

ORIGINAL ARTICLE OPEN ACCESS

The Tomato PR-5 Proteins PR-5x and NP24 Exert Antifungal Activity Against *Fusarium oxysporum*

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Received: 25 October 2024 | **Revised:** 21 January 2025 | **Accepted:** 8 February 2025

Funding: This work was supported by grants PID2022-136922NB-I00 and TED2021-131457B-I00, provided by MCIN/AEI/[10.13039/501100011033](https://doi.org/10.13039/501100011033) and by the 'European Union Next Generation EU/PRTR' (to N.S.C. and M.V.) and by NWO VICI 865.14.003 (to M.S. and F.L.W.T.).

Keywords: gene editing | pathogenesis-related proteins | plant–fungal interaction | transgenic plants | wilt disease

ABSTRACT

Vascular wilt diseases, caused by the soilborne pathogen *Fusarium oxysporum* (Fo), significantly impact agriculture and horticulture. Traditional management strategies are often environmentally harmful and not effective once the pathogen has entered the host. Consequently, understanding the molecular mechanisms underlying resistance to pathogenic Fo strains is crucial for developing novel control strategies. In tomato, resistance to *F. oxysporum* f. sp. *lycopersici* (Fol) can be mediated by *R* genes, such as *I-1*, *I-2* or *I-3*, or by Fo endophytes. Fol inoculation of resistant lines results in high accumulation of pathogenesis-related 5 (PR-5) proteins. However, the role of xylem-localised PR-5 proteins in Fol resistance in tomato plants remains unknown. Here, we demonstrate that apoplastic fluid enriched with recombinant PR-5x or NP24 exhibits antifungal activity against Fol. Using CRISPR/Cas9-mediated gene editing and overexpression studies, the role of *PR-5x* and *NP24* in disease resistance was studied. Loss-of-function mutations in either PR-5 isoform did not compromise Fol resistance. However, overexpression of *NP24* in tomato plants resulted in decreased susceptibility to Fol and surprisingly enhanced susceptibility to *Ralstonia solanacearum*. The role of *PR-5x* in Fol resistance remains undetermined as lines overexpressing the transgene could not be identified. Our findings suggest that PR-5 proteins are involved in restricting Fol proliferation in the xylem vasculature and thereby contribute to disease resistance.

1 | Introduction

Vascular wilt diseases caused by the fungus *Fusarium oxysporum* (Fo) affect over 100 different plant species, including economically significant field crops (e.g., banana), market garden crops (e.g., tomato) and ornamentals (e.g., chrysanthemum) (Edel-Hermann and Lecomte 2019). The pathogen is characterised by its host-specific forms, known as *formae speciales* (ff. spp.) (Michielse and Rep 2009). Typically, pathogenic Fo causes vascular browning, chlorosis, wilting and ultimately

plant collapse (Gordon 2017). Traditional disease control methods, such as soil fumigation, are harmful to the environment and ineffective once the pathogen is established in the crop (Alabouvette 1986; Bawa 2016).

Currently, the most cost-effective and sustainable management strategy is the use of host resistance, primarily through the implementation of resistance (*R*) genes (Chitwood-Brown et al. 2021). *R* genes encode immunity receptors that detect specific avirulence (*Avr*) effector proteins secreted by the pathogen

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to manipulate the host (Takken and Rep 2010). However, monogenic resistance has been identified in only a limited number of crops, including tomato (*Solanum lycopersicum*), melon, cucumber, pea and strawberry (Michielse and Rep 2009; Pincot et al. 2022). The interaction between tomato and *F. oxysporum* f. sp. *lycopersici* (Fol) serves as a model system for studying the molecular mechanisms underlying disease resistance and susceptibility to vascular pathogens. This pathosystem is unique in that four structurally distinct *R* genes have been identified (*I-1*, *I-2*, *I-3* and *I-7*) that all act against the same pathogen. The *I-1* receptor recognises Avr1 and confers resistance to Fol race 1; *I-2* recognises Avr2, providing resistance to Fol race 2; *I-3* recognises Avr3, conferring resistance to Fol race 3; and *I-7* also provides resistance to Fol race 3, though its corresponding Avr is unknown (Catanzariti et al. 2015, 2017; Gonzalez-Cendales et al. 2016; Houterman et al. 2009; Ma et al. 2013; Simons et al. 1998). The loss or mutation of these AVR genes allows the pathogen to bypass *R*-gene-mediated resistance (RMR), leading to an ongoing arms race between plant breeders and pathogens (Takken and Rep 2010). Besides genetically encoded race-specific resistance, non-race-specific resistance to Fol can be induced by certain endophytes, for example, by nonpathogenic Fo isolates (Constantin et al. 2021; de Lamo and Takken 2020; Fuchs et al. 1997). Although endophyte-mediated resistance (EMR) holds great potential, its field application is limited by high variability in effectiveness (De Silva et al. 2019). Understanding the molecular underpinnings of both RMR and EMR can aid the development of novel strategies to increase disease resistance.

Fol can still colonise the vasculature during RMR or EMR, albeit to a lesser extent than in a susceptible plant (de Lamo et al. 2021; van der Does et al. 2019). To elucidate the mechanisms that restrict the fungus within the xylem vessels and prevent disease symptoms, previous studies have focused on analysing the composition of the xylem sap proteome during RMR and EMR. These analyses revealed that, 2 weeks post-inoculation, *I-2* RMR and Fo47 EMR correlated with the accumulation of PR-5x and NP24, respectively—both belonging to the pathogenesis-related protein 5 family (PR-5) (de Lamo et al. 2018). Additionally, 1 week post-inoculation, PR-5x, along with other PR proteins, was shown to differentially accumulate in the xylem sap of *I-1*, *I-2*, *I-3* and *I-7* *R*-gene lines (Šimkovicová et al. 2024). While PR-5x also accumulates in Fol-susceptible plants, it does so at lower levels than in resistant lines (de Lamo et al. 2018). PR-5x also differentially accumulates in the xylem of resistant tomato plants inoculated with *Ralstonia solanacearum*, a bacterial vascular pathogen (Planas-Marquès 2020). Expression of the other PR-5 member was also higher in resistant than in susceptible plants following inoculation with *Fusarium solani*, *Verticillium dahliae* or *R. solanacearum* (Li et al. 2023). Currently, the role of these xylem-localised PR-5 proteins in vascular disease resistance remains unknown.

Members of the PR-5 protein family, also referred to as thaumatin-like or osmotin-like proteins, are involved in plant responses to both biotic and abiotic stresses (de Jesús-Pires et al. 2020) and are essential for callose regulation during pollen development in *Arabidopsis thaliana* (Oh and Park 2024). PR-5 proteins contain highly conserved cysteines that form disulphide bonds, contributing to their high stability under extreme

thermal and pH conditions and conferring protection to proteinases (Fierens et al. 2009; Liu et al. 2010). The apoplastically localised PR-5 proteins are the best characterised. Some exert direct antifungal activity through various mechanisms, including lipid bilayer permeabilisation, hydrolysis of β -1,3-glucans or inhibition of fungal enzymes such as xylanases (Fierens et al. 2009; Grenier et al. 1999; Vigers et al. 1992). In planta overexpression of specific apoplastic PR-5 members enhanced resistance to *Phytophthora infestans*, *Rhizoctonia solani*, *Fusarium graminearum* and *Alternaria alternata* in potato, rice, wheat and tobacco, respectively (Anand et al. 2003; Chen et al. 1999; Datta et al. 1999; Liu et al. 1994).

Given the antifungal properties observed in other PR-5 protein family members, we hypothesised that the xylem-localised proteins NP24 and PR-5x may play a role in restricting Fol proliferation within the xylem of resistant plants, thereby reducing disease progression. Here, the direct antifungal activity of recombinant PR-5x and NP24 on Fol hyphae was assessed. Producing recombinant PR-5 proteins in *Escherichia coli* often results in the formation of inclusion bodies complicating the study of these proteins (Campos et al. 2008). Hence, the recombinant proteins were expressed in *Nicotiana benthamiana*. The presence of a signal peptide resulted in the secretion of the protein into the apoplastic fluid, facilitating its isolation. To determine whether the loss of NP24 or PR-5x affects resistance mediated by endophytic Fo and/or *I-1* or *I-2* genes, the PR5 genes were knocked out using CRISPR/Cas9 technology. Furthermore, susceptible tomato plants were transformed to overexpress PR-5x or NP24 using two different promoters: the constitutive and systemic 35S promoter and the *XSP10* promoter, which is specifically active in the vasculature of the roots and lower stem. The overexpressing and knockout lines were used in disease assays to test whether altered expression affects Fol susceptibility.

2 | Materials and Methods

2.1 | Plant and Fungal Material

Nicotiana benthamiana and *S. lycopersicum* ‘MoneyMaker’ and ‘Motelle’ were used. Plants were grown in a controlled greenhouse environment at 65% relative humidity with a 16-h photoperiod. The temperature was maintained at 25°C for tomato and 21°C for *N. benthamiana*. Wild-type *F. oxysporum* isolates Fol004 (race 1), Fol007 (race 2), Fol029 (race 3), Fo47 and two transgenic strains, Fol007 Δ AVR2 and Fol pGPD:GFP, were used (Duyvesteijn et al. 2005; Houterman et al. 2009; Lemanceau and Alabouvette 1991; Rep et al. 2005).

2.2 | Plasmid Construction and Transient Protein Expression in *N. benthamiana* Leaves

The open reading frames (ORFs) of PR-5x (Solyc08g080620) and NP24 (Solyc08g080640) were amplified from genomic DNA of cv. MoneyMaker using high-fidelity Phusion polymerase and cloned into the NotI- and PacI-linearised expression vector pTRBO (Lindbo 2007). As a control, the mCherry ORF was cloned into pTRBO using a NEBuilder Hifi DNA assembly cloning kit as described (Blekemolen et al. 2018).

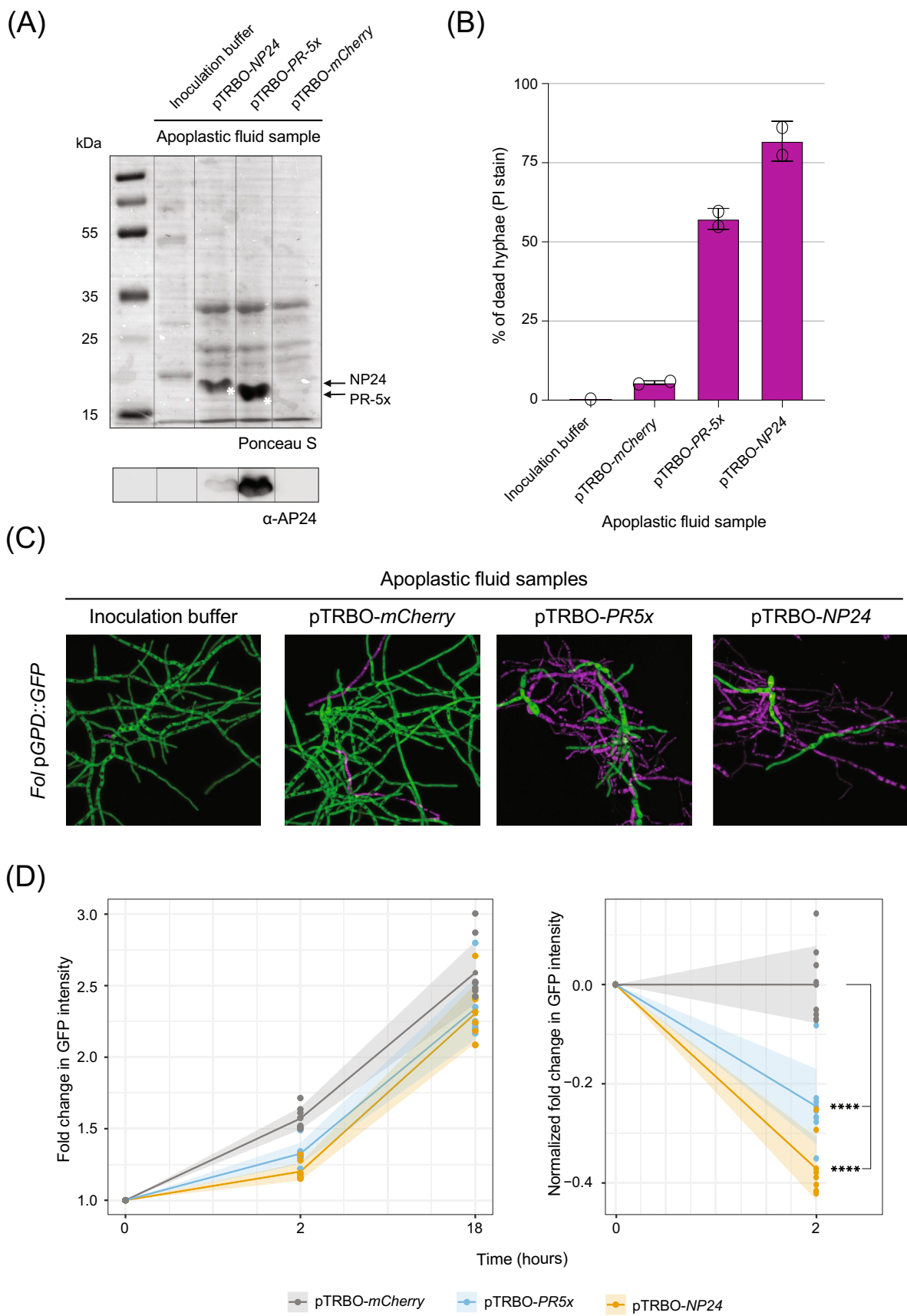


FIGURE 1 | Legend on next page.

FIGURE 1 | Apoplastic fluid (AF) enriched with recombinant NP24 or PR-5x exhibits antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* (Fol). AF was collected from *Nicotiana benthamiana* leaves that had been either mock-treated with buffer or treated with *Agrobacterium tumefaciens* carrying NP24, PR-5x or *mCherry* pTRBO constructs. (A) Western blot of the AF visualised using Ponceau S staining (top), and after probing with an α -AP24 antibody (bottom) revealed specific bands for NP24 and PR-5x proteins. The bands are marked by a white asterisk below each band and an arrow on the right side of the gel. The molecular weight (kDa) sizes of a PageRuler prestained ladder are indicated on the left side of the gel. (B) The antifungal activity of the AF samples was evaluated using Fol007 pGPD::GFP. Quantification was based on the percentage of dead hyphae, stained with propidium iodide (PI). Data from two representative confocal microscopy images are combined. (C) Confocal image of living hyphae of Fol007 pGPD::GFP in green and dead hyphae in magenta following AF treatment. (D) A time series analysis demonstrates the antifungal effects of AF containing either NP24 or PR-5x by reducing GFP fluorescence intensity 2 and 18 h after application. The right panel of this series presents the normalised fold changes in GFP intensity relative to the control, pTRBO-*mCherry*. The statistical significance of the observed differences in fold change, compared to the control, was determined using one-way ANOVA (**** $p \leq 0.0001$). [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

The constructs were sequence-verified and transformed into *Agrobacterium tumefaciens* GV3101 (Berres et al. 1992). The primers used and the constructed plasmids are listed in Table S1.

For transient expression in *N. benthamiana* leaves, *A. tumefaciens* was collected from plates and resuspended in 10 mL 10 mM MES infiltration buffer at pH 5.6 with 10 mM MgCl_2 to an OD_{600} of 1 (Zhang et al. 2024). The suspension was infiltrated into the abaxial sides of fully developed true leaves of 4-week-old *N. benthamiana* plants using a needleless syringe. Seven days post-agroinfiltration, apoplastic fluid (AF) and total protein were isolated. AF was collected from three leaves in a buffer (50 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.5, with 200 mM KCl) using a vacuum pump (Welch, 2515C-75) and desiccator (Nalgene classic). Subsequently, the leaves were dabbed off with tissue paper and rolled up, and placed inside the barrel of a 20 mL syringe placed within a 50 mL Falcon tube. The AF was extracted by centrifugation at 3000g for 10 min at 4°C (Joosten 2012). To remove residual *A. tumefaciens* cells, AF underwent an additional centrifugation step at 16,000g for 10 min. The supernatant of the AF samples was concentrated approximately tenfold using Amicon Ultra-0.5 mL protein concentration units with a 10 kDa molecular weight cutoff (Millipore). Protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Sigma). To maintain protein integrity, 5% glycerol (vol/vol) was added to the samples before snap freezing and storage at -80°C . For total protein extraction, two 0.6 cm diameter leaf discs were pulverised with a pestle and then mixed with 300 μL of urea extraction buffer (9.5 M urea, 10 mM Tris pH 6.8, 2% SDS, 5 mM dithiothreitol) and placed on ice for 15 min. The lysate was centrifuged at 11,750g for 10 min at room temperature. The supernatant was snap-frozen and stored at -80°C .

Proteins from AF and total protein extracts were separated using 11% and 16% SDS-PAGE gels, respectively. Immunoblotting was performed on polyvinylidene difluoride (PVDF) membranes using the iBind Western System (ThermoFisher Scientific). PR-5x and NP24 were detected using a primary antibody raised against the homologous protein AP24 (Syngenta), which has higher sequence similarity to PR-5x than NP24 (Rep et al. 2002; Woloshuk et al. 1991). As a secondary antibody, goat anti-rabbit horseradish peroxidase-conjugated antibody was used (ThermoFisher Scientific). The chemiluminescent signal was developed using Pierce ECL Plus Substrate (ThermoFisher Scientific). The stained SDS-PAGE gels and PVDF membranes

were visualised with a ChemiDoc MP Imaging System (Bio-Rad). The band intensities were calculated using Image Lab software (Bio-Rad).

2.3 | Multi-Well Antifungal Plate Assay and Confocal Microscopy

The spore suspension of Fol pGPD::GFP was prepared as described (Šimkovicová et al. 2024). The spore concentration was adjusted to 2.5×10^5 spores/mL, with a 15% glycerol concentration, snap-frozen and stored at -80°C . For the antifungal assay, 2500 spores from the glycerol stock were added to 125 μL potato dextrose broth (PDB; Difco) in a flat-bottomed dark 96-well plate (Greiner). Surrounding empty wells were filled with 135 μL PDB to maintain consistent conditions across the plate. The spores were germinated in a shaker at 25°C , 70 rpm, and the AF samples (25 μL) were added to a 27-h-old culture. The OD_{750} and GFP (A_{480}/A_{520}) fluorescence were measured just before adding AF, and 2 and 18 h after application using a plate reader (Biotek Synergy H1MF). For confocal microscopy (LSM510; Zeiss), 50 $\mu\text{g}/\text{mL}$ propidium iodide (PI) was added 1 h after AF treatment. Following a 1 h incubation, one well per treatment was analysed using an A1 confocal microscope (Nikon) equipped with a 63 $\times$ water immersion objective lens. GFP was excited at 488 nm with an Ar-ion laser and emission was captured at 505–550 nm; PI stain was excited at 568 nm with an Argon-2 laser, and emission was captured at 585–615 nm. The quantity of green (GFP) and red fluorescence (PI) per image was calculated using Fiji, an ImageJ processing package (Schindelin et al. 2012).

2.4 | Plasmid Assembly and Selection of CRISPR/Cas9-Edited and Overexpressing Transgenic Lines

Vectors for CRISPR/Cas9 mediated gene editing were constructed by Gateway LR cloning using the pCR3-EF/pENTR-Topo donor vector (Staskawicz Laboratory, UC Berkeley) encoding the *At-U6-26* promoter, gRNA, 2 $\times$ 35S promoter, and *Cas9* encoding a nuclear localisation signal into the destination vector pGWB401 (Nakagawa et al. 2007; Waibel and Filipowicz 1990). The gRNAs for targeting PR-5x (3'-TGGGCAGCATCGACCCCGAT-5') and NP24 (3'-TGGGCGGCATCGACTCCGAT-5') were designed using CRISPR-P 2.0 (<http://crispr.hzau.edu.cn/CRISPR2/>; Liu et al. 2017). The binary vectors for stable expression were constructed through Gateway LR cloning of the pDONR207 donor

FIGURE 2 | *I-1*- and *I-2*-mediated resistance were not affected in *pr-5x* knockout lines. Motelle, *pr-5x-1* and *pr-5x-2* seedlings were inoculated with water (mock), Fol004, Fol007 or Fol029. Disease scoring was done 3 weeks post-inoculation. (A) Photographs of five representative plants per treatment. (B) A Tukey boxplot shows fresh weights above the cotyledon level. (C) A bar graph indicates disease index scores based on vascular browning. A one-way ANOVA was employed to compare fresh weights between Motelle and the mutant lines, whereas a Mann–Whitney *U* test assessed differences in disease index ($n=20$, ns=no significance, $*p\leq 0.05$, $****p\leq 0.0001$). The assay was repeated three times (Figures S4 and S5). The data presented in Figures 2 and 3 originate from the same experiment in which the *np24* and *pr-5x* knockout lines were inoculated with *Fusarium* using the same positive and negative controls. As a result, the controls (mock- and Fol029-inoculated Motelle plants) are shared across both figures. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

vector encoding the ORFs of *PR-5x* or *NP24* and destination vector pGWB402 (Nakagawa et al. 2007). A short sequence ACCCAGCTTT was added after the stop codon of both ORFs to be able to distinguish the transgene from endogenous mRNA. Transgene expression was either driven by the 35S promoter or the promoter of the xylem sap specific 10 (*XSP10*) gene (Rep et al. 2004). The promoter sequence of *XSP10* was amplified from the genomic DNA of cv. MoneyMaker using high-fidelity Phusion polymerase. The 35S promoter sequence in pGWB402 was replaced with the *XPS10* promoter sequence using HindIII and XbaI restriction sites. The resulting pGWB401 and pGWB402 vectors were transformed into *A. tumefaciens* EHA105. Cotyledon explants from cv. Motelle were inoculated with *A. tumefaciens* harbouring the constructed vectors and regenerated in vitro using a kanamycin selection medium as described (Cortina and Culiáñez-Macià 2004). The constructed plasmids and the primers used for cloning and characterisation of the lines are listed in Table S1.

Tomato shoots transformed with the pGWB401 CRISPR/Cas9 vector were screened for mutations in the target gene by amplifying and sequencing *PR-5x* or *NP24*. Synthego's Interference of CRISPR Edits (ICE) version 3 (<https://ice.editco.bio/#/>) analysis was used to compare Sanger-sequenced target genes between wild-type and CRISPR/Cas9 lines. This revealed mosaic mutations in *PR-5x* in four shoots and in *NP24* in five shoots. To identify stable lines, T_1 plants were screened for the absence of *Cas9* through PCR, and for the presence of homozygous indels. ICE Synthego analysis confirmed homozygous frameshift mutations in two *pr-5x* and two *np24* T_1 lines. For off-target predictions, the CRISPR-P 2.0 tool was used. The potential off-targets for *NP24* included *PR-5x* and another *PR-5* isoform, *PR-P23* (Solyc08g080650). Predicted off-targets for *PR-5x* were *NP24* and *PR-P23*. The sequences of these potential off-targets were analysed using ICE Synthego and no mutations were observed.

PCR was used to identify transformants containing either the 35S or the *XSP10* promoter sequence. This resulted in seven T_0 p35S:*PR-5x* plants (five of which were fertile), eight T_0 p35S:*NP24* plants (seven fertile), 26 T_0 p*XSP10*:*PR-5x* plants (11 fertile) and 22 T_0 p*XSP10*:*NP24* plants (11 fertile). Homozygous T_1 plants were selected by comparing the band intensity of the transgene to that of the reference gene *TOPELESS 1* (Solyc03G117360) using gel electrophoresis following multiplex PCR (25 cycles) in lines showing a 3:1 segregation pattern. A Fol disease assay was conducted on seven segregating T_1 transformant lines containing an *XSP10* promoter and four lines with a 35S promoter for each transgene. This pilot prescreening of T_1 plants indicated that one quarter of the 20 inoculated tomato seedlings exhibited increased resistance in three p*XSP10*:*PR-5x* lines, two p*XSP10*:*NP24* lines, and two p35S:*NP24* lines, while no differences were observed in any of the p35S:*PR-5x* lines. Three lines per construct were

kept for further analyses. The homozygosity of the lines was confirmed in T_2 plants using the same multiplex PCR technique.

2.5 | Total Root RNA Extraction and Reverse Transcription-Quantitative PCR Assay

To assess the expression levels of *PR-5x* or *NP24* in the transgenic lines, RNA was isolated from the roots of three T_3 tomato seedlings per line. Seedlings were germinated for 10 days in soil, followed by 6 days of cultivation in aluminium-covered Falcon tubes filled with tap water supplemented with 1 mg/mL fertiliser (CalMag Grower, Peters Excel). The roots were freeze-dried and total RNA was extracted using the MagMAX Plant RNA Isolation kit and a KingFisher Apex machine (ThermoFisher Scientific). cDNA was synthesised from 1 µg of RNA using RevertAid H reverse transcriptase (ThermoFisher Scientific) following the supplier's instructions. Real-time quantitative PCR (qPCR) was performed on a QuantStudio3 (ThermoFisher Scientific) using the HOT FIREPol(r) EvaGreen(r) qPCR Mix Plus (Solis Biodyne). The qPCR primer sets were designed to amplify either the transgene mRNA alone or the endogenous and the transgene mRNA simultaneously. Due to the high sequence similarity between *PR-5x*, *NP24* and *PR-P23*, it was not possible to design primers specific for either the endogenous or transgenic *PR-5x*. To amplify transgenic *NP24*, a single nucleotide mismatch was introduced in the third base from the 3' end of the forward primer (Liu et al. 2012). Tomato α -*tubulin* (Solyc04g077020) served as a reference gene for reverse transcription (RT)-qPCR. Primer efficiencies were determined using a serial dilution of mixed cDNA samples and the melting curves were checked for specificity. The qPCR primers used are listed in Table S1.

2.6 | Phenotypic Analyses of *NP24* Overexpression Lines

Tomato fruits were collected weekly from three plants per T_3 of lines p*XSP10*:*NP24*-1, p*XSP10*:*NP24*-2, p35S:*NP24*-1 and p35S:*NP24*-2 for 5 weeks to determine the fruit number, fruit weight and number of seeds. Five randomly picked flowers at similar developmental stages were selected per transgenic line and photographed.

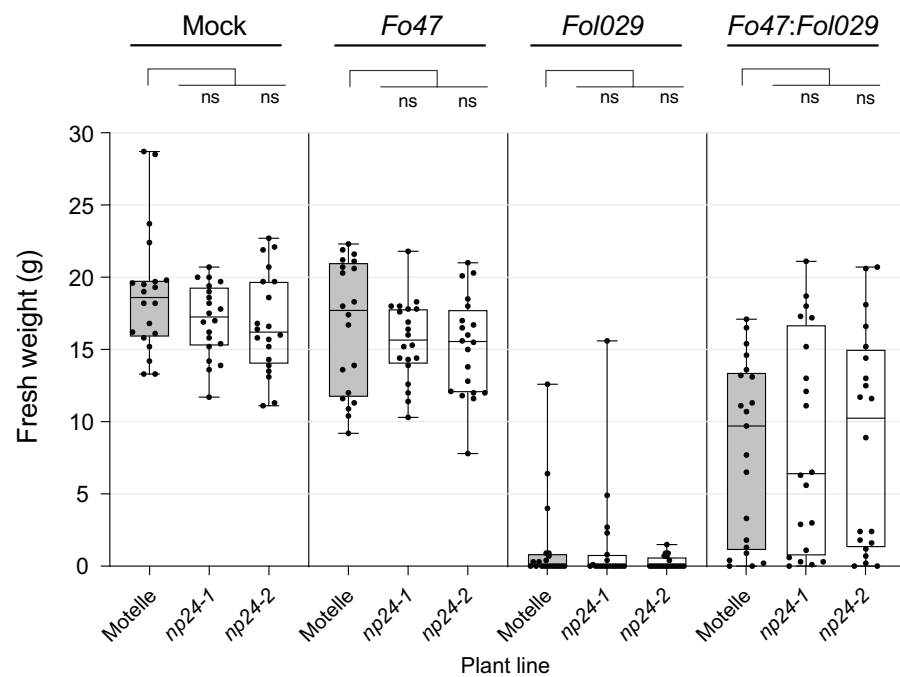
2.7 | *Fusarium* Inoculation Assay

A microconidial Fol spore suspension was prepared as described (Šimkovicová et al. 2024). The roots of 10-day-old tomato seedlings were trimmed to approximately 1 cm in length and inoculated with water (mock) or a 10^7 spores/mL suspension for

(A)



(B)



(C)

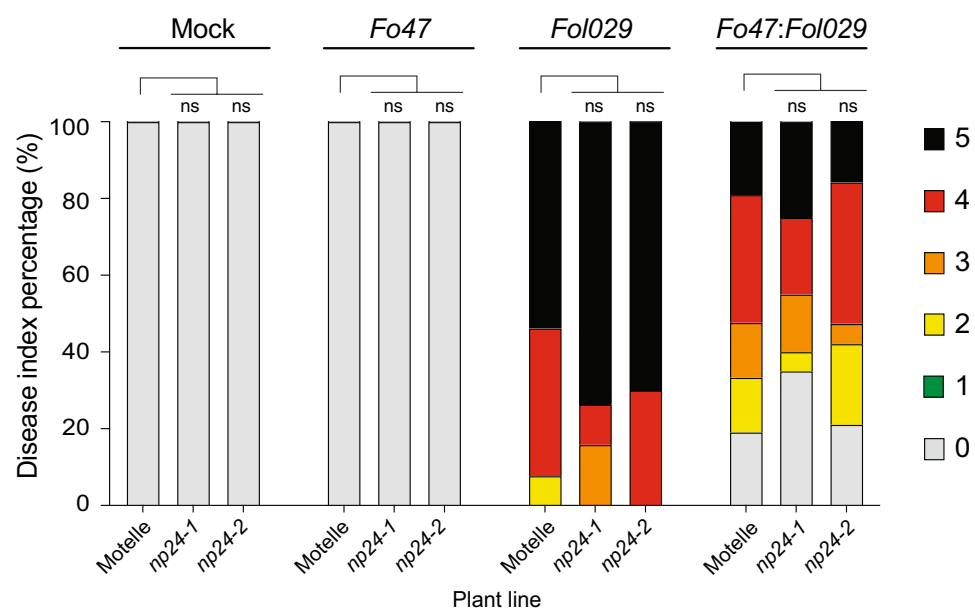


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FIGURE 3 | Endophyte-mediated resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Fol) is not changed in *np24* mutant lines. Seedlings from Motelle, *np24-1* and *np24-2* were inoculated with either water (mock), Fo47, Fol029 or a combination of Fo47 and Fol029. Disease scoring was done 3 weeks post-inoculation. (A) Photographs of five representative plants for each treatment. (B) A Tukey boxplot illustrates the fresh weight. (C) A bar graph presents disease index scores. Differences between Motelle and the mutant lines were evaluated using a one-way ANOVA for fresh weight, and a Mann–Whitney *U* test for the disease index ($n = 10$, ns = not significant). The experiment was repeated three times with similar results (Figures S6 and S7). The data presented in Figures 2 and 3 originate from the same experiment in which the *np24* and *pr-5x* knockout lines were inoculated with *Fusarium* using the same positive and negative controls. As a result, the controls (mock- and Fol029-inoculated Motelle plants) are shared across both figures. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

5 min. For the disease assay with Fol007 Δ AVR2, 11-day-old p35S:NP24-2 seedlings were used to ensure they matched the size of the other seedlings at the time of inoculation. The co-inoculum of Fo47 and Fol029 was prepared in a 1:1 ratio (de Lamo et al. 2021). Three weeks post-inoculation (wpi), the fresh weight (FW) of the foliage above the cotyledon level was determined. Disease index (DI) was scored, where 0 = no brown vascular bundles, 1 = only browning of vascular bundles at the crown level, 2 = less than half of the bundles are brown at the cotyledon level, 3 = at least half of the bundles are brown, 4 = 3/4 of the bundles are brown and 5 = dead plants (de Lamo et al. 2018).

2.8 | *Ralstonia solanacearum* Pathogenicity Assay

Four-week-old tomato plants grown in pots were soil-drench inoculated by pouring 40 mL of 10^7 CFU/mL ($OD_{600} = 0.01$) of bacterial suspension on each pot after making three holes in the soil with a disposable 1-mL pipette tip. Plants were kept in a 12 h light/12 h dark photoperiod and scored for wilting symptoms using a 0–4 scale, where 0 = no wilting, 1 = 25% wilting, 2 = 50%, 3 = 75% and 4 = 100% of the canopy wilted, with $n = 24$ plants of each variety/line used per assay.

3 | Results

3.1 | Antifungal Properties of PR-5x and NP24 Recombinant Proteins

The accumulation of PR-5x and NP24 proteins in the xylem sap of tomato plants signifies their potential involvement in resistance to Fol as observed during RMR and EMR, respectively (de Lamo et al. 2018; Šimkovicová et al. 2024). To investigate their antifungal properties, recombinant PR-5x and NP24 proteins were produced in *N. benthamiana* via *A. tumefaciens*-mediated transformation, employing the TMV-amplicon-based overexpression vector pTRBO (Lindbo 2007). Because both isoforms have a signal peptide for secretion, the apoplastic fluid (AF) was harvested to use for antifungal assays. The presence of PR-5x and NP24 in the apoplast was confirmed by Ponceau S-stained blots and immunoblotting with anti-AP24 (α -AP24) revealing that the band intensity of PR-5x was approximately 25% higher than that of NP24 (Figure 1A). Recombinant proteins were absent in the AF samples of control groups, which included AF samples collected from leaves solely infiltrated with buffer (IB) or in leaves infiltrated with *A. tumefaciens* carrying the pTRBO-*mCherry* vector (accumulating mCherry intracellularly). The concentration of endogenous proteins in AF samples from

leaves treated with pTRBO-NP24 or pTRBO-PR-5x appeared identical to that of the pTRBO-*mCherry* control. However, the protein pattern differed from that of IB AF samples, suggesting that *A. tumefaciens* induced secretion of specific plant or bacterial proteins into the apoplast. Analysis of the total protein extract from infiltrated leaves confirmed the production of mCherry (Figure S1). On a Coomassie-stained SDS-PAGE gel using total protein extracts, a strong band was observed for NP24 and a faint band for PR-5x. The presence of both NP24 and PR-5x in the extracts was confirmed by immunoblotting using α -AP24 (Figure S1).

The antifungal efficacy of AFs enriched with the recombinant proteins was tested using a Fol007 pGPD:GFP strain. To examine the effect of the AF on Fol hyphal morphology, confocal microscopy was used, allowing the visualisation of live (GFP-expressing, green) and dead (propidium iodide stained, purple) mycelium (Figure 1B,C). Image analysis showed 0% of dead hyphae following treatment with IB AF, 5.5% for pTRBO-*mCherry*, 57% for pTRBO-PR-5x and 82% for pTRBO-NP24 (Figure 1B). Additionally, pTRBO-PR-5x and pTRBO-NP24 AF-treated hyphae were deformed and enlarged, indicative of the antifungal activity of AF enriched with recombinant NP24 or PR-5x (Figure 1C). A plate reader was used to quantify GFP fluorescence and OD_{750} at time 0, 2, and 18 h following the addition of AF samples. Suppression of Fol growth was observed in the presence of pTRBO-NP24 or pTRBO-PR-5x AF, relative to pTRBO-*mCherry* AF, with the strongest inhibition for NP24 (Figure 1D; Figure S2).

3.2 | RMR and EMR in *pr-5x* and *np24* Knockout Lines

To investigate a possible requirement of the PR-5x and NP24 genes for Fol resistance, CRISPR/Cas9 was used to produce null alleles in tomato cv. Motelle. The gRNAs targeted a sequence in the 5' of the single exon gene (102–122 bp) to ensure loss-of-function mutants. Out of seven T_0 transgenic lines selected for each of the NP24 and PR-5x genes, five and four, respectively, produced fruit and were genotyped for edits. Two independent stable homozygous lines with frameshift mutations in both target genes were generated (Figure S3). The selected lines did not have mutations in the predicted off-targets.

Motelle carries *I-1* and *I-2* but not *I-3*. To elucidate the role of PR-5x in resistance mediated by *I-1* or *I-2*, *pr-5x-1* and *pr-5x-2* knockout lines were inoculated with the avirulent Fol004 (race 1) and Fol007 (race 2) strains. Additionally, the knockout lines were inoculated with a virulent Fol029 (race 3) to assess if the absence of PR-5x leads to hypersusceptibility. As anticipated, the analysis of fresh

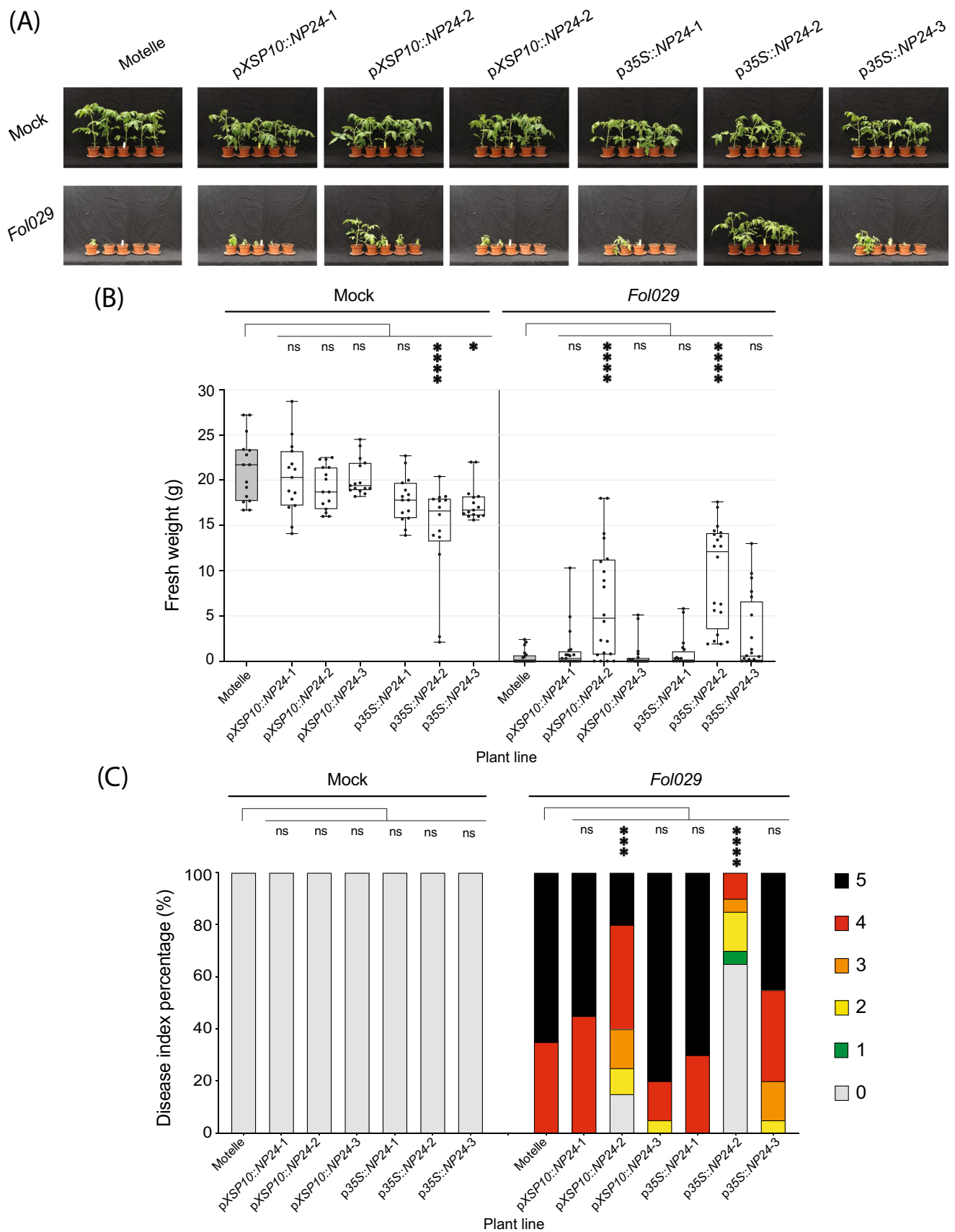


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FIGURE 4 | *NP24* overexpression reduces susceptibility to *Fusarium oxysporum* f. sp. *lycopersici* Fol029. Motelle, pXSP10:*NP24* and p35S:*NP24* tomato seedlings were mock- or Fol029-inoculated. At 3 weeks post-inoculation, photographs were taken of five representative plants per treatment (A), and the disease index was scored. (B) A Tukey boxplot showed the fresh weights from the cotyledon level. (C) The bar graph displays disease index scores. Fresh weight comparisons between Motelle and transgenic lines were made using a one-way ANOVA, and disease index score differences were analysed with the Mann–Whitney *U* test (ns = not significant, * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$). The study was repeated three times, yielding consistent results for the p35S:*NP24* lines but not for the pXSP10:*NP24* lines (Figures S11 and S12). [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

weight and disease index 3 weeks post-inoculation revealed that the wild-type Motelle exhibits resistance to Fol004 and Fol007 and is susceptible to Fol029 (Figure 2; Figures S4 and S5). Resistance to Fol004 was not affected in either *pr-5x* knockout line. Upon inoculation with Fol007, the *pr-5x-1* line exhibited a significant reduction in fresh weight as compared to the mock treatment. However, this reduction was observed in only one of the three repetitions, while the *pr-5x-2* line showed no altered resistance in the three replicates. Inoculation of Motelle and the *pr-5x-1* and *pr-5x-2* lines with the virulent strain Fol029 did not reveal differences in fresh weight or disease index as compared to the progenitor line. These findings imply that the loss-of-function of *PR-5x* does not alter resistance mediated by *I-1* or *I-2*, nor does it affect susceptibility to Fol029.

NP24 accumulates in the xylem sap following Fo47-EMR to Fol (de Lamo et al. 2018). To assess the role of *NP24* in EMR, transgenic lines *np24-1* and *np24-2* were (co-)inoculated with Fo47 and Fol029 (race 3). The fresh weights of mock-inoculated Motelle, *np24-1* and *np24-2* were similar (Figure 3B, Figures S6B and S7B). Also, no differences were observed in fresh weight or disease index between the Motelle and the *np24* mutant lines when inoculated with Fo47, Fol029 or with both the pathogen and the endophyte simultaneously (Figure 3, Figures S6 and S7). These findings imply that Fo47-mediated resistance is not compromised by the loss of *NP24*; neither is susceptibility to Fol029.

3.3 | *NP24* Overexpression in Tomato Plants and Susceptibility to Fol

To assess whether a single xylem-associated PR-5 protein may confer (partial) resistance to Fol, Motelle was transformed to constitutively (over)express *PR-5x* or *NP24*. Besides the 35S promoter, the *XSP10* promoter sequence was used, which is predominantly active in roots, potentially mitigating pleiotropic effects that are often associated with strong promoters driving constitutive and systemic expression (Figure S8A) (Di et al. 2016; Rep et al. 2004).

Expression levels of *NP24* were assessed in the roots of three homozygous plants per construct. qPCR analyses of cDNA from roots revealed that only one line per construct exhibited a modest *NP24* overexpression. Compared to wild-type Motelle, the pXSP10:*NP24-2* line showed a 2.2-fold increase, whereas the p35S:*NP24-2* line displayed a 2.8-fold increase in *NP24* expression (Figure S9A,C). The pXSP10:*NP24-2* and p35S:*NP24-2* roots showed expression levels that were 0.7- and 1.3-fold higher for the *NP24* transgene (Figure S9B,D). The expression level of *PR-5x* could not be determined as gene-specific primers could not be developed. *NP24* and *PR-5x* protein abundance in xylem sap and AF was below the detection

threshold for immunoblotting with the α -AP24 antibody (data not included). Phenotypic analysis indicated a developmental cost associated with the constitutive expression of *NP24* in the p35S:*NP24-2* line, resulting in decreased fruit yield and seed number (Figure S10B–D). This phenotype was not observed in the pXSP10:*NP24-2* line. Flower size was unaffected by *NP24* overexpression in both p35S:*NP24-2* and pXSP10:*NP24-2* overexpression lines (Figure S10A).

The transgenic *NP24* expressing lines were used in disease assays with Fol race 3 (Fol029), to which Motelle is susceptible. The pXSP10:*PR-5x* and p35S:*PR-5x* lines were not included as *PR-5x* expression data were not available and trials on T_1 plants did not indicate (partial) resistance segregating in the Fol-inoculated progeny (data not shown). At 3 wpi, fresh weight and disease index were assessed for the pXSP10:*NP24* and p35S:*NP24* lines. The fresh weight of most mock-inoculated transgenics was identical to that of Motelle, except for line p35S:*NP24-2*, which consistently had a lower fresh weight (Figure 4B, Figures S11B and S12B). Motelle showed high susceptibility to Fol029, with a fresh weight close to zero and a disease index of 4 or 5 (Figure 4, Figures S11 and S12). The first assay revealed that both *NP24* overexpressing lines, pXSP10:*NP24-2* and p35S:*NP24-2*, exhibited increased fresh weight and decreased disease index scores as compared to Motelle (Figure 4); however, this was not confirmed in subsequent repetitions for the pXSP10:*NP24-2* line. However, the p35S:*NP24-2* line consistently showed reduced susceptibility to Fol029 (Figures S11 and S12).

Given that Fol029 is a highly virulent strain, potentially masking a small increase in resistance, the transgenic lines were also inoculated with a Fol007 Δ AVR2 line. This mutant is less virulent and is not affected by the *I-2* gene as it lacks *AVR2*. The fresh weight of Fol-inoculated plants of all transgenic lines was identical to that of Motelle (Figures 5B and 6B, Figures S13B and S16B). Fol007 Δ AVR2-inoculated p35S:*NP24-2* plants had a significantly lower disease index score (Figure 5C, Figures S13C and S14C), unlike the pXSP10:*NP24-2* line, which resembled Motelle (Figure 6C, Figures S15C and S16C). These findings correspond with the previous finding that line p35S:*NP24-2* shows increased resistance to Fol, while no altered resistance is observed in the other transgenics.

To assess the specificity of *NP24*-mediated resistance to Fol, the two overexpressing lines, pXSP10:*NP24-2* and p35S:*NP24-2*, were inoculated with another root-invading vascular pathogen, *R. solanacearum* (Figure 7). Following soil-drenching inoculation, both transgenic lines exhibited significantly increased susceptibility to *R. solanacearum* compared to wild-type Motelle. To conclude, increased expression of *NP24* in the root results in increased susceptibility to *R. solanacearum* and reduces susceptibility to Fol.

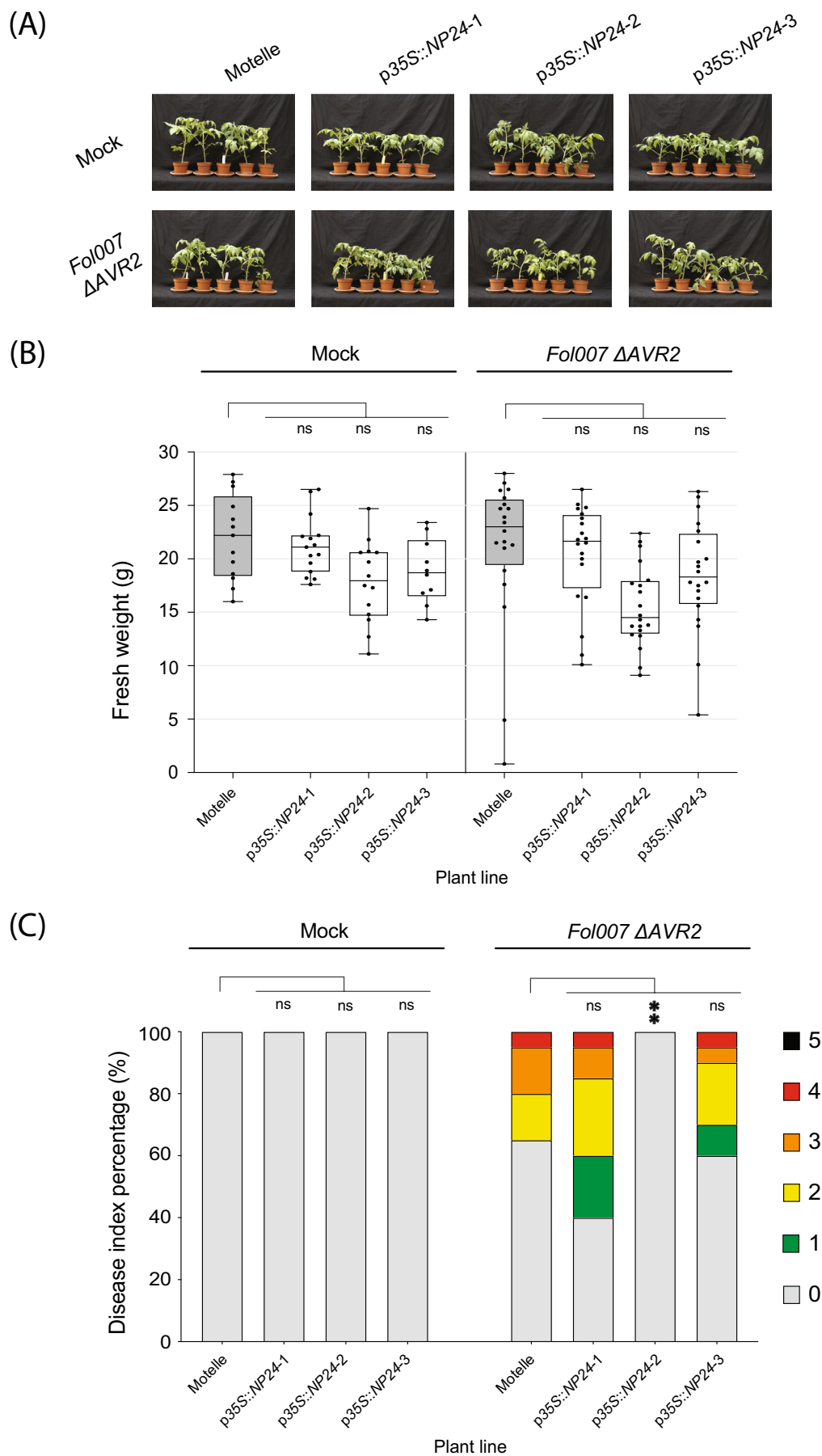


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FIGURE 5 | Increased *NP24* expression in a p35S:*NP24* transgenic line results in a reduction of vascular wilt disease symptoms following *Fusarium oxysporum* f. sp. *lycopersici* Fol007Δ*AVR2* inoculation. Seedlings of Motelle and p35S:*NP24* lines were subjected to either water (mock) or Fol treatment. Disease evaluation at 3 weeks post-inoculation involved capturing images of five representative plants from each group (A) and scoring for disease presence. (B) A Tukey boxplot illustrates the fresh weight at the cotyledon level. (C) A box plot outlines the disease index scores. Statistical comparison of fresh weight between Motelle and the transgenic lines used a one-way ANOVA, whereas the Mann–Whitney *U* test addressed differences in disease indices (ns = not significant, ***p* ≤ 0.01). The experiment was conducted three times with consistent results (Figures S13 and S14). [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

4 | Discussion

Here we demonstrate that AF enriched with recombinant *NP24* or *PR-5x* protein exerts direct antifungal activity against Fol. Many apoplastically localised *PR-5* isoforms have been reported to exhibit antifungal activity, including spore lysis, inhibition of germination and reduced fungal viability of fungi such as *Fusarium*, *Verticillium*, *Trichoderma* and *Alternaria* (Abad et al. 1996; Guo et al. 2016; Mani et al. 2012; Woloshuk et al. 1991). One of the proposed mechanisms is the permeabilisation of the microbial plasma membrane (Abad et al. 1996; Anzlovar et al. 1998; Roberts and Selitrennikoff 1990; Vigers et al. 1992). Confocal microscopy revealed that most Fo hyphae were stained with propidium iodide (PI) within 1 h of exposure to *PR-5*-enriched AF samples (Figure 1C), indicative of damaged plasma membranes (Crowley et al. 2016). Quantitative assessments of OD₇₅₀ and GFP fluorescence showed reduced Fol growth following the application of *NP24*- or *PR-5x*-enriched AF, further supporting the proposed antifungal activity. Interestingly, the antifungal activity of *NP24* appeared stronger than that of *PR-5x*, despite being approximately 25% less abundant in AF than *PR-5x*. The two *PR-5*s share 90% sequence identity, excluding the 21-amino acid sequence at the C-terminus of *NP24*, which is lacking in *PR-5x*. The tail is proposed to direct proteins to the vacuole, potentially explaining the higher *NP24* levels in total protein extracts (Melchers et al. 1993; Neuhaus et al. 1991). The conserved acidic cleft of *PR-5* proteins, formed by domains I and II, is proposed to be crucial for antimicrobial activity and receptor binding. Differences within and around the cleft determine the specificity of *PR-5* to a specific target organism (Ghosh and Chakrabarti 2008; Min et al. 2004; Petre et al. 2011; Sharma et al. 2015). A comparison of the cleft regions revealed a difference at position 210, where *NP24* carries a threonine, capable of forming hydrogen bonds, whereas *PR-5x* contains a leucine (Figures S15 and S16). Additionally, five amino acids located in domains I and II, at the outer part of the cleft, differ in their chemical properties between the two *PR-5* isoforms (Figure S16). Modifications in these amino acids within and around the cleft could show whether these differences are causal to the observed differences in antifungal activity.

Knocking out the *PR-5x* or *NP24* gene did not affect resistance mediated by the *I-1* or *I-2* gene nor by a Fo endophyte. We hypothesise that other *PR* proteins in the xylem sap compensate for the loss of *PR-5x* or *NP24*. Besides *PR-5x*, other *PR* proteins such as *PR-1*, *PR-2*, *PR-3*, *PR-6*, and *PR-7* differentially accumulate in the xylem sap of *I-1*, *I-2*, *I-3*, and *I-7* *R*-gene lines 1 week after Fol inoculation (Šimkovicová et al. 2024). These proteins are associated with defence against numerous pathogens (Balasubramanian et al. 2012; Girhepuje and Shinde 2011; Li et al. 2011; Lopes et al. 2009; Rauscher et al. 1999; Zhang et al. 2024). Likewise, besides *NP24*, one other *PR* protein was

found to accumulate in the xylem sap during EMR (de Lamo et al. 2018). Possibly, knocking out multiple *PR* proteins is required to compromise EMR or *R*-gene-mediated Fol resistance. In correspondence, deletion of four *PR-5* homologues in *A. thaliana* was required to increase susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 infection (Park and Kim 2021). *PR-5x* and *NP24* belong to a cluster of seven *PR-5* genes on chromosome 8 of tomato. Overexpression of two members from the *PR-5* cluster, *SITLP5* and *SITLP6*, resulted in slightly reduced susceptibility to Fol, *F. solani*, *V. dahliae* and *R. solanacearum* in disease assays (Li et al. 2023). It would be interesting to investigate the effect on Fol resistance upon deletion of the entire *PR-5* gene cluster in tomato.

35S promoter-driven overexpression of *NP24* in tomato plants decreased susceptibility to Fol, though it did not confer complete resistance, which is in line with previous studies (Anand et al. 2003; Chen et al. 1999; Li et al. 2023). van Loon et al. (2006) suggested that partial resistance could be due to insufficient *PR* expression levels in transgenic lines. Although we detected the *NP24* transgene in multiple independent lines, increased expression was only observed in two lines. One p35S:*NP24* line showed a 2.8-fold increase in *NP24* expression concomitant with reduced susceptibility to Fol. A pXSP10:*NP24* line exerting a 2.2-fold increase in *NP24* expression did not consistently exhibit reduced Fol susceptibility, which might be due to insufficient *NP24* accumulation. Interestingly, both these lines showed increased susceptibility to *R. solanacearum*. In contrast, tomato lines overexpressing *PR-5* members *SITLP5* or *SITLP6* showed reduced *R. solanacearum* disease symptoms (Li et al. 2023). This aligns with the hypothesis that the accumulation of *PR-5*s in resistant tomato and tobacco cultivars following *R. solanacearum* inoculation contributes positively to plant defence (Li et al. 2021; Planas-Marquès 2020). The enhanced disease symptoms observed in our study may, therefore, be due to a negative effect of *NP24* on plant performance. An indication of such pleiotropy is the observation that increased expression of *NP24* from the 35S promoter resulted in reduced seed and fruit yields and stunted growth. Another indication that enhanced or systemic *PR-5* expression is detrimental to a plant is the observation that from the same number of explants, fewer p35S:*PR5x* transformants than pXSP10:*PR5x* lines were retrieved. Moreover, a recent study revealed that transient *NP24* expression in *N. benthamiana* leaves triggers cell death, potentially explaining the observed pleiotropic effects (Qian et al. 2024). This problem of phytotoxicity might be overcome by using inducible promoters (Borghi 2010). Yet, it is unlikely that the overexpression of a single *PR-5* protein would confer strong resistance to a specialised pathogen, as these probably evolved means to mitigate the antifungal activity, as reported for *V. dahliae*, *Botrytis cinerea* and *Blumeria graminis* f. sp. *hordei* (González et al. 2017; Pennington et al. 2016; Zhang et al. 2019). For Fol, *SIX1* or *SIX6*

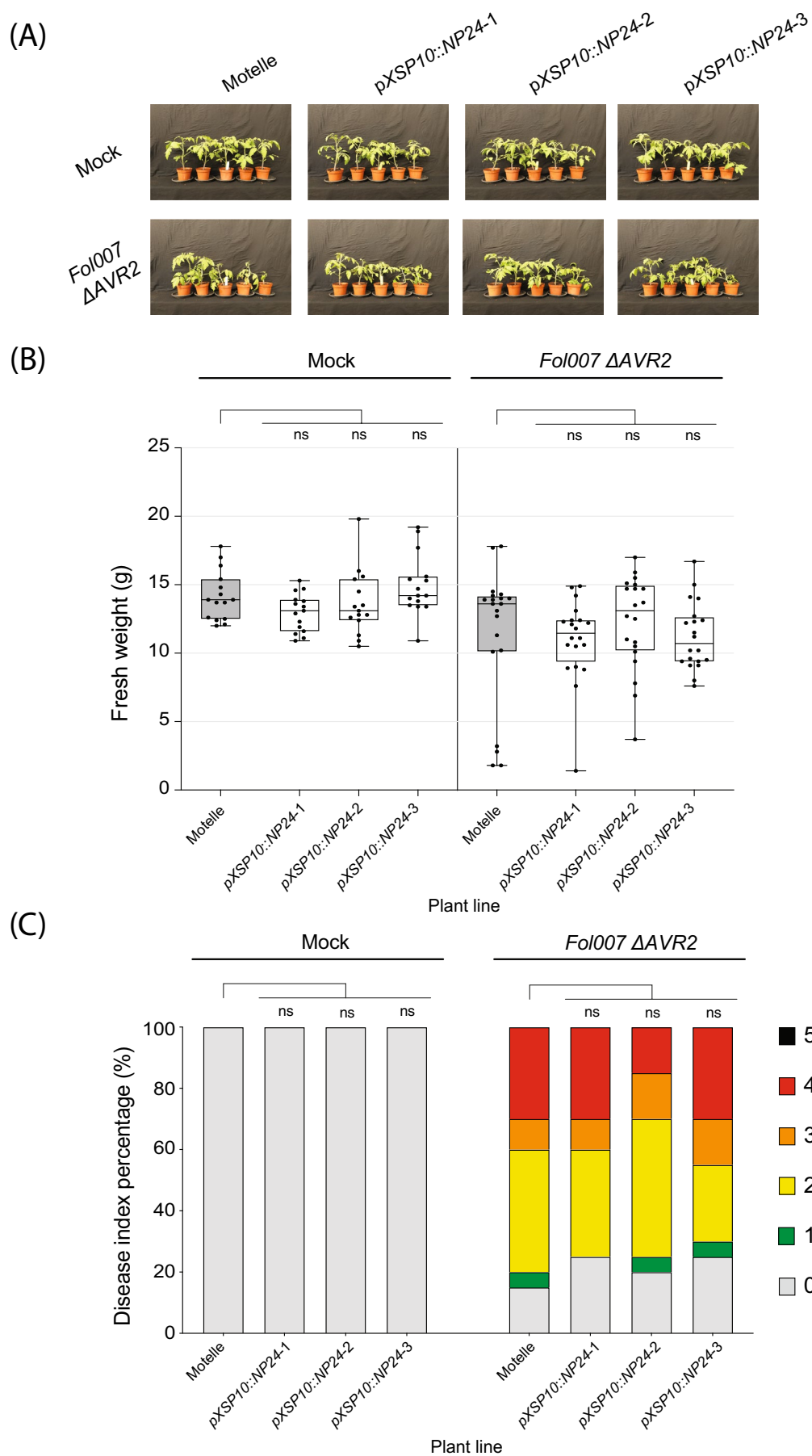


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FIGURE 6 | NP24 overexpression in a pXSP10:NP24 transgenic line does not diminish the disease index following *Fusarium oxysporum* f. sp. *lycopersici* (Fol) Fol007ΔAVR2 inoculation. Motelle and pXSP10:NP24 tomato seedlings underwent either water (mock) or Fol treatment. At 3 weeks post-inoculation, five representative plants were photographed per treatment (A), plants were scored for fresh weight represented in a Tukey box-plot (B), and disease index plotted in a box plot (C). Differences between Motelle and the transgenic lines were statistically analysed with a one-way ANOVA for fresh weight, and Mann–Whitney *U* test for disease index (ns = not significant). Three repetitions of the assay yielded uniform results (Figures S15 and S16). [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

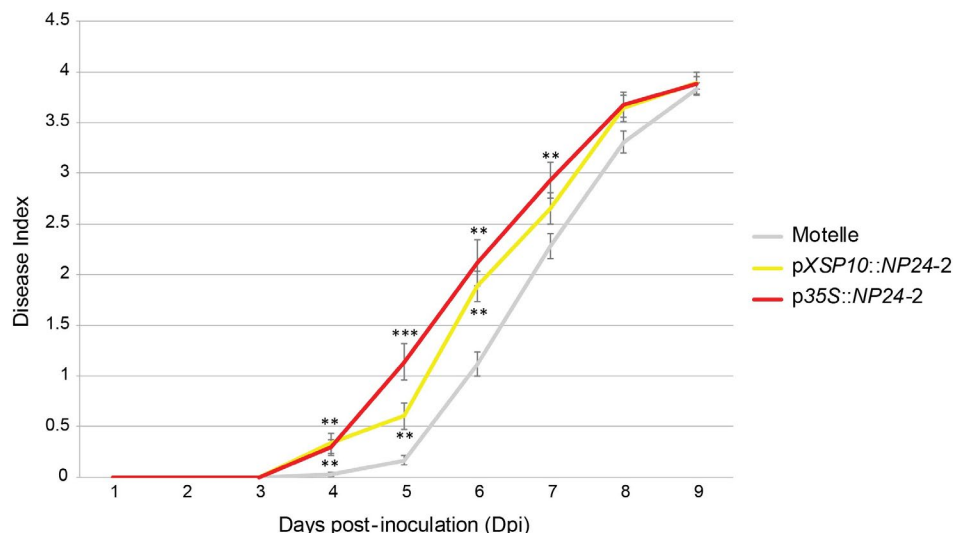


FIGURE 7 | Bacterial wilt symptom development in Motelle and NP24-overexpressing tomato plants. Four-week-old tomato plants were soil-drenched with a *Ralstonia solanacearum* GMI1000 suspension and wilting symptoms were recorded on a 0–4 scale using Motelle (grey) and the pXSP10:NP24-2 (yellow) and p35S:NP24-2 (red) lines. Results combining two independent experiments are shown (48 plants per genotype in total). Error bars indicate standard errors. Significant differences between Motelle and the transgenic lines, based on the *t* test for daily disease index, are marked with asterisks (** $p < 0.01$, *** $p < 0.001$). [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

effector deletions resulted in increased NP24 accumulation in the xylem sap after inoculation with the respective Fol knockout strains (Gawehns et al. 2015). Possibly, these effectors play a role in suppressing the host defence and/or the induction of NP24 expression. It is tempting to speculate that a modest increase in the expression of several PR genes simultaneously, mimicking the natural situation, could confer enhanced resistance without affecting plant performance. The identification of the antifungal activity of xylem-localised PR-5 proteins provides a first step towards such a strategy to develop Fol-resistant varieties.

Acknowledgements

We acknowledge Fran de Lamo and Lisong Ma for constructing the vectors for the knock-out and overexpression lines and Bas Beerens for generating the knock-out lines. We are very thankful to Ilse Bakker and Alicia Invernón Garrido for assembling and testing vectors for transient expression of PR-5 proteins as well as Wijnand Schravensande for his expertise in confocal microscopy and his contributions to imaging. We are grateful to Harrold Lemereis and Ludek Tikovsky for maintaining the transgenic tomato lines in the greenhouse as well as the assistance of our colleagues with the Fusarium disease assays. M.Š. and F.T. received support from the NWO-Earth and Life Sciences—funded VICI Project no. 865.14.003 and the University of Amsterdam. The research conducted by M.L., N.S.C. and M.V. was funded by grants PID2022-136922NB-I00 and TED2021-131457B-I00, provided by MCIN/AEI/10.13039/501100011033 and by the ‘European Union NextGenerationEU/PRTR’ (to N.S.C. and M.V.).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Materials described in this study are available from the corresponding author upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.