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RESEARCH PAPER

Glutamine synthetase of potato (*Solanum tuberosum* L. cv. Désirée) plants: cell- and organ-specific expression and differential developmental regulation reveal specific roles in nitrogen assimilation and mobilization

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Abstract

Potato (*Solanum tuberosum* L. cv. Désirée) glutamine synthetase (GS) (EC 6.3.1.2) gene expression and polypeptide accumulation patterns were analysed in several organs and at several developmental stages. Three GS genes have been identified, one gene encoding plastidic GS (GS2) and two encoding cytosolic GS (GS1) that are differentially expressed in the plant at cellular and organ levels. Specific developmental regulation of different GS genes was also observed. Potato GS seems to be regulated essentially at transcription and/or RNA stability levels. GS2 polypeptides and mRNAs were detected in leaves and their content decreased as leaves senesced. A similar pattern of expression was observed for the GS1 gene *Stgs1a*. GS1 transcripts and polypeptides were present in all organs analysed and are the only GS detected in non-photosynthetic tissues and in the later leaf senescing stages. The increase in GS1 during leaf senescence mainly reflected polypeptide and transcript accumulation of the GS1-encoding gene *Stgs1b*. *In situ* hybridization results point to a cell-specific expression of GS1 genes within the vascular bundles, *Stgs1b* being expressed in the xylem and phloem parenchyma cells, and *Stgs1a* being expressed only in the phloem companion cells. This pattern of spatial distribution and differential developmental regulation of different GS1 genes differs from what has been previously described for genes of other Solanaceae with a high degree of similarity with the ones described here and suggests that distinct GS1 isozymes have specific and possibly distinct roles within the same organ. These new findings highlight the physiological importance of different GS1 isoenzymes in plant nitrogen metabolism.

Key words: Digoxigenin, glutamine synthetase, *in situ* hybridization, nitrogen assimilation, potato, senescence.

Introduction

In higher plants, glutamine synthetase (GS, EC 6.3.1.2) is a key enzyme involved in the assimilation of inorganic nitrogen into organic forms. GS catalyses the ATP-dependent condensation of ammonium with glutamate to yield glutamine, which then provides nitrogen groups, either directly or via glutamate, for the biosynthesis of all nitrogenous compounds in the plant (Forde and Cullimore, 1989). Two groups of GS isoenzymes, plastidic (GS2) and cytosolic (GS1), have been identified in higher plants (Cren and Hirel, 1999). In the majority of higher plants GS2 is predominant in most chlorophyllous tissues and is localized in the chloroplast stroma (Cullimore *et al.*, 1984; Cullimore

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Abbreviations: Dig, digoxigenin; GS, glutamine synthetase; RuBisCO, ribulose bisphosphate carboxylase/oxygenase; SAG, Senescence Associated Genes.

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and Bennett, 1988; Carvalho *et al.*, 1992; Pereira *et al.*, 1992). The isolation of cDNAs encoding GS2 allowed the demonstration that in most plant species this isoenzyme is encoded by a single nuclear gene per haploid genome (Becker *et al.*, 1992; Lightfoot *et al.*, 1988). Previous studies demonstrated that this isozyme is indispensable for the reassimilation of ammonium released from the photorespiratory nitrogen cycle (Wallsgrove *et al.*, 1987). The physiological importance of GS2 was later demonstrated by using transgenic plants overexpressing GS2 in the leaves. These plants revealed an increased photorespiratory capacity when grown under osmotic stress and high light intensity (Palatnik *et al.*, 1999; Hoshida *et al.*, 2000).

The assumption that distinct isoenzymes have different roles in specific organs/tissues of the plant has been further supported by the work of Edwards *et al.* (1990) which concluded that cytosolic and chloroplastic GS have non-overlapping functions in photosynthetic and non-photosynthetic tissues. The non-overlapping localization of GS1 and GS2 was further confirmed by immunogold localization of both isoenzymes in potato (Pereira *et al.*, 1992) and tobacco (Carvalho *et al.*, 1992) leaf tissues. However, the significance of the multiplicity of GS1 genes has not been thoroughly elucidated and very few studies have been performed in order to unravel this subject (Brugière *et al.*, 1999; Carvalho *et al.*, 2000).

GS1 is the predominant isoenzyme present in non-photosynthetic tissues. Immunocytochemical studies demonstrated that GS1 protein is localized in the vascular tissue, in which a high proportion of the protein is concentrated in the phloem companion cells (Carvalho *et al.*, 1992; Kamachi *et al.*, 1992*b*; Pereira *et al.*, 1992, 1996). Its participation in nitrogen translocation can be assumed due to its localization, but the relative physiological role of different GS1 isozymes remains to be elucidated.

In most plant species GS1 is encoded by a multigene family (Cullimore *et al.*, 1984; Cullimore and Bennett, 1988; Bennett *et al.*, 1989; Forde and Cullimore, 1989; Forde *et al.*, 1989; Sakamoto *et al.*, 1989; Sakakibara *et al.*, 1992; Li *et al.*, 1993; Stanford *et al.*, 1993; 1996; Dubois *et al.*, 1996; Cren and Hirel, 1999; Ochs *et al.*, 1999). An important contribution for the elucidation of the meaning of different GS1 isozymes came from the findings that GS1 present in the phloem plays a major role in regulating proline production (an amino acid with increased accumulation in response to osmotic stress) in tobacco plants (Brugière *et al.*, 1999).

During senescence, total leaf GS activity decreases. However, it was possible to detect an increased accumulation of GS1-related mRNAs and polypeptides since the onset of leaf development until the final stages of leaf senescence (Kawakami and Watanabe, 1988; Kamachi *et al.*, 1991, 1992*a*; Pérez-Rodríguez and Valpuesta, 1996).

Plant senescence is a highly regulated and complex developmental stage that results in the co-ordinated degradation of macromolecules with subsequent mobilization of its components to other parts of the plant. During its life span, leaves go through three distinct developmental stages. Initially, they undergo rapid expansion and growth, importing carbon and nitrogen, and are characterized by having high synthesis rates until maximum photosynthetic capacity is achieved. Then the full-expanded leaf contributes to plant growth by supplying carbon- and nitrogen-containing organic compounds. This will continue to occur until internal and external signals induce the onset of senescence. During this final stage there is an intense mobilization of nitrogen, carbon, and minerals from the senescing leaf to the other parts of the plant (Buchanan-Wollaston and Ainsworth, 1997).

Although it is characterized as being a deterioration process, senescence is considered to be a highly evolved genetic process (Nam, 1997). Throughout senescence the great majority of genes decrease their expression while others increase it. These latter are called SAG (Senescence Associated Genes) and include lipases, proteases, and endopeptidases, RNases, and some oxidative stress- and pathogen-related genes whose products participate in cell integrity and protection so that the senescence process can be completed (Gan and Amasino, 1997; Nam, 1997). SAG regulation is highly complex and, according to their temporal expression patterns, SAGs can be grouped into two classes: class I is composed of senescence-specific SAGs because their mRNAs can only be detected during this stage; class II genes are those in which their transcripts can be detected during the early stages of leaf development with a basal expression which increases throughout senescence (Gan and Amasino, 1997).

Most of the nitrogen in C_3 leaves is located in the chloroplast. RuBisCO is the major chloroplastidial protein, contributing to almost 50% of total leaf protein content. During senescence, nitrogen from RuBisCO and other proteins from the chloroplast, has to be reassimilated to be used in the formation of new tissues occurring in other parts of the plant (Feller and Fischer, 1994). Proteins have to be converted into amino acids, in particular, into amides namely glutamine and asparagine, the two major translocated amino acids in the phloem of senescing leaves (Kamachi *et al.*, 1992*a*; Feller and Fischer, 1994).

The observations that GS1 increased through senescence suggests its inclusion into the class II SAGs (Buchanan-Wollaston and Ainsworth, 1997), but the differential GS1 accumulation pattern has never been discriminated in most studies, except for tobacco, where it was possible to verify that a new GS1 isoenzyme appeared in the mesophyll in the latter senescence stages (Brugière *et al.*, 2000). Immunocytolocalization studies performed on tobacco plants suggest a differential role for GS1 isozymes during leaf senescence, however, the *in situ* localization of tobacco GS1-related transcripts was not performed and this assumption relies on the parallelism existing between northern

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blotting and immunolocalization analyses. *In situ* hybridization enables the detection of specific nucleic acid sequences in morphologically preserved biological material, providing their exact locations, allowing the evaluation of differential gene expression in tissues, cells, and subcellular compartments, and to correlate this information with temporal and spatial cell differentiation and specificity.

The focus of this work was the isolation and characterization of members of the GS family in potato. The isolation of three different cDNAs encoding three distinct GS polypeptides in potato plants is reported. These cDNAs were used as templates for the production of antisense RNA probes for GS-related transcript accumulation studies throughout leaf senescence and the whole plant system. One and two-dimensional electrophoresis followed by western blotting analysis were performed in order to study the GS protein accumulation pattern. Synthetic gene-specific oligonucleotide probes chemically labelled with biotin were used for *in situ* hybridization studies. The data obtained in this work suggests that the main regulatory process regarding GS polypeptide accumulation in potato resides at the transcriptional level. The relative physiological role of each GS isozyme in particular physiological situations is discussed.

Materials and methods

Plant material

Potato plants (*Solanum tuberosum* L. cv. Désirée) were grown from sprouting tubers in a ground mixture containing vermiculite under greenhouse conditions. Five weeks after emergence samples were harvested from roots, growing tubers, stems, and leaves. Leaf samples were collected from the second, fourth, sixth, and ninth leaves counting from the apex. Sprouting tubers and their corresponding sprouts were also used in these studies.

The samples from at least three different plants were grinded with liquid N_2 and the resulting powder was stored at −80 °C until used.

GS extraction and assay

Tissue samples were homogenized at 0 °C, with 2 vols (w/v) extraction buffer (25 mM Trizma [pH 8.0], 10 mM magnesium chloride, 1 mM DTT, and 10% glycerol), and 10% (w/v) of polyvinylpolypyrrolidone. The homogenates were centrifuged twice at 12 000 *g* for 15 min. The clear supernatant was recovered to determine GS activity. GS activities, expressed as nkat γ-glutamyl hydroxamate g^{-1} FW, were determined by the transferase assay according to (Shapiro and Stadtman, 1970).

cDNA library screening

A λgt11 *Sfi*I-*Not*I (Promega, USA) library was constructed from potato leaf mRNA according to the manufacturer's instructions. A λZAPII library (Stratagene, USA) constructed from potato growing tubers was kindly provided by Professor Lothar Willmitzer (Max-Planck-Institut für Molekulare Pflanzenphysiologie, Germany). These libraries were screened by the plaque hybridization technique using ^{32}P-labelled probes: a PCR amplified DNA fragment with conserved degenerated primers (GSF1: 5′-(GAA TTC) TCC TGC TGG CGA GCC-3′ as a forward primer and GSR1: 5′-(GAA TTC) TGT GAG CAC CTG CAC C-3′ as a reverse primer) and a cDNA coding the GS1 subunit Gln-γ of *Phaseolus vulgaris* L. kindly provided by Doctor Julie V Cullimore (Bennett *et al.*, 1989). Hybridizations and further washing for removal of excess probe were performed as described (Church and Gilbert, 1984). The cDNA inserts isolated from the λgt11 library (*Stgs2* and *Stgs1b*) were subcloned into pGEM11Zf(+) vector (Promega, USA) at the *Sfi*I–*Not*I orientation and the cDNA isolated from the λZAPII library (*Stgs1a*) was *in vitro* excised according to the manufacturer's instructions into a pBluescript vector (Stratagene, USA) in the *Eco*RI–*Xho*I orientation. The cDNAs were sequenced using the T7 Sequencing and the DYEnamic ET Terminator Cycle Sequencing kits from Amersham Pharmacia Biotech, USA. The DNA sequences were treated and analysed with the freeware application 'pDraw32 1.1.50' by Kjeld Olesen (AcaClone Software). DNA sequences were also analysed and compared by the BLAST application (Altschul *et al.*, 1997). Sequence alignments were carried out using the ClustalW (www.ebi.ac.uk).

GS mRNA and protein accumulation analysis

Total RNA was extracted from frozen tissue according to the instructions provided with the kit 'RNeasy Plant Mini kit' (Qiagen, Germany). Northern blots were performed with 1 μg total RNA using antisense digoxigenin-labelled RNA probes. To prepare the RNA probes, plasmids containing the isolated cDNAs were linearized with *Eco*RI at their 5′ end and the restriction products were separated through agarose gel electrophoresis with 1× TAE as buffer. The linearized plasmids were cut and gel-purified with the 'QIAquick Gel Extraction kit' (Qiagen, Germany), and the isolated DNA spectrophotometricaly quantified. Digoxigenin labelling and nucleic acid hybrids detection were performed with the 'Dig Northern Starter kit' according to the instructions manual provided by Roche (Germany).

Western blot analysis was performed as described in Pereira *et al*. (1992, 1996) with the exception that a 12.5% polyacrylamide gel was used instead of 10%. The bidimensional analysis of the GS-related polypeptides was performed as described in Avila *et al*. (2000, 2001).

GS transcript localization by in situ hybridizations

Petioles from the fourth leaf were fixed in Histochoice NB for 1–2 h. Vibratome sections were hybridized with 5′-end biotin-conjugated oligonucleotide probes specific for the two cytosolic GS isogenes that do not cross-recognize each others GS1 transcripts and with a sense probe. Temperatures of prehybridization and hybridization were 50 °C and 42 °C, respectively. *Stgs1a* 5′-CTT CGC AAT GAA CTC AGG CTT GCT GAG TAC-3′; *Stgs1b* 5′-GCA AAC CAG AAA CAA GCG-3′ and *Stgs1b*-sense 5′-CGC TTG TTT CTG GTT TGC-3′ probes were used at a concentration of 0.1 ng μl^{-1} hybridization solution as described by Borlido *et al*. (2002).

Results

GS activity assays

The GS transferase activities obtained can be visualized in Fig. 1. GS activity was highest in the second leaf and lowest in the ninth leaf, with a progressive decline along leaf age. It was higher in sprouting tubers than in growing tubers. The highest activity was detected in sprouts, followed by the second and fourth leaves and sprouting tubers. Roots, stems, and senescing (ninth) leaves corresponded to the organs with the lowest activities detected.

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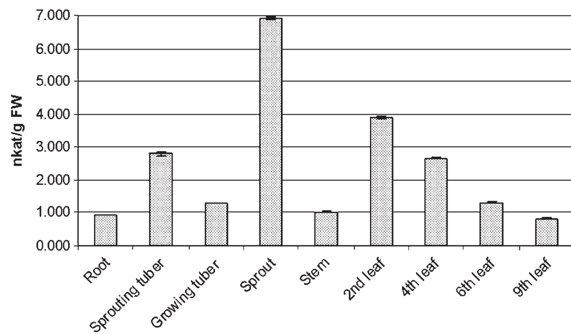

Fig. 1. Graphical representation of total GS transferase activity in the potato organs analysed, expressed as nkat γ-glutamyl hydroxamate g^{-1} FW. Values are means ±SD of at least three independent assays.

Screening of the cDNA libraries and characterization of the isolated clones encoding GS polypeptides

A potato (*S. tuberosum* L. cv. Désirée) leaf library constructed in the λgt11 *Sfi*I–*Not*I vector was screened at low stringency conditions for GS coding sequences with a homologous PCR-amplified DNA fragment. After one round of purification it was possible to isolate one phage that hybridized to the probe used among 2×10^5 phages. At the end of the procedure two cDNA clones encoding GS were isolated and mapped using various restriction enzymes. Both clones presented the same restriction pattern and had an insert of 1.2 kb. The clone *Stgs2* was used for subcloning into pGEM11Zf(+) and sequencing. Sequence analyses revealed that this cDNA, with 1200 bp, encodes a plastidic GS (GS2) isoenzyme. The accession number for retrieving the sequence of this cDNA clone at the GeneBank database is AF302113.

Screening of a potato growing tuber library, with a cDNA encoding the GS1 subunit of *P. vulgaris* L. Gln-γ (Bennett *et al.*, 1989), allowed the isolation, within 3.4×10^6 phages, of one that hybridized with the probe used. At the end of the three rounds of purification, the isolated phage (*Stgs1a*) was excised *in vitro* and the resulting pBluescript plasmid was subjected to restriction analysis and sequencing. Sequence analysis revealed that this cDNA, with 1215 bp, encodes a cytosolic GS (GS1) isoenzyme. Its accession number at the GeneBank database is AF302115. The first library was re-screened using this cDNA as probe.

Screening a total of 1.8×10^6 phages led to the isolation of a third cDNA clone that hybridized to the probe. This cDNA was subcloned into the pGEM11Zf(+) and sequenced. Sequence analysis revealed that this cDNA (*Stgs1b*) (550 bp) (accession number AF302114) encodes a cytosolic GS (GS1) isoenzyme, different from *Stgs1a*. Further screenings were unsuccessful in isolating other GS encoding sequences.

The nucleotide sequence analysis of the cDNA clones encoding GS in potato revealed that the three sequences are different, despite having high homology within the coding regions, the 3′ UTR regions being less conserved. All cDNAs isolated lack the ATG codon and the information upstream and therefore they do not represent complete GS-related mRNA molecules. The analysis of the deduced amino acid sequences revealed that the isolated clones encode three distinct GS polypeptides, all lacking the N-terminal region. The presence of the 16 conserved amino acid residues, common to the C-terminal extension of the GS2 subunits, is present in the deduced amino acid sequence encoded by the *Stgs2* cDNA, and therefore further confirms that this cDNA encodes a GS2-related polypeptide. However, this sequence stretch was not present in the deduced amino acid sequences of the other two GS clones (*Stgs1a* and *Stgs1b*), indicating that these encode GS1-related polypeptides.

These sequences were also compared to others available in databases encoding for GS and this analysis revealed a high degree of similarity with cDNAs encoding GS isozymes from other Solanaceous species. The cDNA *Stgs2* presented higher similarities with cDNAs that encode GS2 polypeptides, the highest homology being obtained with the sequence NSGS2AA that represents a mRNA of the GS2 subunit from *Nicotiana sylvestris* L. (Becker *et al.*, 1992). High homologies were obtained for the cDNA *Stgs1a* related to cDNAs encoding GS1 subunits, the highest being the *Nicotiana tabacum* L. mRNA for cytosolic glutamine synthetase *Gln1-5*. The highest homologies for the other cDNA clone encoding GS1 (*Stgs1b*) were also related to cDNAs encoding GS1 subunits. The cDNA sequence with a higher similarity to this latter isolated cDNA corresponds to another GS1-related cDNA—*Gln1-3* in *N. tabacum* L (Dubois *et al.*, 1996).

Expression of GS in the potato organs

GS activity was detected in all organs analysed. The highest activities could be detected in sprouts, sprouting tubers, and in the younger leaf developmental stages. Roots, growing tubers, stems, and the later leaf developmental stages had the lowest activity. Western blot analysis following SDS-PAGE revealed a differential accumulation pattern for the two GS isozymes present in the potato organs analysed (Fig. 2). GS2 polypeptides appear in the leaf and stem extracts with a molecular weight of 45 kDa. They correspond to the predominant GS isozyme in leaves and its relative content decreases along the progression of leaf senescence, contributing largely to the decrease in the total leaf GS activity observed. The decrease in GS2 content was paralleled by an increase in the relative content of the 42 kDa GS1 polypeptide. The GS1 polypeptides correspond to the only GS isozyme detected in the late senescence stage and in all the other organs examined, being mainly responsible for the GS activity determined therein. A greater relative abundance of GS1 was detected in sprouting tubers when compared with growing tubers, which is correlated with total GS activity detected in those organs.

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Considering that GS is post-translationally regulated by phosphorylation (Finnie *et al.*, 1999; Finnemann and Schjoerring, 2000; Riedel *et al.*, 2001; Comparot *et al.*, 2003) and that the degree of phosphorylation will influence the total net charge leading to different mobilities for the same subunit, the methodology used in this report for potato GS bidimensional analysis was the same as described for pine GS isozymes (Avila *et al.*, 2000, 2001) with a pH separation range from 3 to 10. This way it was guaranteed that the separation of the GS subunits was performed based on their total net charge, resulting mainly from their amino acid composition, and not because of charge fluctuations derived from different phosphorylation degrees which would be detected if the pH range were narrower. The two-dimensional analysis of the GS polypeptides allowed the identification of three distinct isoelectric points (Fig. 3). Only one isoelectric point of 45 kDa, with a pI of 4.7, could be visualized in stem and leaf extracts, suggesting that there is only one population of GS2-related polypeptides in these potato organs. Two lower molecular weight isoelectric points of 42 kDa, with pIs 5.0 and 4.8, could be detected in all organs studied. The polypeptide population with pI 5.0 is more abundant in all the leaf developmental stages analysed, and increased with leaf age. The other GS1 polypeptide, less abundant and with pI 4.8, describes a short increase in its relative abundance from the second to the fourth leaf and then its abundance decreased as leaf age increased, being almost undetected in the sixth leaf extracts. This procedure could not be performed for the ninth leaves because of the low concentration of protein present in the extract. In the non-photosynthetic organs examined it is possible to visualize the same isoelectric points corresponding to the two GS1-related polypeptides.

mRNA accumulation patterns of GS in potato organs

Northern blot analyses of RNA samples taken from potato organs were performed using *Stgs2*, *Stgs1a*, and *Stgs1b* antisense RNA Dig-labelled probes (Fig. 4a, b, c). It was possible to detect GS-related transcripts in all the situations studied. *Stgs2*-related transcripts appear to have an estimated size of 1.6 kb and were detected in leaves and faintly in stems (Fig. 4a). Their relative abundance increased from the second to the fourth leaf and decreased drastically as leaf age increased, not being detected at the ninth leaf level.

The *Stgs1a* mRNAs have an estimated size of 1.4 kb. These transcripts could be detected in all organs and developmental stages analysed (Fig. 4b). It was possible to visualize that the relative abundance of these mRNAs is greater in sprouting than in the developing tubers. At leaf level there is an increase in these mRNAs accumulation from the second to the fourth leaf, decreasing again as leaf age increases in a similar pattern as that obtained for the *Stgs2* probe and the GS1 isoelectric point 4.8.

The *Stgs1b* mRNAs have an estimated size of 1.4 kb and could also be detected in all organs and developmental stages analysed (Fig. 4c). At leaf level an increase of transcript accumulation paralleled leaf ageing in a similar way as described for the GS1 isoelectric point 5.0.

Similar amounts of RNA were loaded in each lane as ascertained by spectrophotometry and agarose gel visualization (Fig. 4d).

GS transcript localization by in situ *hybridizations*

Detection of GS1-related mRNAs by *in situ* hybridization revealed a differential expression and accumulation for the two isogenes in potato petioles (Fig. 5). The biotinilated *Stgs1a*-related probe detected transcript accumulation only in internal phloem companion cells (Fig. 5a) and the *Stgs1b* probe detected transcript accumulation in the internal and external phloem and xylem parenchyma cells, although some labelling could be detected in phloem companion cells and corresponding sieve elements (Fig. 5b). Hybridizations with the *Stgs1b*-sense probe led to no nucleic acid being detected (Fig. 5c).

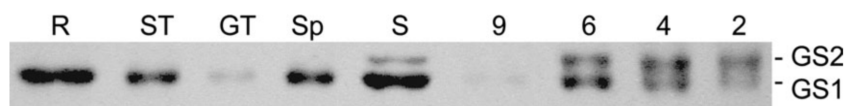

Fig. 2. One-dimensional western blotting analysis of potato GS using GS antibodies raised to bean (*Phaseolus vulgaris* L.) cytosolic GS1. Lanes were loaded with 20 μg of soluble protein. The identity of the GS1 and GS2 polypeptides is based on their M_r. R, roots; ST, sprouting tubers; GT, growing tubers; Sp, sprouts; S, stems; 9, 9th leaf; 6, 6th leaf; 4, 4th leaf; 2, 2nd leaf from the apex.

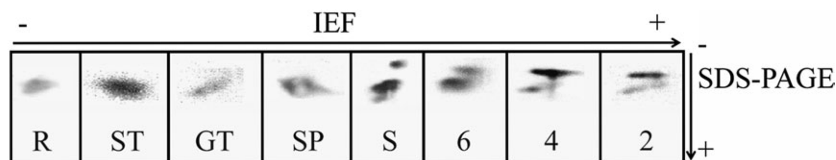

Fig. 3. Two-dimensional western blotting analysis of potato GS using GS antibodies raised to bean (*Phaseolus vulgaris* L.) cytosolic GS1. Lanes were loaded with 50 μg of soluble protein. The identity of the GS1 and GS2 polypeptides is based on their M_r. R, roots; ST, sprouting tubers; GT, growing tubers; Sp, sprouts; S, stems; 6, 6th leaf; 4, 4th leaf; 2, 2nd leaf from the apex.

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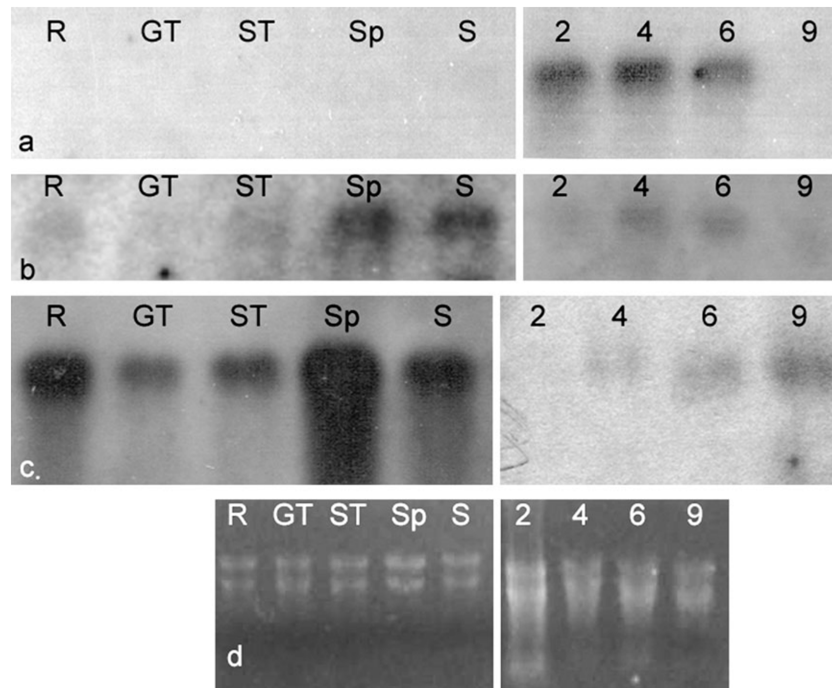

Fig. 4. Transcript accumulation patterns of the GS gene members in the different organs of potato related to the following probes: (a) *Stgs2*, (b) *Stgs1a*, (c) *Stgs1b*, (d) agarose gel electrophoresis of 200 ng of RNA from the loading mixtures used. R, roots; ST, sprouting tubers; GT, growing tubers; Sp, sprouts; S, stems; 2, 2nd leaf; 4, 4th leaf; 6, 6th leaf; 9, 9th leaf from the apex.

Discussion

In this work, three potato GS cDNAs were characterized and their expression pattern analysed. Potato GS may be encoded by a small multigene family composed of at least one GS2-encoding gene and two GS1-encoding genes.

Total GS activity was detected in all the organs analysed. The lower GS activity detected in roots and stems, can be justified by the fact that potato plants assimilate most of their inorganic nitrogen in leaves (Karley *et al.*, 2002; Mäck and Schjoerring, 2002). The high activity rates detected in sprouting tubers (and corresponding sprouts) can be explained by the need of nitrogenous compounds for new plant formation and growth, being GS involved in the remobilization of nitrogen from protein catabolism, producing glutamine to be used as a nitrogenous precursor or as a nitrogen translocation product, since it is known that, in potato, phloem sap glutamine and asparagine correspond to the principal transported nitrogenous compounds (Karley *et al.*, 2002).

The GS-related polypeptide accumulation pattern observed revealed a differential accumulation of the GS isozymes throughout leaf senescence and in the different organs analysed. Western blotting analysis confirmed the existence of two GS-related polypeptide populations with different molecular weights, a larger one, with 45 kDa (GS2) and a smaller one with 42 kDa (GS1) (Pereira *et al.*, 1992), and allowed the observation of a differential accumulation in the different organs analysed. The GS2 relative polypeptide paralleled the changes in total leaf GS activity with leaf age. This pattern was similar for that obtained by northern blotting analysis using the *Stgs2* probe, suggesting that potato GS2 regulation in the leaves resides mainly at the transcriptional and/or mRNA stability levels.

During leaf senescence, the decrease in total GS activity reflected the GS2-related polypeptide content. This decrease in potato GS2 protein content will correspond to the sum of the decreased transcript accumulation, non-enzymatic fragmentation by reactive oxygen species (Ishida *et al.*, 2002), and protein breakdown that is going on in the chloroplast of the senescing leaf (Gan and Amasino, 1997), leading to a large decrease in leaf total GS activity. The reduction in transcript accumulation may result from a regulation mechanism possibly common to the one that regulates the photosynthesis-related genes expression (Oliveira and Coruzzi, 1999). However, the control mechanisms that rule GS2 expression are not fully known (Cren and Hirel, 1999) and the study of GS2 promoters and their *cis* and *trans* regulatory elements will allow a better understanding of the role that this isoenzyme plays during senescence and other metabolic processes that it may be involved in.

GS1 was detected in all organs examined, being the only GS isozyme present in non-photosynthetic tissues. 2-D analysis performed in this study allowed the evaluation of the differential accumulation of distinct GS1 polypeptides. The increase of total GS1 polypeptide content throughout leaf development was mainly due to the increased accumulation of the polypeptides with pI 5.0. This accumulation

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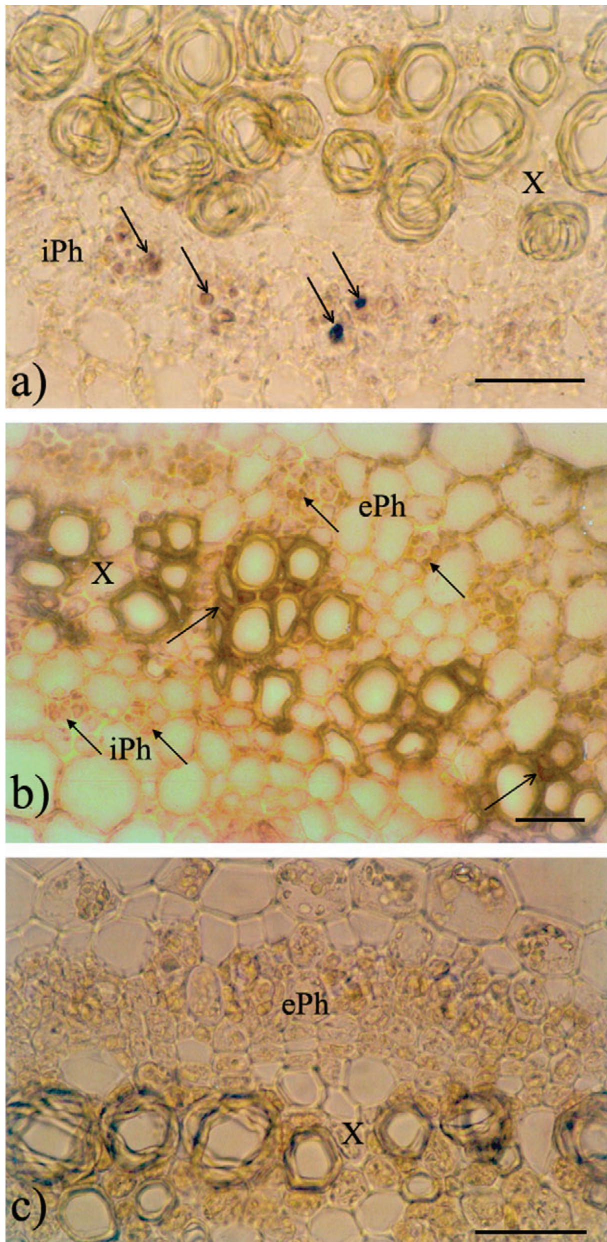

Fig. 5. Specific detection of GS1-related transcripts in potato petioles. (a) *Stgs1a*-related transcripts, arrows indicate phloem companion cells where these GS1-related transcripts were detected (bar=50 μm). (b) *Stgs1b*-related transcripts, light arrows indicate xylem parenchyma cells and full arrows indicate phloem cells (all cells from the phloem bundle) where these GS1-related transcripts were detected (bar=50 μm). (c) *Stgs1b*-sense probe, no transcripts could be detected (bar=50 μm).X, Xylem; iPh, internal phloem; ePh, external phloem.

pattern correlates with the one observed for the *Stgs1b*-related transcripts. These results strongly suggest that the GS1 polypeptides with pI 5.0 are the translational product of the *Stgs1b*-related mRNAs. Similarly, the observed overlap between the accumulation of the GS1 polypeptides with pI 4.8 and the *Stgs1a*-related transcripts suggests that these must be related. The overlapping GS1 mRNAs and polypeptides accumulation patterns observed throughout leaf development suggest that this isoenzyme may be regulated essentially at the transcription and/or mRNA stability levels.

Interestingly, *Stgs1a*-related transcripts and polypeptides described a similar accumulation pattern to that of *Stgs2* (GS2). The possibility that *Stgs1a* may be under the regulation of light/carbon content is supported by previous observations that GS1 transcripts in *Arabidopsis* increased with sugar content *(*Oliveira and Coruzzi, 1999).

Previous studies have shown that, in the Solanaceae species potato and tobacco, GS1 polypeptides accumulate in the phloem companion cells (Carvalho *et al.*, 1992; Pereira *et al.*, 1992, 1996). The *in situ* hybridization analyses revealed that the transcripts related to *Stgs1a* are accumulated only in the phloem companion cells of the petiole, in a similar way as its tobacco homologous (*Gln 1–5*) (Dubois *et al.*, 1996) does.

The GS1 products (mRNAs and polypeptides) related to *Stgs1b* progressively accumulated with leaf age and correspond to the sole GS1 representatives in the later developmental stage analysed. This accumulation pattern is typical of class II SAG genes (Gan and Amasino, 1997), meaning that this particular GS1 gene belongs to this class and will be mainly responsible for the production of glutamine for export from the senescing leaf. *Stgs1b* transcripts could be detected in the petiole phloem and xylem parenchyma cells as well as in phloem companion cells by *in situ* hybridization. This increased transcript accumulation may reflect a higher proportion of vascular parenchyma cells expressing this GS1 gene. The fact that vascular tissues are the last to lose their function during senescence corroborates this hypothesis (Feller and Fischer, 1994).

Unlike that described for the tobacco gene (*Gln1-3*), which had a high nucleotide similarity for *Stgs1b*, the expression of *Stgs1b*-related transcripts was found in vascular tissues: in phloem companion cells and in other cell types such as xylem and phloem parenchyma cells. Furthermore, the expression of this gene (*Stgs1b*) is not senescence induced, as its tobacco homologue, but showed a progressive enhancement throughout leaf development. Other studies in this laboratory (not shown) suggest that this gene is upregulated in response to various stress situations.

The total GS activity obtained in the tubers' physiological conditions examined is correlated with GS1 polypeptide and transcript content, suggesting that GS1 regulation, in these conditions, resides firstly at the transcriptional level. The amino acid content of the growing tubers' phloem sap is mainly constituted by essential amino acids (Karley *et al.*, 2002) implying that ammonium fixation for amino acid synthesis is not an important event during this stage. At this stage GS1 low expression and activity rates contribute to ammonium reassimilation that may be derived from the protein turnover and deamination reactions that are occurring. When tubers begin to sprout, a high metabolic state is imposed, and GS1 becomes more active. The

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reassimilation of large amounts of ammonium derived from protein catabolism and deamination reactions will be performed by GS1 and the glutamine produced will be used as a precursor and translocated to the growing/forming parts of the plant.

In conclusion, potato GS appears to be encoded by a small multigene family composed of one GS2-encoding and two GS1-encoding genes although the possibility can not be ruled out that other genes and pseudogenes can be present in the potato genome.

Plants have evolved extremely diversified gene families as a means of living with variable environmental and developmental conditions. The significance of these multigene families may be related to a need for a fine-tuned regulation of the different members in order to respond to different physiological situations in different organ and cell types. These results strongly contribute to the understanding of the different roles that can be assigned, not only to GS2 and GS1 isoenzymes, but to the individual members of the GS1 gene family.

GS expression is differential at organ and cell type level and is mainly regulated at transcriptional and/or mRNA stability levels in all the organs analysed. GS total activity decreases with leaf age due to a decrease in the accumulation of GS2. The activity detected in the later developmental stages is mainly the responsibility of one GS1 isozyme (*Stgs1b*). The results presented here strongly suggest that potato GS1 isozymes may have specific roles within the leaf regarding ammonium assimilation: *Stgs1a*-related products may be involved in controlling production of proline, and *Stgs1b*-related products may be assimilating the ammonium derived from protein catabolism throughout senescence and/or producing glutamine for translocation.

The high activity detected in sprouting tubers and sprouts is due to both GS1 isozymes and provides glutamine for transport and as a nitrogenous precursor for new potato organs formation.

Although GS2 regulation and expression appears to be conserved among the different Angiosperm species, and GS1 is present in the phloem of the majority of the plant species studied, there is an enormous variability regarding GS expression patterns, leading to great difficulties when trying to interpret and generalize physiological and biochemical events. The main reason resides at the promoter level, where homologies between genes is low, implying a small probability in finding the same *cis* elements across species and, ultimately, leading to different expression patterns. Further studies regarding GS promoters' characterization may contribute to the completion of this gap in the GS expression knowledge.

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