 1996), this shows that GS even under conditions with strongly enhanced photorespiration was present in sufficient amount to keep tissue NH_4^+ at a low level in the antisense plants. Transpiration was not affected by the exposure to elevated O_2 and remained between 1.1 and 1.2 mmol m^{-2} s^{-1} throughout the experiment (Fig. 5). Similarly, leaf conductance was not affected (data not shown). Except for Glu, which was 38% higher in antisense plants compared with the wild type, no differences were found in other key amino acids, e.g. Gln, Gly, and Ser, associated with photorespiration.

At a constant ratio between O_2 and CO_2 (210 mmol O_2 mol^{-1} air versus 365 μmol CO_2 mol^{-1} air), the leaf temperature of 6-week-old antisense and wild-type plants were gradually raised from 14°C to 27°C, with a stepwise increase of 2.5°C every 45 min (Fig. 6). The NH_3 emission from antisense plants was at all leaf temperatures 1.5 to 2.1 times higher than that from the wild type, but the overall curvilinear temperature response was identical and in both cases smaller than that predicted on the basis of the temperature dependence of the relevant thermodynamic equilibria between NH_4^+ and $NH_{3,gas}$. Because NH_4^+ levels in wild-type and antisense plants were similar (Table II), the higher level of NH_3 emission from the latter may have been due to a slightly higher apoplastic pH (≈0.1 unit). The fact that the measured temperature response of NH_3 emission was less steep than that

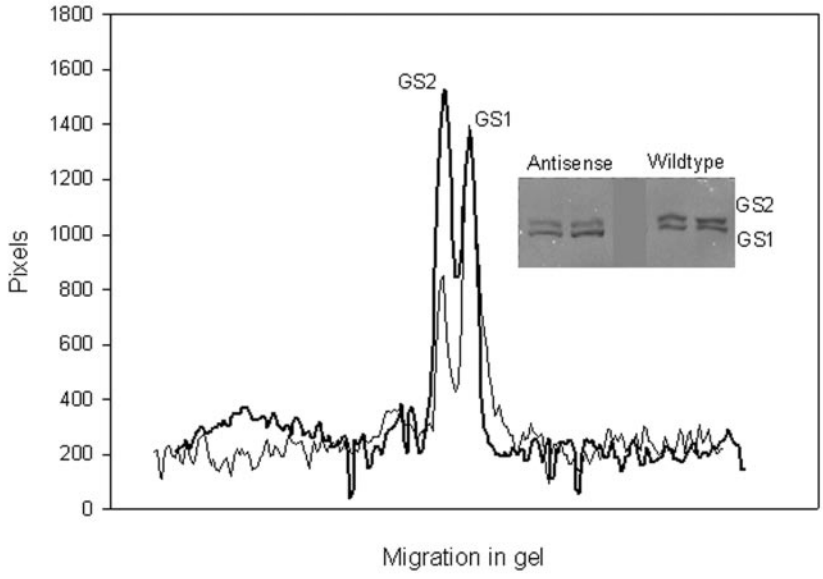

Figure 2. Western blotting of GS isoenzymes (GS1 and GS2) in leaves of 6-week-old wild-type (bold line) and antisense (thin line) plants.

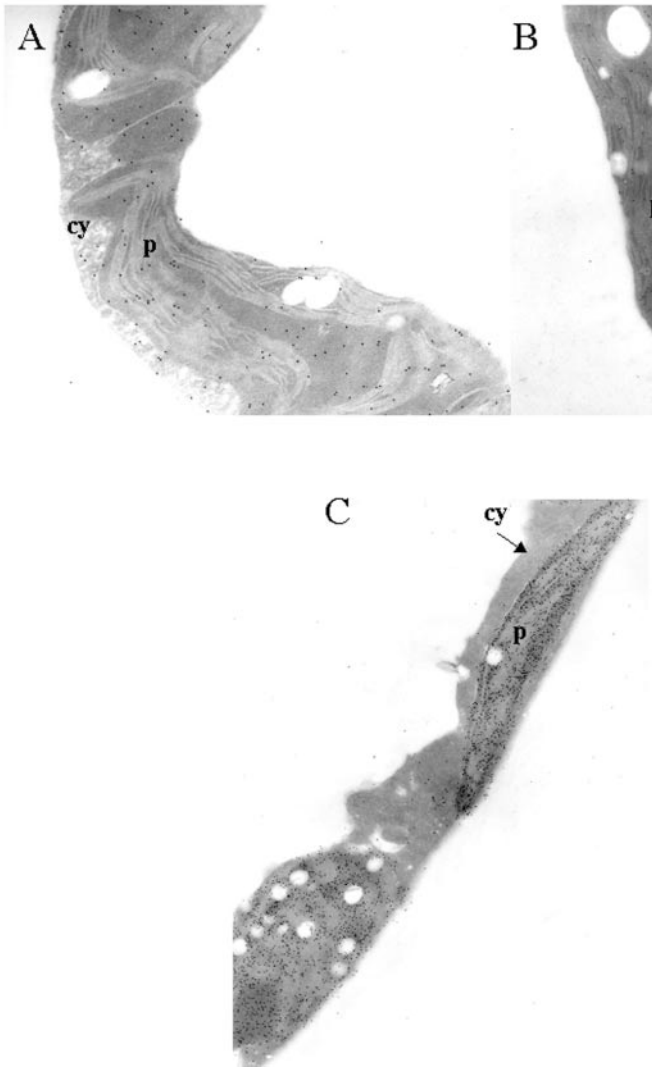

Figure 3. Transmission electron microscopic immunolocalization of GS and Rubisco in thin sections of leaves of 6-week-old wild-type (A) and GS2 antisense (B) oilseed rape plants. cy, Cytoplasm; p, chloroplast. A heavy gold labeling of Rubisco was detected in the mesophyll chloroplast (Fig. 6C), whereas labeling was almost absent in the cytoplasm, indicating that cell organelle integrity was maintained during sample preparation and staining.

predicted on the basis of physicochemical assessments (Fig. 6) confirms that apoplastic NH_4^+ declined rather than increased with temperature (Fig. 1). In addition, this shows that even the low GS activity remaining in the antisense plants (Table II) was thus more than sufficient to cope with the increased NH_4^+ production at elevated leaf temperature and photorespiration.

DISCUSSION

Photorespiration increased 5-fold when the leaf temperature was raised from 15°C to 25°C (Table I). The enhancement of photorespiration with increasing temperature is partly due to a more pronounced decline of CO_2 solubility in water compared with that of O_2 and partly due to favored Rubisco oxygenation relative to carboxylation (Jordan and Ogren, 1984; Brooks and Farquhar, 1985). One mole of NH_4^+ is being produced in photorespiration for every 2 mol of CO_2 being assimilated in photosynthesis. Therefore, it was expected that increasing photorespiration would elevate the steady-state NH_4^+ concentration in the mesophyll tissue. Nevertheless, even under conditions when the amount of GS protein was greatly reduced (Fig. 2), the in planta GS activity was still sufficient to maintain tissue NH_4^+ at a low level. That GS in all cases was in considerable excess relative to the rate of NH_4^+ liberation in photorespiration was corroborated by the in vitro GS assay (Table II). Photorespiration, therefore, does not seem to be the dominating process controlling leaf tissue NH_4^+ levels and NH_3 emission in oilseed rape, at least in the temperature range investigated here. Working up to temperatures of 37°C, Husted and Schjoerring (1996) observed that once above 31°C, oilseed rape leaves started to emit NH_3 in an atmosphere containing 15 nmol NH_3 mol^{-1} air, whereas at lower temperatures the leaves were NH_3 sinks. The dramatic increase in NH_3 compensation point with increasing temperature was, however, not caused by a breakdown of the GS assimilatory pathway, but could be fully ex-

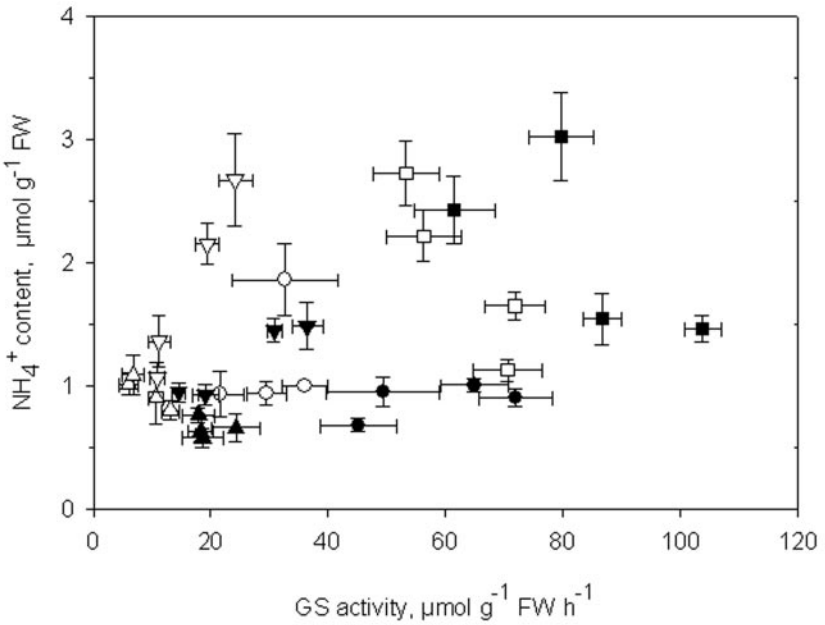

Figure 4. Relationship between NH_4^+ concentration and GS activity in leaves and siliques of wild-type and GS2 antisense oilseed rape plants. Plants were grown at two different N levels (2 mM and 6 mM NO_3^-) and harvested at two different developmental stages, namely end of flowering (10-week-old plants) and end of silique development (13-week-old plants). On each harvest occasion, GS and NH_4^+ were measured in young leaves (leaf 10–11 from the base), old leaves (leaves 7–8 from the base), young siliques (10 most recently developed siliques on axial racemes), and old siliques (siliques 1–10 from base of terminal raceme). Values are means ± SE ($n = 6$). Black symbols, Wild type; white symbols, antisense plants: ● and ○ leaves, 10-week-old plants; ▲ and △ leaves, 13-week-old plants; ■ and □ siliques, 10-week-old plants; and ▼ and ▽ siliques, 13-week-old plants.

plained on the basis of the temperature dependence of the relevant thermodynamic equilibria between NH_4^+ and $NH_{3,gas}$. Thus, oilseed rape leaves seem in general to possess sufficient GS capacity to avoid elevated tissue levels of NH_4^+ during increased photorespiration and leaf temperature. Only when GS is quantitatively inhibited by addition of DL-Met-DL-sulfoximine, tissue and apoplastic NH_4^+ levels and NH_3 emission increase (Husted and Schjoerring, 1995). Changes in other environmental factors than temperature may cause both xylem and tissue levels of NH_4^+ to increase in oilseed rape. This is the case, for example, in increasing NO_3^- levels in the root medium (Husted et al., 2000) or replacement of NO_3^- with NH_4^+ in the root medium (Finnemann and Schjoerring, 1999). Also, drought stress induces a significant increase in leaf NH_4^+ in oilseed rape (data not shown).

The lack of any increase in tissue NH_4^+ concentration in GS2 antisense oilseed rape contrasts with results for barley mutants in which GS2 activity was reduced to between 20% and 47% of that in the wild type (Wallsgrove et al., 1987; Häusler et al., 1994; Mattsson et al., 1997). In the latter study, mutants with only 66% GS activity compared with the wild type had twice the leaf tissue NH_4^+ concentration and 6-fold higher NH_3 emission. However, a further decrease in GS activity to 47% resulted in a much smaller increase in NH_4^+ concentration and NH_3 emission than observed for the 66% GS mutant, suggesting that NH_4^+ release was inhibited by some kind of compensatory reaction in plants with only

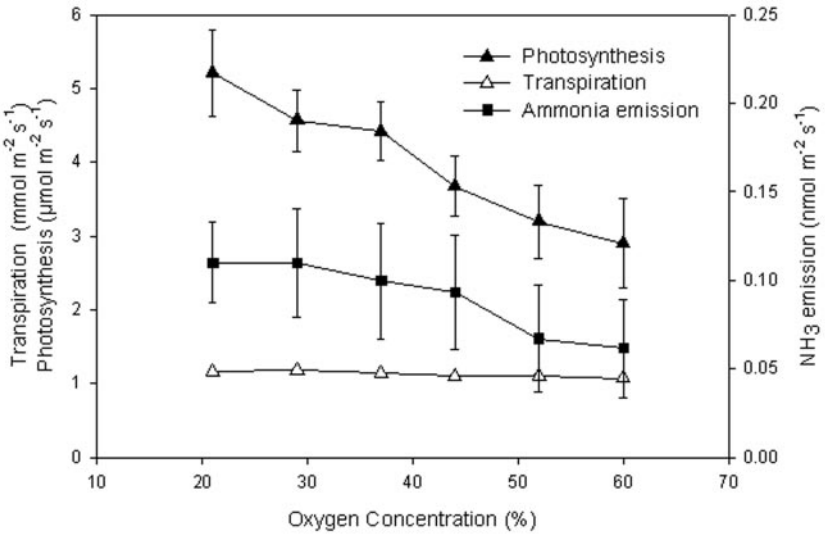

Figure 5. Effect of increasing atmospheric O_2 concentrations on photosynthesis, transpiration, and NH_3 emission of GS2 antisense oilseed rape plants. The external CO_2 concentration was maintained at 365 ± 5 µmol CO_2 mol^{-1} air and the leaf temperature was fixed at 20°C during the experiment. The plants were allowed to adjust to a new O_2 to CO_2 ratio for 45 min before the O_2 level was increased. The experiment was repeated twice on consecutive days and values are means ± SE.

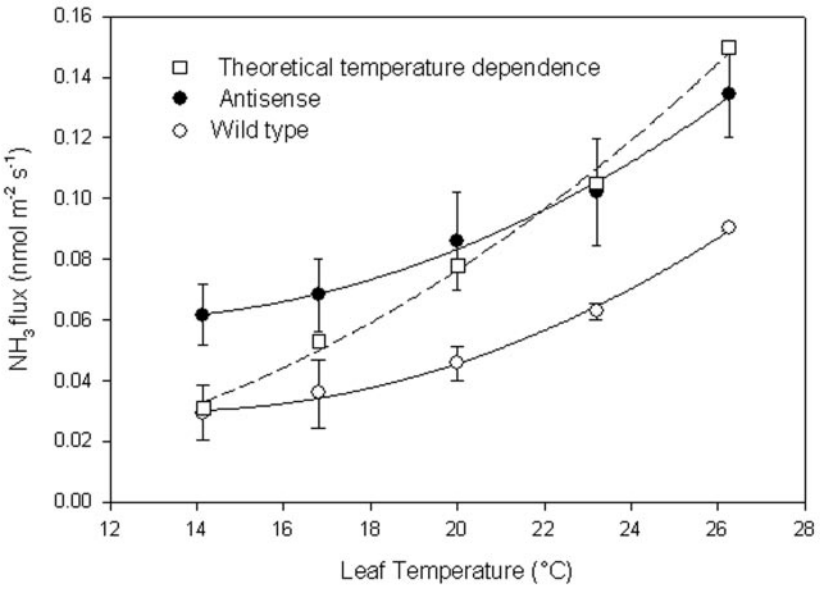

Figure 6. Effect of increasing leaf temperature between 14°C and 27°C on the NH_3 emission from leaves of wild-type and GS2 antisense oilseed rape plants. The experiment was performed over 4 consecutive d with one plant analyzed every day. Values are means ± SE. The theoretical temperature dependence of the NH_3 emission was calculated according to Husted and Schjoerring (1996) using an apoplastic NH_4^+ concentration of 75 μM and a pH value of 5.8.

47% GS activity. Nevertheless, and contrasting with the results presented here (Fig. 6) for GS- antisense oilseed rape, both barley mutants showed a more pronounced increase in NH_3 emission with rising temperatures than did wild-type plants, suggesting that the low GS activity could not keep up with the demand for re-assimilation of photorespiratory NH_3 at the elevated temperatures. In accordance, recalculation of the data from Mattsson et al. (1997) showed that in vitro GS activity and photorespiration were roughly equal in the barley mutants. In addition, wild-type barley plants had a 5-fold lower surplus of in vitro GS activity relative to photorespiration than the wild-type oilseed rape plants used in the present work. Overexpression of a GS2 gene from rice (*Oryza sativa*) in tobacco (*Nicotiana tabacum*) leaves reduced their sensitivity to photoinhibition (Kozaki and Takeba, 1996), indicating that the native GS2 level in this species was insufficient under high-intensity light conditions. Similarly, the NH_3 emission from barley mutants with reduced GS activity was also more enhanced by increasing light intensity than that from the wild type (Mattsson et al., 1997).

The GS2 antisense construct only reduced the amount of GS2 protein, whereas that of GS1 protein was maintained in substantial amounts and at the same level as in the wild type (Fig. 2). Leaf age increases GS1 expression relative to that of GS2 (Finnemann and Schjoerring, 2000), whereas photorespiratory NH_4^+ itself does not directly regulate GS2 expression (Beckmann et al., 1997; Migge et al., 1997). The dominating physiological role of GS1 in leaves is synthesis of transport amides and the enzyme is generally assumed to be located in the phloem companion cells (Dubois et al., 1996; Brugiere et al., 1999), but may be shifted toward the mesophyll during aging in tobacco leaves (Brugiere et al., 2000). Our data (Fig. 3) show that GS was present in the cytoplasm and chloroplasts of both wild-type and antisense plants, even in young mesophyll cells. To what extent GS1 contributed to the NH_4^+ assimilating capacity, compensating for the decrease in GS2 protein, is not known and cannot be immediately assayed because the in vitro GS activity may not truly reflect that in planta due to posttranslational regulation by phosphorylation and 14-3-3 protein interactions (Finnemann and Schjoerring, 2000). However, the fact that the reduction in GS2 activity in 6-week-old antisense plants (Table II) was accompanied by an almost equivalent reduction in GS2 protein (Fig. 2) indicates that only a minor part of the GS1 protein was active in the young leaves. Thus, for the results from the western blotting (Fig. 3) to match those from the activity assays in 6-week-old plants (Table II), only about 15% of the GS1 protein could have contributed to the in vitro GS activity, assuming an activation state of GS2 protein of 0.5 in wild-type and 0.2 in antisense plants.

Based on the theory developed by Brooks and Farquhar (1985) for intact spinach (*Spinacia oleracea*) leaves, the following nonlinear regression model to estimate Γ^*, which is the CO_2 concentration where oxygenation is balanced by carboxylation, has been developed (Sharkey, 1988; Leegood, 1995):

$$\Gamma^* = P(42.7 + 1.68(T - 25) + 0.012(T - 25)^2) \quad (1)$$

P is the atmospheric pressure in bars and T is the leaf temperature in °C. Application of Equation 1 on the data presented in Table I resulted in a specificity factor (ϕ = ratio between oxygenation and carboxylation) of 0.19, 0.29, and 0.43 at 15, 20, and 25°C, respectively, which is very close to the corresponding experimental values of 0.20, 0.31, and 0.42 for oilseed rape (Table I). It is encouraging that values obtained with two different plant species, spinach and oilseed rape, are so close in agreement. The possibility of

predicting ϕ, and thereby photorespiration, without complicated, labor-intensive, and expensive gas exchange measurement is valuable for future work on the relationship between photorespiration and various metabolic processes.

CONCLUSIONS

Antisense oilseed rape plants with 83% reduced GS2 activity had similar photorespiration as wild-type plants and also similar tissue concentrations of NH_4^+ and amino acids. A 5-fold increase in photorespiration caused by raising the air temperature above 15°C or the atmospheric oxygen concentration above 21% did not lead to elevated leaf apoplastic or tissue levels of NH_4^+ in either antisense or wild-type plants. As a consequence, photorespiration does not exert a direct control on leaf atmosphere NH_3 fluxes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of the oilseed rape (*Brassica napus*) were soaked in tap water overnight and transferred to wet filter paper. The seeds were germinated in the dark for approximately 4 d at 20°C. Four seedlings were mounted in the lid of a 4-L high-density polyethylene container and grown in a greenhouse with a day/night period of 16/8 h. To keep the daylight intensity above 400 μmol m^{-2} s^{-1}, supplementary light was given by HQI lamps (Power Star 400W, Osram, Munich). Day/night temperatures were 20°C ± 3°C/15°C ± 2°C. Plants were grown in aerated nutrient solutions consisting of: NO_3^- (3.0 mM), $H_xPO_4^{(3-x)-}$ (0.2 mM), K^+ (1.2 mM), Ca^{2+} (0.9 mM), Mg^{2+} (0.6 mM), SO_4^{2-} (0.5 mM), Na^+ (0.1 mM), Cl^- (0.1 mM), Fe-EDTA (50 μM), Mn^{2+} (7 μM), $B(OH)_3$ (2 μM), Zn^{2+} (0.7 μM), Cu^{2+} (0.8 μM), and MoO_4^{2-} (0.8 μM). The solution was renewed once a week, but after 4 weeks of growth (plant height of approximately 0.35 m), NO_3^- (3 mM), K^+ (0.6 mM), Mg^{2+} (0.3 mM), and Ca^{2+} (0.9 mM) were supplied three times a week. pH was adjusted to 6.0 with a few drops of 5 M HCl when the nutrient solution was renewed. All measurements of photorespiration were performed on 6-week-old plants. Additional analyses of GS activities and tissue NH_4^+ concentrations were carried out in wild-type and antisense plants at the end of flowering (10-week-old plants) and the end of silique development (13-week-old plants). These plants were grown as described above, expect for the NO_3^- concentration, which was 2 or 6 mM, adjusted by varying the amount of $Ca(NO_3)_2$ added to the solution.

GS2 Antisense Plants

The oilseed rape cv Drakkar was transformed with the construct pBINGSL1 antisense. The cDNA of the oilseed rape plastidic GS, GSL1 (Ochs et al., 1993), was cloned in antisense orientation between the 35S promoter and terminator of pRT101 (Töpfer et al., 1987); the chimeric antisense gene was cloned into the *Sma*I site of pBIN19 (Bevan, 1984). The *Agrobacterium tumefaciens* strain GV3101pMP90 was transformed with pBINGSL1 antisense by electroporation. The transformation of hypocotyl explants was performed according to protocol described by Schröder et al. (1994) with minor modifications. After a precultivation period of 3 d, the hypocotyls were cut in 5-mm segments and incubated for 20 min in CIM medium (K3 combined with 20 g L^{-1} Glc, 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid, and 0.1 mg L^{-1} indole-3-acetic acid) containing 1×10^8 *A. tumefaciens* cells mL^{-1}. The cocultivation took 2 d in liquid CIM medium. Selection was carried out on DKW medium (Duchefa Biochemie, Amsterdam) containing 20 g L^{-1} Suc, 1 mg L^{-1} benzylaminopurine, 0.01 mg L^{-1} indole-butyric acid, 0.01 mg L^{-1} GA_3, 25 mg L^{-1} kanamycin, and 500 mg L^{-1} carbenicillin. Kanamycin-resistant regenerates were screened by NPTII ELISA (5′-3′). Primary transformants (T_1 plants) were analyzed for total GS activity and by western blot. The transgenic line F1-40 was selected for further experimentation. The transgene copy number in this primary T_1 transformant was 2, as determined by Southern blot analysis. T_1 and T_2 plants were selfed and T_3 plants selected for low GS activity were used in the experiments.

Photosynthesis and Transpiration

Twenty-four hours before analysis, plants were transferred from the greenhouse to a 0.075-m^3 cuvette made of polycarbonate coated with Margard (General Electric, Pittsfield, MA), ensuring a low water adsorption and an unchanged spectral composition of the photosynthetically active light (Husted and Schjoerring, 1995). The cuvette was mounted in a 1.2-m^3 growth chamber (MB-teknik, Copenhagen), ensuring intimate control of the climatic conditions, and continuously flushed with dry ambient air (relative humidity <10% at 20°C) at 50 L min^{-1}. The PPFD was measured on the mid-leaf and adjusted to 400 μmol m^{-2} s^{-1}. In the same leaf position, the leaf temperature was measured by small 1-mm thermocouples (Testo 925, Lenzkirch, Germany) inserted into the major veins on the abaxial side of the leaf. Growth chamber conditions were adjusted to produce a leaf temperature of 20°C ± 0.5°C. Before measurement of photosynthesis and transpiration, flushing the cuvette with ambient dry air was changed to an artificial atmosphere consisting of 21% (v/v) O_2, 360 μmol CO_2 mol^{-1} air, and 79% (v/v) N_2. Exactly 3 h into the photoperiod, the measurements were taken and usually less than 15 min was needed to achieve stable values.

Photosynthesis and transpiration were also measured at different O_2 to CO_2 ratios (Fig. 2) and leaf temperatures (Table I). Readings were always stable within 15 min but usually 30 to 45 min was allowed before the conditions were adjusted to a new setting because the measurements of the NH_3 emission performed simultaneously responded more slowly (se below).

Differences in mole fractions of CO_2 and water entering and leaving the cuvette were measured with a CO_2/water infrared analyzer (Ciras-1, PP Systems, Hertshire, UK). Transpiration and photosynthesis were calculated according to the method of Leuning (1983). Transpiration (E) was calculated using Equation 2:

$$E = \frac{Q}{a}\frac{(x_{wo} - x_{wi})}{x_{ao}} \quad (2)$$

where Q is the air flow rate (mol s^{-1}), a the leaf area (m^2), x_{wi} and x_{wo} the mole fraction of water vapor at the chamber inlet and outlet, respectively, and x_{ao} the mole fraction of dry air at the chamber outlet, assuming $x_a + x_w = 1$. The rate of photosynthesis (A) was calculated as:

$$A = \frac{Q}{a}\left[(x_{co} - x_{ci}) + \frac{x_{co}}{x_{ao}}(x_{wo} - x_{wi})\right] \quad (3)$$

where x_{ci} and x_{co} are the mole fraction of CO_2 at the chamber inlet and outlet, respectively.

Photorespiration

The rate of photorespiration was determined at leaf temperatures of 15°C, 20°C, and 25°C in the Global genotype. Moreover, photorespiration in Global and GS2 antisense Drakkar genotypes were compared at 20°C.

Determination of photorespiratory rates was based on the theory developed by Laing et al. (1974), von Caemmerer and Farquhar (1981), and Brooks and Farquhar (1985), which uses gas exchange measurements to estimate the specificity factor (ϕ) of Rubisco from the CO_2 compensation point (Γ^*) where oxygenation is balanced by carboxylation (Sharkey, 1988). Plants were exposed to an artificial atmosphere of 21% (v/v) O_2, 79% (v/v) N_2, and five different CO_2 mole fractions ranging from 0 to 100 μmol CO_2 mol^{-1} air. Plants were progressively adjusted to a new CO_2 concentration 15 min after the gas exchange had stabilized. For each leaf temperature, the measurements were performed at three different light intensities, namely 100, 200, and 400 μmol m^{-2} s^{-1}. The point at which the regression lines intersected was used to determine Γ^* and R_d (Brooks and Farquhar, 1985; Atkin et al., 1997) and these values were subsequently used to calculate the rates of oxygenation (v_o) and photorespiration ($v_0/2$; 2 mol of O_2 is used, one in the Calvin cycle and one in the oxidation of glycollate to glyoxylate, for every CO_2 released by Gly decarboxylation).

Using the above-mentioned technique to estimate photorespiration, there is a risk that plants could be carbon starved due to an insufficient CO_2

fixation, especially as the internal CO_2 concentration approaches Γ*. This might potentially affect several metabolic processes including Rubsico and mitochondrial respiration and consequently lead to a severe overestimation of, e.g. R_d, and thereby induce significant errors in the determination of the specificity factor. However, it was observed that photosynthesis could be restored to original values within the 10- to 20-min period needed to stabilize the gas concentrations in the plant cuvette, indicating that the C metabolism and the resulting gas exchange was not affected even if plants had been exposed to low external CO_2 (<100 μmol CO_2 mol^{-1} air) for a period of up 50 min.

Ammonia Gas Exchange

A continuous flow denuder connected to the plant cuvette system described above was used to measure the NH_3 gas exchange from the plants at different O_2 to CO_2 ratios (Fig. 2) and leaf temperatures (Figs. 3 and 4). A detailed description of the development and operation of this system is given by Schjoerring and Husted (1997). The NH_3 emission was always extremely low (<0.2 nmol NH_3 m^{-2} leaf s^{-1}); therefore, 30 to 45 min was needed to stabilize the NH_3 concentrations in the plant cuvette and tubing before NH_3 readings were taken at each new O_2 to CO_2 ratio or leaf temperature.

Ammonium and Amino Acids

Leaves were excised, major veins quickly removed (<30 s), the remaining parts immediately immersed in liquid N_2, and stored at −80°C until analysis (<3 months). Individual samples were then ground to a fine powder with a metal rod in liquid N_2. Approximately 200 mg of accurately weighed tissue was transferred to a chilled mortar, a little quartz sand was added, and the mixture was homogenized with 2 mL of ice-cold 10 mM HCOOH containing 500 μM α-aminobutyric acid, which was used as internal standard for determination of amino acids. The homogenate was centrifuged in small Eppendorf vials at 21,000*g* (4°C) for 10 min and the supernatant was transferred to 2-mL centrifuge filters (polysulphone 0.45 μm, Whatman, Maidstone, UK). The supernatant was filtered at 5,000*g* (4°C) for 5 min and the clear extract was used for analysis of NH_4^+ and amino acids.

The apoplast was extracted with a vacuum infiltration technique described in detail by Schjoerring and Husted (1997). The technique is based on vacuum infiltration of small leaf segments (25 × 50 mm) with an ice-cold 350 mOsm isotonic sorbitol solution (280 mM) in a 50-mL plastic syringe, which is mounted on a hydraulic arm that automatically moves the plunger up and down to infiltrate the leaf, alternating under pressure and vacuum. The infiltrator exposes the leaf discs to 4 atm pressure and vacuum for 10 s, and repeats the procedure eight times, thereby ensuring full infiltration within 2 min. Immediately after infiltration, the leaf apoplast was extracted by centrifugation at 2,000*g* for 10 min at 4°C and the apoplastic solution (20–100 μL) was collected in small Eppendorf vials. The apoplast extracts were stabilized with ice-cold 20 mM HCOOH in a 1:1 (v/v) volume ratio and stored at 2°C for a maximum of 12 h.

The stem was cut 3 to 4 cm above the root and the stump allowed to bleed for a few minutes, after which the first drop of stem bleeding sap was discharged. Thereafter, xylem sap was sampled over a period of 30 min and stabilized as described above.

Ammonium was quantified by a highly sensitive and selective columnless HPLC method described in detail by Husted et al. (2000), using on-line derivatization of NH_4^+ by *o*-phthalaldehyde. Physiological amino acids were determined by reverse-phase HPLC using the AccQ-Tag technique (van Wandelen and Cohen, 1997).

GS Assay and Western Blotting

The activity of GS (synthetase reaction) was measured with the biosynthetic reaction assay, using NH_2OH as artificial substrate, by measuring the formation of γ-glutamyl hydroxamate (GHA; O'Neal and Joy, 1973). Leaves frozen in liquid N_2 were homogenized in extraction buffer [70 mM MOPS, pH 6.80; 10 mM $MgSO_4$; 2 mM dithiothreitol; 5 mM Glu; 0.1% (v/v) Triton X-100; and 10% (v/v) ethanediol] with a little quartz sand using a mortar and a pestle at 2°C. The extracts were centrifuged at 21,000*g* (2°C) for 15 min and the supernatants were analyzed for soluble protein (Bradford, 1976) and GS activity. Leaf GS activity was measured in pre-incubated assay buffer (37°C) consisting of 70 mM MOPS (pH 6.80), 100 mM Glu, 50 mM $MgSO_4$, 15 mM NH_2OH, and 15 mM ATP. The reaction was terminated after 30 min at 37°C by addition of an acidic $FeCl_3$ solution (88 mM $FeCl_3$, 670 mM HCl, and 200 mM trichloroacetic acid). GHA was quantified spectrophotometrically at 498 nm using GHA as standards.

Western-blot analysis was carried out using 15% (w/v) Tris-HCl ready gels according to the manufacturer's protocol (Bio-Rad, Richmond, CA) with 12 μg of protein per lane. Proteins were electroblotted onto a nitrocellulose membrane and GS was immunodetected with a polyclonal GS antibody raised against *Phaseolus vulgaris* root nodule GS (Cullimore and Miflin, 1984). Visualization of GS was achieved with alkaline phosphatase linked to a goat-rabbit IgG (Bio-Rad) and quantified on a Storm 860 Scanner (Molecular Dynamics, Sunnyvale, CA).

Subcellular Localization of GS

The subcellular localization of GS in wild-type and antisense plants was examined by immunogold transmission electron microscopy. The subcellular localization of Rubsico was also examined to ensure the integrity of chloroplasts, thereby verifying that no GS2 had leaked into the cytoplasm. A leaf segment of 7 × 4 mm was cut from the intravenous area with a razorblade. Four replicates of wild-type and antisense plants were used. The sample was fixed and infiltrated in an ice-cold buffer (1% [w/v] paraformaldehyde and 1% [w/v] Suc) with the hydraulic system described above. The infiltration was continued until all segments were at the bottom of the syringe containing the buffer. The leaf sample was rinsed in buffer supplemented with a drop of Tween 20. The sample was dehydrated in a series of ethanol solutions (15%, 30%, 50%, 70%, and 90% [v/v]) and then embedded in a 1:1 (v/v) solution of 90% (v/v) ethanol and LR-white resin (Polysciences, Warrington, PA). Polymerization was carried out in gelatin capsules at 50°C. The sample preparation procedure for immunotransmission electron microscopy was performed using specific anti-GS or anti-Rubisco rabbit serum (Brugiere et al., 2000).

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