Greenhouse-grown conditionally lethal tobacco plants obtained by expression of plastidic glutamine synthetase antisense RNA may contribute to biological safety

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Abstract

A cDNA corresponding to plastidic glutamine synthetase (GS-2), an enzyme involved in photorespiration, was expressed in antisense orientation under the control of a leaf-specific soybean ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene promoter in transgenic tobacco to yield conditionally lethal plants. Three transgenic tobacco lines with decreased (at most 64%) foliar GS-2 activity were obtained. These plants grew normally when maintained in an atmosphere with a CO2 partial pressure sufficiently high (300 Pa CO2) to suppress photorespiration. However, when photorespiration was initiated by the transfer of the plants to air (35 Pa CO2), ammonium accumulated in the leaves. With time, the transgenic plants exhibited severe chlorotic lesions and, eventually, the plants died. A stable atmosphere containing at least 300 Pa CO2 can be established easily in the greenhouse but is unlikely to occur in a natural environment. Therefore, the transgenic tobacco plants with decreased leaf GS-2 activity may contribute to biological safety for production of desired proteins. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Transgenic plants can be used to produce desired proteins. There is, however, an increasing social concern about the use of recombinant DNA technology. The production of desired proteins using transgenic plants may involve, for example, the risk that the genes encoding the desired proteins and/or the genes coding for the antibiotic-resistance, required as selection marker during the generation of the transgenic plants, could be transferred from the transformed plant species to related species growing in the same environment. Such concerns may lead to restrictions on the use of transgenic plants to produce desired proteins. It is, therefore, desirable to develop methods that prevent or at least strongly restrict any involuntary gene transfer from transformed plants used in biotechnology to other plants. We reasoned that transgenic plants that are viable and fertile in a specific greenhouse environment but not under natural conditions may contribute to biological safety if chosen to produce desired proteins.

Conditionally lethal plants can be obtained by deleting the activity of an enzyme involved in photorespiration [1]. In leaves, ribulose-1,5-bisphosphate carboxylase/oxygenase catalyzes both the carboxylation and oxygenation of ribulose-1,5-bisphosphate. Carboxylation results in the formation of two molecules of 3-phosphoglycerate (3-PGA) while oxygenation produces one molecule of 3-PGA and one of glycollate phosphate [2]. The 3-PGA is utilized within the photosynthetic carbon reduction cycle to produce carbohydrates.
Glycollate phosphate is metabolized via the photosynthetic carbon oxidation cycle. The operation of this pathway results in the uptake of O₂ and in the release of both CO₂ and ammonium. This process is designated photorespiration [3]. The photorespiratory CO₂ is in part reassimilated and in part lost to the atmosphere [4] while the ammonium is reassimilated during the photorespiratory nitrogen cycle [5]. The major environmental cues affecting the rate of photorespiration are the ratio of CO₂ to O₂ and temperature [6].

Manipulation of the CO₂ partial pressure of the atmosphere allowed the selection of barley or Arabidopsis thaliana mutants severely deficient in enzymes of photorespiration from populations of seeds treated with mutagens [3]. These photorespiratory mutants grow normally if photorespiration is suppressed by increased ambient concentrations of CO₂. When exposed to air, however, photosynthesis is rapidly impaired. With time, chlorotic lesions appear and, eventually, the mutants die [3].

Conditionally lethal photorespiratory mutants of barley or A. thaliana [3] may potentially contribute to biological safety when chosen to produce desired proteins. However, barley cannot be transformed easily so far while A. thaliana is difficult to handle in a biotechnological production process due to the small size of the plants and the short life cycle of this species. One possibility to overcome these difficulties may be to phenocopy photorespiratory mutants of barley or A. thaliana in transgenic tobacco. We attempted to obtain transgenic tobacco lines with decreased activity of plastidic glutamine synthetase (GS-2). The GS-2 was chosen as the target of our approach due to the reaction catalyzed by GS-2 represents the first step of the fixation of photorespiratory ammonium [7].

In the case of barley, the probability to obtain mutants severely deficient in GS-2 activity from mutagenized seeds was appreciably high because only a single-copy GS-2 gene is present in the barley genome [8]. Using the same approach, the isolation of tobacco mutants with decreased GS-2 activity may, however, be considerably more difficult because two distinct GS-2 genes were detected in the amphidiploid genome of tobacco [9]. We have, therefore, chosen the antisense strategy [10] to obtain transgenic tobacco plants with decreased GS-2 activity. The antisense strategy allows the inhibition of the expression of a multigene family for which no mutants are available and difficult to isolate [11].

2. Material and methods

2.1. Plasmid construction

The GS-2 cDNA insert of pcGS2-17 [9] containing the coding region and the 5'- and 3'-flanking regions was isolated as an EcoRI–EcoRI fragment (1.6 kb) from pBluescript and ligated in reverse orientation into the EcoRI-restricted binary vector pAQ4.1 [12] between the soybean ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene (rbcS) promoter (1.1 kb) [13] and the polyadenylation signal of the octopine synthase gene of Agrobacterium tumefaciens (2.3 kb) [14]. The resulting plasmid (13.6 kb) was designated pGS20. This plasmid also carries the neomycin phosphotransferase II (nptII) and nptIII genes to provide a kanamycin-selectable marker in plants and in bacteria, respectively. After verification of the orientation of the inserted GS-2 gene by restriction analysis, pGS20 was mobilized from E. coli into the disarmed A. tumefaciens strain LB4404 by triparental mating [15].

2.2. Transformation of tobacco

Tobacco (Nicotiana tabacum L. var Petite Havanna SR1; INRA, Versailles, France) was transformed via A. tumefaciens-mediated gene transfer using a leaf disc inoculation technique [16]. Transgenic plants were regenerated and grown in a CO₂-enriched atmosphere (300 Pa CO₂) obtained as previously described [17] by injection of CO₂ into an air stream. Rooted transformants were first maintained for 6 weeks on Murashige Skoog medium with kanamycin (100 μg ml⁻¹) and cefotaxim (500 μg ml⁻¹) and then grown to maturity in garden soil under white light from fluorescent lamps providing a photosynthetically active irradiance of 120 μmol photons m⁻² s⁻¹ and a 16 h light period. The temperature was 25/20°C during the day/night cycle. The plants were supplied every second day with a nutrient solution containing 11 mM nitrate [18].
2.3. Assay of GS activity

Total soluble tobacco leaf protein was extracted at 0°C using a potassium phosphate buffer (200 mM, pH 7.5) supplemented with 5 mM EDTA, 12.5 mM 2-mercaptoethanol and 2 mM phenylmethyl sulfonyl fluoride (tissue to volume ratio is 0.5). In the semi-biosynthetic GS assay [19], the physiological substrate ammonium is replaced by hydroxylamine. The GS catalyses the formation of γ-glutamylhydroxamate (GHA) from hydroxylamine and glutamate. The GHA was determined colorimetrically at 540 nm after complexation with acidified ferric chloride. Our reaction mixture contained 100 μl imidazole buffer (450 mM, pH 7.2), 100 μl MgCl₂ (450 mM), 100 μl NH₂OH·HCl (60 mM), 100 μl ATP (80 mM), 100 μl L-glutamate (870 mM) and 300 μl H₂O. The reaction was started by adding 50 μl protein extract and terminated after incubation for 15 min at 30°C by adding 850 μl of a solution containing 0.37 M FeCl₃, 0.2 M trichloroacetic acid and 0.67 N HCl.

2.4. Ammonium analysis

Leaf samples were homogenized in 50% (v/v) ethanol (pH 3). The extract was acidified with HCl prior to the removal of the liquid phase under vacuum at 40°C. The residue was resuspended in water. Ammonium was isolated from this solution by isothermic distillation and then determined colorimetrically at 436 nm using the Nessler reagent [20]. Our reaction mixture contained 200 μl NaOH (2 N), 200 μl K-Na-tartrate (10% [w/v]) and 9 ml H₂O. After adding 300 μl of the sample and incubation for 10 min at 25°C, 400 μl Nessler reagent were added. The reaction mixture was incubated for 30 min at 25°C. The Nessler reagent was prepared by mixing solution A (5 g KJ/5 ml H₂O) with solution B (2.3 g HgCl₂/36 ml H₂O) followed by stirring for 5 min prior to the addition of solution C (4 g NaOH/20 ml H₂O).

3. Results and discussion

In tobacco, the genes corresponding to GS-2 are predominantly expressed in the leaves (i.e. at the site of photorespiration) [9]. We have, therefore, attempted to decrease the activity of GS-2 specifically in the leaves of transgenic tobacco by employing a strong and leaf-specific promoter to drive a chimeric GS-2 antisense gene. In transgenic tobacco, the soybean rbcS promoter [13] is strongly preferentially active in the leaves [12]. Therefore, a binary vector containing a full-length tobacco GS-2 cDNA [9] in reverse orientation downstream of the soybean rbcS promoter [13] was constructed and introduced into tobacco by A. tumefaciens-mediated gene transfer. Transgenic tobacco plants were regenerated in an atmosphere containing 300 Pa CO₂ to ensure the survival of potentially obtained transgenic lines with decreased foliar GS-2 activity. Previously, we have shown that a CO₂ partial pressure of 300 Pa is sufficient to suppress or at least strongly impair photorespiration [17,21]. Three transgenic tobacco lines (designated GSc, GSd and GSe) were obtained that express a GS-2 gene in reverse orientation under the control of the soybean rbcS promoter.

To verify the degree of antisense inhibition, the activity of GS-2 was measured in leaf extracts of transformants grown under non-photorespiratory conditions in a high CO₂ atmosphere (300 Pa CO₂). In plant leaves, the GS may be represented by two groups of proteins, one being located in the chloroplasts (GS-2), the other (GS-1) being restricted to the cytosol of the phloem companion cells [22]. In tobacco, however, the abundance of GS-1 is at the limit of detection by immunoblot analysis [9]. Therefore, the (total) GS activity measured in tobacco leaf extracts predominantly reflects the activity of GS-2. As shown in Fig. 1, the foliar GS-2 activity of the individual transgenic tobacco lines was decreased to 45% (GSc), 36% (GSd) and 56% (GSe) of wild-type levels. In each transgenic line, the decrease in GS-2 activity was paralleled by a decreased accumulation of GS-2 specific transcripts (data not shown). Taken together, these results show that a substantial degree of antisense inhibition with respect to GS-2 gene expression was achieved in transgenic tobacco.

In tobacco, the leaves are the major site of primary ammonium assimilation [23]. Despite the rather strong inhibition of foliar GS-2 activity, the transgenic tobacco plants were phenotypically indistinguishable from wild-type plants when grown in a high CO₂ atmosphere (300 Pa CO₂). Plant height, leaf number and leaf size were unaltered. In addition, no appreciable differences in chlorophyll or soluble protein content between wild-type
and transgenic plants were observed under high CO₂ conditions (data not shown). Thus, under non-photorespiratory conditions, transgenic tobacco plants tolerate more than 60% reduction in the activity of leaf GS-2 without any growth retardation. This result indicates that the residual foliar GS-2 activity has sufficient capacity for ammonium assimilation via the GS-2/Fd-GOGAT cycle to provide adequate input of nitrogen for normal growth of the transgenic plants, if photorespiratory ammonium production is suppressed. A previous study with barley mutants has shown that these mutants even tolerate a decrease of 90% in leaf GS-2 activity without any growth retardation provided the plants are maintained in a high CO₂ atmosphere [24].

The rate at which CO₂ and ammonium are released during photorespiration at ambient CO₂ partial pressure (35 Pa CO₂) in the leaves of C₃-plants has been estimated to be as high as 30–40% of the rate of net CO₂ uptake [4,25]. Therefore, a strong accumulation of ammonium in the leaves was expected upon transfer to air of the transgenic tobacco plants with decreased foliar GS-2 activity. However, upon illumination in air (35 Pa CO₂), the high CO₂-grown (300 Pa CO₂) transgenic tobacco GSd plants accumulated only about 2.7-fold more ammonium in the leaves than wild-type plants (Fig. 2). Similar results were obtained in identical experiments with GSc or GSe plants (data not shown). Our data are consistent with previous observations with barley mutants with reduced GS-2 activity, which suggested that a threshold of about 40% GS-2 activity exists below which ammonium accumulation takes place [7]. There is, therefore, a clear similarity between the C₃ plants barley and tobacco.

Photosynthesis is inhibited rapidly following exposure of barley mutants severely deficient in GS-2 to air [26] and, in turn, the generation of photorespiratory ammonium may be impaired under these circumstances [5]. The (putative) inhibition of photorespiratory ammonium release may, at least in part, explain the relatively small accumulation of ammonium in the leaves following illumination in air of high CO₂-grown transgenic tobacco with reduced foliar GS-2 activity. In addition, some of the photorespiratory ammonium may have been lost from the plants, since even wild-type plants have an ammonium compensation point and lose some ammonium to the atmosphere [27]. Finally, the residual leaf GS-2 activity may have still been sufficient to re-assimilate a considerable amount of the photorespiratory ammonium generated upon illumination in air.
The transfer of high CO₂-grown transgenic tobacco GSd plants with reduced leaf GS-2 activity into air resulted in the appearance of severe chlorotic lesions (Fig. 3) and, eventually, the transgenic plants died. Prior to that, premature flowering was observed (Fig. 3). This response may have been triggered directly or indirectly by the increased foliar ammonium concentration [28]. The flowers were, however, lost before seeds were produced. Seeds derived from high CO₂-grown transgenic tobacco with reduced leaf GS-2 activity did not yield viable seedlings when germinated in air (data not shown).

We have obtained transgenic tobacco plants with reduced foliar GS-2 activity and shown that these plants are conditionally lethal with respect to the ambient CO₂ partial pressure. Stable atmospheric CO₂ concentrations that are high enough to allow these plants to grow normally are unlikely to occur in a natural environment. Such conditions can, however, be established and maintained in the greenhouse [29]. The conditionally lethal transgenic tobacco plants with reduced leaf GS-2 activity are unable to survive any prolonged exposure to air, e.g. in case that these plants are involuntarily removed from the high CO₂ greenhouse atmosphere.

The escape of transgenic seeds is another major concern, because such seeds would rapidly yield transgenic plants that express the transgene of interest. Seeds of GSD plants, however, would not develop into viable transgenic plants if germination took place outside the high CO₂ greenhouse atmosphere. Transgenic pollen, once escaped from the greenhouse, could lead to pollination of related plant species in the surrounding area. The frequency of such a pollination event is probably very low. The antisense marker may be recessive and, therefore, the resulting transgenic heterozygotes could survive. This possibility is not eliminated by the technology based on conditionally lethal transgenic plants with reduced foliar GS-2 activity. Nevertheless, these plants may contribute to biological safety if employed to produce desired proteins in greenhouse conditions.

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