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Research article

Knockout mutants as a tool to identify the subunit composition of *Arabidopsis* glutamine synthetase isoforms

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ABSTRACT

Glutamine synthetase (GS) is a key enzyme in nitrogen assimilation, which catalyzes the formation of glutamine from ammonia and glutamate. Plant GS isoforms are multimeric enzymes, recently shown to be decamers. The *Arabidopsis* genome encodes five cytosolic (GS1) proteins labeled as GLN1;1 through GLN1;5 and one chloroplastic (GS2) isoform, GLN2;0. However, as many as 11 GS activity bands were resolved from different *Arabidopsis* tissues by Native PAGE and activity staining. Western analysis showed that all 11 isoforms are composed exclusively of 40 kDa GS1 subunits. Of five GS1 genes, only *GLN1;1*, *GLN1;2* and *GLN1;3* transcripts accumulated to significant levels in vegetative tissues, indicating that only subunits encoded by these three genes produce the 11-band zymogram. Even though the GS2 gene also had significant expression, the corresponding activity was not detected, probably due to inactivation. To resolve the subunit composition of 11 active GS1 isoforms, homozygous knockout mutants deficient in the expression of different GS1 genes were selected from the progeny of T-DNA insertional SALK and SAIL lines. Comparison of GS isoenzyme patterns of the selected GS1 knockout mutants indicated that all of the detected isoforms consist of varying proportions of GLN1;1, GLN1;2 and GLN1;3 subunits, and that GLN1;1 and GLN1;3, as well as GLN1;2 and GLN1;3 and possibly GLN1;1 and GLN1;2 proteins combine in all proportions to form active homo- and heterodecamers.

1. Introduction

Glutamine synthetase (GS, EC 6.3.1.2) is the key enzyme involved in the assimilation of ammonia derived from nitrate reduction, photorespiration, amino acid catabolism, N_2 fixation in legumes, phenylpropanoid metabolism and other metabolic processes in plants (Lam et al., 1996; Hirel and Lea, 2011; Betti et al., 2012). Higher plants contain two types of GS isoforms: GS2 located in the chloroplasts and GS1 located in the cytosol. A small gene family encodes different cytosolic isoforms in higher plants, while in most plants a single gene encodes the plastidic GS2 isoform (Betti et al., 2012). GS1 isoforms are present in all plant tissues, and are mainly involved in primary ammonium assimilation in the root, nitrogen remobilization during leaf senescence, and synthesis of Gln for transport in the vasculature (Bernard and Habash, 2009). The chloroplastic GS2 isoform is abundant in photosynthetic tissues, with a primary role in reassimilation of photorespiratory ammonia (Wallsgrove et al., 1987; Orea et al., 2002). Differential expression of GS1 genes during ontogenesis, and in response to external cues, indicates specific roles of GS1 isoforms during the plants life cycle (Lam et al., 1996; Bernard and Habash, 2009).

Based on biochemical and electron microscopy experiments, plant GSs have been assigned an octameric structure (McParland et al., 1976; Pushkin et al., 1985; Llorca et al., 2006), but recent crystallographic evidence revealed their decameric composition (Unno et al., 2006). Since GS1 genes encode for proteins that share high sequence homology, it is not surprising that heteromeric GS isoforms have been detected in several plant species (Bennett and

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; GS, glutamine synthetase; GS1, cytosolic glutamine synthetase; GS2, chloroplastic glutamine synthetase; *GLN1;1* through *GLN1;5*, different GS1 genes; *GLN1;1*KO through *GLN1;5*KO, knockout mutants of GS1 genes; KO, knockout mutant; LB, T-DNA left border; MS, Murashige and Skoog plant growth medium; MS½, half strength Murashige and Skoog plant growth medium; MSO, methionine sulfoximine; PAGE, polyacrylamide gel electrophoresis; PPT, phosphinothricin; RB, T-DNA right border; wt, wild type.

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Cullimore, 1989; Cai and Wong, 1989; Temple et al., 1996; Carvalho et al., 1997; Mäck, 1998; Brechlin et al., 2000). The *Arabidopsis* genome contains five GS1 genes, labeled as *GLN1;1* through *GLN1;5*, (Arabidopsis Genome Initiative, 2000; Ishiyama et al., 2004; Lothier et al., 2011) which encode for proteins with sequence homology ranging between 79 and 92%, and one GS2 gene, labeled *GLN2;0*.

Development of in-gel assay for GS activity (Simonović et al., 2004) enabled profiling of active GS isoforms in several species, including maize (Simonović et al., 2004), spinach (Dragićević et al., 2011) and *Lotus corniculaturs* (Dragićević et al., 2013). While spinach has one GS1 gene and correspondingly a single GS1 activity, maize has five GS1 genes, just like *Arabidopsis*, and yet they comigrated on nondenaturing gels as a single activity band (Simonović et al., 2004; Simonović and Anderson, 2007). On the contrary, preliminary analyses of *Arabidopsis* protein extracts revealed as many as 11 GS activities, suggesting that different GS subunits combine to form active heteromers. The use of knockout mutants in combination with in-gel activity assays has been successfully applied to resolve the subunit composition of few enzymes, including glutamate dehydrogenase (Fontaine et al., 2012, 2013) and catalase (Hu et al., 2010). Hereby we present the analysis of subunit composition of *Arabidopsis* GS isoforms, using knockout mutants lacking different GS1 genes.

2. Materials and methods

2.1. Plant material

The *Arabidopsis* seeds were obtained from The Nottingham *Arabidopsis* Stock Centre (NASC). *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia (NASC: N60000), were used as wild type (wt) plants. To obtain *Arabidopsis* GS1 knockout mutants, plants homozygous for the T-DNA insertions were selected from T1 progeny of SAIL_86_B04 (NASC: N870927, insertion in *GLN1;1*), SALK_102291 (NASC: N602291, insertion in *GLN1;2*), SALK_148604C (NASC: N669232, insertion in *GLN1;3*), SALK_042546C (NASC: N662238, insertion in *GLN1;4*), and SALK_086579C (NASC: N660998, insertion in *GLN1;5*) lines, derived from respective SAIL (McElver et al., 2001; Sessions et al., 2002) and SALK (Alonso et al., 2003) libraries. All mutants had T-DNA insertions in the exons of the GS1 genes (Fig. 1.)

2.2. Plant culture conditions

Seeds of wt and mutant lines were surface sterilized with 90% ethanol and 10% commercial bleach for 30 s, washed three times with sterile water and stratified for 48 h at 4 °C. Seeds were distributed on plates with half MS medium (MS½, containing 10.3 mM NH_4^+ and 19.7 mM NO_3^- Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.7% agar adjusted to pH 5.8. The cultures were grown under 16 h/8 h photoperiod, under photon flux rate of ≈40 μmol m^{-2} s^{-1} at the level of the plants, at 23 ± 2 °C. Leaves from two week old plants were used for DNA isolation for selection of homozygotic T-DNA insertions in the GS1 genes. The selected homozygous plants were transferred to fresh MS½ in Magenta vessels (#V8505, Sigma–Aldrich, St. Louis), one plant per container, where they self-pollinated and developed siliques which were harvested. In further experiments the knockout mutant seeds were sterilized, stratified, sown and grown for two weeks on solidified MS½, prior to DNA, RNA and protein extraction.

In an attempt to achieve better electrophoretic resolution of active GS isoforms, plants were also grown submerged in liquid MS media, as well as callus cultures. For *Arabidopsis* liquid culture, sterile and stratified seeds were grown in 50 ml MS (containing 20.6 mM NH_4^+ and 39.4 mM NO_3^-), with 3% sucrose, adjusted to pH 5.8 in 250 ml Erlenmeyer flasks. The cultures were grown under 8 h/16 h photoperiod, under photon flux rate of ≈10 μmol m^{-2} s^{-1} at the level of plants and were continuously shaken for aeration of the medium for 4 weeks at 23 ± 2 °C. RNA and proteins were extracted from roots and leaves of the obtained plants.

Calli were induced by sub-cultivation of root explants excised from two week old plants grown on solid MS½. The root explants were cultured for five days on MS½ supplemented with 0.7% agar, 3% sucrose and 5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The calli were further propagated on solid MS½ with 3% sucrose, 1 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin.

2.3. Protein isolation, GS activity staining and Western analysis

Soluble proteins were extracted from 0.5 to 1 g of plant tissue by grinding in liquid nitrogen followed by homogenization in protein extraction buffer (50 mM Tris–HCl, pH 8, 1 mM EDTA, 1.5% w/v polyvinylpolypyrrolidone, 10 mM dithiothreitol, 1 mM

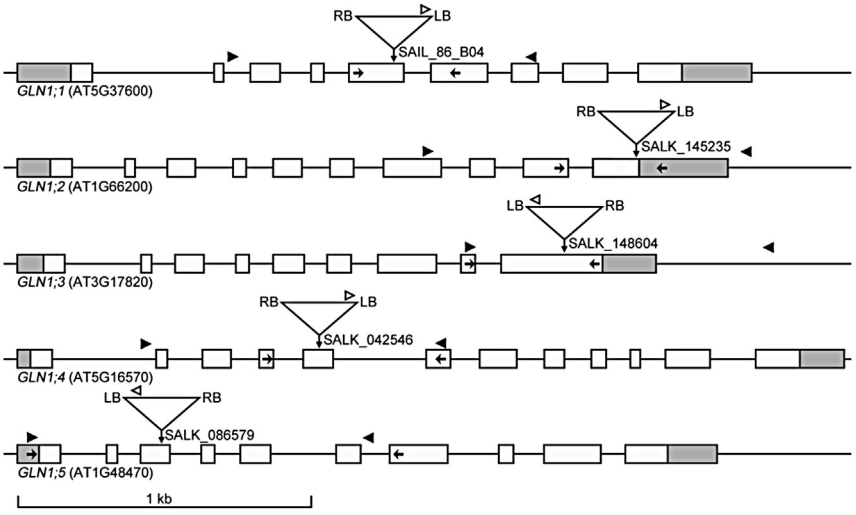

Fig. 1. Locations of the T-DNA insertions and PCR primers in *Arabidopsis* GS1 genes in the selected insertional mutants. White boxes represent exons, while grey boxes represent 5′ and 3′ untranslated regions of the genes. Indicated are positions of gene-specific primers (►, ◄) and T-DNA-specific primers at the left border (▷, ◁) used for selection of homozygous mutants. Primers used for RT-PCR and qRT-PCR analyses are indicated as arrows (→, ←). LB and RB – left and right T-DNA borders. Sequences of all primers are given in Table 1.