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Ectopic expression of soybean leghemoglobin in chloroplasts impairs gibberellin biosynthesis and induces dwarfism in transgenic potato plants

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Abstract We have characterized potato (*Solanum tuberosum* L.) plants expressing a soybean leghemoglobin that is targeted to plastids. Transgenic plants displayed a dwarf phenotype caused by short internode length, and exhibited increased tuberization in vitro. Under in vivo conditions that do not promote tuberization, plants showed smaller parenchymal cells than control plants. Analysis of gibberellin (GA) concentrations indicated that the transgenic plants have a substantial reduction (approximately 10-fold) of bioactive GA_1 concentration in shoots. Application of GA_3 to the shoot apex of the transformed plants completely restored the wild type phenotype suggesting that GA-biosynthesis rather than signal transduction was limiting. Since the first stage of the GA-biosynthetic pathway is located in the plastid, these results suggest that an early step in the pathway may be affected by the presence of the leghemoglobin.

Keywords Gibberelin · Dwarfism · Leghemoglobin · Plant stature

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Introduction

Introduction of dwarfism into plants is one of the prime targets for crop improvement because it allows better management and higher yields while avoiding lodging in cereals (Hedden 2003). The use of dwarfing genes in wheat and rice breeding dates from the beginning of the 20th century in the far-east and has spread worldwide as a consequence of the Green Revolution. The recognition that *Reduced height* (*Rht*) genes of wheat cause impairment of gibberellin (GA) signal transduction (Peng et al. 1999), while the rice *semi-dwarf1* (*sd1*) genes disrupt GA biosynthesis (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002) demonstrate the importance of GAs for the control of plant architecture. Thus, genetic manipulation of GA biosynthesis or signal transduction offers a very promising strategy for crop improvement (Phillips 2004).

GAs are an important class of diterpenoids that can act as plant growth promoters, affecting numerous development processes, including organ growth, flower and fruit development, and the mobilization of seed reserves (for review see Richards et al. 2001). GA biosynthesis is a complex pathway mediated by several distinct enzyme classes located in three different subcellular compartments: the plastid, the endoplasmic reticulum (ER) and the cytosol (Hedden and Philips 2000a). In the last few years, the isolation and characterization of most of the genes involved in GA biosynthesis have increased understanding of GA-biosynthetic pathways and their regulation in several plant species (Hedden and Philips 2000a; Olszewski et al. 2002). The first reactions involve two plastid-located enzymes, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) that convert geranylgeranyl diphosphate into the highly hydrophobic *ent*-kaurene. The oxidation steps from *ent*-kaurene to GA_{12} are catalysed by two cytochrome P450 mono-oxygenases, the first of which, *ent*-kaurene oxidase (KO), oxidizes *ent*-kaurene to *ent*-kaurenoic acid and has been shown to be located in the plastid envelope (Helliwell et al. 2001a). Finally, the last stages of GA biosynthesis are catalysed by small families of cytosolic 2-oxoglutarate-dependent dioxygenases, which catalyse 20-oxidation (GA20ox) and 3β-hydroxylation (GA3ox) reactions to produce the active GAs, and 2-oxidation (GA2ox), which causes inactivation (Thomas et al. 1999).

Suppression of GA biosynthesis and enhancement of GA inactivation have been shown to be effective strategies for controlling plant stature (reviewed in Pimenta Lange and Lange 2006). Identification of new approaches to achieving this aim, ideally through expression of dominant transgenes would increase the option for breeders to obtain dwarf varieties of agriculturally important crops, such as rice, wheat and trees for use in arboriculture, horticulture and forestry (Busov et al. 2003).

In the present work, we demonstrate that targeting of soybean leghemoglobin to potato chloroplasts reduces GA_1 production resulting in dwarfism, short internode length, reduced cell size and increased tuberisation in vitro and in vivo. The wild type phenotype could be fully restored by spraying the dwarf plants with GA_3, indicating that GA-depletion is the major, if not sole cause of dwarfism in these plants. The implication for GA biosynthesis of the presence of a high-affinity oxygen-binding protein inside the chloroplast is discussed.

Materials and methods

Plant material and growth conditions

Transgenic and control (untransformed) potato plants cv. Bintje (Chaparro-Giraldo et al. 2000) were propagated by in vitro culture of single node stem cuttings in MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose and 0,7% (w/v) Agar (Acros Organics). Culture media was adjusted to pH 5.7 with NaOH prior to autoclaving. The plantlets were grown for four weeks in tissue culture media under long days (16 h of light, 8 h of dark) before being transplanted to a commercial substrate and transferred to the glasshouse. Plants were propagated and grown under a photoperiod of 15 h daylight and a temperature of 25/30°C.

Phenotypic assessment of transgenic potato plants

Analysis of phenotypic traits was performed on control plants (WT) and three independent transformants (B1, B2, B3). Measurements of plant height and internode length were made after two and four weeks of in vitro growth. The tubers were recovered from three months-old in vitro plants.

GA_3 treatment

Transgenic (B1, B2 and B3) and control plants were grown in vitro for four weeks and then transferred to the glasshouse. After one month, plants were sprayed with 100 μM GA_3. This procedure was repeated after one week. As a control, another equivalent group of plants was sprayed with water.

Histological sections

Longitudinal sections of the 6th internode were performed on four week-old potato plants. Samples were fixed on Karnovsky solution (Karnovsky 1965) under slight vacuum and dehydrated through an ethanol series. Sections were cut at 5 μm and stained with 0.05% toluidine blue according to Sakai (1973). Permanent slides were mounted in synthetic resin.

Quantitative analysis of GA

Young expanding leaves were excised from 4 week-old plants grown in vitro, frozen in liquid nitrogen, freeze-dried, homogenized in a ball-mill to a fine powder and stored at −20°C. Aliquots (1 g) were analysed for GA content by GC-MS using [$^2H_2$]GA internal standards as described previously (Coles et al. 1999), except that full scans were acquired and quantification was based on mass chromatogram traces.

Results and discussion

We have shown previously that soybean leghemoglobin is stably expressed, efficiently imported and correctly processed into potato chloroplasts (Chaparro-Giraldo et al. 2000). The transgenic plants presented reduced stature, increased tuberization in vitro and enhanced sprouting. In potato (*Solanum tuberosum* L.), GAs suppress tuberization (Carrera et al. 2000) and conditions that promote tuberization, such as short days, are negatively correlated with GA content (Carrera et al. 2000). Transgenic potato plants expressing leghemoglobin in chloroplasts initiated tubers earlier than wild-type plants (Chaparro-Giraldo et al. 2000).

Comparison of three independent transgenic lines with the wild-type revealed substantially decreased stem length (Fig. 1a) associated with a reduction in internode length (Table 1). The number of leaves was unchanged. The differences in internode length seen at 14 and 28 days (Table 1) were maintained throughout the plant life cycle (not shown). Additionally, expression of leghemoglobin in potato chloroplasts also affected the cell size and organization of parenchyma cells as observed in leaf cross section (Fig. 2). Cell size

Fig. 1 (**a**) Transgenic potato plants (B1, B2, B3) and control plant (WT), after 2 weeks of in vitro culture. (**b**) Reversion of the dwarf phenotype in greenhouse-grown plants by application of GA_3. The photograph represents a typical response observed in all different transformants (TRA) in at least three replicate plants in each of the GA and control experiments

Table 1 Characterization of phenotypic traits of transgenic and untransformed potato

Age/Trait	WT	B1	B2	B3
Height 14d (cm)	24.3 ± 6.3	15.6 ± 1.7	11.4 ± 1.6	9.3 ± 4.2
Height 28d (cm)	43.6 ± 7.8	29.7 ± 2.6	20.4 ± 3.4	18.8 ± 5.6
Internode 2a (cm)	8.7 ± 1.9	3.8 ± 1.0	3.0 ± 0.7	2.8 ± 1.3
Internode 3a (cm)	4.1 ± 2.2	1.8 ± 0.1	1.6 ± 0.4	1.5 ± 0.7
Internode 2b (cm)	10.2 ± 1.8	5.0 ± 1.4	4.2 ± 0.4	4.5 ± 1.6
Internode 3b (cm)	8.8 ± 1.1	3.7 ± 0.4	3.2 ± 0.6	3.3 ± 0.9
Internode 4b (cm)	5.2 ± 0.5	3.6 ± 0.5	2.3 ± 0.6	2.7 ± 0.9
Parenchyma cell length (cm)	6.48 ± 1.41	4.35 ± 1.51	3.46 ± 0.92	3.08 ± 1.07

Plant height was measured at 14 and 28 days from plants grown in vitro. Internode 2, 3 and 4 length was measured at 14 (a) and 28 (b) days of in vitro grown plants. Data represent mean values of 12 plants of each line ± SE. Measurements of cell length represent mean values from 100 parenchyma cells ± SE

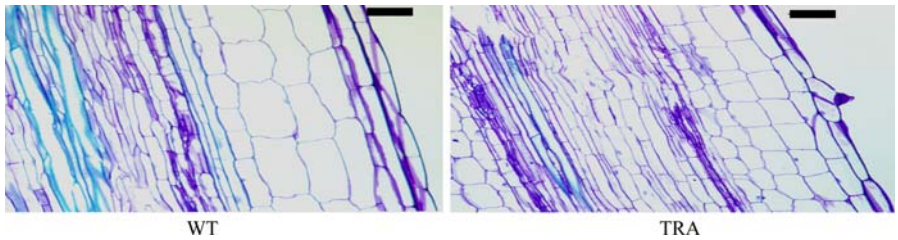

Fig. 2 Longitudinal section of the 6th internode of control (WT) and transgenic (TRA) B3 plant. Bar represents 10 μm

in the parenchyma layer of transformed plants was reduced by 80% in comparison with wild-type plants (Table 1). On the other hand, cells in the pith were not significantly affected. Most of the traits observed in the transgenic potato plants were similar to those previously reported for GA-deficient mutants (Reid and Ross 1993; Carrera et al. 2000; Rosin et al. 2003).

To determine whether the dwarf phenotype was due to GA deficiency, 100 μM GA_3 was applied twice with a one-week interval to the shoot apex of a transgenic line, resulting in complete rescue of the wild-type phenotype (Fig. 1b). This is consistent with the phenotypic alterations seen in the transgenic lines being a result of decreased GA content rather than reduced GA signal transduction. We confirmed this by analysis of the concentration of C_{19}-GAs in the shoots.

In potato plants, the early 13-hydroxylated pathway seems to be the predominant route for GA biosynthesis (van den Berg et al. 1995). This pathway involves 13-hydroxylation of GA_{12} to GA_{53}, followed by oxidative removal of C-20 to produce GA_{20} and then 3β-hydroxylation to form bioactive GA_1. The C_{19}-GAs GA_{20} and GA_1 are inactivated by 2β-hydroxylation to form GA_{29} and GA_8, respectively. Quantification of the C_{19}-GA concentrations in shoots of transgenic and untransformed potato plants indicated substantial changes in GA levels (Table 2). The bioactive GA (GA_1) was reduced in transformed plants to 5–9% of the concentration in untransformed plants, while levels of GA_{20}, the immediate precursor of GA_1, were 1.8–2.4-fold higher in the transformed plants. The concentrations of GA_{29} and GA_8 were reduced approximately 10- and 2-fold, respectively, in the transgenic plants.

Table 2 GA content in leaves of transgenic and untransformed potato

GA	WT	B1	B2	B3
GA_1	19.0	1.0	2.7	3.6
GA_{20}	1.9	3.4	3.9	4.5
GA_{29}	61.1	5.7	5.3	6.7
GA_8	7.9	2.9	3.3	4.6

GA levels are represented in ng g^{-1} DW

These results could be explained by a reduction in biosynthetic flux due to a restriction early in the pathway. The concentration of the intermediate GA_{20} may be maintained by enhanced GA 20-oxidase activity and reduced 2β–hydroxylation as a result of changes in expression of genes encoding the enzymes responsible for these activities in response to the low bioactive GA concentration (Hedden and Phillips 2000b). A major question from this work is why expression of soybean hemoglobin in plastids would interfere with GA biosynthesis. Plant hemoglobins are widespread in the plant kingdom, generally displaying a high affinity for oxygen (Arredondo-Peter et al. 1998). The first two steps of GA biosynthesis are plastid-localised, and involve two enzymes (*ent*-copalyl diphosphate synthase and *ent*-kaurene synthase), which have no requirement for oxygen. However, it has been shown that *ent*-kaurene oxidase (KO) is present in the plastid envelope and may link the plastid and endoplasmic reticulum steps of GA biosynthesis (Helliwell et al. 2001b). This enzyme is a cytochrome P450 mono-oxygenase, a heme-containing protein with an oxygen requirement. The leghemoglobin and P450 could compete for the heme moiety or for oxygen, leading to reduced *ent*-kaurene oxidase activity. We cannot rule out the possibility that other oxygen-requiring processes or those requiring heme-containing proteins inside the plastid are also affected, but the ability to achieve full recovery of the wild-type phenotype by GA application indicates that GA biosynthesis is the primary target. It is also possible that, despite its plastid targeting, a small amount of the chimeric proteins could accumulate in the cytosol or other non-chloroplastic plastids. Our previous observation based on immunological

analyses of subcellular fractions from leaves of transgenic tobacco and potato plants showed that very little if any mistargeting occurs. Targeting efficiency is difficult to evaluate precisely since some organellar proteins are released during homogenization. However, most of the oxygenation reactions of GA biosynthesis occur outside the plastid and it is possible that a small amount of leghemoglobin mistargeting might affect these reactions. Interestingly, tobacco plants expressing the same leghemoglobin inside chloroplasts did not show any developmental change or alteration in stature (Barata et al. 2000). This could reflect the levels of transgene expression in the two species, differences in the oxygen affinities of the respective *ent*-kaurene oxidases or in the availability of heme. Although speculative, these assumptions can be tested experimentally. Further experiments will aim to determine precisely which GA-biosynthetic steps are affected by the presence of plastid-localised leghemoglobin and what are the limiting factors limiting enzyme activity.

A number of different strategies have been described for restricting plant growth through genetic manipulation of the GA biosynthetic or signal transduction pathways (Phillips 2004). These include silencing GA-biosynthesis genes (Bulley et al. 2005), ectopic expression of the GA-inactivating 2-oxidase genes, as for example in rice (Sakamoto et al. 2003), wheat (Appleford et al. 2007) and tobacco (Gallego-Giraldo et al. 2007), or expressing mutant forms of the DELLA growth repressors that are not degraded in responsive to GA. An example of this last approach is the ectopic expression of the dominant mutant form of the Arabidopsis *GAI* gene in rice (Fu et al. 2001), which is analogous to the *Rht* gene in wheat (Peng et al. 1999). Despite the different genes targeted by these approaches, their overall effects on plant phenotype are very similar and mimic those of GA-biosynthesis inhibitors that are widely used in agriculture as growth retardants (Rademacher 2000). The results reported here demonstrate a further strategy for manipulating GA signaling in plants that offers new possibilities for introducing useful agronomic traits into crops. In addition, the work has provided novel information on factors that may limit GA-biosynthesis.

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