Plant Physiol. (1987) 85, 845-849
0032-0889/87/85/0845/05/$01.00/0

# Tabtoxinine-β-Lactam Transport into Cultured Corn Cells[1]

## UPTAKE VIA AN AMINO ACID TRANSPORT SYSTEM

Received for publication May 17, 1987 and in revised form August 3, 1987

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### ABSTRACT

**Tabtoxinine-β-lactam (T-β-L), a unique amino acid, is a toxin produced by several closely related pathovars of *Pseudomonas syringae*. These chlorosis-inducing pathogens establish themselves in the apoplastic space of their hosts where they release the toxin. We have examined the transport of T-β-L into cultured corn (*Zea mays* cv Black Mexican) cells using [$^{14}$C]T-β-L. The pH optimum of the uptake of the toxin was between 4.0 and 5.5 pH units. Toxin uptake was inhibited by the protonophore, carbonyl cyanide *m*-chlorophenyl hydrazone, and by the sulfhydryl reagent, *N*-ethylmaleimide. Tabtoxinine-β-lactam transport exhibited saturation kinetics that were described by the Michaelis-Menton equation for toxin concentrations of 1 millimolar and less. However, the transport of toxin in concentrations greater than 1 millimolar was not described by Michaelis-Menten kinetics. Glutamate and alanine exhibited similar transport kinetics with a transition to non-Michaelis-Menten kinetics when the amino acid concentration exceeded 1 millimolar. Hill numbers for glutamate, alanine, and T-β-L ranged from 0.6 to 0.8. Methionine, alanine, tyrosine, glutamine, glutamate, and arginine were inhibitors of toxin transport. Alanine was a competitive inhibitor of the transport of T-β-L and of glutamate. The data are consistent with T-β-L being transported into the plant cell through an amino acid transport system.**

Tabtoxinine-β-lactam is a toxin produced by several closely related pathovars of *Pseudomonas syringae*. This compound is directly involved in eliciting the chlorotic symptoms these pathogens induce in their hosts, which include such economically important crops as corn, pea, beans, soybeans, oat, and tobacco (9). The invading bacteria multiply in the apoplastic space of the plant where they synthesize a pretoxin dipeptide, tabtoxin, consisting of threonine or serine and T-β-L[3] (Fig. 1) (2-amino-4-[3-hydroxy-2-oxo-azacyclobutan-3-yl]butanoic acid) (23). T-β-L is released from tabtoxin by either bacterial or plant hydrolyases (26). The biochemical target of T-β-L is GS [EC 6.3.1.2] (21, 24), for which T-β-L is an irreversible inhibitor (12, 13). The inactivation of GS by T-β-L deprives the plant of an important metabolite, glutamine. However, a second detrimental effect results from the loss of GS activity. Ammonia concentrations in infected tissue reach toxic (mM) levels (21, 25). This ammonia arises because of the loss of GS function in the reassimilation of ammonia released by photorespiration (8, 25). The high rate of nitrogen flux (up to 80 μmol $NH_3$ fresh weight·h) (15) through the photorespiratory N-cycle results in a rapid accumulation of unassimilated ammonia. Ultrastructural changes observed in diseased plant tissue are similar to those resulting from nutritionally imposed ammonia toxicity (7, 18). Thus, the inactivation of GS by T-β-L not only prevents the synthesis of a key host metabolite, glutamine, it also results in structural and metabolic disruptions attributable to toxic concentrations of ammonia.

Although the inactivation of GS by T-β-L has been studied in detail, transport of the toxin into plant cells has not been examined. Uptake of T-β-L is a required step in the induction of chlorosis caused by pathovars of *P. syringae* that possess tabtoxin-hydrolyase activity (26) and thus release T-β-L into the apoplastic space. Therefore, knowledge of T-β-L transport into the plant cell is necessary to fully understand its role in disease causation. In a previous study of the *in vivo* inactivation of GS by T-β-L (1), we showed that cells treated in 50 μM toxin lost greater than 50% of their initial GS activity within 60 min. This result suggested T-β-L was transported into the cell via a very efficient transport system. Since the toxin is structurally similar to common amino acids (Fig. 1), we hypothesized that it is translocated into the cell via an amino acid transport system. In support of this hypothesis, we reported that several amino acids included in toxin treatment solutions were able to inhibit the *in vivo* inactivation of GS, presumably by blocking T-β-L uptake. In this paper, we report on a quantitative description of [$^{14}$C]T-β-L transport into cultured corn cells. The results show T-β-L is transported into the plant cell via an amino acid transport system.

### MATERIALS AND METHODS

**Plant Material.** We chose suspension cultures of corn as our experimental tissue because corn is a host plant and because cultured cells minimize several complications associated with transport experiments in the highly differentiated and multilayered tissues of intact plant organs (2). Cultures of *Zea Mays* cv Black Mexican were grown in Murashige and Skoog's medium (16) supplemented with 1 mg/L 2,4-D. The cells were subcultured (25 ml cells and medium into 50 ml fresh medium) at 4-d intervals. The cultures were maintained on an orbital shaker (160 rpm) in complete darkness at 27°C.

**Tabtoxinine-β-Lactam Transport.** Corn cells were used in transport experiments 4 d after subculturing. The cells were harvested and pretreated for 5 h in 10 mM Mes-BTP (pH 5.5),

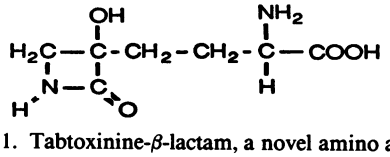

FIG. 1. Tabtoxinine-β-lactam, a novel amino acid.

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[3] Abbreviations: T-β-L, tabtoxinine-β-lactam; BTP, bis-tris-propane; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; GS, glutamine synthetase; NEM, *N*-ethylmaleimide; MSO, methionine sulfoximine.

0.5 mM $CaCl_2$, and 1% sucrose (10 g cells/300 ml). This pretreatment stabilizes cultured cell amino acid transport at maximum rates (10), and preliminary results showed toxin uptake was maximum after this treatment. Unless otherwise stated, transport solutions (7 ml per 0.1 g tissue) contained 10 mM Mes-BTP (pH 5.5), 0.5 mM $CaCl_2$, 0.02 to 0.05 $\mu$Ci [$^{14}$C]T-$\beta$-L, and unlabeled T-$\beta$-L to the desired final concentration (0.14–4.08 mM). Under our experimental conditions (transport periods ≤ 30 min), the cells never removed more than 5% of the available T-$\beta$-L. Transport experiments were initiated by adding cells to well-stirred uptake solutions at 25° C. Aliquots of cells and solution were collected at regular intervals on Whatman No. 1 filter paper mounted on a Millipore filtration apparatus. The uptake solution was aspirated into a 2-L vacuum flask, and the cells were rinsed three times with 10 ml volumes of 10 mM Mes-Tris (pH 5.5), 1 mM $CaCl_2$, and 1 mM glutamine. This rinsing protocol effectively eliminated extracellular toxin, as judged with time zero samples taken immediately after adding the cells to solutions containing $^{14}$C-labeled toxin. The rinsed cells were weighed and placed in a scintillation cocktail, and the accumulated radioactivity was determined with scintillation spectrometry. Known amounts of radioactivity were added to untreated samples to measure the quench associated with this technique.

**Amino Acid Transport.** The same methods used in the toxin transport experiments were used to examine amino acid transport. Briefly, transport solutions (7 ml per 0.1 g tissue) contained 10 mM Mes-BTP (pH 5.5), 0.5 mM $CaCl_2$, up to 0.1 $\mu$Ci [U-$^{14}$C] amino acid and unlabeled amino acid to the desired final concentration. Uniformly labeled glutamate and alanine were obtained from NEN Research Products with specific activities of 294 mCi/mmol and 168 mCi/mmol, respectively. Two time points (10 and 25 min) were routinely taken in the amino acid transport experiments to ensure transport linearity.

**Purification of Tabtoxinine-$\beta$-Lactam.** *Pseudomonas syringae* pv *tabaci* (Pt 113) was grown with agitation at 24°C in a modified Woolley's medium (27) in which 1% glucose was substituted for sucrose as the carbon source. After 42 h growth, $ZnCl_2$ was added (4 mg/L) to the medium to ensure the hydrolytic release of T-$\beta$-L from the pretoxin, tabtoxin (6). Six h after adding the $ZnCl_2$, the cells were removed by centrifugation (9000*g* for 15 min) and T-$\beta$-L was purified from the resulting supernatant using the procedure of Thomas *et al.* (24). The identity of the purified T-$\beta$-L was confirmed by its biological activity (chlorosis induction on tobacco leaves) and co-migration with an authentic T-$\beta$-L standard on an amino acid analyzer. $^{14}$C-labeled T-$\beta$-L was produced using the above protocol with the exception that 5 mCi/L [U-$^{14}$C]glucose was included in the growth medium. The specific activity of the purified T-$\beta$-L was 80 $\mu$Ci/mmol.

**Experimental Replication.** All experiments were repeated at least twice with replicates included in each experiment. The results presented are from one representative experiment. Standard errors never exceeded ±5% of treatment averages.

## RESULTS

**General Characteristics of T-$\beta$-L Transport.** The time course of T-$\beta$-L uptake was linear for up to 30 min (Fig. 2). Consequently, the transport experiments reported here never exceeded this limit, and at least two time points per treatment (generally 15 and 30 min) were used to compute transport rates. T-$\beta$-L uptake exhibited an acidic pH optimum with a sharp drop between pH 6 and 7 (Fig. 3). This optimum corresponds to the pH of the apoplastic space (22). T-$\beta$-L uptake was inhibited by CCCP, a protonophore, and *N*-ethylmaleimide (NEM, a sulfhydryl reagent) (Table I). This result shows that translocation into the cell is linked to metabolic free energy and suggests that toxin uptake may occur via a specific transport system.

We examined T-$\beta$-L transport kinetics to differentiate between simple diffusion and protein-mediated uptake. T-$\beta$-L transport exhibited saturation kinetics, implicating a proteinaceous pathway into the cell (Fig. 4). The Lineweaver-Burk plot of T-$\beta$-L transport curved downward with substrate concentrations exceeding 1 mM (Fig. 5a). This result shows that T-$\beta$-L transport does not fit the simple Michaelis-Menten kinetic model.

**Transport via An Amino Acid System.** T-$\beta$-L is a novel amino acid containing a $\beta$-lactam ring (Fig. 1). Because of its structural similarity to common amino acids, we hypothesized (1) that T-$\beta$-L is transported into the cell via an amino acid transport

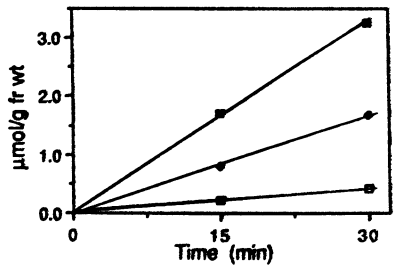


FIG. 2. Tabtoxinine-$\beta$-lactam transport into cultured corn cells as a function of time. The uptake solution 10 mM Mes-BTP (pH 5.5) and 0.1 mM $CaCl_2$. Toxin concentrations were (■), 4 mM; (◆), 1 mM; and (□), 0.14 mM.

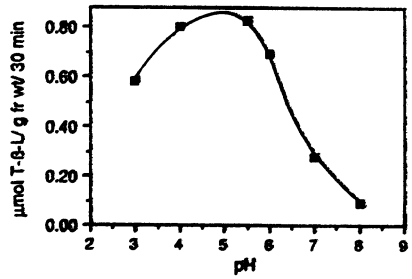


FIG. 3. Tabtoxinine-$\beta$-lactam transport into cultured corn cells as a function of pH. The uptake solution included 0.34 mM T-$\beta$-L and 0.1 mM $CaCl_2$. To adjust the solution pH to the desired levels, specific treatments included: (a) pH 3 and 4, HCl; (b) pH 5.5 and 6, 10 mM Mes with BTP used to adjust the final pH; (c) pH 7 and 8, 10 mM BTP with Mes used to adjust the final pH.

Table I. *Inhibitor and Amino Acid Competition Effects on Tabtoxinine-$\beta$-Lactam Transport*

The transport solutions included 10 mM Mes-BTP (pH = 5.5), 0.1 mM $CaCl_2$, and 0.34 mM $^{14}$C-labeled T-$\beta$-L (0.02 $\mu$Ci/treatment). For inhibitor experiments, the cells were pretreated for 15 min before adding the toxin and with *N*-ethylmaleimide, this inhibitor was washed out before measuring toxin transport. Control toxin transport was 0.82 $\mu$mol T-$\beta$-L/g fresh weight/30 min.

| Treatment | T-$\beta$-L Transport |
|---|---|
| | % |
| Control (0.34 mM T-$\beta$-L only) | 100 |
| Inhibitors | |
| 0.34 mM T-$\beta$-L + 10 $\mu$M CCCP | 10 |
| 0.34 mM T-$\beta$-L + 0.3 mM *N*-ethylmaleimide | 10 |
| Amino Acids | |
| 0.34 mM T-$\beta$-L + 0.3 mM methionine | 31 |
| 0.34 mM T-$\beta$-L + 1.0 mM methionine | 9 |
| 0.34 mM T-$\beta$-L + 1.0 mM alanine | 13 |
| 0.34 mM T-$\beta$-L + 1.0 mM tyrosine | 10 |
| 0.34 mM T-$\beta$-L + 1.0 mM glutamine | 26 |
| 0.34 mM T-$\beta$-L + 1.0 mM glutamate | 66 |
| 0.34 mM T-$\beta$-L + 1.0 mM arginine | 76 |

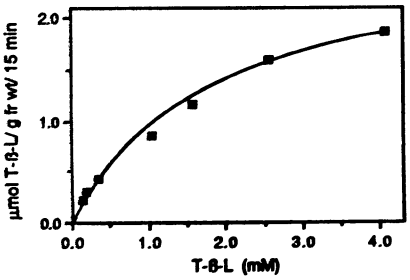


FIG. 4. Tabtoxinine-β-lactam transport into cultured corn cells as a function of concentration. The uptake solution included 10 mM Mes-BTP (pH 5.5), 0.1 mM $CaCl_2$, and T-β-L.

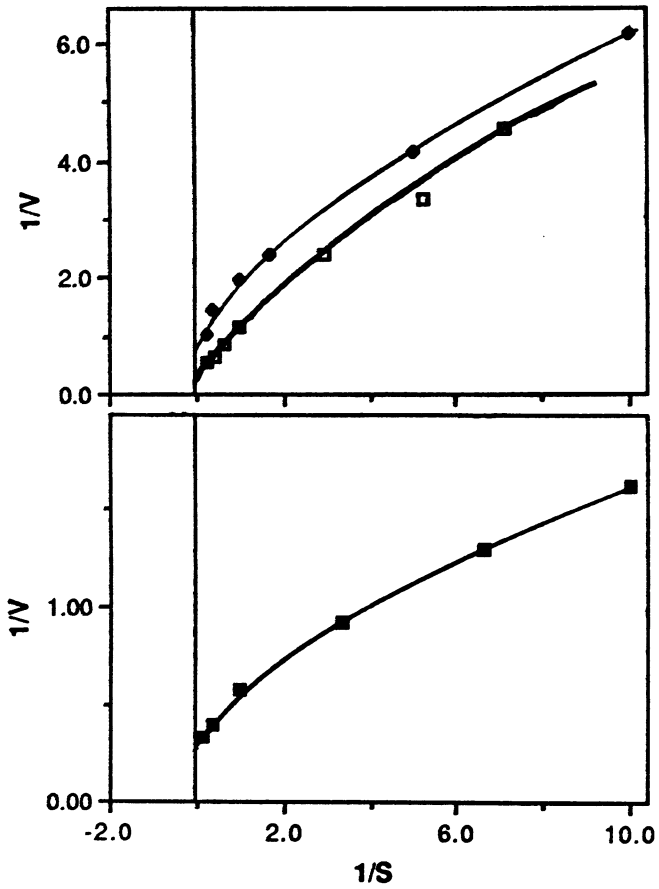


FIG. 5. Lineweaver-Burk plots of tabtoxinine-β-lactam (□), alanine (■), and glutamate (♦) transport into cultured corn cells. The uptake solutions included 10 mM Mes-BTP (pH 5.5), 0.01 mM $CaCl_2$, and $^{14}C$-labeled transport substrate. V = μmol/g fresh weight/15 min; S = mM.

system. The general characteristics of T-β-L transport reported here, *e.g.* acidic pH optimum, a link to metabolic free energy, and complicated kinetics, are similar to the transport characteristics of most common amino acids (19). These observations are consistent with the notion that T-β-L is entering the cell via an amino acid transport system. To probe this correlation in more detail, we examined the effects of several amino acids on T-β-L uptake, and we determined the transport kinetics of alanine and glutamate for comparison with the toxin.

Lineweaver-Burk plots of glutamate and alanine transport (Fig. 5, b and c) exhibited the same downward curvature at high substrate concentrations as seen for T-β-L. We studied these two amino acids because of their abilities to inhibit toxin transport (Table I). Hill numbers for glutamate, alanine, and T-β-L ranged

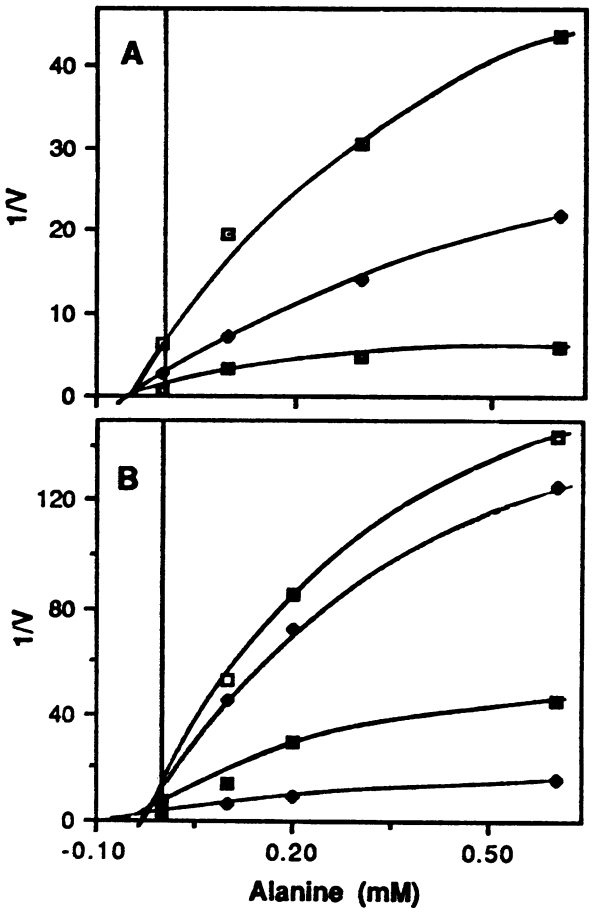


FIG. 6. Dixon plots of alanine competition with tabtoxinine-β-lactam (A) and glutamate (B) transport. The uptake solutions included 10 mM Mes-BTP (pH 5.5), 0.1 mM $CaCl_2$, and $^{14}C$-labeled T-β-L or glutamate at the desired concentrations. A, T-β-L concentrations were: (□), 0.14 mM; (♦), 0.34 mM; and (■), 0.64 mM. B, Glutamate concentrations were: (□), 0.06 mM; (♦), 0.1 mM; (■), 0.2 mM; and (◇), 0.6 mM. V = μmol/g fresh weight/15 min.

from 0.6 to 0.8. These data support the hypothesis that T-β-L is transported via an amino acid transport system.

If T-β-L is transported via an amino acid transport system, one or more common amino acids may compete with T-β-L for that system and, thereby, effectively decrease net toxin uptake. Several amino acids representing a range of structural and charged configurations were tested for their ability to inhibit T-β-L transport. Each amino acid tested inhibited T-β-L uptake (Table I). The relative capabilities of these amino acids to inhibit T-β-L transport were approximately the same as their respective abilities to inhibit lysine transport into cultured tobacco cells (10). These results are consistent with the amino acid transport hypothesis. However, the type of inhibition involved (*e.g.* competitive, noncompetitive, other) cannot be deduced from the results in Table I. Therefore, alanine inhibition of T-β-L and glutamate transport was examined in detail to determine the type of inhibition involved.

Inhibition of T-β-L and glutamate transport by alanine was measured over a range of concentrations for both the amino acid and the inhibitor. Dixon plots of the data were used to differentiate between competitive, noncompetitive, and uncompetitive inhibition (Fig. 6, a and b). Alanine was a competitive inhibitor of both T-β-L and glutamate transport ($K_i$ 20 μM), as shown by the intersection of the curves in the Dixon plot. However, the curvilinear nature of alanine inhibition suggests that an addi-

tional interaction is involved. The Lineweaver-Burk plots of T-β-L, glutamate, and alanine transport began to curve downward when the substrate concentration exceeded 1 mM. Therefore, the alanine inhibition experiments were executed with the summed amino acid concentrations below this transition point and in the linear range of the double reciprocal plots of the individual amino acids (Fig. 5). Even under these conditions, however, the presence of alanine resulted in non-Michaelis-Menten kinetics (Fig. 6), suggesting alanine is affecting glutamate and T-β-L uptake in an undefined manner.

## DISCUSSION

Tabtoxinine-β-lactam uptake and the subsequent inactivation of glutamine synthetase are required steps in the induction of the chlorotic symptoms caused by T-β-L-producing pathovars of *P. syringae*. In this paper, we report the results of a quantitative examination of [$^{14}$C]T-β-L transport into plant cells and conclude that this toxin is transported via an amino acid transport system. Evidence supporting that conclusion is: (a) the general transport characteristics of T-β-L (*e.g.* acidic pH optimum, inhibitor sensitivity, and non-Michaelis-Menten kinetics) were similar to those reported for amino acids (19); (b) the double reciprocal plots of T-β-L transport were similar to those of glutamate and alanine transport; (c) several amino acids inhibited T-β-L uptake; and (d) alanine was a competitive inhibitor of T-β-L and glutamate transport.

The sensitivity of T-β-L and amino acids (5) transport to CCCP and NEM is consistent with several lines of evidence that suggest amino acids are transported into the plant cells via a proton-amino acid symport (19). Since both CCCP and NEM affect the proton motive force across the plasma membrane, CCCP as a protonophore and NEM as an inhibitor of the plasma membrane H-pumping ATPase (11), T-β-L accumulation appears to be mediated by this free energy gradient. In support of that conclusion, we recently presented evidence of proton gradient-dependent amino acid transport in plasma membrane vesicles isolated from zucchini hypocotyls (3).

Complex amino acid transport kinetics in plant tissue have been frequently observed, and consequently, several transport models have been proposed (19). The nonlinear Lineweaver-Burk plots we showed (Fig. 5) are consistent with two interpretations. Downward curving, double reciprocal plots can be diagnostic of negative cooperativity in a complex protein system (17). Alternatively, the presence of two transport systems, one with a low $K_m$ and large $V_{max}$ and the other with a high $K_m$ and low $V_{max}$, could also produce these kinetics (20). Both interpretations have been proposed as models of amino acid transport in plants (19). However, no data have been presented to date that unequivocally differentiate between these two possibilities. Nevertheless, the strong correlation between T-β-L and amino acid transport in these data supports the hypothesis that T-β-L is translocated into the plant cell via an amino acid transport system.

Inhibition of T-β-L uptake by methionine is consistent with a previous study of methionine sulfoximine-resistant tobacco mutants (4). Methionine sulfoximine is a structural analog of T-β-L and also a glutamine synthetase inhibitor. The MSO-resistant tobacco mutants were also resistant to the development of chlorotic symptoms when inoculated with a toxin-producing *P. syringae* pathovar. The protection mechanism employed by the mutant tobacco plants was not determined. However, the mutant tobacco plant contained elevated levels of methionine and normal levels of glutamine, suggesting normal glutamine synthetase function. Carlson (4) suggested that the elevated methionine pool may have protected against MSO uptake. Our results and Carlson's suggestion are consistent with the findings of Meins and Abrams (14) who showed that methionine blocks cellular uptake of MSO, a structural analogue of T-β-L.

Decreased transport of T-β-L in the presence of CCCP and NEM (Table I) suggests that metabolic free energy was expended to accumulate the toxin against a concentration gradient. In support of that conclusion, we estimate (assuming 90% fresh weight equals cell water) T-β-L accumulation at the end of a 30 min experiment to be greater than three times that predicted by concentration alone. This observation is particularly important with regard to the effectiveness of the toxin. If the plant cell did not concentrate the toxin, high extracellular concentrations of T-β-L would be required to ensure an intracellular concentration high enough to inactivate glutamine synthetase. However, since the plant cell accumulates T-β-L against its concentration gradient, the extracellular concentration of toxin can be much lower than that needed to inactivate glutamine synthetase.

T-β-L producing pathovars of *P. syringae*, such as pv tabaci, are capable of devastating a crop within a few days. The ecological success of these pathogens lies not only in the pivotal role of the biochemical target of T-β-L, but also in the exploitation of an amino acid transport system for toxin uptake.

*Acknowledgments*—We thank Dr. Paul Jackson for generously providing the original inoculation of corn cells.

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