Nicotiana benthamiana asparagine synthetase associates with IP-L and confers resistance against tobacco mosaic virus via the asparagine-induced salicylic acid signalling pathway

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Abstract
Asparagine synthetase is a key enzyme that catalyses the conversion of amide groups from glutamine or ammonium to aspartate, which leads to the generation of asparagine. However, the role of asparagine synthetase in plant immunity remains largely unknown. Here, we identified a Nicotiana benthamiana asparagine synthetase B (NbAS-B) that associates with tomato mosaic virus coat protein-interacting protein L (IP-L) using the yeast two-hybrid assay and examined its role in tobacco mosaic virus (TMV) resistance. The association of IP-L with NbAS-B was further confirmed by in vivo co-immunoprecipitation, luciferase complementation imaging, and bimolecular fluorescence complementation assays. IP-L and NbAS-B interact in the nucleus and cytosol and IP-L apparently stabilizes NbAS-B, thus enhancing its accumulation. The expressions of IP-L and NbAS-B are continuously induced on TMV-green fluorescent protein (GFP) infection. Co-silencing of IP-L and NbAS-B facilitates TMV-GFP infection. Overexpression of NbAS-B in tobacco reduces TMV-GFP infection by significantly improving the synthesis of asparagine. Furthermore, the external application of asparagine significantly inhibits the infection of TMV-GFP by activating the salicylic acid signalling pathway. These findings hold the potential for the future application of asparagine in the control of TMV.

Keywords
asparagine, asparagine synthetase B, CP-interacting protein-L, salicylic acid signalling pathway, tobacco mosaic virus

1 | INTRODUCTION
Over thousands of years, plants and pathogens have co-evolved antagonistically. This co-evolutionary battle enables plants to develop multilayered defence mechanisms and allows the pathogen to subvert host defences with a corresponding arsenal of counterstrategies (Bent & Mackey, 2007, Ma et al., 2012a). Plant pathogens have evolved virulence factors that interact with host proteins to interfere with host physiological processes and accelerate their infections (Regenmortel & Mahy, 2009). Plant viruses, as the second major pathogens of plants, cause significant annual yield losses ranging from 10% to 15% worldwide (Regenmortel & Mahy,
Plant viruses use host nutrient metabolism to complete their replication, intercellular movement, and systemic movement, resulting in the development of symptoms in the host plant (Maarouf et al., 2009; Zhao et al., 2016).

**Tobacco mosaic virus** is a member of the *Tobamovirus* genus and has a close serological relationship with *Tomato mosaic virus*. Tobacco mosaic virus (TMV) infects a wide range of plants, including many crops such as Solanaceae, brassicas, and cucurbits (Brunt, 1986; Pfiznner, 2006). A significant increase in the accumulation level of TMV coat protein (CP), accounting for 7% of the total plant protein, after TMV infection indicates that CP is crucial for TMV infection (Asumendi et al., 2007; Brunt, 1986; Pfiznner, 2006). The viral CP protein as a virulence factor plays an important role in the process of virus infection. It can protect viral nucleic acid and recognize the host cell, as well as participating in the long-distance transport of virions (Carrington et al., 1996). CP is required for the protective coat and long-distance movement to promote virus infection (Heinlein, 2003; Waignmann et al., 2007). A number of studies have shown that CP can affect host disease resistance and thereby regulate virus infection. Tobacco mosaic virus (ToMV) CP can interact with ferredoxin I (FdI), resulting in reduced accumulation of FdI that is associated with the development of the mosaic symptom in tobacco (Ma et al., 2008; Sun et al., 2013). The CP of the cucumber mosaic virus (CMV)-M strain associates with the precursor of FdI in the cytoplasm and impedes the transport of FdI into chloroplasts, resulting in the inhibition of FdI functions during viral infection (Qiu et al., 2018). Potato virus X (PVX) CP is able to interact with NbPCIP1, which functions as a susceptibility factor to promote PVX infection (Park et al., 2009). A recent study showed that TMV CP negatively regulates the salicylic acid (SA) signalling pathway to enable long-distance transport of the virus (Venturuzzi et al., 2021).

**CP-interacting protein-**L (IP-L) is a host factor that interacts with ToMV CP (Li et al., 2005; Zhang et al., 2008), which might facilitate the long-distance movement of virus, and participates in photosynthesis, electron transport, and adenosine triphosphate (ATP) synthesis (Karlsson et al., 2015; Shikanai, 2014). The N-terminal helical region (amino acid residues 3–18) of IP-L is required for the interaction between IP-L and ToMV CP, while two α-helical domains (amino acid residues 21–31 and 142–147) of ToMV CP are involved in this interaction (Zhang et al., 2008). ToMV infection significantly induces the expression and accumulation of IP-L protein in *Nicotiana benthamiana* leaves (Peng et al., 2017). IP-L localizes in the thylakoid membrane, while ToMV CP and IP-L colocalize in the cytosol of *N. benthamiana* (Peng et al., 2017; Zhang et al., 2008). More recently, Liu et al. found that the expression of IP-L is regulated by light and IP-L regulates viral infection and photosynthesis by affecting the expression of *PsbO* (Liu et al., 2021). Furthermore, IP-L homologous protein NbPCIP1 can interact with PVX CP and positively regulates PVX RNA replication in *N. benthamiana* (Park et al., 2009). However, the role of IP-L in host disease resistance remains uncovered.

Asparagine synthetase (AS) is a catalytic enzyme that converts aspartic acid to asparagine by using the energy of ATP hydrolysis and glutamine as a nitrogen source (Boehlein et al., 1994; Larsen et al., 2000). Asparagine, as one of the main amino acids, plays an important role in long-distance nitrogen transport in both the xylem and phloem of plants (Wang et al., 2005). Thus, AS is regarded as a key enzyme that participates in nitrogen assimilation. In plants, only asparagine synthetase-B (AS-B) is found, and it can be subdivided into two classes in accordance with its expression under light (Luo et al., 2018; Qu et al., 2019). Previous studies showed that nitrogen, as one of the basic plant metabolisms, is involved in plant-pathogen interactions (Delledonne et al., 2001; Qiao & Fan, 2008; Wendehenne et al., 2001). Expression of AS is rapidly induced in tomato leaves in response to *Pseudomonas syringae*, as well as the accumulation of a single AS polypeptide being observed (Lea et al., 2006; Olea et al., 2004). The glutamate-dependent AS assimilates ammonium, which is released from both protein degradation and amino acid deamination into asparagine in the diseased tissue (Olea et al., 2004). Silencing AS in *N. benthamiana* compromises the hypersensitive response (HR) mediated by *Cladosporium fulvum* 4 (CF-4)/Avr4 and induced by the nonadapted bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo), indicating that AS might contribute to plant immune response (Xu et al., 2012). So far, several studies have reported that AS is involved in the biological processes in response to stress (Gaufichon et al., 2015; Joy et al., 1983; Olea et al., 2004; Xu et al., 2012). However, the antiviral function of AS remains unknown.

In this study, we report the identification of *N. benthamiana* AS-B (NbAS-B) as an interacting protein of IP-L. Cosilencing of IP-L and NbAS-B promotes TMV infection, while transient overexpression of NbAS-B enhances the resistance against TMV, suggesting that NbAS-B positively regulates plant defense against TMV infection. NbAS-B functions as an asparagine synthetase that increases asparagine content, resulting in asparagine-mediated resistance against TMV by activating the SA signalling pathway.

## RESULTS

### 2.1 IP-L is continuously induced by TMV infection

Previous studies showed that IP-L interacts with ToMV CP and contributes to the long-distance movement of ToMV (Li et al., 2005). To investigate the role of IP-L in TMV infection, we quantified the expression changes of IP-L in *N. benthamiana* after inoculation with different viruses, including green fluorescent protein (GFP)-tagged TMV (TMV-GFP), GFP-tagged tobacco rattle virus (TRV-GFP), GFP-tagged PVX (PVX-GFP), and GFP-tagged turnip mosaic virus (TuMV-GFP) at 3, 6, and 9 days postinoculation (dpi). First, we determined the systemic spread of examined viruses from inoculated leaves to upper leaves. Reverse transcription PCR (RT-PCR) analysis showed that upper leaves were systemically infected by TMV at 3 dpi, but by TRV, PVX, and TuMV at 6 dpi (Figure S1). Reverse transcription quantitative PCR (RT-qPCR) was used to detect IP-L expression on systemically infected leaves at 6 and 9 dpi, and phosphate-buffered saline (PBS) was used as a negative control. We observed that the mRNA expression of IP-L is significantly induced by TMV-GFP infection.
at 6 and 9 dpi (Figure 1a). However, the expression levels of IP-L decreased at 6 days after PVX and TuMV infections (Figure 1b–d). Interestingly, at 9 dpi the expression of IP-L was significantly induced by TRV and TuMV infections (Figure 1c,d). In addition, we found that TMV U1 infection resulted in increased accumulation of Myc-tagged IP-L (Myc:IP-L) protein, but not GFP (Figure 1e), implying that IP-L is stabilized during TMV U1 infection. Next, we used the TRV-mediated silencing technique to silence N. benthamiana IP-L and found that silencing IP-L inhibited TMV-GFP infection, while PVX-mediated overexpression of IP-L promoted TMV-GFP infection, suggesting that IP-L promotes TMV-GFP infection (Figure S2).

2.2 IP-L interacts with NbAS-B in vitro and in vivo

To identify IP-L-interacting proteins from N. benthamiana, IP-L was used as a bait for a yeast two-hybrid (Y2H) protein screening against a prey cDNA library prepared from N. benthamiana. Nineteen IP-L-interacting candidate clones were obtained from the screening. Sequencing analysis showed that 11 clones had identical sequences, and NCBI BLAST database searching found that they exhibit high homology with asparagine synthetase B (AS-B). A SMART study showed that it contains three conserved domains: unknown function 3,200 (DUF 3,200), GATase 6, and GATase 7, which are similar to other AS-B proteins like Glycine max AS-B (GMU55874) and Morus alba AS (HQ025955). In addition, RT-qPCR showed that the highest expression of NbAS-B was observed in N. benthamiana leaves (Figure S3).

To confirm the interaction between IP-L and NbAS-B, Y2H was performed. Figure 2a showed that yeasts co-expressing the pGBK-T7:IP-L and pGADT7:NbAS-B combinations developed blue colouration on selective medium, even at a dilution ratio of 10⁻³, indicating that an interaction occurs between both proteins. To further confirm the interaction of IP-L with NbAS-B in vivo, a co-immunoprecipitation (Co-IP) assay was performed. 7Myc-tagged NbAS-B (Myc:NbAS-B) co-immunoprecipitated with eGFP-tagged IP-L fusion protein (eGFP:IP-L) (Figure 2b). Next, the interaction between IP-L and NbAS-B in plants was validated by luciferase complementation imaging (LCI) assay. N-terminal luciferase-tagged IP-L (nLUC:IP-L) was transiently co-expressed with C-terminal luciferase-tagged NbAS-B (NbAS-B:cLUC) in N. benthamiana. Strong luciferase (LUC) activity was detected in the combination of IP-L and NbAS-B after adding luciferin, the substrate for firefly LUC (Figure 2c), and the fluorescence intensity of the nLUC:IP-L + NbAS-B:cLUC was seven times higher as compared with the control groups (Figure S4). Finally, the interaction was confirmed using bimolecular fluorescence complementation (BiFC) in planta. As shown in Figure 2d, marked increases of yellow fluorescent signals were observed in cells co-expressing C-terminal yellow fluorescent protein (YFP)-tagged IP-L (IP-L:yFP) and N-terminal YFP-tagged NbAS-B (nYFP:NbAS-B) as well as in the positive control.
co-expressing AC2:cYFP and nYFP:AV1, but not with the negative controls: IP-L:cYFP and nYFP, and cYFP and nYFP:NbAS-B. The fluorescence intensity of nYFP:AV1 + AC2:cYFP and IP-L:cYFP + nYFP:NbAS-B was 50 times higher than the controls (Figure S5). We transiently expressed NbAS-B tagged with mCherry (mCherry:NbAS-B) and eGFP:IP-L together or in a separate way in the leaves of *N. benthamiana*. Plasma membrane marker AtROP10 tagged with a cyan fluorescent protein (AtROP10:CFP) was included as a positive control (Chen et al., 2017). Figure 3 showed that mCherry:NbAS-B and eGFP:IP-L colocalize in the plant cytosol and nucleus. Collectively, these findings strongly support that NbAS-B physically associates with IP-L, and both colocalize in the plant nucleus and cytosol.
2.3 | NbAS-B is continuously induced by TMV infection

To investigate the transcriptional changes of NbAS-B in *N. benthamiana* upon virus infection, we quantified the expression of NbAS-B on systemically infected leaves with different viruses, including TMV-GFP, TRV-GFP, PVX-GFP, and TuMV-GFP at 6 and 9 dpi. PBS was used as a negative control. We observed that the expression of NbAS-B was significantly induced at 6 dpi on TMV-GFP infection and continuously induced at 9 dpi (Figure 4a). However, after PVX-GFP, TRV-GFP, and TuMV-GFP infection, the expression of NbAS-B first decreased at 6 dpi and then significantly increased.
at 9 dpi (Figure 4b–d). In addition, Agrobacterium tumefaciens carrying Myc:NbAS-B was infiltrated into the leaves of N. benthamiana at 2 days after TMV infection, and the accumulation level of Myc:NbAS-B protein was determined by western blotting. Figure S6 showed that the accumulation level of Myc:NbAS-B protein in TMV U1-inoculated leaves was much higher than that in the leaves of the mock-inoculated plant. Our observations indicate that NbAS-B is continuously induced by TMV infection, and NbAS-B protein can be stabilized during TMV infection, resulting in increased accumulation of NbAS-B protein in the infected leaves.

2.4 | Silencing of NbAS-B enhances TMV infection

To further study the role of NbAS-B in TMV infection, TRV-induced gene silencing was employed to knock down the expression of NbAS-B in N. benthamiana. Fourteen days after infiltration of A. tumefaciens carrying either TRV:00 (empty vector) or TRV:NbAS-B, the expression of NbAS-B was quantified by RT-qPCR. As shown in Figure 5b, the expression level of NbAS-B in TRV:NbAS-B silenced plants was only 28% of that in the control plants. Next, we inoculated TMV-GFP by rubbing and the infection of TMV-GFP was observed under ultraviolet (UV) light at 2, 4, and 7 dpi. Figure 5a showed that at 2 dpi, GFP fluorescence signals were not visualized in the systemic leaves of silenced and control plants. The amount of TMV-GFP transcripts in the silenced plant was significantly higher than that in the control plants (Figure 5c). At 4 dpi, GFP fluorescence expanded to the upper leaves in the silenced plant, while no GFP signals were observed in the upper leaves of control plants. At 7 dpi, the GFP signals fully expanded to the upper leaves in the silenced plant. However, in the upper leaves of the control plant, limited GFP signals were visualized (Figure 5a). RT-qPCR analysis showed that a significant amount of TMV-GFP transcripts accumulated in the leaves of the silenced plant compared to that in the control plant at 4 and 7 dpi (Figure 5c). Furthermore, the western blotting analysis showed that the accumulation of TMV-GFP protein in the leaves of the silenced plant at 4 dpi was much higher than that in the control plant (Figure 5d). Based on these observations, we concluded that NbAS-B positively regulates the plant defence against TMV-GFP infection.
2.5 | Overexpression of NbAS-B reduces TMV infection

To understand the further role of NbAS-B in TMV infection, we employed the PVX-mediated overexpression system to transiently overexpress NbAS-B in *N. benthamiana* plants. Empty vector PVX:00 was used as a control. Fifteen days after agroinfiltration, RT-qPCR was used to quantify the expression of NbAS-B in infiltrated plants. As shown in Figure 5f, NbAS-B showed a significantly high level of expression in the PVX:NbAS-B plants compared to that in the control plant inoculated with PVX:00, indicating that the increased expression of NbAS-B was successfully achieved in *N. benthamiana*. Subsequently, we inoculated TMV-GFP to PVX:NbAS-B and PVX:00 plants, and observed TMV-GFP infection under UV light in the inoculated leaves at 2 dpi and in the upper leaves at 4 and 7 dpi. Figure 5e showed that at 2 dpi marked GFP fluorescent spots were observed in the inoculated...
leaves of PVX:00 plants compared with that in the PVX:NbAS-B plant. RT-qPCR also showed that the number of TMV-GFP transcripts in PVX:00 was significantly higher than that in PVX:NbAS-B (Figure 5g). At 4 dpi, strong GFP signals largely expanded to the upper leaves of the PVX:00 plant, while weak and limited GFP signals were observed in the upper leaves of the PVX:NbAS-B plant (Figure 5e). At 7 dpi, GFP signals fully spread to the upper leaves of the PVX:00 plant and reached the fourth leaf, but the GFP signals in the upper leaves of the PVX:NbAS-B plant were not fully expanded and were confined to the third leaf (Figure 5e). Furthermore, RT-qPCR analysis showed that the relative abundance of TMV-GFP transcripts in the upper leaves of the PVX:NbAS-B plant was significantly lower than that in the PVX:00 plant at 4 and 7 dpi (Figure 5g). Similarly, the western blot analysis confirmed that a reduced amount of TMV-GFP protein was detected in the leaves of the PVX:NbAS-B plant compared with that in the PVX:00 plant at 4 dpi (Figure 5h). These findings indicate that overexpression of NbAS-B can significantly reduce the infection of TMV.

2.6 | Cosilencing of IP-L and NbAS-B promotes TMV infection

IP-L promotes TMV infection, but NbAS-B inhibits TMV infection. To test whether cosilencing IP-L and NbAS-B influences TMV infection, we generated a IP-L/NbAS-B cosilencing construct (Figure 6a) and transiently silenced them in N. benthamiana. Figure 6c,d showed that 14 days after agroinfiltration, the amounts of IP-L and NbAS-B transcripts were reduced to about 20% and 17% of that in the control plant, respectively. The silenced plants were inoculated with TMV-GFP by rubbing and the development of TMV-GFP infection was observed under UV light at 4, 6, and 8 dpi. Interestingly, we found that increased GFP signals were observed in the systemic leaves of the cosilenced plant at 4, 6, and 8 dpi, indicating that cosilencing of IP-L and NbAS-B promoted TMV infection (Figure 6b). Furthermore, the accumulation of TMV-GFP transcripts and protein was determined by RT-qPCR and western blotting, respectively. The results showed that the amount of TMV-GFP transcripts significantly increased in the leaves of the cosilenced plant compared to that in the control plant at 4, 6, and 8 dpi (Figure 6e). Similarly, the accumulation of TMV-GFP protein increased in the leaves of the cosilenced plant at 4 dpi (Figure 6f). These findings are consistent with the observations of the single silencing of NbAS-B, suggesting that silencing NbAS-B can overcome the consequences of silencing IP-L.

2.7 | NbAS-B is a functional asparagine synthetase and can be stabilized by IP-L

Under the action of AS, asparagine (Asn) is synthesized by the conversion of amide groups from glutamine or ammonium to aspartate (Boehlein et al., 1994). As TMV infection increased the expression of NbAS-B, we examined the AS activity both in the systemic leaves of N. benthamiana plants inoculated with TMV at 5 dpi and in the leaves transiently overexpressing NbAS-B or NbAS-B and IP-L together by using Naismith’s reagent (Chai et al., 1996; Shanghai Plant Physiology Society, 1982). As shown in Figure 7a, the activity of AS significantly increased on TMV infection as well as in the leaves with the increased accumulation of NbAS-B protein or the increased accumulation of NbAS-B and IP-L protein compared to the leaves overexpressing empty vector Myc:00. Asn content in the systemic leaves of PVX-mediated overexpression of NbAS-B plants significantly increased compared to that in the control plant (Figure 7b). These findings strongly confirm that NbAS-B has asparagine synthetase activity and is responsible for the increased content of Asn.

Based on the presented evidence that IP-L interacts with NbAS-B, we hypothesized that IP-L might stabilize NbAS-B accumulation. To support our hypothesis, we studied the stabilization analysis of NbAS-B by IP-L. mCherry:NbAS-B and eGFP:IP-L were co-expressed in N. benthamiana using agroinfiltration. Co-expression of eGFP:IP-L and mCherry:00 or eGFP:00 and mCherry:NbAS-B served as controls. As shown in Figure 7c, mCherry:NbAS-B accumulated to a significantly higher level in the presence of eGFP:IP-L than the GFP control, but IP-L displayed similar accumulations in the absence or presence of NbAS-B. Ubiquitin/26S proteasome is one of the main
pathways involved in plant protein degradation (Dreher & Callis, 2007; Li et al., 2019; Wang et al., 2020). We investigated the accumulation of NbAS-B in the presence or absence of MG132, a proteasome inhibitor. Dimethyl sulphoxide (DMSO) was used as a negative control. *N. benthamiana* leaves co-expressing Myc:NbAS-B and eGFP:IP-L or eGFP were treated with proteasomal inhibitor MG132 or DMSO at 24 h after infiltration. As shown in Figure 7d, Myc:NbAS-B accumulated at a similar level in the leaves co-expressing eGFP:IP-L and Myc:NbAS-B in the presence of MG132 compared with the leaves co-expressing eGFP:IP-L and Myc:NbAS-B and treated with DMSO (Figure 7d and Figure S7). MG132 application resulted in a slightly increased accumulation of Myc:NbAS-B in the leaves co-expressing eGFP and Myc:NbAS-B. However, a significantly decreased accumulation of Myc:NbAS-B was observed in the leaves co-expressing eGFP and Myc:NbAS-B without application of MG132 (Figure 7d and Figure S7), suggesting that IP-L can stabilize NbAS-B by preventing its degradation through the ubiquitin-mediated 26S proteasome pathway.

2.8 | Asparagine induces resistance against TMV by activating the SA signalling pathway

To further investigate the role of NbAS-B in anti-TMV, we determined the anti-TMV activity of Asn. The leaves of *N. benthamiana* plants were sprayed with exogenous asparagine (Asn) or water, and 3 days after spraying TMV was inoculated by rubbing. The development of TMV-GFP infection was observed under UV light at 3, 5, and 7 dpi. As shown in Figure 8a, at 3 dpi the GFP fluorescent intensity in the inoculated leaves of Asn-treated plants was significantly lower than that of the water-treated plants. At 5 dpi, sporadic fluorescence signals appeared in the systemic leaves of water-treated plants, but
no fluorescence signals appeared in the systemic leaves of Asn-treated plants. At 7 dpi, the GFP fluorescence in the Asn-treated leaves expanded to half of the second young leaf, while the fluorescence of water-treated plants fully expanded to the second and third young leaves. RT-qPCR and western blotting were employed to determine the accumulation of TMV-GFP transcripts and protein, respectively. Figure 8b showed that the relative abundance of TMV-GFP transcripts significantly decreased in the leaves treated with Asn compared to that in the control plant at 3, 5, and 7 dpi. Similarly, the accumulation of TMV-GFP protein decreased in the leaves treated with Asn.
with Asn at 5 dpi (Figure 8c). Collectively, these results suggest that Asn has anti-TMV activity.

To further determine the molecular mechanism underlying the Asn-induced resistance against TMV, we quantified the expressions of SA-responsive genes, including nonexpressor of pathogenesis-related gene 1 (NPR1), pathogenesis-related gene 1 (PR1), pathogenesis-related gene 2 (PR2) (Zhu et al., 2012), jasmonic acid (JA)-responsive gene MYC1α, JA receptor coronatine insensitive 1 (COI1), and ethylene response factor 1 (ERF1), by RT-qPCR in the NbAS-B silenced plants and Asn-treated plants, respectively (Liu et al., 2020; Yan et al., 2009). Interestingly, the expressions of SA-responsive genes PR1 and PR2 were significantly down-regulated in NbAS-B silenced plants, while Asn application significantly increased the expressions of PR1 and PR2 genes (Figures 8d,e, S8 and S9). In addition, NbAS-B was induced on the application of methyl salicylate (MeSA), a derivative of SA, at 2 h after treatment, peaked at 6 h and decreased to normal level at 10 h (Figure 8f). To gain further insights into whether SA is involved in Asn-induced TMV resistance, we pretreated the NahG transgenic N. benthamiana plants, overexpressing a bacterial NahG gene encoding SA hydroxylase to reduce
Asparagine participates in anti-TMV activity by activating the salicylic acid (SA) signalling pathway. (a) External application of asparagine (Asn) inhibited TMV-GFP development. The plants were sprayed with Asn or water and TMV was inoculated at 3 days after spraying. The green fluorescence signals representing TMV infection in the inoculated and systemic leaves were visualized at 3, 5, and 7 days postinoculation (dpi) under ultraviolet (UV) light. Representative pictures are shown. (b, c) The number of TMV-GFP transcripts and the accumulation of TMV-GFP protein significantly decreased in the Asn-treated plants compared to the control plants. The expression of TMV-GFP was quantified by reverse transcription quantitative PCR (RT-qPCR) at 3, 5, and 7 dpi in the systemic leaves and TMV-GFP protein accumulation was detected by western blot at 5 dpi. Coomassie brilliant blue (CBB) showed an equal amount of protein loaded in each well. (d) Silencing NbAS-B reduced the expression of SA-responsive genes PR1 and PR2. The expressions of PR1 and PR2 were quantified by RT-qPCR and actin was used as an internal reference. (e) External application of Asn induced the expressions of PR1 and PR2. The plants were treated with Asn and water-treated plants were included as the negative control. (f) Methyl salicylate (MeSA) treatment induced the expression of NbAS-B. The plants were treated with 0.1 mM MeSA and the expression of NbAS-B was quantified by RT-qPCR at 0, 2, 4, 6, 8, and 10 h after treatment. (g) Asn-treated NahG transgenic Nicotiana benthamiana plants, overexpressing a bacterial NahG gene encoding SA hydroxylase to reduce the endogenous SA levels, displayed similar TMV development with water-treated plants. TMV-GFP was inoculated to NahG plant at 3 days after Asn and water treatments. The infection of TMV was observed at 3, 5, and 7 dpi under UV light. Representative pictures were showed. (h) RT-qPCR analysis showing the expression profile of TMV-GFP in the inoculated leaves of NahG N. benthamiana at 3 dpi and in the systemic leaves at 5 and 7 dpi. Actin was used as an internal reference gene. (i) The accumulation of TMV-GFP protein was detected by western blotting at 5 dpi in the leaves of NahG plants. Coomassie brilliant blue (CBB) showed an equal amount of protein loaded in each well. Values represent mean ± SE from three biological replicates. The statistical analyses were performed using Student’s t test (p < 0.05, "p < 0.01, """p < 0.001)
required for virus systemic and long-distance transport, probably by stabilizing DELLAs proteins (Venturuzzi et al., 2021). Therefore, we propose a model in which NbAS-B is stabilized by IP-L and promotes asparagine content, which induces the SA signalling pathway to resist TMV infection (Figure 9). Conversely, TMV could hijack IP-L by using TMV CP that indirectly interacts with IP-L or with unknown mechanisms, resulting in the destabilization of NbAS-B and the inhibition of the SA signalling pathway, thus promoting TMV long-distance movement (Figure 9). We cannot rule out the possibility that there is another role of IP-L that is not associated with the SA signalling pathway in promoting TMV infection because silencing of IP-L alone delays TMV infection (Figure S2).

On TMV infection, the number of IP-L transcripts increased (Figure 1a), resulting in increased accumulation of IP-L protein and NbAS-B protein (Figures 1e and 7). NbAS-B is predicted to be an asparagine synthetase B. Asparagine synthetase B is one of the asparagine synthetases that is widespread in prokaryotes and eukaryotes. It catalyses the biosynthesis of asparagine using ammonium or glutamine as substrates, which can enhance ammonium assimilation and the reorientation of nitrogen from source tissue to reservoir tissue in the plant (Rédei, 2008; Zhang et al., 2018). Our findings showed that transient overexpression of NbAS-B enhanced the asparagine content in tobacco leaves (Figure 7b), indicating that NbAS-B has asparagine synthetase activity. Asparagine synthetase (AS) participates in the regulation of various biological functions by regulating nitrogen metabolism in the plant (Gaufichon et al., 2010; Tsai et al., 2020). For example, overexpression of AS1 in Arabidopsis thaliana enhances the nitrogen status in seed and the transport of nitrogen from source tissue to reservoir tissue, thus improving nitrogen content (Lam et al., 2003). Cosilencing of Ast1 and Ast2 in potatoes reduces the acrylamide-forming potential of tubers, but the silenced plants produce small and deformed tubers (Rommens et al., 2008). Silencing of Ast1 reduces the asparagine content by 80% in potato tubers without interfering with the potato field yield (Chawla et al., 2012). The role of AS in plant disease resistance has been reported in several previous studies. The expression of the AS gene is significantly induced in tomato leaves infected by P. syringae pv. tomato and strong immunostaining of AS is observed in phloem cells of the main vascular bundles and in secondary veins of the infected leaf (Olea et al., 2004). CaAS1 is induced in pepper leaves infected with Xanthomonas campestris pv. vesicatoria (Xcv) and silencing of CaAS1 enhances the susceptibility to Xcv (Hwang et al., 2011). Overexpression of CaAS1 in A. thaliana confers enhanced resistance to P. syringae pv. tomato DC3000 and Hyaloperonospora arabidopsidis, indicating that CaAS1 is required for asparagine synthesis and disease resistance in pepper (Hwang et al., 2011). Our results showed that silencing of NbAS-B increased the susceptibility of tobacco to TMV, while overexpression of NbAS-B enhanced the resistance of tobacco against TMV (Figure 5). These observations suggest that NbAS-B acts as a positive regulator of plant defence against TMV. However, there was no direct interaction between NbAS-B and TMV CP (Figure S11), so we hypothesize that NbAS-B delays TMV infection due to the activation of basal host defence responses. To support this hypothesis, we further found that the expression levels of SA-related genes PR1 and PR2 were significantly lower in the NbAS-B silenced plants than that in wild-type plants (Figure 8d). In contrast NbAS-B was significantly induced after external application of MeSA (Figure 8f). Similarly, the SA-responsive genes PR1 and PR2 were induced after external application of Asn (Figure 8e), and Asn-treated NahG N. benthamiana is not resistant to TMV infection (Figures 8g–i), suggesting that the asparagine-induced SA signalling pathway mediates the resistance against TMV. Previous studies showed that nitrogen might mediate plant systemic acquired resistance (SAR) by influencing the production of nitric oxide (NO) (Piterková et al., 2009; Sun et al., 2020). Our studies provide the first example that a tobacco AS gene NbAS-B is involved in the resistance against a plant virus, suggesting that nitrogen metabolism might play a role in TMV resistance. In the future, generating transgenic tobacco overexpressing NbAS-B and testing

![Figure 9](image-url) The proposed working model of IP-L and NbAS-B in TMV infection. On TMV infection, the continuously induced expression of IP-L and NbAS-B results in increased accumulation of NbAS-B protein via the stabilization by IP-L by preventing 26S proteasome degradation. The increased NbAS-B protein promotes the synthesis of asparagine and induces the salicylic acid (SA) signalling pathway to resist TMV infection. However, TMV might employ coat protein (CP) or other unknown mechanisms to hijack IP-L to inhibit the SA signalling pathway for promoting TMV long-distance movement and a successfully deferred infection. On the other side, IP-L could have a role unrelated to the SA signalling pathway in promoting TMV infection.
its anti-TMV activity will provide additional support for understanding the role of NbAS-B against TMV.

Asparagine, as the major nitrogen compound, can be transported from source to sink organs in the phloem of most nonleguminous plants and translocated in the xylem from roots to leaves of legumes (Gaufichon et al., 2010; Shi et al., 1997). Numerous studies have shown that nitrogen supply and metabolism play an important role in plant disease by impacting the pathogen and host defence (Qiao & Fan, 2008). Accumulating evidence from studies showed that amino acids, as the low molecular substances in plants, play an important role in plant growth, metabolism, and disease response (Webb, 2019; Wu, 2020). The tryptophan pathway is involved in the defence response of rice against pathogens and insects (Ishihara et al., 2008; Lu et al., 2018). Tryptophan as the substrate is synthesized as a new antiviral substance that induces SA and an antioxidant system (Lv et al., 2020). Our studies found that the external application of asparagine on tobacco leaves inhibited TMV infection by activating the SA signalling pathway (Figure 8). Therefore, these findings support that amino acids can regulate plant resistance and trigger a plant defence response, which provides a potential for synthesizing amino acids and applying them to plants in response to biotic stresses in the future.

To the best of our knowledge, this is the first report that describes an asparagine synthetase involved in plant resistance against TMV. Our results showed that IP-L was continuously induced on TMV infection and increased accumulation of IP-L interacted with and stabilized NbAS-B. NbAS-B functions as an asparagine synthetase to improve the asparagine content in tobacco leaves. Silencing and overexpression of NbAS-B altered the tobacco response to TMV infection. In addition, our studies support that an asparagine synthetase is involved in TMV resistance by applying asparagine on tobacco leaves, which triggered a high level of resistance against TMV through the SA signalling pathway. The research presented here expands our knowledge about the molecular mechanism of plants against TMV and highlights the importance of asparagine as a potential agent in the control of TMV.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials

*N. benthamiana* plants were grown in a chamber maintained at 25°C (14 h light, 10 h dark, 75% relative humidity), and the plant growth conditions were the same for the virus-induced gene silencing (VIGS) and virus-mediated gene overexpression. Plants at the five-leaf stage were used for infection. All experiments were repeated at least three times.

4.2 | RNA extraction and cDNA synthesis

The leaves of the plant were kept at −80°C and ground into powder in liquid nitrogen. RNA was extracted using an Eastep Super Total RNA Extraction Kit (Promega). The RNA sample was then reverse transcribed with a PrimeScript RT reagent kit (TaKaRa) in a 10 μl reaction.

4.3 | Vector constructions

*IP-L ORF* (AB040409) was amplified using *IP-L*-primers containing the *EcoRI* and *Sall* restriction sites. The obtained PCR fragment was digested with *EcoRI/Sall* and cloned into the pSTT91 vector digested with the same restriction enzymes. The resulting plasmid was named pSTT91:*IP-L* and used as bait for subsequent Y2H assay.

The eGFP, mCherry, and 7Myc-fusion plant expression vectors were generated following the methods described previously (Liu et al., 2020). In brief, the eGFP, mCherry, and 7Myc fragments were inserted into the pKannibal vector using *Xhol* and *EcoRI* restriction sites, respectively. The coding sequence of the NbAS-B (MT900576) or *IP-L* was inserted into the pKannibal vector using the *EcoRI* and *XbaI* sites. Next, the eGFP, mCherry, and 7Myc-fusion fragments containing the CaMV 35S promoter and terminator were released from pKannibal with NotI sites, respectively. The obtained fragment was cloned into pART27 vector digested with NotI. The obtained plasmids and the empty control plasmid were transformed into *Agrobacterium tumefaciens* GV3101 following the method described previously (Ma et al., 2012b).

For VIGS, a relatively conserved fragment of approximately 300 bp of NbAS-B or *IP-L* was selected according to the SGN VIGS tool (Fernandez-Pozo et al., 2015). The PCR-amplified fragment carrying the *PstI* was cloned into the Pash18 vector. The obtained recombinant plasmid, TRV:NbAS-B, TRV:*IP-L* or TRV:*IP-L/NbAS-B*, was transformed into *A. tumefaciens* GV3101. The empty vector, TRV:00 (empty vector), was used as a negative control.

For virus-mediated gene overexpression, the full-length *IP-L* or NbAS-B/6His fusion fragment containing *Ciol* and *Sall* restriction sites was cloned into the pgR107 vector digested with the same restriction sites. The empty vector, PVX:00, was used as a negative control. The resulting plasmids were transformed into *A. tumefaciens* GV3101 for agroinfiltration. All the specific primers used in this study are listed in Table S1.

4.4 | Yeast two-hybrid library screening and assay

The yeast strain L40 was transformed following the method as described previously (Qiu et al., 2018). Briefly, yeast transformants harboring the pSTT91:*IP-L* and empty pGAD424 vectors were selected on the plates containing synthetic minimal medium lacking Leu and Trp (SD−W−L) at 30°C for 2 days. Single colonies were picked from SD−W−L and transferred to the fresh SD−W−L plates supplemented with different concentrations of 3-amino-1,2,4-triazole (3-AT) for examining the autoactivation of pSTT91:*IP-L*. The yeast two-hybrid library screening was performed following the method as described previously (Qiu et al., 2018). Briefly, 7 μl of the pSTT91:*IP-L* bait
vector, 7 μl of salmon sperm DNA, and 7.5 μl of the N. benthamiana cDNA library were used to transform 100 μl of competent yeast cells. The transformants were selected on the plates containing synthetic minimal medium lacking Leu, Trp, and His (SD−W−L−H) at 30°C for 3–7 days. The positive colonies were transferred to the SD−A−H−W−L plates supplemented with 40 μg/ml X-α-Gal. After 3–5 days of incubation, the plates were photographed. Plasmid DNA was isolated from positive yeast colonies and transformed into Escherichia coli DH5α cells for sequencing. Sequence analysis was performed using bioinformatics methods.

4.5 BiFC assay

YFP-based bimolecular fluorescence complementation (BiFC) assay was used (Han et al., 2015). To generate the BiFC constructs, IP-L fragment was cloned into the vector pCV-cYFP, and NbAS-B fragment was inserted into the vector pCV-nYFP. The resulting plasmids pCV-IP-L:cYFP and pCV-nYFP:NbAS-B were used for Agrobacterium transformation. Four- to five-week-old N. benthamiana leaves were agroinfiltrated with agrobacteria harbouring the indicated plasmids at a final OD₆₀₀ of 1.0. Leaf tissue was isolated from the infiltrated leaves at 48 h after infiltration and observed under an LSM780 (Zeiss) confocal laser scanning microscope.

4.6 LCI assay

LCI assay was performed as described previously (Chen et al., 2008). All combinations tested were agroinfiltrated into N. benthamiana leaves. The infiltrated leaves were detached at 48 hpi, sprayed with 1 mM luciferin, and observed under a low-light cooled charge-coupled device imaging apparatus (iXon; Andor Technology). Photographs were taken at 5 min after exposure to luciferin.

4.7 Co-immunoprecipitation

Co-IP was performed as previously described (Ma et al., 2018). Briefly, 2 days after agroinfiltration, N. benthamiana leaves were detached and ground under liquid nitrogen with a mortar and pestle. Total protein fractions were extracted with lysis buffer (10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl) supplemented with 2% (wt/vol) polyvinylpolypyrrolidone, 10 mM dithiothreitol, 13 protease inhibitor cocktail (Sigma), and 0.1% Tween 20 (Sigma), and incubated for 1.5 h at 4°C in a rotator with GFP-Trap agarose beads (Chromotek) or anti-Myc beads. After washing the beads four times with lysis buffer supplemented with 0.1% Tween 20, immunoprecipitated proteins were separated by 12% SDS-PAGE and blotted onto polyvinylidene fluoride membrane (Bio-Rad) overnight using wet blotting. The luminescent signal was visualized by Immobilon Western Chemiluminescent HRP Substrate using a ChemiDoc imager (Bio-Rad) with protein-specific antibodies.

4.8 Confocal observation

Agrobacterium-mediated transient expression was performed following the methods described previously (Ma et al., 2012b). Briefly, A. tumefaciens GV3101 was transformed with the pART27-N-eGFP:IP-L, pART27-N-mCherry:NbAS-B, pART27-N-7Myc:NbAS-B, pCV-cYFP:IP-L, and pCV-nYFP:NbAS-B. N. benthamiana leaves at the four-leaf stage were infiltrated and leaf discs were isolated from the infiltrated leaves and visualized using a LSM780 confocal laser scanning microscope equipped with a 40×/1.2 water-immersion objective (Zeiss). Enhanced citrine YFP-derived fluorescence was acquired using a 514-nm laser and emission was captured with 519–587-nm filters. Enhance GFP-derived fluorescence was excited at 488 nm and emission was captured with a 505–530-nm filter. Excitation of mCherry-derived occurred at 543 nm with a HeNe laser and emission was captured with a 590–620-nm filter.

4.9 Virus inoculation

For TMV-GFP and TMV U1, the leaves containing TMV-GFP or TMV U1 were mixed with 1% (wt/vol) carborundum, and then homogenized in PBS. Next, the homogenates were centrifuged at 5,000 × g for 10 min and the supernatant was adjusted to OD₆₅₀ = 0.8. One hundred microlitres of supernatant was applied to each leaf for inoculation. For TRV-GFP, PVX-GFP, and TuMV-GFP, agroinfiltration was used for inoculation (Liu et al., 2002). The Agrobacterium containing the above virus was adjusted to OD₆₅₀ = 0.8 for inoculation. GFP fluorescence was visualized with a handheld UV lamp (model no. B-100AP; UVP). For systemically infected leaves, the whole leaves of the three independent plants were mixed and collected. In contrast, for inoculated leaves, a quarter of the leaves in the lower right corner of the three independent plants were mixed and collected for subsequent TMV-GFP content detection. Each experiment was repeated three times.

4.10 Quantitative PCR

qPCR was performed using qTOWER3.0 real-time PCR (Analytikjena) and QuantilNova SYBR Green PCR Kit (Qiagen) to determine the relative expression levels of target genes. Gene-specific primers were designed according to the coding sequences of each gene using Primer 5.0 software. Actin was selected as an internal control. Quantification of the relative changes in gene transcript levels was performed using the 2−ΔΔCt method (Liu et al., 2021).

4.11 Plant treatment with asparagine and MeSA

N. benthamiana plants at the six-leaf stage were sprayed with 0.1 mM asparagine (Aladdin) and 0.1 mM methyl salicylate (MeSA; Aladdin),
respectively (Peng et al., 2019). Control plants were sprayed with sterile water. Samples were collected at 2-h intervals up to 10 h and immediately frozen in liquid nitrogen and stored at −80°C for RNA isolation.

### 4.12 | Determination of asparagine content

Samples (0.2 g) from treatment and control groups were weighed and ground with hydrochloric acid, hydrolysed with nitrogen, and freeze-dried. The dried samples were extracted by hydrochloric acid, n-leucine, triethylamine, phenyl isothiocyanate, and n-hexane, and then the content of asparagine was analysed by high-performance liquid chromatography (HPLC) by Suzhou Grace Bio-technology Co., Ltd.

### 4.13 | Measurement of asparagine synthetase activity

Asparagine synthetase catalyses the amidation reaction from glutamine or ammonium to aspartate to form asparagine. The asparagine synthetase activity was determined to measure the decreased rate of ammonium detected by Naismith’s reagent following the methods previously described (Brears et al., 1994; Brouquisse et al., 1992; Chai et al., 1996; Shanghai Plant Physiology Society, 1982).

### 4.14 | Statistical analysis

All experiments and data presented here involved at least three repeats. The data are presented as means and SD. The statistical analysis was performed with SPSS v. 22.0 software using Student’s t test and one-way analysis of variance (ANOVA, LSD test). ImageJ was employed to quantify the western blot bands.

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### CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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