

## Up-Regulation and Localization of Asparagine Synthetase in Tomato Leaves Infected by the Bacterial Pathogen *Pseudomonas syringae*

Francisco Olea<sup>1</sup>, Alejandro Pérez-García<sup>1</sup>, Francisco R. Cantón<sup>2</sup>, M. Eugenia Rivera<sup>1</sup>, Rafael Cañas<sup>2</sup>, Concepción Ávila<sup>2</sup>, Francisco M. Cazorla<sup>1</sup>, Francisco M. Cánovas<sup>2</sup> and Antonio de Vicente<sup>1,3</sup>

<sup>1</sup> Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, E-29071 Málaga, Spain

<sup>2</sup> Departamento de Biología Molecular y Bioquímica-Unidad Asociada UMA-CSIC, Facultad de Ciencias-Instituto Andaluz de Biotecnología, Universidad de Málaga, E-29071 Málaga, Spain

Nitrogen metabolism is one aspect of basic metabolism, which is still quite unknown in the field of plant–pathogen interactions. Evidence derived from previous studies conducted in our laboratory strongly suggests that during microbial pathogenesis an important nitrogen mobilization process takes place in diseased tissues. Here we describe the expression pattern of asparagine synthetase (AS; EC 6.3.5.4) in tomato leaves infected by the bacterial pathogen *Pseudomonas syringae* pv. tomato. Using an homologous AS cDNA probe isolated by RT-PCR from infected leaves, we have observed a high level induction of AS expression during the course of infection. Concomitantly, a single AS polypeptide also accumulated in response to bacterial infection. Furthermore, immunohistochemical analysis of AS in infected leaves revealed a strong immunostaining in phloem cells of the main vascular bundles and in secondary veins of the leaf blade. These data correlate with those previously reported for expression of a cytosolic isoform of glutamine synthetase (GS1) also induced during development of the infectious process. Taken together, our results suggest the existence of a GS1/AS pathway representing a metabolic route for transferring ammonium released from protein catabolism into asparagine, an amino acid that may have a major role in nitrogen mobilization from diseased tissues.

**Keywords:** Asparagine synthetase (EC 6.3.5.4) — Bacterial pathogenesis — Glutamine synthetase — Nitrogen mobilization — *Pseudomonas syringae* — Tomato (*Lycopersicon esculentum* Mill.).

Abbreviations: AS, asparagine synthetase; GS1, cytosolic glutamine synthetase; GS2, chloroplastic glutamine synthetase.

The nucleotide sequence reported in this paper (LeAS1) has been submitted to NCBI database under accession number AY240926.

### Introduction

It is well known how pathogens affect plant physiological functions, such as photosynthesis and respiration (Agrios 1997,

Lucas 1998); however, much less is known about the effect of pathogens on other important plant processes. One of those basic functions poorly understood during compatible interactions is plant nitrogen metabolism. Nitrogen is a main limiting nutrient for most plant species and other living organisms; therefore, it is not surprising that this essential element is efficiently economized by plants in a tightly regulated process known as nitrogen mobilization. For instance, during seed germination nitrogen is mobilized from seed storage proteins to build up all components of photosynthetic apparatus of the seedling (Callis 1995). Moreover, during plant senescence nitrogen is also mobilized from senescent tissues and transferred to developing parts of the plant wherein it can be conveniently employed for the biosynthesis of a vast variety of plant molecules (Buchanan-Wollaston 1997). Similar evidence derived from studies conducted in our laboratory on the interaction between tomato and the bacterial pathogen *Pseudomonas syringae* pv. tomato, strongly suggests that during bacterial infection a nitrogen mobilization process also takes place in diseased tissues (Pérez-García et al. 1995, Pérez-García et al. 1998a, Pérez-García et al. 1998b).

*P. syringae* pv. tomato causes bacterial speck, an important disease of tomato that is characterized by the presence of small necrotic lesions surrounded by chlorotic halos on leaves. With regard to the biochemical and molecular basis of disease, it has been suggested that the accumulation of ammonium ions and the evolution of gaseous ammonia may contribute to the bacterial speck symptoms (Bashan et al. 1980). In fact, it has also been demonstrated that protease activity increases during *P. syringae* pv. tomato infection, which suggests that catabolism of released amino acids could contribute to ammonium accumulation and, therefore, play an important role in the development of bacterial speck disease (Bashan et al. 1986). The destination of such ammonium-derived nitrogen released during infectious process is a relevant issue to be addressed. An obvious destination could be the increase of bacterial biomass itself. On the other hand, since nitrogen is a key element for plant metabolism, plants should have evolved efficient mechanisms for the reassimilation of this essential element.

In healthy plants ammonium is assimilated into amino acids by the coordinated action of two enzymes, glutamine syn-

<sup>3</sup> Corresponding author: Fax: +34-952132000. E-mail: adevicente@uma.es

thetase (GS) and glutamate synthase (GOGAT), in the so-called GS/GOGAT cycle (Lea 1999). It is well established that angiosperms contain two different isoforms of GS, a cytosolic enzyme (GS1) and a chloroplast-located isoenzyme (GS2) (McNally and Hirel 1983). In a previous work, we have shown that in tomato leaves infected by *P. syringae*, the progressive degeneration of chloroplasts associated with chlorosis symptom development makes the reassimilation of the released ammonium by the GS2/GOGAT cycle no longer possible (Pérez-García et al. 1995). In the same work we also reported that during the bacterial infection there is a change in the pattern of tomato GS isoforms; GS2 is down-regulated whereas GS1 is induced and appears as the predominant isoform (Pérez-García et al. 1995). Furthermore, we have also shown that expression of this pathogenesis-induced GS gene was regulated at the transcriptional level in a light-dependent fashion and that its corresponding polypeptide was specifically accumulated in the cytosol of mesophyll cells (Pérez-García et al. 1998a, Pérez-García et al. 1998b). Thus, to counteract the infection effect on ammonium assimilation the expression of a gene encoding a cytosolic GS during pathogenesis could assure the reassimilation of nitrogen released from chloroplast disassembly and other cellular processes. Glutamine could then be used as a vehicle for nitrogen transport; however, high levels of asparagine were observed in infected leaves, which would indicate that it is not glutamine but possibly asparagine the amino acid mainly involved in the transport of nitrogen derived from the infected to the healthy tissues of the plant (Pérez-García et al. 1998b).

Glutamine and asparagine are central intermediates in nitrogen metabolism and contribute to nitrogen transport in many higher plants. In some species, asparagine is preferred over glutamine for nitrogen transport and/or transient storage of nitrogen, because it has a higher nitrogen/carbon ratio compared with glutamine (Lea and Mifflin 1980, Lam et al. 1996). The conversion of aspartate into asparagine by a glutamine-dependent asparagine synthetase (AS; EC 6.3.5.4) is now generally accepted as the main route for asparagine biosynthesis in plants (Siecichowicz et al. 1988, Eason et al. 1996). Asparagine synthetase cDNAs have been characterized from many plant species including pea (Tsai and Coruzzi 1990), asparagus (Davies and King 1993), *Arabidopsis* (Lam et al. 1994), maize (Chevalier et al. 1996), rice (Watanabe et al. 1996), soybean (Hughes et al. 1997), alfalfa (Shi et al. 1997) and common bean (Osuna et al. 1999). The full-length clones isolated so far indicate that plant ASs are proteins composed of 579–591 amino acid residues and a molecular mass of approximately 65 kDa, with a glutamine binding site as the most remarkable domain (Shi et al. 1997). Furthermore, in most of the species AS seems to be encoded by a small gene family (Nakano et al. 2000). Specific regulation of the different AS isogenes and their particular function are poorly understood, but it is known that AS expression appears to be mainly regulated by the carbon/nitrogen status in plant tissues (Lam et al. 1998).

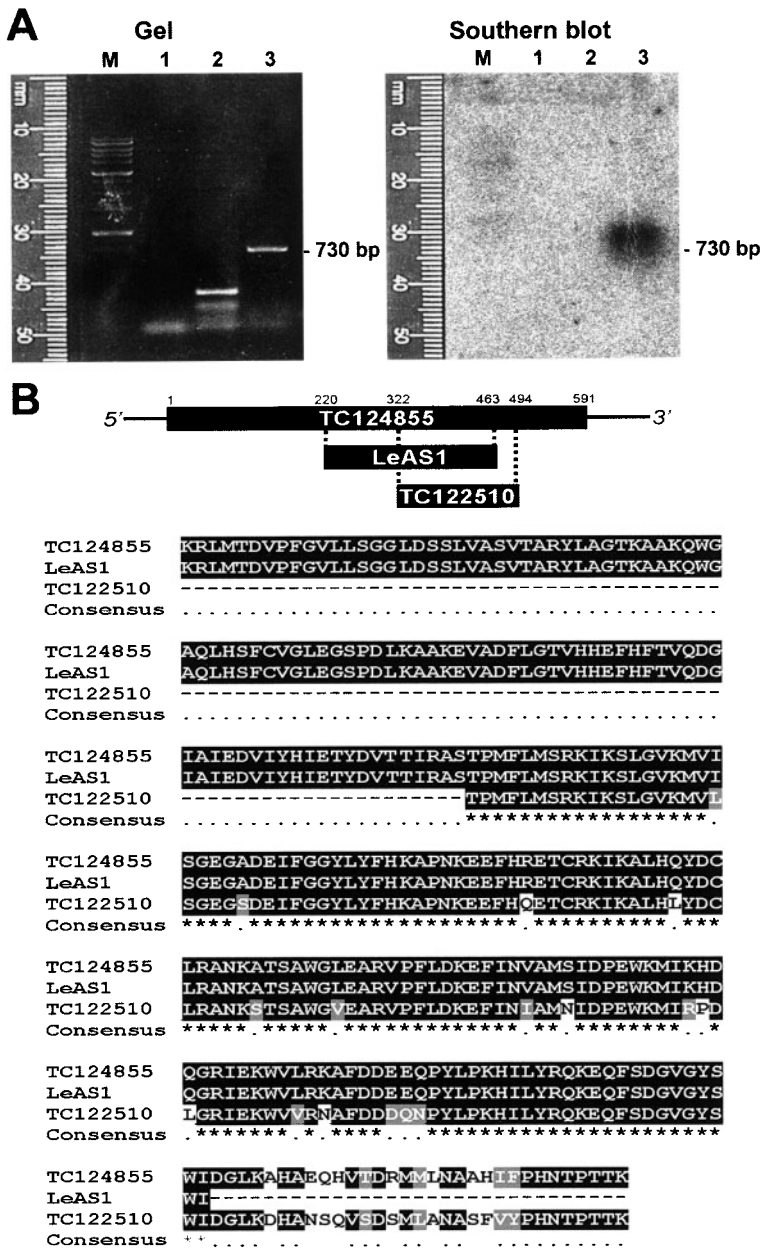
To establish the metabolic origin of the high levels of asparagine detected in tomato leaves infected by *P. syringae* pv. tomato, in this work we have undertaken the study of the expression and localization of asparagine synthetase, using an in vitro-maintained detached leaf model system. The possible role of cytosolic GS and AS enzymes in nitrogen mobilization from infected tissues is discussed.

## Results

### *Isolation of a tomato asparagine synthetase cDNA and expression analysis*

As a previous step to the characterization of the expression of tomato AS during bacterial pathogenesis, we proceeded to isolate by RT-PCR a tomato AS cDNA fragment to be used as homologous probe. For these assays we used total RNA extracted from *P. syringae*-infected leaves harvested 7 d after inoculation. A single RT-PCR fragment of the expected size (730 bp) was obtained and its homology to plant AS was first tested by Southern blot analysis, using a maize AS cDNA (pZSS1) as heterologous probe (Chevalier et al. 1996). A strong hybridization band was observed at the size of the amplification product in the gel (Fig. 1A). This cDNA fragment was then cloned and sequenced; the predicted amino acid sequence of this RT-PCR product revealed a partial open reading frame of 243 amino acids highly homologous to other plant AS proteins (data not shown). This cDNA was designated as LeAS1 and was also compared with The TIGR Tomato (*Lycopersicon esculentum*) Gene Index (LeGI). The database includes two different tentative consensus sequences (TC) for tomato asparagine synthetase. The first one is represented by TC124855, a full-length cDNA of 2,190 bp deduced from more than 20 different ESTs and expressed in ovary, callus tissue and flowers. According to amino acid sequence alignments, TC124855 is similar to ASN1 from *Arabidopsis*. The second available consensus sequence is represented by TC122510, a partial cDNA of 519 bp, much less represented among the total tomato EST sequences and expressed in ovary and flower buds. The deduced amino acid sequence of this gene was close to ASN2 from *Arabidopsis*. We compared our cDNA LeAS1 with both TC sequences and the results are shown in Fig. 1B. We found that LeAS1 was 100% identical in amino acids to TC124855 whereas it showed only 77% identity to TC122510. As LeAS1 corresponds with only one of the two possible AS genes expressed in tomato, we also included in our expression studies another tomato AS probe, ASP2-4, isolated from young tomato fruit (C. Chevalier, unpublished). This cDNA was nearly 100% identical to TC122510.

Southern blot analyses were performed to determine the genomic organization and the gene copy number of AS in tomato. Extractions of genomic DNA from tomato leaves were digested with different restriction enzymes and hybridized with LeAS1 and ASP2-4 probes at high stringency conditions (Fig. 2). When LeAS1 was used, only a single hybridization

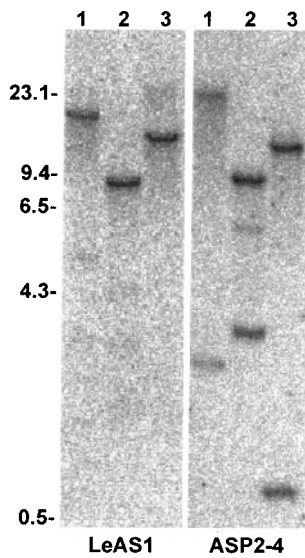


**Fig. 1** Isolation of a tomato cDNA clone encoding asparagine synthetase. (A) Agarose gel showing the amplification band obtained from tomato total RNA by RT-PCR, using degenerate primers for AS (Gel). The identity of this band was first tested by hybridization using a maize AS cDNA (ZSS1) probe (Southern blot). M, molecular weight markers; lane 1, RT-PCR negative control; lane 2, RT-PCR positive control; lane 3, RT-PCR assay using total RNA from tomato leaves infected with *P. syringae* pv. tomato 7 d after inoculation. (B) Schematic representation of the relative position of the predicted amino acid sequence encoded by the partial tomato AS cDNA LeAS1 and its comparison with the deduced proteins of the two TC sequences for tomato AS present in the TIGR Tomato Gene Index (TC124855 and TC122510). Numbers on top refer to the amino acid residue position of each partial sequence with respect to the complete sequence of TC124855. We include a Boxshade of a ClustalW 1.8 alignment corresponding to the overlapped sequences of the three cDNAs. Black boxes indicate identical amino acids and grey boxes indicate conservative amino acid changes.

fragment was detected in each lane, which suggests that the probe used is highly specific for just one AS gene in the tomato genome. In order to get further insight in the genomic organization of AS, we rehybridized the same membrane under the same experimental conditions with the tomato AS probe ASP2-4. In this case, several distinct hybridization fragments were detected in each lane, which were different from those obtained with LeAS1. These results further support the existence of at least two different genes for AS in tomato.

For the expression studies, total RNA was isolated from non-infected and infected tomato leaflets at different times after inoculation with *P. syringae* pv. tomato, and the relative abundance of AS transcripts was compared by Northern blot,

using both LeAS1 and ASP2-4 cDNAs as molecular probes (Fig. 3). In the case of the LeAS1 probe, accumulation of AS mRNA was observed under both experimental conditions; however, the time course of transcript appearance and their relative abundance differed considerably. In infected leaves, AS transcripts were detectable 2 d after inoculation, reaching very high levels after 4 d which stood until the end of the examined period. In control non-infected leaves, transcripts were barely detected until 6 d post-inoculation and their relative abundance increased slightly in days 8 and 10. In contrast, with the ASP2-4 probe very low expression levels were observed in both healthy and infected leaves. These results show clear differences in the expression pattern of AS. While the gene recog-



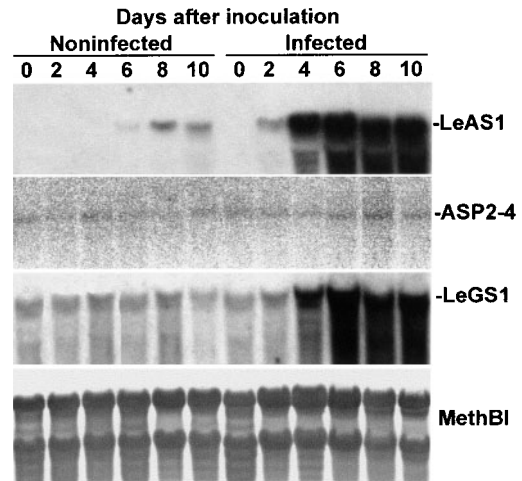
**Fig. 2** Southern blot analysis of AS genes in the tomato genome. Genomic DNA isolated from tomato leaves (30  $\mu$ g) was digested with *Bam*HI (lane 1), *Eco*RI (lane 2) and *Hind*III (lane 3). The filter was first hybridized with the tomato AS cDNA LeAS1 probe and subsequently rehybridized with the tomato AS cDNA ASP2-4. Numbers at left indicate the position of the molecular weight markers in kilobases.

nized by LeAS1 was strongly induced upon infection, AS expression detected with ASP2-4 was very low and no significant differences were observed between healthy and infected samples.

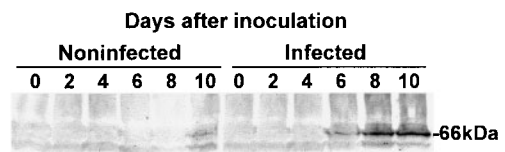
As previously mentioned, the main route for asparagine biosynthesis in plants is glutamine-dependent and since induction of a tomato GS1 had been described in response to *P. syringae*, we examined GS expression in the same RNA samples in which AS expression was analysed. Thus, filters were rehybridized with a cDNA probe encoding for a tomato GS1 (LeGS1), isolated by RT-PCR using primers designed from a previously reported sequence (Pérez-Rodríguez and Valpuesta 1996). As expected, the expression pattern of tomato GS1 observed during bacterial pathogenesis was very similar to LeAS1 (Fig. 3).

#### Accumulation and tissular localization of asparagine synthetase polypeptides

To determine whether the accumulation of AS transcripts observed in infected leaves was correlated with the accumulation of their corresponding polypeptides, we examined the steady-state levels of AS protein by immunoblotting. Total soluble proteins were extracted from non-infected and infected tomato leaflets at different times after inoculation, separated by SDS-PAGE, transferred to nitrocellulose filters and immunoprobed with AS antiserum raised against pine AS (Fig. 4). A major cross-reacting band of approximately 66 kDa was detected, which is in agreement with the size of the AS protein deduced from the TC124855. AS polypeptide contents



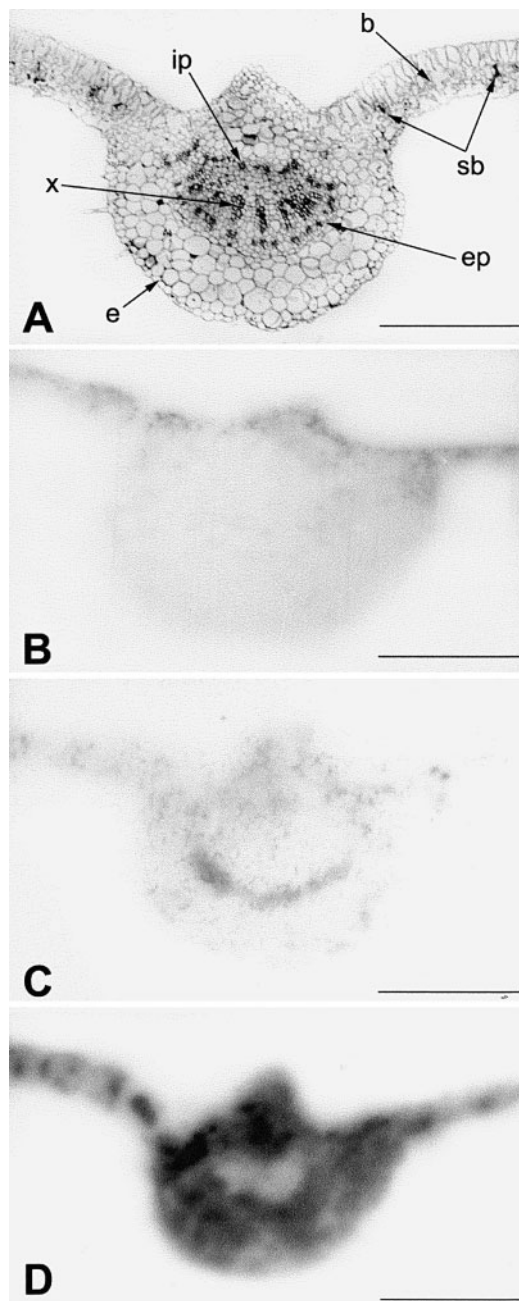
**Fig. 3** Northern blot analyses of tomato AS and GS1 mRNA levels during the course of infection with *P. syringae* pv. tomato. Total RNA extracted from non-infected (inoculated with sterilized distilled water) and infected tomato detached leaflets was electrophoresed on 1% formaldehyde agarose gels and analysed by Northern hybridization using as molecular probes two tomato AS cDNAs (LeAS1 and ASP2-4) and a tomato GS1 cDNA (LeGS1). The loading of equivalent amounts of RNA (20  $\mu$ g) was verified by spectrophotometric determination of RNA concentrations and staining of rRNA with methylene blue (MethBI).



**Fig. 4** Western blot analysis of AS polypeptides in tomato leaves during the course of infection by *P. syringae* pv. tomato. Soluble proteins extracted from non-infected (inoculated with sterilized distilled water) and infected tomato leaves were separated by SDS-PAGE and immunoprobed with AS antibodies. The size of the major cross-reacting band is indicated on the right. The same amount of protein (50  $\mu$ g) was loaded per lane.

increased during the bacterial infection and the highest levels were observed during the final stages of the symptoms development (8 and 10 d after inoculation). In non-infected leaflets only a slight accumulation of the AS polypeptide was detected after 10 d in MS medium. Faint bands were detected below the main band of AS, which may be possibly due to proteolytic degradation of the 66 kDa polypeptide (data not shown).

Tissue printing experiments were used to characterize AS tissue distribution in tomato leaflets. The assays were performed with non-infected and infected tomato leaflets after 7 d of inoculation. Prints were probed with a partially purified AS antiserum raised against pine AS. A leaf cross-section stained with toluidine blue is included to show leaf anatomy (Fig. 5A) and a print incubated without primary antibody is shown as a negative control (Fig. 5B). In non-infected leaves immunostain-



**Fig. 5** Tissue-print localization of AS in tomato leaves infected with *P. syringae* pv. tomato. (A) Leaf cross-section stained with toluidine blue to show the anatomy of a non-infected leaf. (B) Negative control; tissue print of a leaf cross-section from an infected leaflet incubated without primary antibody. Similar results were obtained with non-infected leaves (data not shown). (C and D) Tissue prints of leaf cross-sections from tomato leaves non-infected (inoculated with sterilized distilled water) and infected with *P. syringae* pv. tomato 7 d after inoculation, respectively. Prints were incubated with anti-AS antibodies and labelling was developed with peroxidase-conjugated anti-rabbit IgG. Immunolabeling was detected in the external phloem of non-infected leaves, whereas in infected leaves it was also strongly detected in the parenchymal cells of leaf blades. No staining was observed in the xylem vessels. b, leaf blade; e, epidermis; ip, internal phloem; ep, external phloem; sb, secondary bundles; x, xylem. Bars: 500  $\mu$ m.

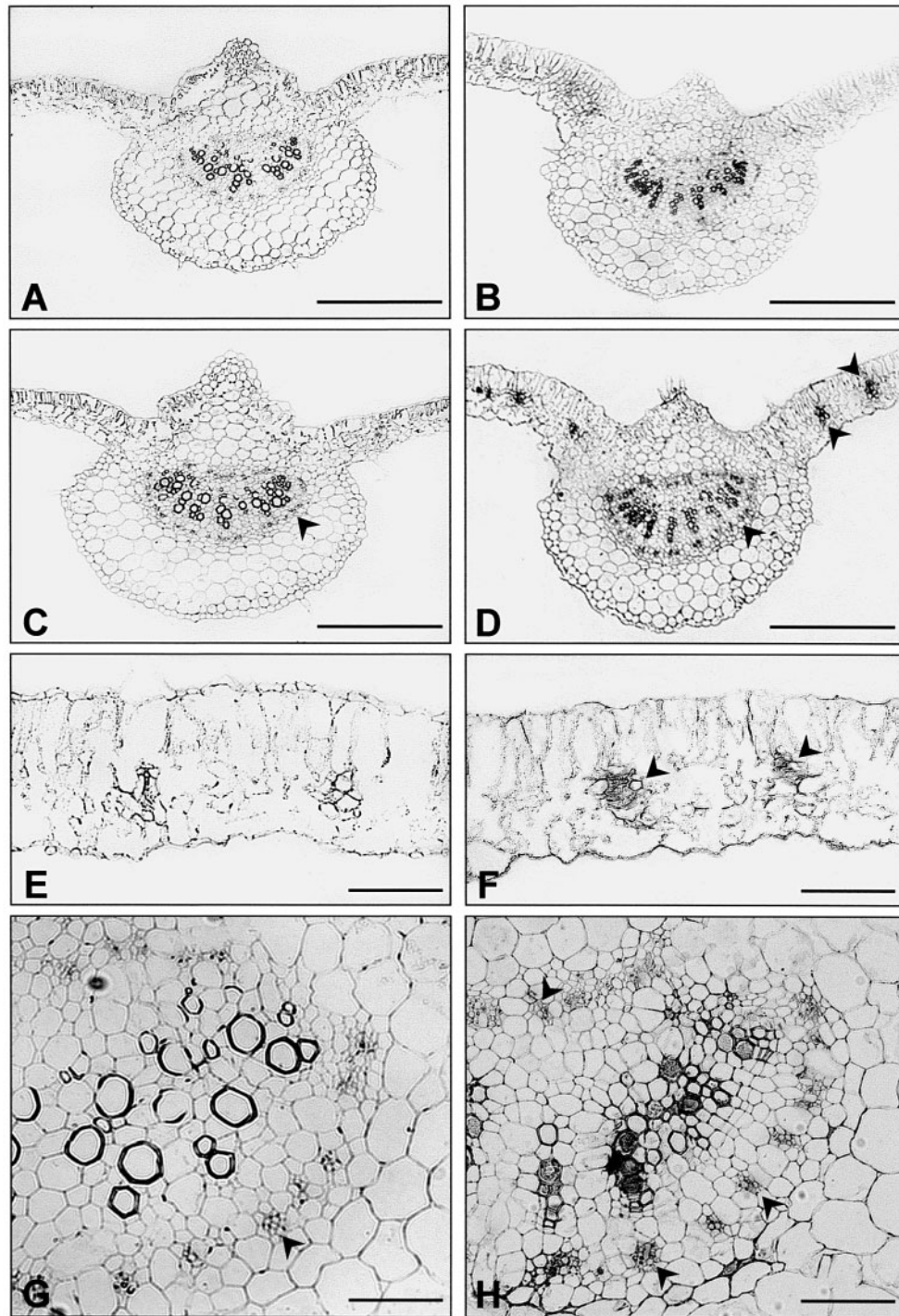
ing was detected only in the external phloem (Fig. 5C), whereas in infected leaves staining was observed in the whole central vein except for the xylem, being also very prominent in the leaf blade (Fig. 5D). With these results we could not determine precisely the tissular localization of AS in infected leaves; nevertheless, they confirm and highlight the great differences in the levels of AS polypeptides observed between healthy and infected leaves.

Immunohistochemical localization on thin tissue sections was employed to characterize the spatial distribution of asparagine synthetase and glutamine synthetase in both non-infected and infected tissues. As for tissue printing analysis, leaflets taken 7 d after inoculation were used in the immunolocalization experiments. Tissue sections were incubated with AS and GS antibodies raised against pine proteins. No signals were obtained in either non-infected or infected control sections, with the exception of an unspecific staining that was detected in xylem vessels and cuticle (Fig. 6A, B). In non-infected leaves signals for AS protein were only detected in phloem cells of the main vascular bundles (Fig. 6C, G). In infected leaves, however, staining was observed in the same cell type, although the signal was enhanced, and it was also detected in the secondary veins of the leaf blade (Fig. 6D, F, H). Other cell types showed very little if any staining. With respect to GS, non-infected leaves displayed a general distribution of the staining due to the fact that GS antiserum is able to recognize both chloroplastic and cytosolic GS isoforms. The mesophyll cells of leaf blade showed the most intense staining, but it was also detected in vascular bundles and epidermis (Fig. 7A, C, E). In infected leaves a similar distribution was observed, although staining was less pronounced in the leaf blade, due to chloroplastic disassembly (Fig. 7B, D, F).

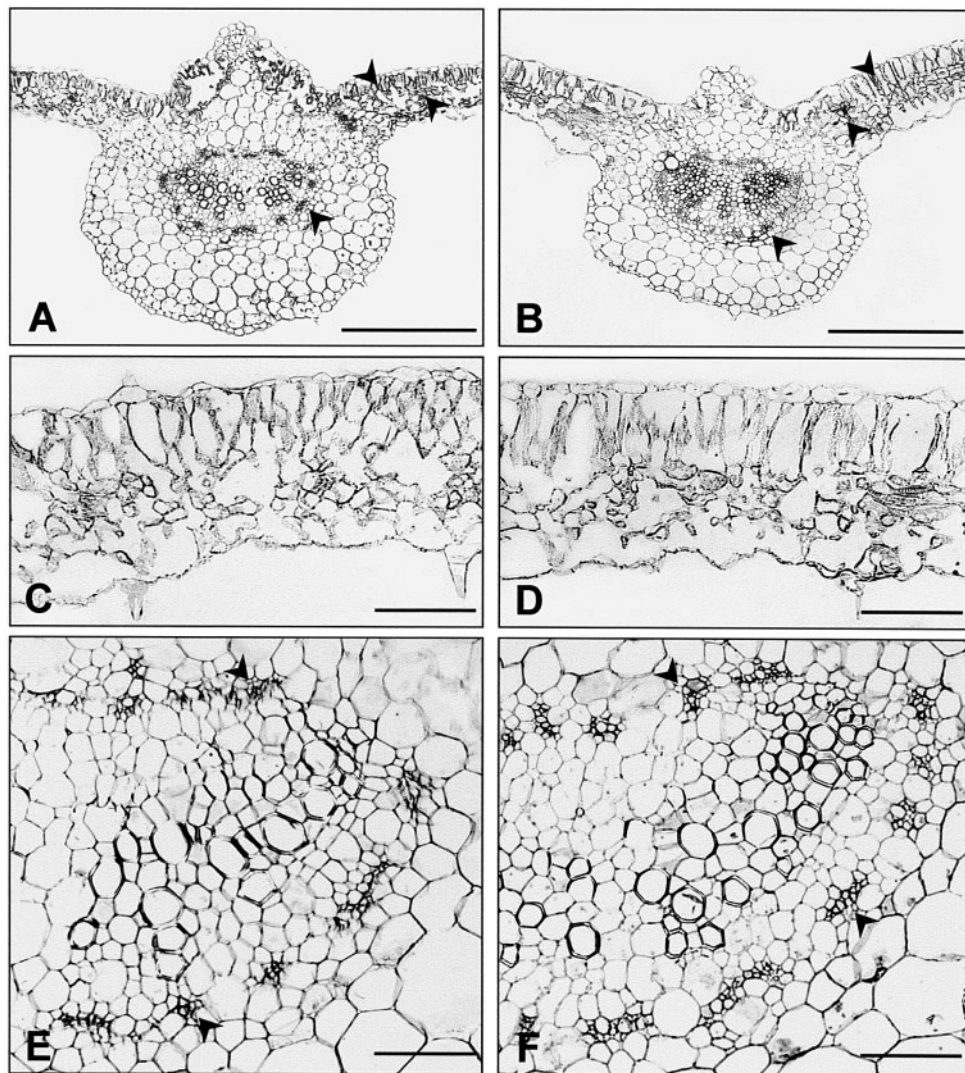
## Discussion

It is well documented that microbial infections trigger a wide range of metabolic changes in plants. Most of the studies concerning host-pathogen compatible interactions are related to the molecular bases of microbial pathogenicity. By contrast, much less is known about the impact of microbial pathogens on plant basic metabolism. Our research is centred on plant nitrogen metabolism due to the fact that nitrogen is a common limiting nutrient for growth of both plants and pathogens and, therefore, competence for the uptake of this element should have a special relevance during the course of their interactions.

We have paid close attention to the nitrogen mobilization phenomenon that appears to take place in plant diseased tissues. This study has been focused on asparagine biosynthesis for two main reasons. First, determinations of the relative abundance of several amino acids in *P. syringae*-infected tomato leaves had revealed, as the most remarkable difference, a strong increase of asparagine levels in comparison with control non-infected leaves (Pérez-García et al. 1998b). And second, asparagine is the main vehicle for nitrogen transport in plants, and it



**Fig. 6** Immunohistochemical localization of AS protein in tomato leaves infected with *P. syringae* pv. tomato. Sections of tomato leaflets non-infected (inoculated with sterilized distilled water) and infected with *P. syringae* pv. tomato 7 d after inoculation were stained with primary pine AS antisera. The immunoreacted peptides were incubated with anti-rabbit IgG alkaline phosphatase conjugate antibodies and enzyme activity was detected in TBS containing NBT/BCIP as substrate resulting in blue staining. (A and B) Transversal sections of non-infected and infected leaves incubated without primary antibody as negative controls. (C and D) Transversal sections of non-infected and infected leaves incubated with AS antisera. (E–H) Magnification pictures of leaf blades (showing secondary bundles) and main veins of non-infected (E and G) and infected leaves (F and H). Arrowheads indicate the immunostaining detected. Bars: 500  $\mu$ m (A–D) or 100  $\mu$ m (E–H). For leaf anatomy description see Fig. 5.

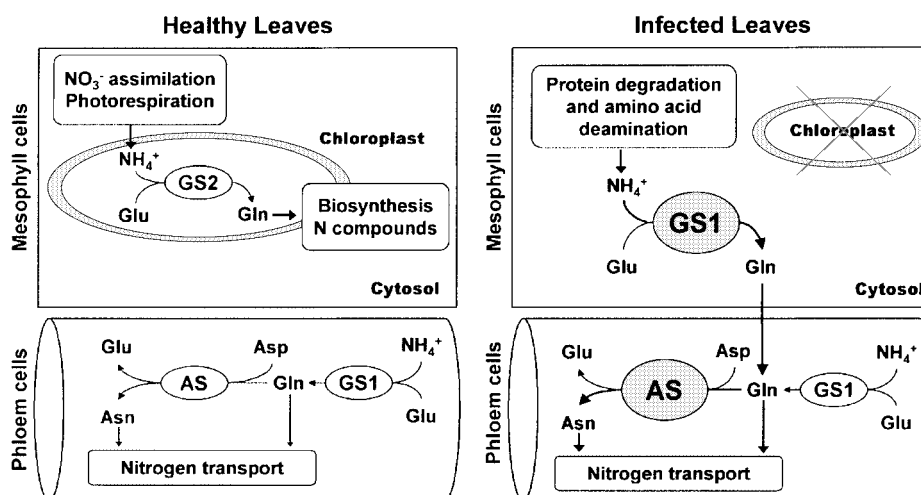


**Fig. 7** Immunohistochemical localization of GS protein in tomato leaves infected with *P. syringae* pv. tomato. Sections of tomato leaflets non-infected (inoculated with sterilized distilled water) and infected with *P. syringae* pv. tomato 7 d after inoculation were stained with primary pine GS antisera. Immunolocalization was done as described in Fig. 6 and negative controls incubated without primary antibody are shown in the same figure. A and B, Transversal sections of non-infected and infected leaves. C-F, Magnification pictures of leaf blades showing secondary bundles and main veins of non-infected (C and E) and infected leaves (D and F). Arrowheads indicate the immunostaining detected. Bars: 500  $\mu\text{m}$  (A-B) or 100  $\mu\text{m}$  (C-F). For leaf anatomy description see Fig. 5.

has been involved in other nitrogen mobilization events such as natural senescence (Lea and Mifflin 1980), sugar starvation (Brouquisse et al. 1992), dark/light transitions (Sieciechowicz et al. 1988), or post-harvest conditions (King et al. 1990). Furthermore, the main route of asparagine biosynthesis in plants takes place via a glutamine-dependent asparagine synthetase (Sieciechowicz et al. 1988) and interestingly, the induction of a novel glutamine synthetase isoform and its accumulation in the cytosol of mesophyll cells of tomato leaves infected by *P. syringae*, had been previously documented (Pérez-García et al. 1995, Pérez-García et al. 1998b).

Expression studies of AS during bacterial pathogenesis were carried out using a tomato AS homologous probe cloned

by RT-PCR from total RNA isolated from infected leaves (LeAS1), and another tomato AS homologous probe (ASP2-4). Probes were only 74% identical to each other and they corresponded with two different tomato consensus sequences for AS present in the TIGR Tomato Gene Index (TC124855 and TC122510, respectively). Furthermore, Southern blot analyses carried out with the two different tomato AS cDNA probes indicated that at least two different AS genes are present in tomato genome, which agrees with what it has been described for other plant species (Nakano et al. 2000). The results obtained demonstrate a strong accumulation of AS transcripts during the course of infection when LeAS1 was used as molecular probe, which occurs in parallel with induction of cytosolic



**Fig. 8** Schematic representation of the physiological roles of GS1 and AS in healthy leaves and the proposed mechanism for nitrogen remobilization in *P. syringae*-infected tomato leaves. Ammonium ions released from protein degradation and amino acid deamination are reassimilated via GS1/AS into asparagine, an amino acid involved in transport and/or transient storage of nitrogen. This process would be compartmentalized in the cytoplasm of mesophyll cells and the vascular tissue of infected leaves.

GS mRNA. However, when ASP2-4 was used as molecular probe, changes in the levels of AS transcripts were not detected. The differential expression patterns observed for tomato AS with both probes may be related to the different physiological events in which asparagine is involved. We postulate that high expression of LeAS1 (TC124855) could be related with nitrogen remobilization process during pathogenesis, whereas ASP2-4 (TC122510) expression seems to be involved in the basal production of asparagine for protein biosynthesis.

Western blot assays showed a concordance with the AS expression since we observed the accumulation of an AS polypeptide during the infection process. In order to complete the molecular studies, we also tried to perform determinations of AS activity in infected leaves. Unfortunately, we were unable to detect specific AS activity with the analytical approaches that we attempted (data not shown). It has been reported that immunochemical and standard biochemical studies of asparagine biosynthesis in plants have been hampered by the instability of the AS enzyme, the presence of AS inhibitors in plant extracts, and the rapid turnover of asparagine by asparaginase (Lam et al. 1996).

In healthy plants, AS induction has been associated with a variety of physiological conditions. AS expression is enhanced when light-grown plants are dark adapted; when plants are subject to sugar starvation; during natural senescence and in response to an exogenous supply of glutamine (Lam et al. 1994, Devaux et al. 2003, Eason et al. 2000, Kawachi et al. 2002). Conversely, AS transcription is repressed by light and sugar supply (Lam et al. 1994). In addition, sucrose repression of AS can be partially reversed by supplying exogenous asparagine, glutamine or glutamate (Lam et al. 1994), which indicates a role for free amino acids in the regulation of AS. These findings suggest that the ratio organic nitrogen/carbon could be

a major factor controlling AS gene expression in plants. Thus, under conditions where levels of carbon skeletons are low with regard to organic nitrogen, asparagine synthesis appears to store the excess of nitrogen as an inert nitrogen reserve (Lam et al. 1996).

Chlorosis is one of the typical symptoms of tomato bacterial speck. In a previous work, we have shown a direct correlation between *P. syringae* growth and chloroplast degeneration, that was demonstrated by a progressive decrease in the contents of chlorophylls and several chloroplastic protein markers during the course of infection (Pérez-García et al. 1995). In this sense, an important accumulation of ammonium ions occurs in infected leaves, which seems to be a consequence of the remarkable proteolytic activity and amino acid deamination that takes place during the progress of the disease (Bashan et al. 1986, Pérez-García et al. 1998a). The parallel induction of GS1 and AS transcripts during pathogenesis suggests that GS1 could be responsible for reassimilation of ammonium and biosynthesis of glutamine, an amino acid that should be a substrate for asparagine biosynthesis by the action of AS.

An important issue to be addressed was to determine the specific cell type in which AS was accumulated in response to bacterial infection. As a first approach for the localization of AS, we carried out tissue-printing assays in *P. syringae*-infected and non-infected leaves. The results showed important differences with regard to AS protein localization. In non-infected leaves, AS protein was restricted to phloem cells of the main vein; in infected leaves, however, the strong immunostaining observed suggested that AS could be also accumulated in other cell types. To determine the specific tissue localization of AS protein we carried out immunohistochemical analyses in non-infected and infected leaves. In parallel we also attempted the GS tissue localization.



With respect to immunolocalization of AS protein in both non-infected and infected leaflets, staining was mainly observed in phloem cells of main vascular bundles, but in infected leaflets, staining was also detected in secondary bundles of the leaf blade. These results agree with other reports, which have found expression and localization of AS associated with the vascular bundles (Brears et al. 1993, Nakano et al. 2000). The localization of AS in vascular elements suggests a functional role of the enzyme in the synthesis of asparagine for long distance transport via phloem. When GS was immunolocalized, we obtained a general staining because GS antibodies are able to recognize both chloroplastic and cytosolic GS isoforms. However, in a previous work immunocytochemical localization of GS revealed that in infected leaves, GS protein was present not only in the cytosol of phloem companion cells but also in the cytoplasm of mesophyll cells (Pérez-García et al. 1998b). At that time, we hypothesized that the coordinated action of both enzymes could represent a metabolic route for transferring nitrogen released from protein catabolism into asparagine, the amino acid mainly involved in the transport of nitrogen from infected tissues. The results showed here indicate that these enzymes are compartmentalized in different cellular types. Thus far, the current hypothesis would involve also a transport of glutamine from mesophyll cells where GS1 is present, to the phloem cells of the vascular bundles where AS would convert glutamine into asparagine. Then, asparagine would be transported via phloem sieve elements to healthy developing tissues in an effort to complete the plant life cycle. This hypothesis is schematically summarised in Fig. 8. Amino acid transport plays a key role in a wide array of physiological activities as leaf senescence and seed germination (Ortiz-López et al. 2000). In all these situations, an efficient mechanism of amino acid transport is necessary to mobilize as many of the amino acids released by proteolysis as possible. This might ensure that amino acids produced in excess and released by the mesophyll cells can not accumulate in the apoplast and serve as a source of nutrients for potential pathogens (Fischer et al. 1998). Furthermore, expression of amino acid transporters in response to some stress conditions such as drought or salt stress has been also reported (Fischer et al. 1998). In addition, evidence has been provided indicating that amino acid transporter gene expression is sensitive to regulation in response to the N or C status of the plant (Ortiz-López et al. 2000). These data support the hypothesis that together with the coordinated expression of GS1 and AS, amino acid transporter genes should be also expressed in response to infection to mobilize nitrogen in the form of asparagine from diseased tissues to other parts of the plant.

## Materials and Methods

### *Bacterial strain, plant material and experimental design*

*P. syringae* pv. tomato strain UMAF4007 was routinely cultured on King's medium B (KB) at 27°C (Pérez-García et al. 1995). Suscep-

tible tomato plants cv. Hellfrucht Frühstamm (seeds obtained from the Experimental Station "La Mayora", CSIC, Málaga, Spain) were grown from seed in a greenhouse under natural light. All experiments were performed using leaflets from 6-week-old tomato plants, that were detached, disinfected and maintained under in vitro culture conditions onto Murashige and Skoog agar medium plates (Pérez-García et al. 1995, Cazorla et al. 2000). Inoculations were carried out with bacterial suspensions adjusted to  $10^6$  (CFU ml<sup>-1</sup>) as previously described (Pérez-García et al. 1995). Non-infected detached leaflets inoculated with sterile water were included in all experiments as controls. All experiments were repeated at last three times.

### *RT-PCR and cDNA cloning*

To obtain an homologous AS cDNA probe for expression studies, a RT-PCR strategy was undertaken. Degenerate oligonucleotide primers AS1 (5'-CAATCCAACRTAWCCAAC-3') and AS2 (5'-AARAG-GTTGATGACWGATGT-3'), based on conserved domains of the protein, were used. RT-PCR reactions were performed with the Enhanced Avian RT-PCR kit (Sigma, St. Louis, MO, U.S.A.) using 0.5 µg of total RNA isolated from leaves inoculated with *P. syringae* and taken 7 d after inoculation. The amplified cDNA fragments were analysed by Southern blot with a maize AS cDNA probe (pZSS1) (Chevalier et al. 1996) following standard procedures (Sambrook and Russell 2001). A hybridizing fragment of 730 bp was ligated into the vector pGEM-T using the pGEM-T Easy Vector System I kit (Promega, Madison, WI, U.S.A.), resulting in the plasmid pLeAS1, and subsequently sequenced.

### *Isolation and analyses of nucleic acids*

Total RNA was isolated from freshly infected and non-infected tomato leaflets as described by Chang et al. (1993). Genomic DNA was isolated from tomato leaves as described by Dellaporta et al. (1983). Northern and Southern blotting procedures were performed essentially according to standard methods (Sambrook and Russell 2001). To confirm that in Northern blot assays equals amounts of total RNA had been loaded in each lane, filters were stained with methylene blue prior hybridizations (Wilkinson et al. 1991). Probes used were LeAS1 (this study); ASP2-4, a young tomato fruit AS cDNA obtained by RT-PCR (C. Chevalier, unpublished), which corresponds to TC122510 sequence; and LeGS1, a tomato cytosolic GS cDNA obtained by RT-PCR with primers based on the sequence previously reported (Pérez-Rodríguez and Valpuesta 1996). Probes were labelled with High Prime Labelling System (Roche, Grezsch-Whyhlen, Germany), including 20 µCi α[<sup>32</sup>P]dCTP (Amersham, Buckinghamshire, U.K.).

### *Protein extraction and Western blot analysis*

Leaf tissue (1–2 g) for protein isolation was removed from Petri dishes, frozen in liquid nitrogen and stored at –80°C until required. Tissue was ground with a mortar and pestle in liquid nitrogen and soluble proteins were extracted in 100 mM Tris HCl, 0.1 mM EDTA, 10 mM MgCl<sub>2</sub>, 0.1 mM ATP, 2 mM Asp, 20% glycerol, 0.5 mM DTT and 67 mM β-mercaptoethanol (pH 7.8) as described by Romagnì and Dayan (2000). Protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. For protein immunoblotting (Cánovas et al. 1991), 50 µg of soluble proteins from crude extracts of tomato leaves were analysed. Polyclonal AS antibodies raised against AS from pine (R. Cañas, unpublished) were used at 1 : 2,000 dilution. Immunocomplexes were detected using peroxidase-conjugated antibodies. Peroxidase activity was revealed by incubation in 0.02% (v/v) H<sub>2</sub>O<sub>2</sub> and 4 mM 4-chloro-1-naphthol in phosphate-buffered saline (PBS).

### Tissue print, immunoblot and immunohistochemical localization

Tissue prints were obtained by pressing freshly cut sections of tomato leaflets onto a nitrocellulose membrane. Antibody staining for AS on the tissue prints was performed as described above for Western blots, except to the fact that endogenous peroxidases were first blocked by treatment of tissue prints with 1% periodic acid for 30 min (Pereira et al. 1992). Antigen-antibody complexes on the tissue prints were located with a light microscope.

For immunolocalization plant material was fixed and embedded in paraffin as described by Jackson (1992) and AS protein was immunolocalized essentially as described by Nylander et al. (2001). Plant material was fixed overnight at 4°C in 4% (w/v) paraformaldehyde in 0.1 M PBS (pH 7.2). Fixed material was dehydrated in a graded ethanol series (50–100%) and afterwards absolute ethanol was replaced by histological clearing agent (Histoclear, National Diagnostic, U.K.) and tissues were embedded in paraffin. Microtome 10-µm-thick sections were affixed on poly-L-lysine-coated glass slides and deparaffinized in Histoclear and rehydrated in decreasing concentrations of ethanol (100–70%) followed by Tris-buffered saline (TBS). Endogenous alkaline phosphatase activity was blocked by boiling in 10 mM sodium citrate (pH 6.0) for 5 min at 750 W in a microwave oven. After the solution had cooled down microscope slides were rinsed with water and TBS. After that, sections were blocked for 30 min in TBS containing 5% (w/v) BSA and incubated for 2 h in primary antibody diluted in TBS with 3% (w/v) BSA. Primary antibodies were pine AS and GS antisera (R. Cañas, unpublished; Cantón et al. 1996) used at 1 : 1,000 and 1 : 500 dilutions, respectively. After several washes in TBS, sections were then incubated for 1 h with the secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate) (Sigma, St. Louis, MO, U.S.A.) diluted 1 : 100 in TBS with 3% (w/v) BSA. Excess antibodies were washed off as described above. Development was performed in darkness in TBS with MgCl<sub>2</sub> 50 mM (pH 9.4), 75 µg ml<sup>-1</sup> BCIP (5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt) (Roche, Germany) and 150 µg ml<sup>-1</sup> NBT (4-nitro blue tetrazolium chloride) (Roche, Germany). Reactions were stopped by a 10-min rinse in water. For observation of the leaflet anatomy, deparaffinized and rehydrated tissue sections were stained for 1 min in 0.1% (w/v) toluidine blue and rinsed in water. All sections were dehydrated, mounted in rapid mounting media (Entellan, Merck, Germany) and observed under a light microscope.

### Acknowledgments

We thank Dr. Christian Chevalier (Institut de Biologie Végétale Moléculaire et Institut National de la Recherche Agronomique (INRA), Centre de Recherche de Bordeaux, Villenave d'Ornon Cedex, France) for providing us with the maize root AS (pZSS1) and the young tomato fruit AS (pASP2-4) cDNA probes and for his helpful comments. This work was supported by grants from Ministerio de Ciencia y Tecnología AGL2001-1837-C02-01 and BMC2003-4772.

### References

- Agrios, G.N. (1997) *Plant Pathology*, 4th edn. Academic Press, San Diego, CA.
- Bashan, Y., Okon, Y. and Henis, Y. (1980) Ammonia causes necrosis in tomato leaves infected with *Pseudomonas tomato* (Okabe) Alstalt. *Physiol. Plant Pathol.* 17: 111–119.
- Bashan, Y., Okon, Y. and Henis, Y. (1986) A possible role for proteases and deaminases in the development of the symptoms of bacterial speck disease in tomato caused by *Pseudomonas syringae* pv. *tomato*. *Physiol. Mol. Plant Pathol.* 28: 15–31.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of micrograms quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.
- Brears, T., Liu, C., Knight, T.J. and Coruzzi, G.M. (1993) Ectopic overexpression of asparagine synthetase in transgenic tobacco. *Plant Physiol.* 103: 1285–1290.
- Brouquisse, R., James, F., Pradet, A. and Raymond, P. (1992) Asparagine metabolism and nitrogen distribution during protein degradation in sugar starved maize root tips. *Planta* 188: 384–395.
- Buchanan-Wollaston, V. (1997) The molecular biology of leaf senescence. *J. Exp. Bot.* 48: 181–199.
- Callis, J. (1995) Regulation of protein degradation. *Plant Cell* 7: 845–857.
- Cánovas, F., Cantón, F.R., Gallardo, F., García-Gutiérrez, A. and de Vicente, A. (1991) Accumulation of glutamine synthetase during early development of maritime pine (*Pinus pinaster*) seedlings. *Planta* 185: 372–378.
- Cantón, F.R., García-Gutiérrez, A., Crespillo, R. and Cánovas, F.M. (1996) High-level expression of *Pinus sylvestris* glutamine synthetase in *Escherichia coli*. Production of polyclonal antibodies against the recombinant protein and expression studies in pine seedlings. *FEBS Lett.* 393: 205–210.
- Cazorla, F.M., Pérez-García, A., Rivera, M.E., Codina, J.C., Torés, J.A. and de Vicente, A. (2000) Bacterial diseases of tomato in Southern Spain: application of a detached tissue assay to evaluate bacterial pathogenicity. *EPPO Bulletin* 30: 351–356.
- Chang, S., Puryear, J. and Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* 11: 113–116.
- Chevalier, C., Borgeois, E., Just, D. and Raymond, P. (1996) Metabolic regulation of asparagine synthetase gene expression in maize (*Zea mays* L.) root tips. *Plant J.* 9: 1–11.
- Davies, K.M. and King, G.A. (1993) Isolation and characterization of cDNA clone for a harvest-induced asparagine synthetase from *Asparagus officinalis* L. *Plant Physiol.* 102: 1337–1340.
- Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) A plant DNA miniprep: version II. *Plant Mol. Biol. Rep.* 1: 19–21.
- Devaux, C., Baldet, P., Joubès, J., Dieuaidé-Noubhani, M., Just, D., Chevalier, C. and Raymond, P. (2003) Physiological, biochemical and molecular analysis of sugar-starvation responses in tomato roots. *J. Exp. Bot.* 54: 1143–1151.
- Eason, J.R., Johnston, J.W., de Vré, L., Sinclair, B.K. and King, G.A. (2000) Amino acid metabolism in senescing *Sandersonia aurantiaca* flowers: cloning and characterization of asparagine synthetase and glutamine synthetase cDNAs. *Aust. J. Plant Physiol.* 27: 389–396.
- Eason, J.R., O'Donoghue, E.M. and King, G.A. (1996) Asparagine synthesis and localization of transcripts for asparagine synthetase in tips of harvested asparagus spears. *J. Plant Physiol.* 149: 251–256.
- Fischer, W.N., André, B., Rentsch, D., Krolkiewicz, S., Tegeder, M., Breikreuz, K. and Frommer, W.B. (1998) Amino acid transport in plants. *Trends Plant Sci.* 3: 188–195.
- Hughes, C.A., Beard, H.S. and Matthews, B.F. (1997) Molecular cloning and expression of two cDNAs encoding asparagine synthetase in soybean. *Plant Mol. Biol.* 33: 301–311.
- Jackson, D.P. (1992) In-situ hybridization in plants. In *Molecular Plant Pathology: A Practical Approach*. Edited by Gurr, S.J., McPherson, M. and Bowles, D.J. pp. 163–174. Oxford University Press, Oxford.
- Kawachi, T., Sueyoshi, K., Nakajima, A., Yamagata, H., Sugimoto, T. and Oji, Y. (2002) Expression of asparagine synthetase in rice (*Oryza sativa*) roots in response to nitrogen. *Physiol. Plant.* 114: 41–46.
- King, G.A., Woollard, D.C., Irving, D.E. and Borst, W.M. (1990) Physiological changes in asparagus spear tips after harvest. *Physiol. Plant.* 80: 393–400.
- Lam, H.M., Coschigano, K.T., Oliveira, I.C., Melo-Oliveira, R. and Coruzzi, G.M. (1996) The molecular genetics of nitrogen assimilation into amino acids in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 569–593.
- Lam, H.M., Hsieh, M.H. and Coruzzi, G.M. (1998) Reciprocal regulation of distinct asparagine synthetase genes by light and metabolites in *Arabidopsis thaliana*. *Plant J.* 16: 345–353.
- Lam, H.M., Peng, S.S.-Y. and Coruzzi, G.M. (1994) Metabolic regulation of the gene encoding glutamine-dependent asparagine synthetase in *Arabidopsis thaliana*. *Plant Physiol.* 106: 1347–1357.
- Lea, P.J. (1999) Nitrogen Metabolism. In *Plant Biochemistry and Molecular Biology*. Edited by Lea, P.J. and Leegood, R.C. pp. 163–191. John Wiley & Sons, Chichester.

- Lea, P.J. and Mifflin, B.J. (1980) Transport and metabolism of asparagine and other nitrogen compounds within the plant. In *The Biochemistry of Plants*, vol. 5. Edited by Stumpf, P.K. and Conn, E.E. pp. 569–607. Academic Press, NY.
- Lucas, J.A. (1998) *Plant Pathology and Plant Pathogens*, 3rd edn. Blackwell Science, Oxford.
- McNally, S.F. and Hirel, B. (1983) Glutamine synthetase isoforms in higher plants. *Physiol. Vég.* 21: 761–774.
- Nakano, K., Suzuki, T., Hayakawa, T. and Yamaya, T. (2000) Organ and cellular localization of asparagine synthetase in rice plants. *Plant Cell Physiol.* 41: 874–880.
- Nylander, M., Svensson, J., Palva, E.T. and Welin, B.V. (2001) Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*. *Plant Mol. Biol.* 45: 263–279.
- Ortiz-López, A., Chang, H.-C. and Bush, D.R. (2000) Amino acid transporters in plants. *Biochim. Biophys. Acta* 1465: 275–280.
- Osuna, D., Gálvez-Valdivieso, G., Piedras, P., Pineda, M. and Aguilar, M. (1999) Cloning characterization and mRNA expression analysis of PVAS1, a type I asparagine synthetase gene from *Phaseolus vulgaris*. *Planta* 213: 402–410.
- Pereira, S., Carvalho, H., Sunkel, C. and Salema, R. (1992) Immunocytolocalization of glutamine synthetase in mesophyll and phloem of leaves of *Solanum tuberosum* L. *Protoplasma* 167: 66–73.
- Pérez-García, A., Cánovas, F.M., Gallardo, F., Hirel, B. and de Vicente, A. (1995) Differential expression of glutamine synthetase isoforms in tomato detached leaflets infected with *Pseudomonas syringae* pv. *tomato*. *Mol. Plant-Microbe Interact.* 8: 96–103.
- Pérez-García, A., de Vicente, A., Cantón, F.R., Cazorla, F.M., Codina, J.C., García-Gutiérrez, A. and Cánovas, F.M. (1998a) Light-dependent changes of tomato glutamine synthetase in response to *Pseudomonas syringae* infection or phosphinothricin treatment. *Physiol. Plant.* 102: 377–384.
- Pérez-García, A., Pereira, S., Pissarra, J., García-Gutiérrez, A., Cazorla, F.M., Salema, R., de Vicente, A. and Cánovas, F.M. (1998b) Cytosolic localization in tomato mesophyll cells of a novel glutamine synthetase induced in response to bacterial infection or phosphinothricin treatment. *Planta* 206: 426–434.
- Pérez-Rodríguez, J. and Valpuesta, V. (1996) Expression of glutamine synthetase genes during natural senescence of tomato leaves. *Physiol. Plant.* 97: 576–582.
- Romagni, J.G. and Dayan, F.E. (2000) Measuring asparagine synthetase activity in crude plant extracts. *J. Agric. Food Chem.* 48: 1692–1696.
- Sambrook, J. and Russell, D.N. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. CHSL Press, NY.
- Shi, L., Twary, S.N., Yoshioka, H., Gregerson, R.G., Miller, S.S., Samac, D.A., Gantt, J.S., Unkefer, P.J. and Vance, C.P. (1997) Nitrogen assimilation in alfalfa: isolation and characterization of an asparagine synthetase gene showing enhanced expression in root nodules and dark-adapted leaves. *Plant Cell* 9: 1339–1356.
- Sieciechowicz, K.A., Joy, K.W. and Ireland, R.J. (1988) The metabolism of asparagine in plants. *Phytochemistry* 27: 663–671.
- Tsai, F.-Y. and Coruzzi, G.M. (1990) Dark-induced and organ-specific expression of two asparagine synthetase genes in *Pisum sativum*. *EMBO J.* 9: 323–332.
- Watanabe, K., Higuchi, T., Sakai, T. and Yamaya, T. (1996) Nucleotide sequence of cDNA encoding asparagine synthetase (Accession no. D83378) from rice callus (PGR96-020). *Plant Physiol.* 111: 347.
- Wilkinson, M., Doskow, J. and Lindsey, S. (1991) RNA blots: staining procedures and optimisation of conditions. *Nucleic Acids Res.* 19: 679.

(Received January 26, 2004; Accepted April 6, 2004)