

# Differential Expression of Glutamine Synthetase Isoforms in Tomato Detached Leaflets Infected with *Pseudomonas syringae* pv. *tomato*

Alejandro Pérez-García,<sup>1</sup> Francisco M. Cánovas,<sup>2</sup> Fernando Gallardo,<sup>2</sup> Bertrand Hirel,<sup>3</sup> and Antonio de Vicente<sup>1</sup>

<sup>1</sup>Departamento de Microbiología; <sup>2</sup>Laboratorio de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Málaga, E-29071 Málaga, Spain; and <sup>3</sup>Laboratoire du Métabolisme, I.N.R.A., route de St-Cyr, 78026 Versailles, France

Received 25 July 1994. Accepted 24 October 1994.

Expression of glutamine synthetase (GS) isoforms of tomato leaflets was investigated during the infection by *Pseudomonas syringae* pv. *tomato*. Glutamine synthetase activity decreased markedly when it was determined by synthetase assay, but when GS activity was evaluated by transferase assay a not so strong decline was observed. Two GS molecular forms were separated by ion-exchange chromatography in noninfected and in chlorotic detached leaves. In noninfected leaves the major peak eluted at about 0.22 M KCl; however, in chlorotic leaves the major peak eluted at about 0.15 M KCl. A GS polypeptide of 45 KDa was the major species detectable by Western blot analysis of noninfected leaves; however, a second band of 39 KDa was the major GS species detectable in chlorotic leaves. Western and Northern blot analysis from infected detached leaflets in different stages of pathogenesis showed a decrease of GS polypeptide of 45 KDa and its specific mRNAs and an increase of GS species of 39 KDa and its transcripts. Our results suggest that during the infection there is a change in the GS isoform pattern and cytosolic GS (GS-1) replace chloroplastic GS (GS-2).

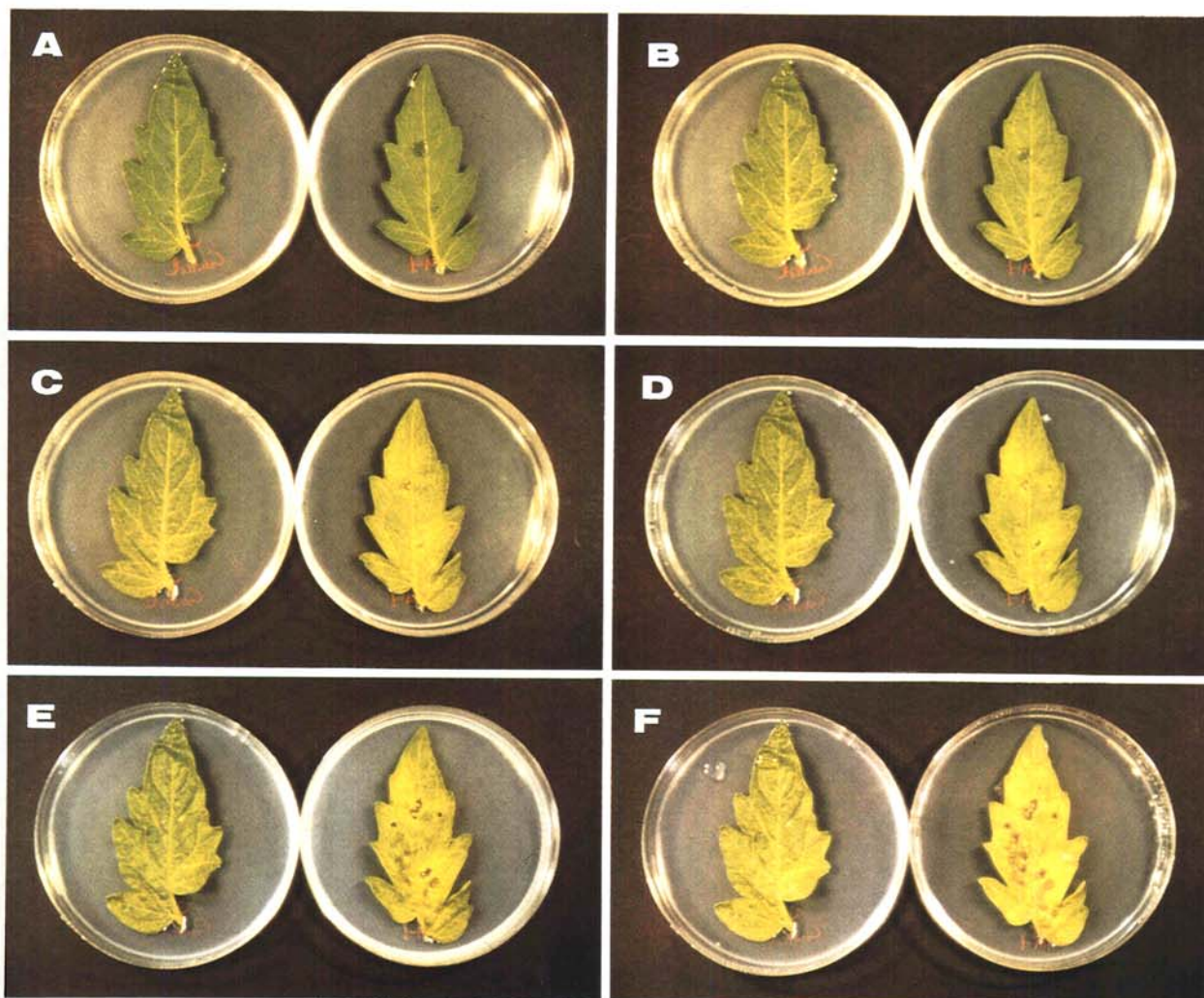
Se ha investigado la expresión de glutamina sintetasa (GS) en hojas de tomate infectadas por *Pseudomonas syringae* pv. *tomato*. Durante la infección, la actividad GS disminuyó drásticamente cuando se determinó mediante el ensayo sintetasa, pero con el ensayo transferasa no se observó un descenso tan acusado. Mediante cromatografía de intercambio iónico se separaron dos formas moleculares de GS tanto en hojas sanas como en infectadas. En hojas control el pico mayoritario de actividad GS eluyó a 0.22 M de KCl, mientras que en hojas infectadas lo hizo a 0.15 M. El inmunoanálisis reveló un polipéptido de GS de 45 kDa como la forma mayoritaria en hojas sanas, mientras que una segunda banda de 39 KDa fue la especie mayoritaria en hojas infectadas. El análisis mediante Western y Northern blot durante la infección, reveló un descenso de los niveles del polipéptido de GS de 45 KDa y de su mRNA específico, y un aumento de los niveles de la especie de 39 kDa y de su correspondiente mRNA.

Nuestros resultados sugieren que durante la infección hay un cambio en el patrón de isoformas de GS, y la forma citosólica de la enzima (GS-1) reemplaza a la isoforma cloroplástica (GS-2).

*Additional keywords:* ammonia assimilation, chloroplast degeneration, plant defense.

*Pseudomonas syringae* pv. *tomato* causes bacterial speck, an economically important disease of tomato that is characterized by small necrotic lesions surrounded by chlorotic halos on tomato leaves. Like other leaf-spotting *P. syringae* pathogens, this pathogen cannot penetrate the plant cell directly; it must enter its host through natural openings or wounds on the plant surface. The primary infection sites are the stomata and the bases of the leaf trichomes from which bacterial masses burst out at later stages of disease development (Bashan *et al.* 1981). In the field, *P. syringae* pv. *tomato* survived as an epiphyte on symptomless tomato plants for extended periods to produce disease under conducive environmental conditions (Smitley 1982). At present, no absolute resistant variety of tomatoes and no effective chemical treatments are available. Studies concerning biochemical and molecular changes in tomato plants in response to this pathogen are rare (Pautot *et al.* 1991; Koch *et al.* 1992; Martin *et al.* 1993); however, several reports concerning other pathogens have shown that pathogen attack leads to drastic metabolic changes such as accumulation of defense proteins (Granell *et al.* 1987; Vera and Conejero 1988; Christ and Mössinger 1989).

Glutamine synthetase (GS) (L-glutamate ammonia ligase, ADP forming E.C. 6.3.1.2) catalyzes the first reaction in the main path of ammonia assimilation in higher plants: the glutamine synthetase-glutamate synthase cycle (Lea *et al.* 1990). It is well established that green tissues of plants contain two different forms of GS, a cytosolic enzyme (GS-1) and a chloroplast located isoenzyme (GS-2) (McNally and Hirel 1983). Both isoforms are constituted by polypeptides of different molecular sizes which are encoded by homologous nuclear genes (Lightfoot *et al.* 1988; Tingey *et al.* 1988). The enzyme of tomato leaves has been purified (Cánovas *et al.* 1984) and its specific localization in the chloroplast has been determined by electron microscopy (Botella *et al.* 1988). It has been sug-



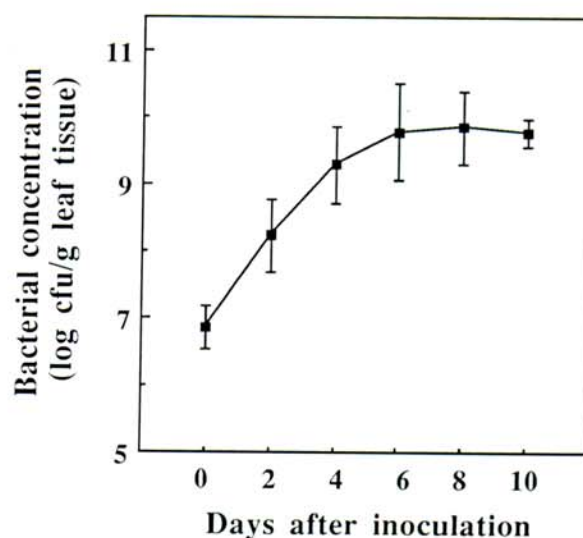
**Fig. 1.** Disease development in tomato detached leaflets infected with *Pseudomonas syringae* pv. *tomato* HM-1. A, B, C, D, E, and F, Noninfected (left) and infected (right) tomato detached leaves of 0, 2, 4, 6, 8, and 10 days after inoculation, respectively. Tomato leaflets were detached, disinfected, placed in MS medium and inoculated with bacterial suspensions as described in the text.

gested that the accumulation of ammonium ions and the evolution of gaseous ammonia may contribute to the bacterial speck syndrome in tomato plants caused by *Pseudomonas syringae* pv. *tomato* (Bashan *et al.* 1980). On the other hand, three pathovars of *P. syringae*: *P. s.* pv. *tabaci*, *P. s.* pv. *coronafaciens*, and *P. s.* pv. *garcae*, have been described as producers of tabtoxinine- $\beta$ -lactam, toxin that irreversibly inhibits glutamine synthetase (Gross 1991). However, to our knowledge, very little is known about glutamine biosynthesis during pathogenesis. In this paper we show that during the course of infection, directly correlated with chloroplast degeneration, there is a change in the GS isoform pattern and cytosolic GS replace chloroplastic GS. The possible role of cytosolic GS in infected tissues as defense-related protein is also discussed.

## RESULTS

### Disease development and chloroplast degeneration.

The time course of development of chlorotic and necrotic symptoms (Fig. 1) was parallel with the bacterial growth in infected tissues (Fig. 2). Symptoms enlarged slowly during



**Fig. 2.** Time course of the growth of *Pseudomonas syringae* pv. *tomato* HM-1 in tomato detached leaves. At the indicated times, leaflet disks were harvested and bacterial populations measured as described in Materials and Methods. Each vertical bar represents the standard error of the sample mean.



the first days and reached important chlorosis after about 6 days when population levels of pathogen were the highest. The determinations of chlorophyll (Chl) contents in infected tissues showed that both *Chl a* and *Chl b* decreased markedly during the final stages of development of symptoms (Fig. 3). Detached leaflets survived quite well under experimental conditions for about 10 days allowing adequate time for the production of fully developed symptoms, although a slight decrease in chlorophyll amounts of noninfected material maintained under experimental conditions was observed.

Total soluble proteins were extracted from healthy and infected leaves, separated by SDS-PAGE and stained with Coomassie blue. The stained gels showed that a noticeable change occurs in the pattern of soluble polypeptides during infection. A number of abundant proteins in tomato leaves disappear and many others accumulate gradually during pathogenesis (Fig. 4A). Duplicate gels were transferred to nitrocellulose filters and immunoprobed with antibodies raised against large subunit (LSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) and glutamate-synthase (Fd-GOGAT) from *Pinus pinaster* seedlings (García-Gut-

ierrez *et al.* 1993a, 1993b), and chloroplastic glutamine synthetase (GS-2) from *Nicotiana tabacum* (Hirel *et al.* 1984) (Fig. 4B). The antibodies raised against GS-2 have been used to identify this protein in different plant species and it has been shown that they cross-react with cytosolic and chloroplastic polypeptides (Tyngey *et al.* 1987; Snustad *et al.* 1988). Figure 4B shows that a major 55 kDa LSU polypeptide was detected in all stages of pathogenesis analyzed, but its steady-state level decreased during the process (lanes 5

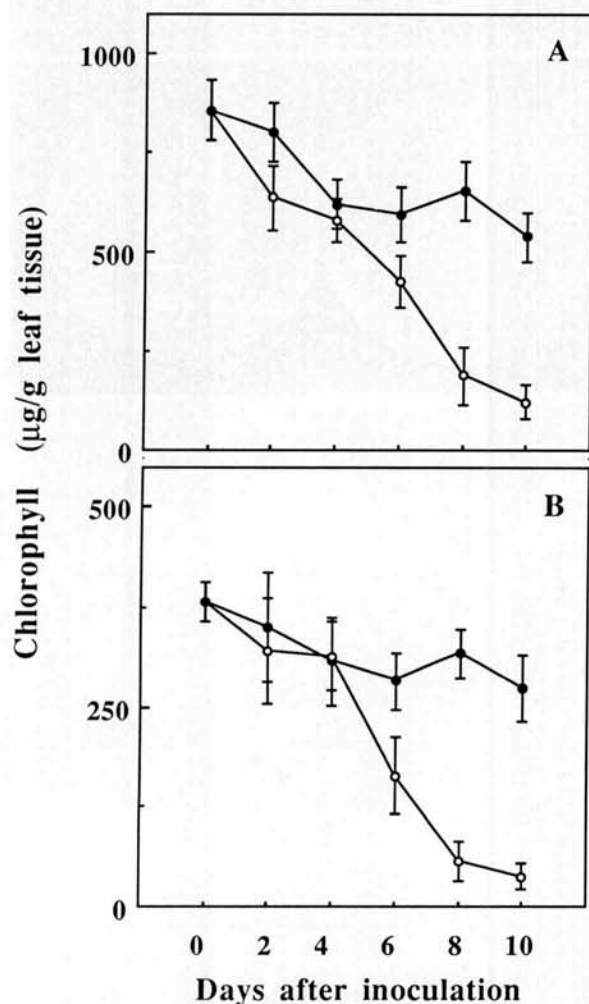


Fig. 3. Time course of chlorophyll contents in noninfected tomato detached leaves (○) and infected with *Pseudomonas syringae* pv. *tomato* HM-1 (●). A and B, chlorophyll *a* and *b* contents, respectively. Values shown are the mean of three independent determinations, and the bars show the standard deviation from the mean.

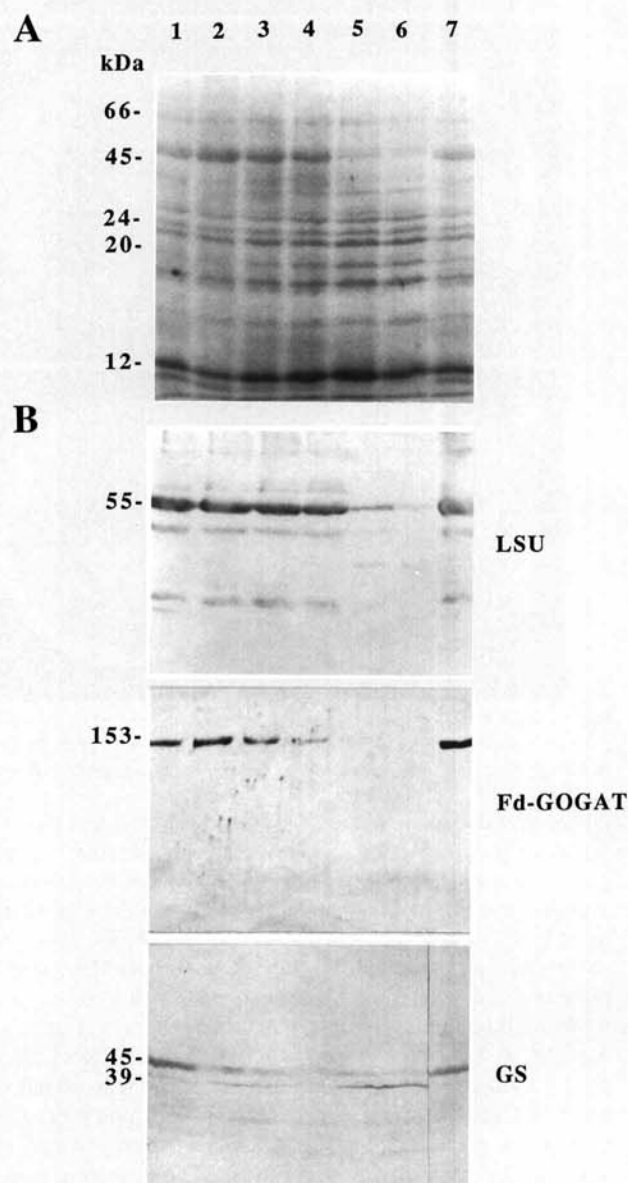
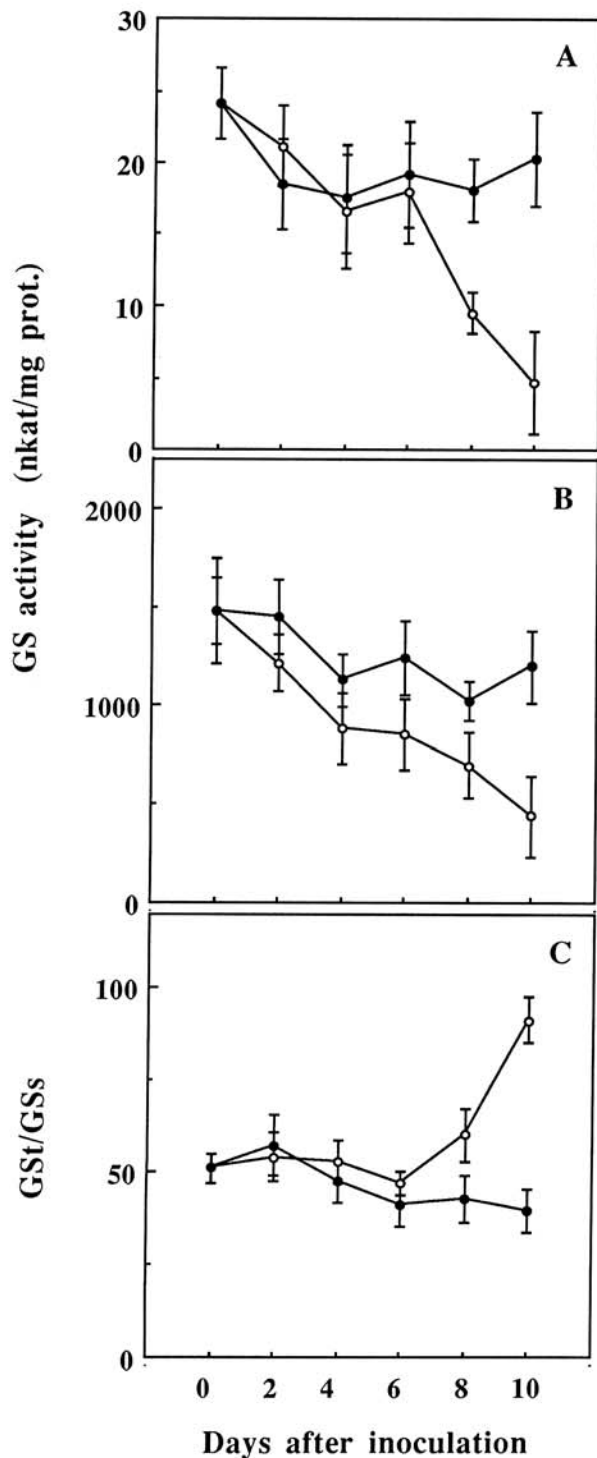


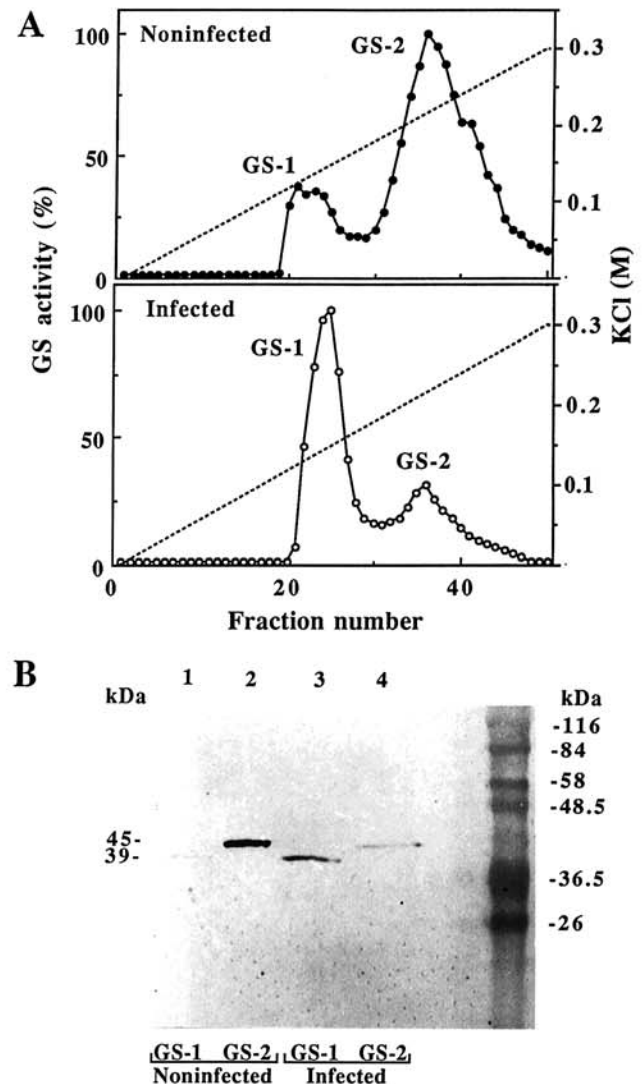
Fig. 4. Analysis of soluble polypeptides during the course of infection with *Pseudomonas syringae* pv. *tomato* HM-1. Soluble proteins extracted from noninfected and infected tomato detached leaves were separated by SDS-PAGE, and stained with Coomassie blue (A) or immunoprobed with antibodies against LSU, Fd-GOGAT or chloroplastic GS (B). Lanes 1, 2, 3, 4, 5, 6, correspond in the order given, to infected leaves of 0, 2, 4, 6, 8 and 10 days after inoculation. Noninfected tomato detached leaves maintained 10 days in MS medium were used as controls (lane 7). The  $M_r$ s of protein markers and the size of the major cross-reacting bands are indicated on the left in kDa. The same amount of protein (25 µg) was loaded per lane.

and 6). A single Fd-GOGAT species of 153 kDa was found in the first stages of pathogenesis disappearing at the end of infection (lanes 5 and 6). A major 45-kDa GS polypeptide was detected in noninfected leaves corresponding to chloro-



**Fig. 5.** Time course of glutamine synthetase activities in noninfected tomato detached leaves (●) and infected with *Pseudomonas syringae* pv. *tomato* HM-1 (○). **A**, Glutamine synthetase activity was determined by the synthetase assay (GS<sub>s</sub>). **B**, Glutamine synthetase activity was determined by the transferase assay (GS<sub>t</sub>). **C**, Ratio GS<sub>t</sub>/GS<sub>s</sub>. Data points represent the mean of three experiments ± SE.

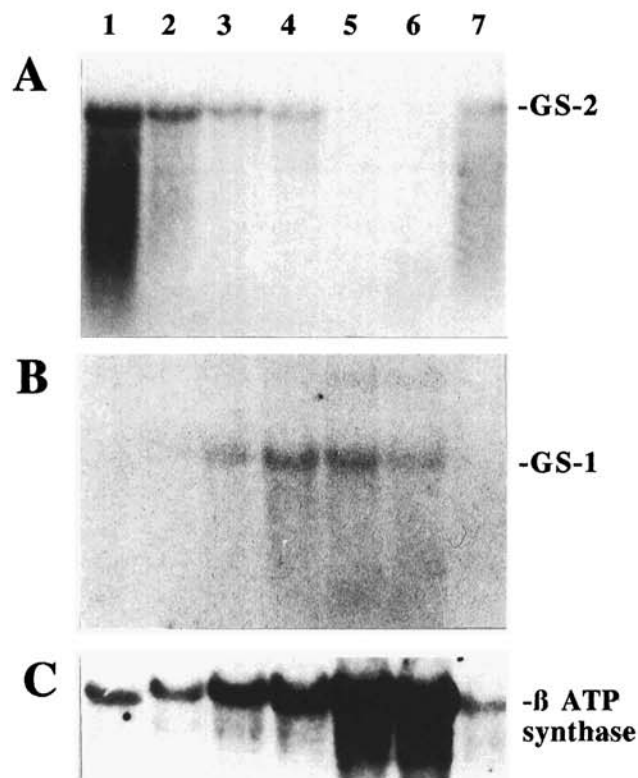
plastic GS (GS-2) (lanes 1 and 7). The steady-state level of this polypeptide decreased during infection (lanes 5 and 6). A second GS polypeptide showing an apparent  $M_r$  of 39 kDa was also detected. The steady-state level of the 39 kDa band increased during the course of infection. Concomitant with *Chl* content, LSU, Fd-GOGAT, and GS-2 contents were drastically reduced during the last phase of infection (days 8 and 10). Decrease in four chloroplast markers: chlorophylls, LSU, Fd-GOGAT, and GS-2, represent a chloroplast degeneration associated with the course of infection.



**Fig. 6.** Elution profiles of GS activity from DEAE-Sephadex columns and immunoblot analysis of GS polypeptides. **A**, Soluble extracts obtained from noninfected tomato detached leaves and infected with *Pseudomonas syringae* pv. *tomato* HM-1, were separated by ion-exchange chromatography and enzyme activity was determined by transferase assay. Maximum GS values were 23.25 nkat.mL<sup>-1</sup> from noninfected leaves, and 37.36 nkat.mL<sup>-1</sup> from infected leaves. **B**, Immunoblot analysis of GS polypeptides in protein fractions with the highest GS activity from the different peaks of GS activity eluted from DEAE-Sephadex columns. Protein fractions were concentrated, separated by SDS-PAGE and immunoprobed with GS antibodies. The  $M_r$ s of protein markers and the size of the major cross-reacting bands are indicated on the right and left, respectively, in kDa. Detached leaves maintained 10 days in MS medium were used in these experiments.

### Glutamine synthetase isoform pattern.

To determine if accumulation of GS polypeptide of 39 kDa could be due to a proteolytic degradation of the 45 kDa band corresponding to chloroplastic GS, or may be attributed to the expression and accumulation of cytosolic GS, we performed different experiments to study the glutamine synthetase activity and GS isoform pattern during the pathogenesis. Parallel with chloroplast degeneration, GS activity decreased markedly in tomato detached leaves during infection when its compared with levels detected in noninfected detached leaves (Fig. 5). The specific activity of GS decreased about fivefold in synthetase assay ( $GS_s$ ) (Fig. 5A) and about twofold in transferase assay ( $GS_t$ ) (Fig. 5B). The ratio  $GS_t/GS_s$  increased during the final stages of pathogenesis (Fig. 5C), suggesting the existence of change in the GS complement during the pathogenesis. To test this hypothesis, the GS isoenzyme pattern was examined by DEAE-Sephadex chromatography in noninfected and infected detached leaves (Fig. 6A). In noninfected detached leaves a major peak of GS activity was detected, which eluted from the columns at a saline concentration of 0.22 M KCl, which corresponded to chloroplastic GS (GS-2) detected in mature tomato leaves (Cánovas *et al.* 1984). However, in chlorotic detached leaves, lower saline strength was required to elute most of the enzyme activity



**Fig. 7.** Northern blot analysis of tomato GS mRNA levels during the course of infection with *Pseudomonas syringae* pv. *tomato* HM-1. Total RNA from noninfected and infected tomato detached leaves was electrophoresed on 1% formaldehyde agarose gels and analyzed by Northern hybridization. Lanes 1, 2, 3, 4, 5, and 6 correspond in the order given, to infected detached leaves of 0, 2, 4, 6, 8, and 10 days after inoculation. Noninfected tomato detached leaves maintained 10 days in MS medium were used as controls (lane 7). **A**, With GS-2 probe. **B**, With GS-1 probe. **C**, With mitochondrial  $\beta$ -ATP synthase probe used as an endogenous control of mRNA.

(0.15 M KCl); we named this peak GS-1 because its chromatographic behavior was different from that of GS-2 detected in noninfected leaves. This finding may represent the detection of a cytosolic GS associated to pathogenesis.

For the identification of GS polypeptides associated to GS isoforms in both infected and noninfected tomato detached leaves, we performed immunoblot analysis of protein fractions isolated from DEAE-Sephadex columns with the highest GS activity (Fig. 6B). Protein fractions were concentrated by inverse dialysis, separated by SDS-PAGE and immunoprobed with anti-GS-2 antibodies. Noninfected leaves contained a major GS polypeptide of 45-kDa (lane 2) and a second band of 39 kDa (lane 3) was detected as the most abundant form in infected tissues.

### mRNA levels of glutamine synthetase isoforms.

To determine whether changes in glutamine synthetase polypeptides were correlated with changes in glutamine synthetase mRNA levels, we examined the steady-state levels of mRNA encoding the two different isoforms. GS mRNA levels were determined by Northern blot analysis using as molecular probes two cDNA probes isolated from tobacco corresponding to the GS-2 (Becker *et al.* 1992) and GS-1 (B. Hirel, unpublished) isoforms. Total RNA was isolated from infected leaves at various times after inoculation with *P. syringae* pv. *tomato* HM-1 and the relative abundance of GS-2 and GS-1 mRNA was compared. A decrease in mRNA levels coding for GS-2 (Fig. 7A) and a differential accumulation of GS-1 mRNAs was observed (Fig. 7B). During infection, GS-2 mRNA was detectable until 6 days after inoculation (lane 4), and was undetectable in days 8 and 10 after infection (lanes 5 and 6). GS-2 mRNA levels of detached control leaves (lane 7) were lower than undetached leaves (lane 1).

GS-1 mRNAs were barely detected until 4 days postinoculation (lane 3). By 6 days of infection, GS-1 mRNA accumulated to high levels, which continued increasing until 8 days after inoculation (lane 5). These GS-1 transcripts were not detected in control detached (lane 7) and undetached leaves (lane 1). Mitochondrial  $\beta$ -ATP synthase mRNA was used as an endogenous control and was detected in all stages examined. The steady-state levels of this transcript increased during the infection, showing the highest abundance in the latest stages (Fig. 7C).

### DISCUSSION

It is well documented that infection by bacterial pathogens induces in plants a wide range of metabolic changes. Most of the studies concerning the host-pathogen system tomato-*P. syringae* are related to pathogenicity of the bacterium (Bashan *et al.* 1986; Bender *et al.* 1987); biochemical events of plant response to infection have been poorly documented (Pautot *et al.* 1991; Koch *et al.* 1992; Martin *et al.* 1993). The experiments presented in this paper demonstrate that during infection, directly correlated with the increase of pathogen population levels, there was chloroplastic degeneration, because a decrease in contents of chloroplastic markers such as chlorophylls, LSU, Fd-GOGAT, and chloroplastic GS was observed.

Leaf protein analysis by electrophoresis showed that the major change in the protein pattern of infected leaves was

concerned with LSU. The drastic decline observed for LSU levels during the course of infection may be result of a decrease of RuBPCase synthesis and/or increase of protein degradation. Bashan *et al.* (1986) demonstrated that the protease activity increased in response to *P. syringae* pv. *tomato* infection, suggesting that proteases play an important role in the development of bacterial speck disease. The major PR proteins induced by CEV in tomato plants, P-69, is an endoproteinase which degrades RuBPCase (Vera and Conejero 1988). We have also detected P-69 protein in tomato leaves infected by *P. syringae* (data not shown). In melon plants infected with *Colletotrichum lagenarium*, RuBPCase gradually disappeared during the different stages of infection and a rapid decrease in the rate of RuBPCase synthesis was observed (Ranty *et al.* 1987). Degradation and synthesis rates of RuBPCase in infected tomato leaves were not assayed in this work, nevertheless a negative regulation of such a quantitatively important protein during infection could be expected, and it might be an essential step in nitrogen mobilization that is required for the biosynthesis of defense proteins by the host plant.

Total GS activity in detached leaves showed a decrease when the leaflets were inoculated with *P. syringae* and incubated for 10 days. In infected leaves, the ratio  $GS_2/GS_1$  increased during the final stages of infection, indicating changes in the isoform complement, because variations in the relative proportions of GS isoforms may contribute to the alteration of the  $GS_2/GS_1$  ratio (Padilla *et al.* 1987). This assumption is confirmed by the GS elution profiles from DEAE-Sephadex columns; a different chromatographic pattern was detected in chlorotic leaves. Western blot analysis are also consistent with this conclusion. Immunochemical analysis of GS isoforms indicated that GS-2 protein, GS species of 45 kDa, constituted the major fraction of total GS proteins in non-infected detached leaves and decreased during pathogenesis. In chlorotic leaves, a major GS polypeptide of 39 kDa (GS-1) was detected, whose amount increased during infection. These data indicate that the increase in the steady-state level of the 39-kDa polypeptide during the infection is correlated to the replacement of chloroplastic GS by the cytosolic isoenzyme as the major GS isoform in infected leaves. A similar observation has been reported for radish (*Raphanus sativus*) cotyledons during senescence (Kawakami and Watanabe 1988).

Under these infection conditions, a strong decrease in the level of GS-2 mRNA and accumulation in mRNA coding for GS-1 were also observed in the leaflets of this tomato variety susceptible to *P. s.* pv. *tomato*. These results show that the expression of GS isoforms genes during infection are regulated at the transcriptional and/or posttranscriptional levels. A similar effect, induction of a gene encoding cytosolic GS, has been reported by Hirel *et al.* (1992) in tobacco leaves by forcing expression of a soybean root glutamine synthetase gene.

Hirel *et al.* (1987) have described that the expression of genes for nodule-specific GS are induced by ammonium provided externally or by symbiotic nitrogen fixation in developing bean root nodules. The accumulation of ammonium ions in response to *Pseudomonas* infection has been proved in tomato plants, as a consequence of proteolytic activity and amino acid deamination (Bashan *et al.* 1986). Turner and Debbage (1982) described the implication of tabtoxin, a bacterial toxin, in ammonia accumulation released in photorespiration during

pathogenesis. It is possible that GS-1 gene could be induced by ammonia which could be produced in protein catabolism and could also accumulate during pathogenesis as a consequence of photorespiratory nitrogen cycle interruption by effect of toxin production from bacteria.

During infection, RuBPCase, which represents 50% of total leaf proteins, and other chloroplast polypeptides are degraded to their structural units, the amino acids, which can be subsequently deaminated. Carbon skeletons can be used as precursors to biosynthesis of molecules involved in plant defense, while ammonium can be reassimilated by GS enhancing the formation of glutamine. Expression of cytosolic GS when chloroplast has been disorganized can be an important factor to maintain plant nitrogen economy during pathogenesis, because the drastic disappearance of chloroplastic GS and Fd-GOGAT during the infection does not allow the reassimilation of ammonia by the GS/GOGAT cycle. Then, glutamine can be an important vehicle for nitrogen transport to healthy parts of plant where it can be used for the biosynthesis of defense-related proteins that permit plant response. The accumulation of cytosolic GS may be due to a synthesis either in the phloem cells or in the mesophyll cells, but there is more and more evidence that cytosolic GS in the phloem is probably playing an important role in ammonia remobilization and transport. These nonoverlapping roles for chloroplast and cytosolic glutamine synthetase have been reported by cell-specific expression in transgenic plants (Edwards *et al.* 1990).

The results presented in this paper provide experimental evidence that during infection of tomato leaves by *P. syringae* the isoform pattern change of GS may be correlated with the chloroplastic degeneration associated to pathogenesis. In addition our data suggest that cytosolic GS could be considered as defense-related protein during pathogen stress.

## MATERIALS AND METHODS

### Bacterial strain and growth conditions.

*Pseudomonas syringae* pv. *tomato* strain HM-1 used in this study was isolated from infected tomato leaves, and characterized further by biochemical tests proposed by Hildebrand *et al.* (1988) and hypersensitivity and pathogenicity tests towards tobacco and tomato, respectively. This strain was routinely grown in King's medium B (KB) (King *et al.* 1954) at 27° C.

### Plant material and inoculation.

*Lycopersicon esculentum* Mill. cv. Hellfrucht Frühstamm was grown from seed in a greenhouse under natural light. Prior to inoculation, tomato leaflets from 10-wk-old plants were detached, disinfected in 0.1% (w/v) HgCl<sub>2</sub> solution, and placed in petri dishes with their petioles immersed in Murashige and Skoog medium (MS) (SIGMA 9274). Bacterial suspensions were adjusted to 10<sup>8</sup> colony-forming units per milliliter with sterile distilled water. Detached leaflets were inoculated with a 100-fold dilution of bacterial suspension by injection and maintained at 22° C under a 16 hr-photoperiod, with relative humidity not less than 80%. To decrease error due to plant-to-plant variation, four to six tomato plants were used for each experimental time point of treatment. All experiments were repeated at least three times. Noninfected detached leaflets inoculated with sterile distilled water and



maintained in MS medium were included in all experiments as controls.

#### Bacterial growth in leaf tissue.

Leaflet disks were taken with a sterilized cork borer and then homogenized in sterile phosphate-buffered saline. Bacterial populations were determined by serial dilutions on plates of KB medium after incubation at 27° C during 48 hr.

#### Chlorophyll content.

Total chlorophylls were extracted from tomato detached leaflets in 80% (v/v) acetone. Chlorophylls *a* and *b* were spectrophotometrically quantified by readings at 647 and 664 nm, according to Graan and Ort (1984).

#### Protein extraction and glutamine synthetase determination.

Leaf tissue (1–2 g) for protein isolation was harvested 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 50 mM Tris (pH 8.0), 2 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM PMSF and 10% (v/v) glycerol as described Cánovas *et al.* (1991). Protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin as standard. Glutamine synthetase activity was evaluated by the synthetase and transferase assays as described earlier (Cánovas *et al.* 1991).

#### Ion-exchange chromatography.

Glutamine synthetase isoenzymes were separated essentially as described elsewhere (Gálvez *et al.* 1990). The crude extracts were applied to a Sephadex G-25 (5.8 × 1.5 cm) columns equilibrated in running buffer 2 mM EDTA, 10 mM 2-mercaptoethanol, 5 mM MgSO<sub>4</sub>, and 50 mM Tris-HCl (pH 8.0). The desalted preparations were loaded on a DEAE-Sephadex A-25 (7 × 1.9 cm) columns equilibrated with the same buffer. The enzyme was eluted by the application of a linear gradient of 0–0.3 M KCl at 20 ml.hr<sup>–1</sup>. Fractions of 1.6 ml were collected and assayed for GS activity by the transferase assay.

#### Electrophoresis and Western blot analysis.

SDS-polyacrylamide gel electrophoresis and Western blot analysis was performed as described by Cánovas *et al.* (1991). After electrophoresis, the polypeptides were visualized by staining Coomassie blue. Duplicate gels were electrophoretically transferred to nitrocellulose membranes. LSU (1:10,000), Fd-GOGAT (1:8,000), and GS (1:1,000) antibodies raised against LSU and Fd-GOGAT from *Pinus pinaster* seedlings (García-Gutiérrez *et al.* 1993a; 1993b) and GS from tobacco leaves (Hirel *et al.* 1984), respectively, were used. Immunocomplexes were detected with a biotin-avidin peroxidase kit following the manufacturer's directions. Peroxidase activity was viewed 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.

#### RNA isolation and Northern blot analysis.

Total RNA was isolated, with minor modifications, as described by Cantón *et al.* (1993) from frozen samples by extraction in liquid nitrogen and homogenization in 1% (w/v)

triisopropyl naphthalene sulphonic acid, 0.5% (v/v) 2-mercaptoethanol, 6% (w/v) *p*-aminosalicylic acid, 1% (w/v) NaCl, and 6% (v/v) phenol, and size-fractionated on formaldehyde agarose gels. Equal RNA loading (20 µg) was based on spectrophotometric determination of RNA concentration and ethidium bromide staining. After destaining, RNA was transferred to positively charged nylon membranes and hybridized with radioactive probes. cDNAs were <sup>32</sup>P-labeled using the random primer method. GS-2 and GS-1 probes are from *Nicotiana tabacum*, and they are 1.5 and 1.4 kb *Eco*RI fragments of the plasmids pcGS2-17 (Becker *et al.* 1992) and pGS1 NT 5 (B. Hirel, unpublished), that contain the coding region of these genes. A mitochondrial β-ATP synthase probe from *Nicotiana plumbaginifolia* of 1.25 kb (Boutry and Chua, 1985) was used as control in the Northern blot experiments. Hybridizations were carried out at 42° C in 50% formamide, 6× SSC (1× SSC is 15 mM sodium citrate, 150 mM NaCl pH 7.0), 5× Denhardt's solution, 0.5% (w/v) SDS and 100 µg/ml salmon sperm DNA. Filters were washed at 42° C in 4× SSC/0.2% SDS, 2× SSC/0.2% SDS, and 1× SSC/0.2% SDS.

#### ACKNOWLEDGMENTS

We wish to thank A. García-Gutiérrez, Universidad de Málaga, for providing the antibodies to pine LSU and Fd-GOGAT. This work was supported by grants from the Fundación para la Investigación Agraria de la Provincia de Almería (FIAPA) and the Dirección General de Investigación Científica y Técnica (Grant PB92-0423).

#### LITERATURE CITED

- Bashan, Y., Okon, Y., and Henis, Y. 1980. Ammonia causes necrosis in tomato leaves infected with *Pseudomonas tomato* (Okabe) Alstatt. *Physiol. Mol. Plant Pathol.* 17:111-119.
- Bashan, Y., Okon, Y., and Henis, Y. 1986. A possible role for proteases and deaminases in the symptoms of bacterial speck disease in tomato caused by *Pseudomonas syringae* pv. *tomato*. *Physiol. Mol. Plant Pathol.* 28:15-31.
- Bashan, Y., Sharon, E., Okon, Y., and Henis, Y. 1981. Scanning electron and light microscopy of infection and symptom development in tomato leaves infected with *Pseudomonas tomato*. *Physiol. Plant Pathol.* 19:139-144.
- Becker, T. W., Caboche, M., Carrayol, E., and Hirel, B. 1992. Nucleotide sequence of a tobacco cDNA encoding plastidic glutamine synthetase and light inducibility, organ specificity and diurnal rhythmicity in the expression of the corresponding genes of tobacco and tomato. *Plant Mol. Biol.* 19:367-379.
- Bender, C. L., Stonbe, H. E., Sims, J. J., and Cooksey, D. A. 1987. Reduced fitness of *Pseudomonas syringae* pv. *tomato* mutants defective in coronatine production. *Physiol. Mol. Plant Pathol.* 30:273-283.
- Botella, J. R., Verbelen, J. P., and Valpuesta, V. 1988. Immunocyto-localization of glutamine synthetase in green leaves and cotyledons of *L. esculentum*. *Plant Physiol.* 88:943-946.
- Boutry, M., and Chua, N. M. 1985. A nuclear gene encoding the beta subunit of the mitochondrial ATP synthase in *Nicotiana plumbaginifolia*. *EMBO J.* 4:2159-2165.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities utilising the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Cánovas, F., Valpuesta, V., and Núñez de Castro, I. 1984. Characterization of glutamine synthetase from tomato leaves. *Plant Sci. Lett.* 37:79-85.
- Cánovas, F.M., Cantón, F.R., Gallardo, F., García-Gutiérrez, A., and de Vicente, A. 1991. Accumulation of glutamine synthetase during the early development of maritime pine (*Pinus pinaster*) seedlings. *Planta* 185:372-378.
- Cantón, F.R., García-Gutiérrez, A., Gallardo, F., de Vicente, A., and Cánovas, F. M. 1993. Molecular characterization of a cDNA clone en-

- coding glutamine synthetase from a gymnosperm, *Pinus sylvestris*. *Plant Mol. Biol.* 22:819-828.
- Christ, U., and Mössinger, E. 1989. Pathogenesis-related proteins of tomato: I. Induction by *Phytophthora infestans* and other biotic and abiotic inducers and correlations with resistance. *Physiol. Mol. Plant Pathol.* 35:53-65.
- Edwards, J. W., Walker, E. L., and Coruzzi, G. M. 1990. Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase. *Proc. Natl. Acad. Sci. USA* 87:3459-3463.
- Gálvez, S., Gallardo, F., and Cánovas, F. 1990. The occurrence of glutamine synthetase isoenzymes in tomato plants is correlated to plastid differentiation. *J. Plant. Physiol.* 137:1-4.
- García-Gutiérrez, A., Cantón, F. R., Gallardo, F., and Cánovas, F. M. 1993. Immunochemical analysis of chloroplast polypeptides from maritime pine. *Phytochemistry* 34:337-341.
- García-Gutiérrez, A., Cantón, F. R., Sánchez-Jiménez, F., and Cánovas, F. M. 1993. Immunocharacterization of ferredoxin-dependent glutamate synthase in pine. *Plant Physiol.* 102(suppl.):127.
- Graan, T., and Ort, D. R. 1984. Quantitation of the rapid electron donors to P<sub>700</sub>, the functional plastoquinone pool, and the ratio of the photosystems in spinach chloroplasts. *J. Biol. Chem.* 259:14003-14010.
- Granell, A., Bellés, J. M., and Conejero, V. 1987. Induction of pathogenesis-related proteins in tomato by citrus exocortis viroid, silver ion, and ethephon. *Physiol. Mol. Plant Pathol.* 31:83-89.
- Gross, D. C. 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. *Annu. Rev. Phytopathol.* 29:247-278.
- Hildebrand, D. C., Schroth, M. N., and Sands, D. C. 1988. *Pseudomonas*. Pages 60-80 in: *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. N. W. Schaad, ed. American Phytopathological Society, St. Paul, MN.
- Hirel, B., Bouet, C., King, B., Layzell, D., Jacobs, F., and Verma, D. P. S. 1987. Glutamine synthetase genes are regulated by ammonia provide externally or by symbiotic nitrogen fixation. *EMBO J.* 5:1167-1171.
- Hirel, B., Marsolier, M. C., Hoarau, A., Hoarau, J., Brangeon, J., Schaferm R., and Verma, D. P. S. 1992. Forcing expression of a soybean root glutamine synthetase gene in tobacco leaves induces a native gene encoding cytosolic enzyme. *Plant Mol. Biol.* 20:207-218.
- Hirel, B., Weartherly, C., Cretin, C., Bergouinoux, C., and Gadal, P. 1984. Multiple subunit composition of chloroplastic glutamine synthetase of *Nicotiana tabacum* L. *Plant Physiol.* 74:448-450.
- Kawakami, N., and Watanabe, A. 1988. Senescence-specific increase in cytosolic glutamine synthetase and its mRNA in radish cotyledons. *Plant Physiol.* 88:1430-1434.
- King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
- Koch, E., Meier, B. M., Eiben, H. G., and Slusarenko, A. 1992. A lipoxygenase from leaves of tomato (*Lycopersicon esculentum* Mill.) is induced in response to plant pathogenic pseudomonads. *Plant Physiol.* 99:571-576.
- Lea, P. J., Robinson, S. A., and Stewart, G. R. 1990. The enzymology and metabolism of glutamine, glutamate and asparagine. Pages 121-159 in: *The Biochemistry of Plants*, vol. 16. B. J. Mifflin and P. J. Lea, eds. Academic Press, New York.
- Lightfoot, D. A., Green, N. K., and Cullimore, J. V. 1988. The chloroplast-located glutamine synthetase of *Phaseolus vulgaris* L. Nucleotide sequence, expression in different organs and uptake into isolated chloroplast. *Plant Mol. Biol.* 11:191-201.
- Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganai, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* 262:1432-1436.
- McNally, S. F., and Hirel, B. 1983. Glutamine synthetase isoforms in higher plants. *Physiol. Vég.* 21:761-774.
- Padilla, J. E., Campos, F., Conde, V., Lara, M., and Sánchez, F. 1987. Nodule-specific glutamine synthetase is expressed before the onset of nitrogen fixation in *Phaseolus vulgaris* L. *Plant Mol. Biol.* 9:65-74.
- Pautot, V., Holzer, F. M., and Walling, L. L. 1991. Differential expression of tomato proteinase inhibitor I and II genes during bacterial pathogen invasion and wounding. *Mol. Plant-Microbe Interact.* 4:284-292.
- Smitley, D. R. 1982. Spread of *Pseudomonas syringae* pv. *tomato* and role of epiphytic populations and environmental conditions in disease development. *Plant Dis.* 66:713-717.
- Snustad, D. P., Hunsperger, J. P., Chereskin, B. M., and Messing, J. 1988. Maize glutamine synthetase cDNAs: Isolation by direct genetic selection in *Escherichia coli*. *Genetics* 120:1111-1124.
- Tingey, S. V., Walker, E. L., and Coruzzi, G. M. 1987. Glutamine synthetase genes of pea encode distinct polypeptides which are differentially expressed in leaves, roots, and nodules. *EMBO J.* 6:1-9.
- Tingey, S. V., Tsai, F. Y., Edwards, J., Walker, E. L., and Coruzzi, G. M. 1988. Chloroplast and cytosolic glutamine synthetase are encoded by homologous nuclear genes which are differentially expressed in vivo. *J. Biol. Chem.* 263:9651-9657.
- Turner, J. G., and Debbage, J. M. 1982. Tabtoxin-induced symptoms are associated with the accumulation of ammonia formed during photorespiration. *Physiol. Plant Pathol.* 20:223-233.
- Vera, P., and Conejero, V. 1988. Pathogenesis-related proteins of tomato. P-69 as an alkaline endoprotease. *Plant Physiol.* 87:58-63.