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# Modulating plant primary amino acid metabolism as a necrotrophic virulence strategy

## The immune-regulatory role of asparagine synthetase in *Botrytis cinerea*-tomato interaction

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**Keywords:** *Botrytis cinerea,* asparagine synthetase, senescence, necrotrophic pathogen, gray mold, senescence-associated genes, amino acid metabolism

**Abbreviations:** AS, asparagine synthetase; GDH, glutamate dehydrogenase; SAG, senescence-associated gene; hpi, hours post inoculation

The fungal plant pathogen *Botrytis cinerea* is the causal agent of the "gray mold" disease on a broad range of hosts. As an archetypal necrotroph, *B. cinerea* has evolved multiple virulence strategies for inducing cell death in its host. Moreover, progress of *B. cinerea* colonization is commonly associated with induction of senescence in the host tissue, even in non-invaded regions. In a recent study, we showed that abscisic acid deficiency in the *sitiens* tomato mutant culminates in an anti-senescence defense mechanism which effectively contributes to resistance against *B. cinerea* infection. Conversely, in susceptible wild-type tomato a strong induction of senescence could be observed following *B. cinerea* infection. Building upon this earlier work, we here discuss the immune-regulatory role of a key senescence-associated protein, asparagine synthetase. We found that infection of wild-type tomato leads to a strong transcriptional upregulation of asparagine synthetase, followed by a severe depletion of asparagine titers. In contrast, resistant *sitiens* plants displayed a strong induction of asparagine throughout the course of infection. We hypothesize that rapid activation of asparagine synthetase in susceptible tomato may play a dual role in promoting *Botrytis cinerea* pathogenesis by providing a rich source of N for the pathogen, on the one hand, and facilitating pathogen-induced host senescence, on the other.

### Results and Discussion

The amino acid asparagine is one of the major metabolic products in senescing leaves in plants.[1] Asparagine has the highest N:C ratio (2:4) among all amino acids, which makes it an efficient metabolite for nitrogen transport during the process of senescence.[2,3] The major route of asparagine synthesis is governed by the ATP-dependent transfer of the amino group of glutamine to a molecule of aspartate to form glutamate and asparagine, a reaction catalyzed by the enzyme asparagine synthetase (AS).[3] AS is also known as a key senescence associated gene (SAG),[4] with possible roles in plant-pathogen interactions.[5,6] It has been proposed that AS is involved in metabolic alterations that facilitate host cell death during plant-pathogen interactions; hence promoting resistance against biotrophic pathogens. However, such metabolic alterations may also render susceptibility to necrotrophic infections.[7] For instance, an early (5 hpi) induction of *AS1* in pepper was shown to be involved in resistance against the hemibiotrophic bacterial pathogen *Xanthomonas campestris* pv *vesicatoria*, while later (15–20 hpi) induction of the enzyme (during the necrotrophic phase of the pathogen infection) was associated with susceptibility.[8]

Taking advantage of the abscisic acid (ABA)-deficient *sitiens* mutant of tomato, which displays high levels of immunity against the necrotrophic fungus *Botrytis cinerea* (*B. cinerea*),[9-11] our research aims to decipher the molecular underpinnings of necrotroph resistance in plants. In our latest study, we showed that the observed resistance response in *sitiens* is dependent on timely restructuring of the central C/N metabolism, particularly the GABA-shunt.[12] The GABA shunt is a cytosolic-mitochondrial pathway that connects amino acid metabolism to the tricarboxylic acid (TCA) cycle, with possible roles in plant defense mechanisms against phytopathogens.[7] According to our proposed model, concurrent hyper-activation of the cytosolic

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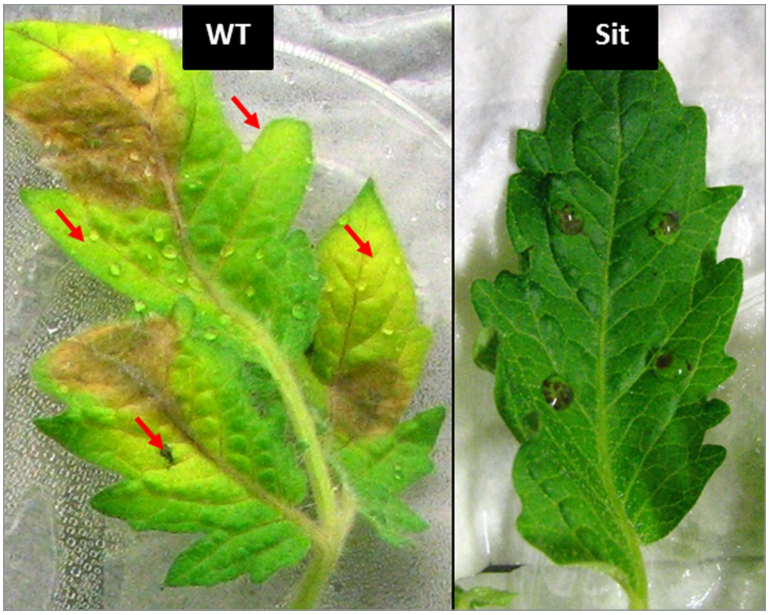



**Figure 1.** Induction of senescence in non-inoculated areas (red arrows) of an infected wild-type (WT) leaf, compared with an inoculated leaf of the *sitiens* (Sit) mutant at 72 hpi. Fifth or sixth leaves of 5-wk-old plants (7 leaf stage) were inoculated according to Curvers et al. (2010)[11] by carefully placing 4 droplets (per leaflet) containing 10-µL of conidial suspension ($5 \times 10^5$ spores $mL^{-1}$ in 0.01 M $KH_2PO_4$ and 6.67 mM Glucose) on the adaxial leaf surface. Inoculated leaves were placed in enclosed trays to retain high relative humidity and incubated at 22 °C under continuous dark conditions.

glutamine synthetase (GS1) and the GABA-shunt in *sitiens* are involved in maintaining basic metabolism in the challenged tissue to tightly control the extent and localization of cell death.[12] As a result, the extent of senescence is spatiotemporally more restricted in the resistant *sitiens* mutant compared with susceptible wild-type tomato plants[12] (**Fig. 1**). In agreement with this, we also found lower levels of total protein

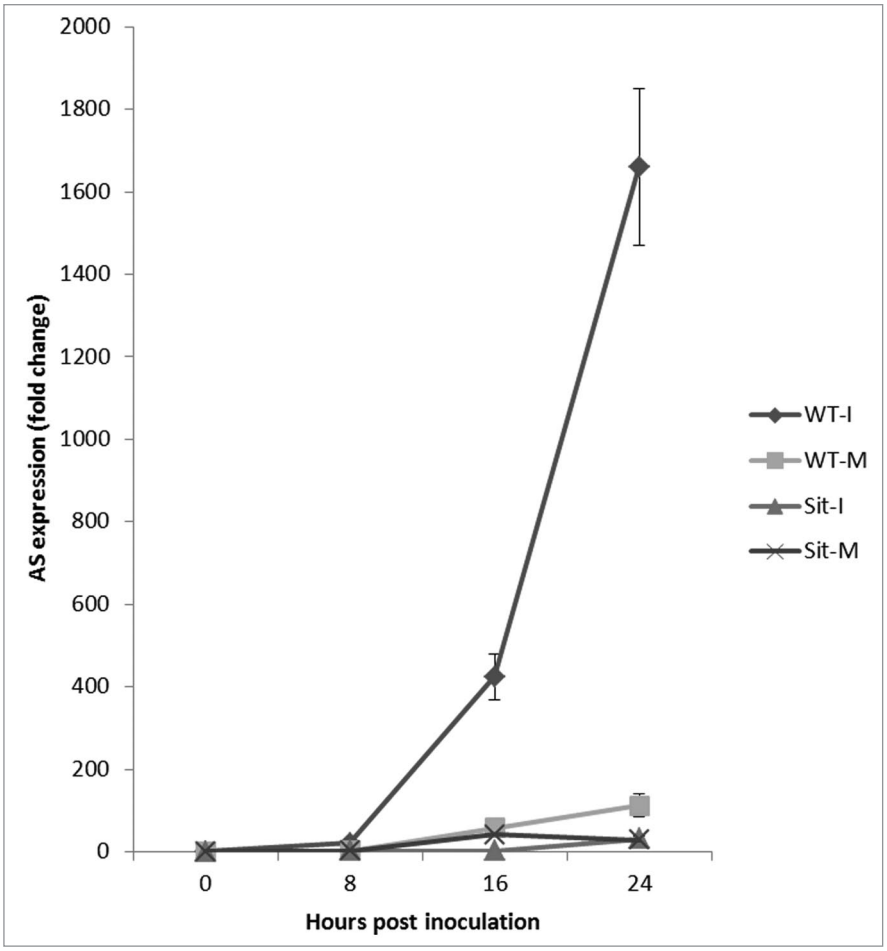
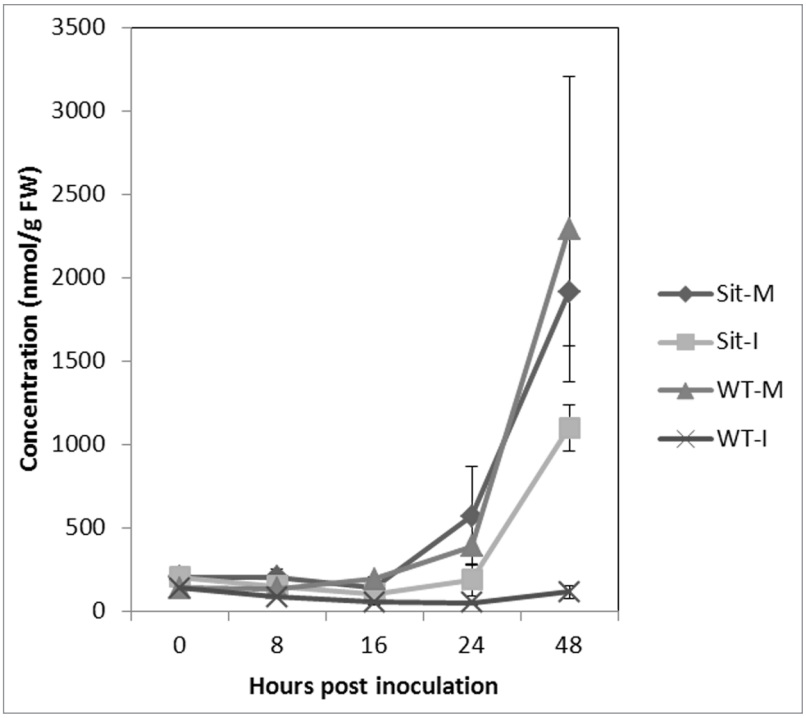



**Figure 2.** Expression of asparagine synthetase in wild type (WT) and *sitiens* (Sit) plants following infection with *B. cinerea* (M: mock; I: infected). The qPCR analysis was performed following the protocol described previously[12] using *AS1*-specific primers; Forward: TCCCCTTTGG TGTTCTGCTC TCG, Reverse: TGCTCCCCAT TGCTTAGCAG C. Error bars represent ± SE of 3 biological replicates.

**Figure 3.** Alterations in asparagine levels in mock-treated (M) and Botrytis-inoculated (I) *sitiens* (Sit) and wild-type (WT) tomato plants at different time points post inoculation. Metabolic analysis of free amino acid levels was conducted as described before.[12] Error bars represent ± SE of 3 biological replicates.




degradation and reduced activation of the SAG, glutamate dehydrogenase (*GDH*), in *sitiens* vs. wild-type seedlings.

Building upon these earlier findings, we sought to extend our analysis by monitoring the expression of another key SAG, asparagine synthetase 1 (AS1; GeneBank accession number AY240926.1). As shown in **Figure 2**, *AS1* showed a tremendous upregulation in the wild-type tomato in response to *B. cinerea* infection, whereas such strong induction was not seen in the *sitiens* mutant (**Fig. 2**). **Figure 3** indicates that the levels of asparagine increased in all mock-treated plants, showing the effect of dark incubation on asparagine accumulation, as suggested previously.[13] Moreover, asparagine titers in *B. cinerea*-inoculated *sitiens* plants

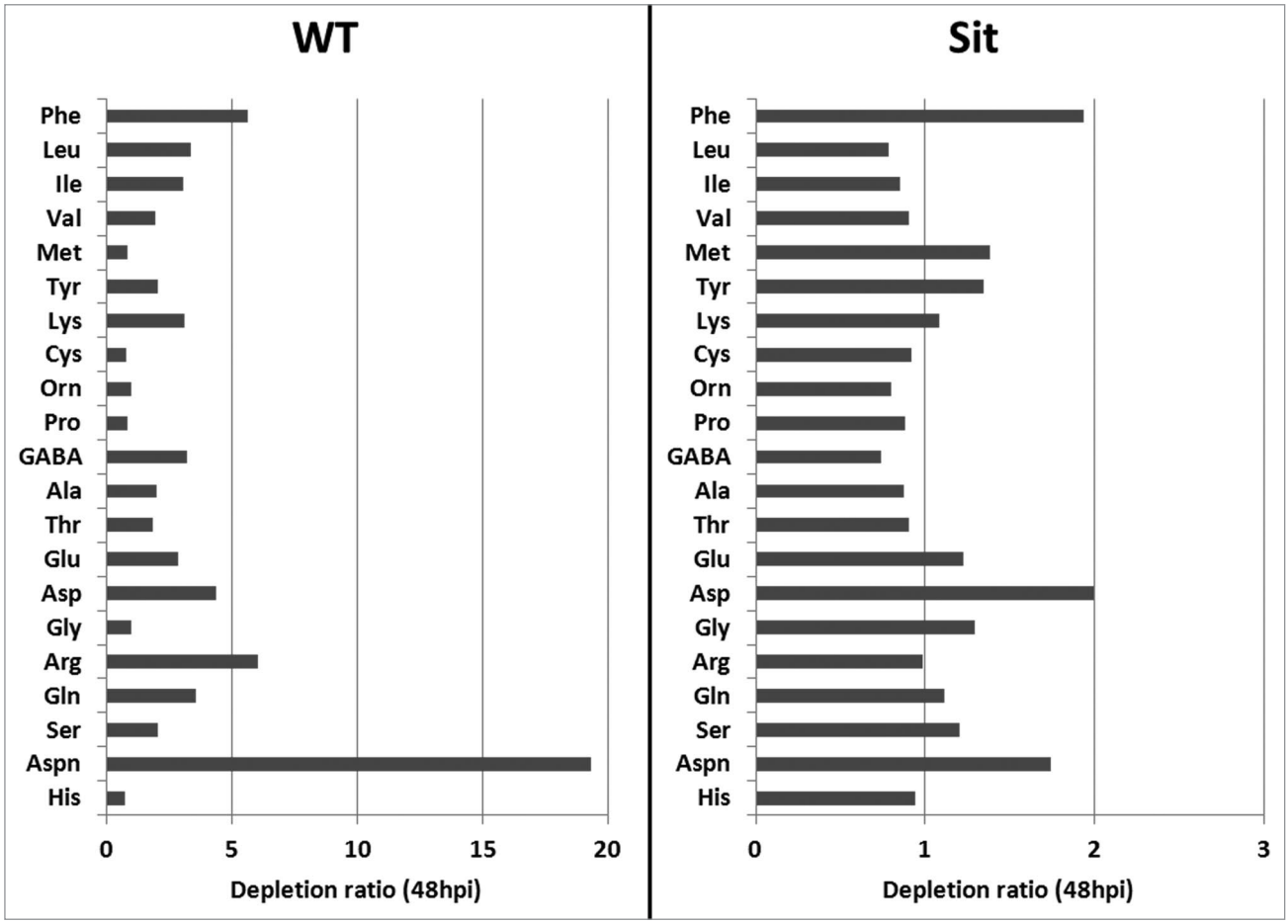



**Figure 4.** Amino acid depletion ratios (levels in mock vs. infected samples) in Botrytis-infected *sitiens* (Sit) and wild-type (WT) tomato at 48 hpi. Among all amino acids, asparagine displays the most marked depletion in the wild-type plant.

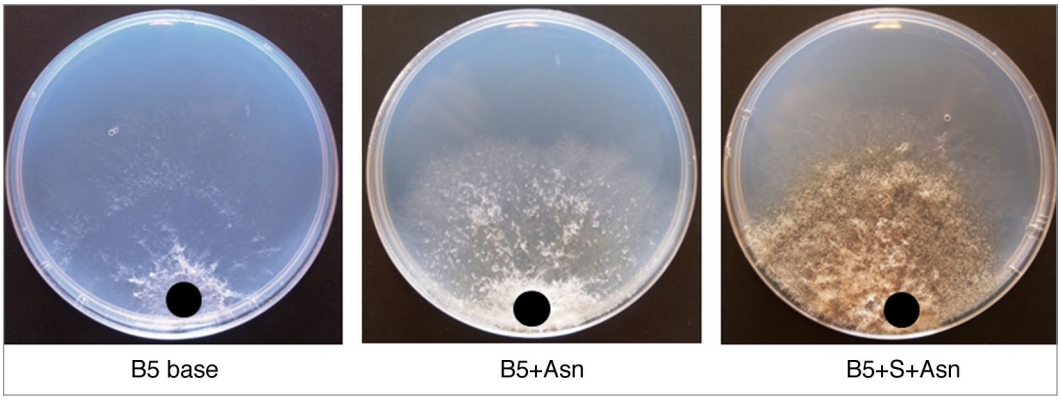



**Figure 5.** In vitro effect of asparagine (Asn, 5mM) on the growth and sporulation of *B. cinerea* grown on Gamborg B5 salt base medium (B5 base: with no carbon and nitrogen sources). Sucrose (S) was used as carbon source. The black circles indicate the place of the inoculation plaque.

increased significantly during the course of infection (**Fig. 3**), whereas infected wild-type seedlings showed a remarkable drop in asparagine content (**Fig. 3**). Particularly, the highest level of depletion of asparagine can be seen at 48 hpi in the wild-type plant, while such a severe depletion did not occur for other amino acids (**Fig. 4**). Finally, an in vitro assay revealed that asparagine is a perfect nitrogen (N) source for *B. cinerea*, resulting in rapid mycelial growth and dense fungal sporulation (**Fig. 5**).

Induction of senescence in the host is a well-studied pathogenicity mode for the necrotrophic pathogen *B. cinerea*.[14] Accordingly, it appears that in wild-type tomato susceptibility to *B. cinerea* is strongly correlated with induction of classic SAGs, including AS. AS has also been shown to be an ABA-responsive gene that is upregulated in response to different abiotic stresses.[15,16] This might explain why in the ABA-deficient *sitiens* mutant, compared with the wild-type plant, the AS gene is only slightly induced upon *B. cinerea* infection. Moreover, the relatively low level of AS activation in *sitiens* might consequently prevent the cell N reservoir (glutamine and aspartate) from being converted to transportable forms of amino acid (asparagine), thus providing more cytosolic supply for the GABA-shunt-mediated, anti-senescence defense response seen in the mutant.[12]

Previous studies revealed that, depletion in sugar levels in tomato plants subjected to prolonged dark conditions also result in strong activation of the AS gene.[17] Therefore, the strong induction of AS in *B. cinerea*-infected wild-type tomato (**Fig. 2**) could be explained through both catabolization of the host apoplastic sugar content by the pathogen, and continuous incubation of tomato leaves in darkness. Interestingly, *B. cinerea*-induced upregulation of AS in the wild-type plant seems to be followed by strong depletion of asparagine content in the infected leaves, suggesting a causal link between both phenomena. Having the highest N content among all amino acids, asparagine may serve as a rich N source for *B. cinerea* in vitro (**Fig. 5**), and possibly also in planta. In view of these findings, it is tempting to speculate that strong induction of AS in susceptible tomato plants plays a dual role in the pathogenicity of *B. cinerea* by both facilitating pathogen-initiated host senescence and providing a rich N reservoir to support fungal growth in planta. Future studies should be focused on exploring the molecular mechanism(s) by which *B. cinerea* targets AS and manipulates the plant's primary metabolism for its own benefit.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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