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Raheleh Jahnbin-Navrod

University of Guilan

Reza Shirzadian-Khorramabad

r.shirzadian@guilan.ac.ir

University of Guilan

Vahid Motaghitalab

University of Guilan

Sara Royan

University of Guilan

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Floral-bud transformation enables rapid assessment of NaD1-associated Fusarium wilt resistance in tomato

Raheleh Jahnbin-Navrod¹, Reza Shirzadian-Khorramabad^{2*}, Vahid Motaghitalab³, Sara Royan⁴

¹ Department of Agricultural Biotechnology, Faculty of agricultural sciences, University of Guilan, Rasht, Iran.

^{2*} Department of Agricultural Biotechnology, Faculty of agricultural sciences, University of Guilan, Rasht, Iran.

³ Department of Chemical Engineering, Faculty of Engineering, University of Guilan, Rasht, Iran.

⁴ Department of Agricultural Biotechnology, Faculty of agricultural sciences, University of Guilan, Rasht, Iran.

* Corresponding author: r.shirzadian@guilan.ac.ir; ORCID number: 0000-0002-8413-6241

Abstract

The emergence of new races of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) can overcome resistance conferred by single dominant resistance genes, highlighting the need for durable, race-nonspecific strategies. Here, we report that heterologous expression of the *Nicotiana glauca* defensin NaD1, a membrane-active antimicrobial peptide, enhanced resistance to *Fol* in the susceptible tomato cultivar *SV4129*. Using a vacuum-assisted floral-bud transformation approach, we generated independent T1 plants and identified line *TSV4129-2*, which exhibited a 12.3-fold reduction in the area under the disease progress curve (AUDPC), a final disease severity of 6% compared with 89% in wild-type plants, and restricted vascular browning to <15 cm, with no evident late-stage disease breakthrough over 28 days.

Mechanistically, *TSV4129-2* displayed a tissue-associated defense pattern characterized by localized changes in root oxidative and phenolic status alongside preservation of shoot physiology. Roots showed increased MDA (~33 nmol/g FW) and phenolic accumulation (~188 mg GAE/g FW), whereas shoots maintained higher chlorophyll a and b contents, low ion leakage (3–4%), and low foliar lipid peroxidation (<1 nmol/g FW). These contrasting responses were consistent with localized root defense accompanied by preservation of photosynthetic tissues during *Fol* infection. Together, these findings support a working model in which *NaD1* expression is associated with local oxidative reinforcement in roots while maintaining physiological stability in shoots. This tissue-coordinated response may contribute to the sustained resistance observed throughout the 28-day assessment period. Beyond providing a candidate transgenic resource for resistance studies, the modified floral-bud vacuum infiltration approach provides a practical route for functional validation in tomato and supports local oxidative reinforcement coupled with systemic photosynthetic preservation as a working model for NaD1-associated vascular resistance to *Fol*.

Keywords:

NaD1; *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*); *Solanum lycopersicum*; Floral-dip transformation; AUDPC, Antimicrobial peptide, disease resistance

Key Message:

NaD1-mediated protection was associated with event-specific disease restriction and distinct tissue responses, linking enhanced root defense with preservation of leaf physiological homeostasis.

37 **Abbreviations**

38	AMP – Antimicrobial peptide
39	AR(1) – Autoregressive covariance structure of order one
40	AIC – Akaike information criterion
41	AUDPC – Area under the disease progress curve
42	BAP – 6-Benzylaminopurine
43	BSA – Bovine serum albumin
44	CaMV – Cauliflower mosaic virus
45	CMC – Carboxymethyl cellulose
46	ddPCR – Droplet digital polymerase chain reaction
47	DNA – Deoxyribonucleic acid
48	dpi – Days post-inoculation
49	ELISA – Enzyme-linked immunosorbent assay
50	<i>Fol</i> – <i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>
51	FW – Fresh weight
52	GAE – Gallic acid equivalents
53	HSD – Honestly significant difference
54	H ₂ O ₂ – Hydrogen peroxide
55	LB – Luria–Bertani (medium)
56	LS-means – Least-squares means
57	MDA – Malondialdehyde
58	MES – 2-(N-morpholino)ethanesulfonic acid
59	MS – Murashige and Skoog (medium salts)
60	NaD1 – Nicotiana glauca defensin I
61	NHEJ – Non-homologous end joining
62	<i>nptII</i> – Neomycin phosphotransferase II
63	OCS-ter – Octopine synthase terminator
64	OD ₆₀₀ – Optical density at 600 nm
65	PDA – Potato dextrose agar
66	PDB – Potato dextrose broth
67	PCR – Polymerase chain reaction
68	PIP ₂ – Phosphatidylinositol 4,5-bisphosphate
69	POD – Peroxidase
70	PYGA – Peptone–yeast extract–glycerol–agar (medium)
71	qPCR – Quantitative polymerase chain reaction
72	qRT-PCR – Quantitative reverse transcription polymerase chain reaction
73	R – Resistance (gene)
74	RNA – Ribonucleic acid
75	RT-PCR – Reverse transcription polymerase chain reaction
76	SE – Standard error
77	SD – Standard deviation
78	TBA – Thiobarbituric acid
79	TCA – Trichloroacetic acid
80	T-DNA – Transfer DNA
81	TMEJ/MMEJ – Theta-mediated end joining / microhomology-mediated end joining
82	UV – Ultraviolet

83 **1.Introduction**

84 Tomato (*Solanum lycopersicum* L.) is an economically important crop widely cultivated for fresh consumption
85 and processing, with substantial nutritional and agricultural value. Global tomato production reached approximately
86 189 million metric tons in 2021(Szabo *et al.* 2025). Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici*
87 (*Fol*), is one of the most destructive soil-borne diseases of tomato, with yield losses of up to 80% in severe cases
88 (Panno *et al.* 2021). Management is complicated by multiple physiological races of the pathogen and the emergence
89 of fungicide-resistant strains (Lei *et al.* 2021; Yin *et al.* 2023). Conventional control strategies, including crop rotation,

soil solarization, and fungicide application, have shown limited long-term efficacy due to the pathogen's persistence in soil, its race diversity, and environmental concerns (Alengebawy *et al.* 2021; Maurya *et al.* 2019). These challenges highlight the need for sustainable strategies to enhance host resistance. Among emerging biotechnological solutions, antimicrobial peptides (AMPs) offer a promising approach. Defensins, a subgroup of AMPs characterized by six conserved cysteine residues forming three disulfide bridges, serve as important components of plant defense against fungal infections (Bulet *et al.* 2004; Gadwala *et al.* 2021). *NaD1*, a 47-amino-acid defensin from *Nicotiana glauca*, is a potent vacuolar-localized peptide with antifungal activity (Lay *et al.* 2003). It disrupts fungal membranes through oxidative stress and interaction with phosphatidylinositol 4,5-bisphosphate, contributing to fungal cell death (Payne *et al.* 2016; Royan *et al.* 2024). This antifungal activity positions *NaD1* as a promising candidate for crop protection.

To deploy *NaD1* effectively, efficient genetic transformation methods are essential. Floral dip and related in planta transformation methods target reproductive tissues directly, potentially reducing the need for conventional tissue culture-based regeneration and associated somaclonal variation (Bastaki and Cullis 2014; Rodin *et al.* 2014). Optimized floral inoculation approaches incorporating viscous suspending agents, acetosyringone, and vacuum infiltration have been developed to improve contact between *Agrobacterium* and plant tissues and facilitate transformation (Honda *et al.* 2021; Yasmeen 2009; Sharada *et al.* 2017). Here, we optimized a vacuum-assisted floral-bud infiltration protocol to accelerate the evaluation of *NaD1* efficacy in tomato.

Following recognition of phenolic compounds and other wound- or tissue-derived signals from the plant, the *vir* genes in *A. tumefaciens* are activated (Stachel and Zambryski 1986; Lee *et al.* 1992). Subsequently, the *VirD1–VirD2* proteins introduce site-specific nicks at the T-DNA border sequences and generate a single-stranded DNA molecule termed the T-strand, with *VirD2* remaining covalently attached to its 5' end (Ward and Barnes 1988; Young and Frank 1996; Herrera-Estrella *et al.* 1988). The T-strand, together with the *VirD2* and *VirE2* proteins, is transferred into the plant cell via the type IV secretion system assembled by the *VirB/VirD4* complex; *VirE2* coats and protects the T-strand from degradation, and nuclear localization signals within *VirD2* and *VirE2* facilitate nuclear import of the T-complex (Citovsky *et al.* 1994; Citovsky *et al.* 1992; Gelvin 2017). After nuclear entry, the T-DNA may be transiently expressed or integrated into the plant genome through host DNA break-repair pathways, particularly non-homologous end joining (NHEJ) and DNA polymerase θ -mediated end joining (TMEJ/MMEJ); when such integration occurs in germline or meristematic cells, the transgene can be transmitted to the next generation (Van Kregten *et al.* 2016; Gelvin 2017; Gelvin 2021).

Most studies report T-DNA-encoded genes such as *GUS* and *GFP* reporters as being primarily detected in tissues associated with the inoculation site. However, detection of transgene sequences or transcripts in tissues distal to the inoculation site does not, by itself, establish systemic movement of T-DNA or *Agrobacterium* through the vascular system. *Agrobacterium*-induced effects can nevertheless extend beyond the directly inoculated zone. For example, systemic signaling and mobile silencing phenomena have been documented following local *Agrobacterium*-mediated expression or viral agroinfection (Voinnet and Baulcombe 1997; Himber *et al.* 2003). These observations provide a biological context for examining whether transgene detection in distal tissues occurs following floral-bud inoculation, while distinguishing such detection from direct evidence of bacterial or T-DNA movement.

Despite the well-documented in vitro antifungal activity of *NaD1*, important questions remain regarding its ability to enhance resistance to *Fol* in tomato and the feasibility of rapid floral-bud transformation for functional evaluation of candidate resistance genes. In particular, determining whether *NaD1* expression is associated with suppression of Fusarium wilt and with tissue-specific physiological responses would provide complementary evidence for its potential role in host defense.

We hypothesized that *NaD1* expression would be associated with tissue-dependent changes in defense-related oxidative and phenolic responses, accompanied by reduced disease development and preservation of leaf physiological homeostasis. We further hypothesized that enhanced defense responses in roots would coincide with reduced disease development and maintenance of physiological status in distal leaves. To test these hypotheses, we employed an optimized floral-bud vacuum infiltration protocol to generate putative transformed tomato plants in the susceptible cultivar *SV4129* and evaluated their molecular, phenotypic, and biochemical responses to *Fol* infection. This approach was used to assess the in-planta efficacy of *NaD1* and to provide a rapid framework for functional evaluation of candidate resistance genes in tomato.

2. Materials and Methods

2.1. Plant materials and growth conditions

Seeds of commercial tomato cultivars (*Solanum lycopersicum* ‘SV4129’, *Rio Grande*, and *Galaxy*) were sourced from Mobin Kesht (Tehran, Iran). Seeds were surface sterilized by sequential immersion in 1% (v/v) sodium hypochlorite for 5 minutes and 70% ethanol for 30 seconds, with sterile distilled water rinses after each step. Sterilized seeds were germinated on wet filter paper in sterile Petri dishes. Seedlings were then transplanted into pots (7 × 7 cm) containing a sterile substrate of cocopeat, vermiculite, and perlite (1:1:1, v/v/v). Plants were maintained in a growth chamber at 24°C, 70% relative humidity, and a 16/8 h (light/dark) photoperiod with a light intensity of approximately 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Ofori *et al.* 2022).

2.2. Fungal Isolation, Culture, and Inoculum Preparation

Isolates of *Fol* were obtained from symptomatic root and stem tissues of wilted tomato plants collected from a greenhouse in Rasht, Iran. Collected tissues were surface sterilized by sequential immersion in 3% sodium hypochlorite for 15 minutes and 70% ethanol for 1 minute, followed by rinses in sterile water. Small tissue segments (0.5 cm) were then aseptically placed onto a semi-selective Potato Dextrose Agar (PDA) medium. The medium was supplemented with 0.1% Triton X-100, 1% gallic acid, streptomycin (20 mg/L), and chloramphenicol (20 mg/L) to inhibit bacterial growth (Segalin and Reis 2010). Plates were incubated at 25 ± 2°C under continuous light for 10 days. Colonies with morphological characteristics consistent with *Fol* species were selected for microscopic examination of microconidia, macroconidia, and chlamydospores. From these, pure isolates were established, and monosporic cultures were prepared for subsequent experiments. For long-term preservation, isolates were stored on filter paper in a 30% (v/v) glycerol solution at -80°C (Carmona *et al.* 2020). For inoculum preparation, the confirmed *Fol* isolate was grown on PDA at 25°C for 10 days. Spores were harvested by gently scraping the colony surface in 5 mL of sterile water containing 0.01% Tween 20. This suspension was used to inoculate 200 mL of Potato Dextrose Broth (PDB), which was then incubated on a shaker (150 rpm) for 5 days to promote microconidia proliferation. The resulting culture was centrifuged (7500 rpm), the pellet was resuspended in sterile distilled water, and the final concentration was adjusted to 1×10^6 microconidia/mL using a hemocytometer (Catanzariti *et al.* 2015).

2.3. Cultivar Pathogenicity Screening

Seedlings of the three tomato cultivars were cultivated for 25 days in a substrate mix of cocopeat, vermiculite, and perlite (1:1:1, v/v/v). For inoculation, seedlings were carefully uprooted, and their root systems were immersed for 15 minutes in a freshly prepared *Fol* spore suspension (1×10^6 microconidia/mL). Following inoculation, plants were replanted and maintained under controlled environmental conditions: 25°C day/22°C night temperature, 70% relative humidity, and a 16-hour photoperiod with a light intensity of approximately 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were irrigated with Hoagland solution every five days. Disease severity was assessed at four weeks post-inoculation to determine the susceptibility of each cultivar. Three biological replicates were used per cultivar in each of two independent experiments, with each biological replicate consisting of three individual plants. The biological replicate, rather than the individual plant, was considered the experimental unit.

2.4. Bacterial Strain and Vector Verification

For plant transformation, *A. tumefaciens* strain GV3101, containing the helper plasmid pMP90, was utilized. This strain harbored the recombinant binary vector pGSA1285:*NaD1*. The T-DNA region of this vector contained the *NaD1* coding sequence driven by the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter and terminated with the octopine synthase terminator (OCS-ter) (Royan *et al.* 2024). The construct also contained the neomycin phosphotransferase II (*nptII*) gene as a selectable marker for kanamycin resistance. A schematic illustration of this T-DNA construct is presented in Fig. 2a.

2.5. Agrobacterium-mediated Transformation

2.5.1. Preparation and Verification of *Agrobacterium tumefaciens*

A. tumefaciens strain GV3101, harboring the recombinant binary vector pGSA1285:*NaD1*, was grown overnight at 28°C with agitation (225 rpm) in Luria-Bertani (LB) medium supplemented with selective antibiotics (50 mg/L

kanamycin, 50 mg/L rifampicin, and 64 mg/L gentamicin). For verification, the overnight culture was streaked onto solid LB agar plates with the same antibiotics and incubated at 28°C for 48 hours to obtain single colonies. Plasmid DNA was extracted from selected colonies using a miniprep kit, and colony PCR was performed using *NaDI*-specific primers (Table 1) to verify the presence of the expected construct. The corresponding PCR results are presented in the Results section.

2.5.2. Inoculation and Plant Transformation

The floral-bud transformation procedure was adapted from the *A. tumefaciens*-mediated floral-bud smearing method (Honda *et al.* 2021) and modified for the tomato cultivar ‘SV4129’. The modifications included replacing the starch-based spreading agent with carboxymethyl cellulose (CMC), extending the inoculation area to include the floral bud, flower, and proximal pedicel, and incorporating vacuum-assisted infiltration. A single positive *A. tumefaciens* GV3101 (pMP90, pGSA1285:*NaDI*) colony was used to inoculate 5 mL of LB broth containing kanamycin, rifampicin, and gentamicin. The culture was incubated overnight at 28°C with shaking at 225 rpm. The overnight culture was diluted to an OD600 of 0.3 in LB broth supplemented with 200 µM acetosyringone and incubated for 4–6 h at 28°C. When the optical density at 600 nm (OD600) reached approximately 0.6, the bacterial cells were harvested, washed once with liquid MS medium, and resuspended at an OD600 of 0.6 in tomato infection medium.

The infection medium contained MS salts (4.6 g/L), 2-(N-morpholino)ethanesulfonic acid (MES; 250 mg/L), sucrose (50 g/L), 1 N KOH (800 µL/L), 6-benzylaminopurine (BAP; 0.01 mg/L), Tween-20 (200 µL/L), and acetosyringone (100 µM), with the pH adjusted to 6.0. CMC was added at 0.5% (w/v) to increase the viscosity of the bacterial suspension (Honda *et al.* 2021).

The bacterial suspension was applied to immature floral buds and the proximal pedicel region of 60-day-old SV4129 plants bearing 6–8 fully expanded true leaves and measuring approximately 20–23 cm in height. The inoculation area extended to within approximately 0.5 cm of the receptacle, and 5–6 floral buds were treated per plant. Following application of the bacterial suspension, the inoculated plants were subjected to vacuum infiltration at 100 mbar for 10 min. The treated plants were then covered with plastic wrap and maintained for 24 h at 22°C under a 16-h photoperiod, after which they were returned to standard growth conditions (Fig.1).

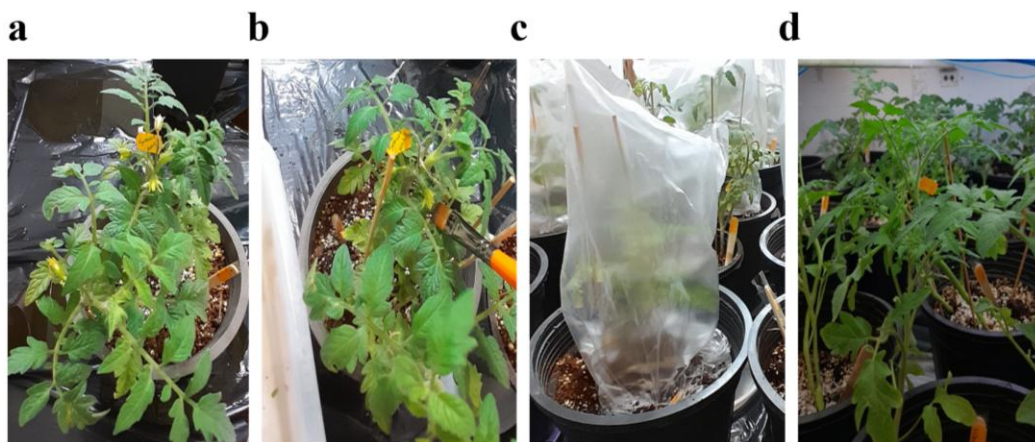


Fig. 1 *A. tumefaciens*-mediated transformation/inoculation of tomato floral buds (smearing method) (a) Selection of healthy SV4129 tomato plants at the flowering stage (60 days old, 6–8 true leaves, 20–23 cm height). (b) Inoculation by smearing the viscous *Agrobacterium* suspension containing 0.5% CMC directly onto the proximal pedicel region (within 0.5 cm of the receptacle) of immature floral buds. (c) Vacuum infiltration of the inoculated plants for 10 min at 100 mbar, followed by covering with plastic wrap for 24 h at 22°C under a 16-h photoperiod. Plants were subsequently transferred to the growth chamber under standard growth conditions for an additional incubation period of up to two weeks before molecular analysis and resistance testing

2.6. Sampling of Tissues Distal to the Inoculation Site

To assess the presence of the introduced *NaDI* sequence in tissues spatially separated from the floral inoculation site, tissue samples were collected from the upper portion of the plants. The two youngest fully expanded leaves (leaves 7 and 8, counted

acropetally from the basal leaves) were sampled from nodes located approximately 8–12 cm above the inoculated floral pedicels, which were positioned at nodes 6–7.

The sampled distal leaves were used for molecular analyses. Leaf and stem tissues collected from regions distal to the inoculation site were additionally used for microbiological assessment of residual *Agrobacterium*. The sampling strategy was therefore used to distinguish detection of the introduced *NaD1* sequence in distal plant tissues from the presence of viable or detectable bacterial cells.

2.7. Molecular Analysis of Putative Transformed Plants

2.7.1. PCR Detection of *NaD1*

Genomic DNA was extracted from the two youngest fully expanded leaves (leaves 7 and 8) of putative T₁ transformed plants using the Dellaporta method (Dellaporta *et al.* 1983). Non-transgenic *SV4129* plants were used as negative controls. The presence of the introduced *NaD1* sequence was assessed by PCR using *NaD1*-specific primers (Table 1). PCR products were separated by electrophoresis on a 2% (w/v) agarose gel and visualized under UV illumination. Plants producing the expected 106-bp *NaD1* amplification product were classified as PCR-positive and were subsequently subjected to tests for detectable *Agrobacterium* contamination and *NaD1* transcript detection.

2.7.2. RNA Extraction and RT-PCR Analysis

Total RNA was extracted from young leaf tissues of PCR-positive, *Agrobacterium*-negative T₁ plants and non-transgenic controls using TRIzol® reagent. RNA integrity was assessed by visualization of ribosomal RNA bands on a 1% agarose gel, and RNA concentration and purity were determined spectrophotometrically using a NanoDrop™ system. To remove residual genomic DNA, RNA samples were treated with RNase-free DNase I before cDNA synthesis. First-strand cDNA was synthesized from DNase-treated RNA using the Cinaclon First Strand cDNA Synthesis Kit (Iran). The presence of *NaD1* transcripts was assessed by endpoint RT-PCR using *NaD1*-specific primers (Table 1). The tomato *elongation factor 1-alpha (elf1a)* gene was amplified as an internal control to verify cDNA integrity and successful amplification. RT-PCR products were separated on a 2% (w/v) agarose gel and visualized under UV illumination.

Table 1 Primer sequences used for colony PCR, genomic PCR, RT-PCR, and *virD2* PCR**

Gene name	Primer Sequence (5'→3')	Annealing temperature (°C)	Size (bp)
<i>NaD1</i>	Forward: 5'-AGGATCTCATCATCACCA-3' Reverse: 5'-AGCTTTTCTACATGGTGGCT-3'	57	106
<i>elf1a</i>	Forward: 5'-TCTTAACATACCAGCATCACC-3' Reverse: 5'-TGAACCATCCAGGACAGATG-3'	57	177
<i>VirD2</i>	Forward: 5'-ATGTCGCAAGGCAGTAAG-3' Reverse: 5'-CAAGGAGTCTTTCAGCATG-3'	51	441

** The same *NaD1* primer pair was used for colony PCR, genomic PCR, and endpoint RT-PCR.

2.8. Confirmation of *Agrobacterium*-free Status

To determine whether the *NaD1*-positive plants contained detectable residual *A. tumefaciens*, two independent approaches were used: PCR-based detection of the bacterial *virD2* gene followed by microbiological culture. This order was aligned with the sequence of these analyses presented in the Results section.

2.8.1. PCR-based Detection of *virD2*

Genomic DNA extracted from the same distal tissue samples was subjected to PCR using *virD2*-specific primers (Table 1), which amplify a 441-bp fragment of the *virD2* gene. The *virD2* sequence was used as a bacterial-specific molecular marker to assess the presence of detectable residual *Agrobacterium* DNA in plant tissues (Johnson *et al.*, 2013).

PCR was performed in a 25-μL reaction containing 50–100 ng of template DNA under the following thermal profile: initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 45 s, 56°C for 60 s, and 72°C for 60 s; followed by a final extension at 72°C for 5 min. DNA from *A. tumefaciens* GV3101 and sterile water were included as positive and negative controls, respectively. The tomato *elf1a* gene was used as an internal plant DNA control.

Samples were considered negative for detectable *Agrobacterium* DNA when no 441-bp *virD2* amplification product was observed while the plant internal control and the positive control produced the expected amplification products. All assays were performed in triplicate.

2.8.2. Microbiological Assay

Leaf and stem tissues were collected from regions distal to the floral inoculation site, including the upper leaf tissues used for molecular analysis. The tissues were surface-sterilized with 70% ethanol, washed with sterile detergent solution, and the epidermal surfaces were aseptically removed. Tissue pieces were then macerated in sterile distilled water and cultured on PYGA medium containing 3 g/L peptone, 5 g/L yeast extract, 10 mL/L glycerol, and 20 g/L agar, supplemented with rifampicin, kanamycin, and gentamicin at the concentrations used for *Agrobacterium* selection.

To increase the sensitivity of bacterial detection, a subset of tissue samples was additionally enriched in liquid PYGA medium without agar for 48 h at 28°C before being transferred to selective solid medium (Domínguez *et al.* 2004). All plates were incubated at 28°C for 3–5 day. Samples were considered negative when no *Agrobacterium*-like colonies were recovered from direct or enrichment cultures.

2.9. Assay for Fusarium Wilt Resistance in Transformed and Wild-Type Plants

For the resistance assay, putative primary transformed T₁ tomato plants generated through *Agrobacterium*-mediated floral inoculation and confirmed by PCR for the presence of the *NaD1* transgene were used. The two independent putative transformed plants were designated *TSV4129-1* and *TSV4129-2*. Non-transgenic *SV4129* plants were used as wild-type controls. At 21 days of age, plants were inoculated by immersing their roots for 30 min in a *Fol* spore suspension containing 1×10^6 microconidia/mL. Following inoculation, seedlings were transplanted into pots containing a mixture of perlite, cocopeat, and vermiculite and maintained under controlled greenhouse conditions at approximately 25°C. Disease progression was monitored for 28 days after inoculation. Disease severity was assessed at 11, 18, 22, and 28 days post-inoculation using a 0–6 disease severity scale (Lebeda and Buczkowski 1986). To provide an integrated measure of disease development over time, the area under the disease progress curve (AUDPC) was calculated according to the following formula:

$$\text{AUDPC} = \sum_i [(D_i + D_{i-1}) \times (t_i - t_{i-1})] / 2.$$

Four biological replicates consisting of individual plants were evaluated for each transformed event (*TSV4129-1* and *TSV4129-2*). Thus, “clonal replicates” has been removed because individual T₁ plants should not be described as clonal replicates unless they were demonstrably clonally propagated. For the pooled analysis presented in Fig. 4b, the two putative transformed plants were combined into a single transgenic group (n = 8) and compared with the wild-type control (n = 3). For the event-wise analysis presented in Fig. 4c, each transformed event (n = 4) was analyzed separately against the wild-type control (n = 3).

2.10. Physiological Evidence of Defense Response to Fusarium Wilt

Physiological and biochemical analyses were performed using the same two youngest fully expanded leaves used for distal-tissue molecular analyses (leaves 7 and 8, counted acropetally from the basal leaves), unless otherwise specified. These leaves were selected from non-inoculated tissues located approximately 8–12 cm above the inoculated floral pedicels. The same leaf positions were used consistently across experimental groups. For root-based analyses, root tissues collected from the corresponding plants were analyzed as described below.

2.10.1. Quantification of Protein Content via Bradford Assay

Total soluble protein was extracted from the leaf tissues of both the high-resistance putative transgenic (*SV4129*, *TSV4129-2*) and wild-type (*SV4129*) plants in three biological replicates. For each sample, 50 mg of fresh

leaf tissue was frozen in liquid nitrogen and ground to a fine powder in a pre-chilled mortar and pestle. The powder was then homogenized in 1 mL of ice-cold extraction buffer (50 mM potassium phosphate buffer, pH 7.0). The homogenate was subjected to a two-step centrifugation process at 4°C to clarify the extract. First, it was centrifuged at 14,000 rpm for 15 minutes. The resulting supernatant was carefully collected and then centrifuged again at 14,000 rpm for an additional 10 minutes. The final supernatant, containing the total soluble protein, was used for quantification. Protein concentration was determined using the Bradford method (Bradford 1976). Bovine serum albumin (BSA) was used to generate a standard curve, and the absorbance was measured at 595 nm.

2.10.2. Peroxidase (POD) Activity Assay

POD activity was determined spectrophotometrically following the method of Chance and Maehly (Chance and Maehly 1955). Three technical measurements were performed for each biological replicate. The reaction mixture contained 2.3 mL of phosphate buffer (0.02 M, pH 6.4), 0.5 mL of guaiacol (12 mM), and 0.2 mL of the protein extract. The reaction was initiated by the addition of 20 µL of 3% H₂O₂. Immediately after initiation, the increase in absorbance at 470 nm, resulting from the formation of tetraguaiacol, was monitored continuously for 2 minutes. The linear rate of change in absorbance ($\Delta A_{470}/\text{min}$) was used to calculate the enzyme activity. The activity was quantified using the molar extinction coefficient for tetraguaiacol ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed as µmol of tetraguaiacol formed per minute per mg of protein ($\mu\text{mol min}^{-1} \text{ mg}^{-1}$).

2.10.3. Chlorophylls and Carotenoid Content Determination

Chlorophyll and carotenoid pigments were extracted and quantified following a modified method based on (Arnon 1949). Briefly, 100 mg of fresh leaf tissue from leaves 7 and 8 was accurately weighed and thoroughly homogenized in a pre-chilled mortar and pestle with 20 mL of 80% (v/v) acetone. The resulting homogenate was transferred to centrifuge tubes and centrifuged at 3,000 rpm for 15 minutes at 4°C to separate the supernatant from the cellular debris. The supernatant, containing the extracted pigments, was carefully decanted and retained. The remaining pellet was subjected to successive re-extractions, each with 5 mL of 80% (v/v) acetone, until the pellet became colorless, indicating complete pigment extraction. All collected supernatants from the initial extraction and subsequent re-extractions were combined to ensure the recovery of total pigments. The absorbance of the combined pigment extract was measured spectrophotometrically at wavelengths of 663, 645, and 475 nm. The concentrations of chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids were then calculated using the specific equations provided by Arnon (1949) (Arnon 1949).

$$\begin{aligned}\text{Chl a (mg/g)} &= 12.7 \times (A_{663}) - 2.69 \times (A_{645}) \times V/1000 \times W \\ \text{Chl b (mg/g)} &= 22.9 \times (A_{645}) - 4.68 \times (A_{663}) \times V/1000 \times W \\ \text{Carotenoids} &= [1000 \times A_{470} - 2.860 (\text{Chl a}) - 85.9 (\text{Chl b})]/245\end{aligned}$$

where V is the final extract volume (mL) and W is the fresh weight (g).

2.11. Determination of Phenolic Accumulation in Root and Leaf Tissues

The total phenolic content of the extracts was determined using the Folin-Ciocalteu colorimetric method, with minor modifications (Policegoudra and Aradhya 2007). In brief, 100 µL of the methanolic extract was mixed with 2 mL of 10% (w/v) aqueous sodium carbonate solution. After 5 minutes, 100 µL of 50% (v/v) Folin-Ciocalteu reagent was added. The reaction mixture was incubated at room temperature for 30 minutes, and the absorbance was measured at 750 nm using a spectrophotometer (Biochrom Libra S22 UV/Vis, USA). A standard curve was prepared using gallic acid, and each extract was assayed in triplicate. The total phenolic content was expressed as milligrams of gallic acid equivalents per gram of fresh weight (mg GAE/g FW).

2.12. Lipid Peroxidation (LPO)

Lipid peroxidation (LPO) level, determined by measurement of malondialdehyde (MDA) content, was assessed using the thiobarbituric acid (TBA) reaction method (Ohkawa *et al.* 1979). Each treatment involved homogenizing 0.1 g of leaf or root tissue in 2.0 mL of 20% trichloroacetic acid (TCA) solution (w/v) containing 1% TBA (w/v) for 30 minutes at 95°C. The reaction was halted by cooling the samples on ice for 10 minutes, followed by centrifugation at 10,000 rpm for 15 minutes to clarify the solution. The absorbance of the supernatant was measured at 532 and 600 nm. The absorbance at 600 nm was subtracted from the 532-nm reading to correct for non-specific turbidity. The

concentration of MDA was then calculated using the molar extinction coefficient for the MDA-TBA adduct ($\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$), and the MDA content was quantified as nmol MDA per gram fresh weight of the sample.

2.13. Quantification of H_2O_2

The H_2O_2 content of leaf tissues (leaves 7 and 8) was quantified based on the method of (Velikova *et al.* 2000) Velikova *et al.* (2000). Fresh leaf tissue (0.1 g) was homogenized in an ice bath with 2.0 mL of 0.1% (w/v) TCA. The homogenate underwent centrifugation at $12,000 \times g$ for 15 minutes, and 0.5 mL of the resulting supernatant was combined with 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide solution, followed by a 5-minute incubation at room temperature. The absorbance of the mixture was measured at 390 nm. The H_2O_2 concentration was calculated by comparison to a standard curve prepared with known H_2O_2 concentrations and was expressed as nmol H_2O_2 per gram fresh weight (nmol/g FW).

2.14. Experimental Design and Statistical Analysis

A completely randomized design was used throughout the study. For cultivar susceptibility screening, three biological replicates were used for each cultivar in each of two independent experiments, with each biological replicate consisting of three individual plants. The biological replicate, rather than the individual plant, was considered the experimental unit for cultivar screening.

For disease-resistance assays, individual plants were treated as biological replicates, with four biological replicates per transformed event and three wild-type control plants. For physiological and biochemical analyses, the number of biological replicates varied among assays and is reported in the corresponding Results and figure legends. Technical replicates were used where applicable and were averaged before statistical analysis. Molecular analyses, including PCR, endpoint RT-PCR, microbiological culture, and *virD2* PCR, were performed using independent biological samples with technical replication of PCR assays.

2.15. Statistical Analysis

Data from the cultivar susceptibility screening were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test at $\alpha = 0.05$.

For physiological and biochemical parameters, comparisons between transformed and wild-type plants were performed using two-tailed Mann-Whitney U tests (Wilcoxon rank-sum tests), with exact two-sided *P* values used where applicable. These nonparametric analyses were selected because several assays involved small sample sizes and did not provide sufficient support for parametric assumptions.

For the time-course disease-severity data collected at 11, 18, 22, and 28 days post-inoculation, a linear mixed-effects model for repeated measures was used. Treatment, time, and the treatment $\times$ time interaction was included as fixed effects, while biological replicate was included as a random effect. An autoregressive covariance structure of order one [AR(1)] was selected to account for the correlation among repeated measurements over time. Adjusted least-squares means (LS-means) were compared using the Tukey-Kramer procedure.

For AUDPC data, normality of model residuals was assessed using the Shapiro-Wilk test, and homogeneity of variances was assessed using Levene's test. Because neither assumption was significantly violated, one-way ANOVA was used to compare AUDPC among the three groups, followed by Tukey's HSD test for pairwise comparisons at $\alpha = 0.05$. For the AUDPC analysis, the three groups consisted of wild-type *SV4129* ($n = 3$), *TSV4129-1* ($n = 4$), and *TSV4129-2* ($n = 4$). Where indicated, event-wise comparisons were performed between each transformed event and the wild-type control.

For pairwise AUDPC comparisons, effect sizes were expressed as Cohen's *d*, and 95% confidence intervals were calculated using bootstrap resampling with 10,000 iterations. All statistical analyses were performed using SAS software. Graphs and data visualizations were generated using Microsoft Excel and R. Unless otherwise indicated, quantitative data are presented as mean $\pm$ standard error (SE), and statistical significance was considered at $P < 0.05$. Statistical analyses were selected according to the experimental design and distributional characteristics of each dataset. Normality was assessed using the Shapiro-Wilk test where applicable. For normally distributed datasets

satisfying variance assumptions, one-way ANOVA followed by Tukey's HSD test was used for comparisons among multiple groups. Where variance heterogeneity was detected, an appropriate heteroscedastic procedure was used. Non-parametric datasets were analyzed using the Kruskal–Wallis test followed by an appropriate post-hoc comparison or the Wilcoxon test, as applicable. Disease progression over multiple time points was analyzed using an appropriate repeated-measures/mixed-effects model, with plant considered the experimental unit. Statistical analyses were performed using SAS 9.3, whereas graphs and data visualizations were generated using R and Microsoft Excel. A significance threshold of $\alpha = 0.05$ was applied. Data are presented as mean $\pm$ standard error (SE), unless otherwise indicated.

3. Results and discussion

3.1. Identification of a Susceptible Tomato Cultivar for Genetic Transformation

To establish a sensitive genetic background for evaluating *NaDl*-associated resistance to *Fol*, three tomato cultivars, *Galaxy*, *SV4129*, and *Rio Grande*, were screened for susceptibility. The screening was performed in two independent experiments, with three biological replicates per cultivar in each experiment; each biological replicate consisted of three individual plants. *Rio Grande* maintained comparatively vigorous growth with limited visible symptoms (Fig. 2a), whereas *Galaxy* and *SV4129* displayed pronounced wilting, extensive chlorosis, and foliar necrosis (Fig. 2b,c). These differences in disease phenotype were consistent with the quantitative disease severity scores at 28 days post-inoculation (dpi). *Galaxy* and *SV4129* exhibited mean disease severity scores of 4.0 ± 0.3 and 3.7 ± 0.2 , respectively, whereas *Rio Grande* showed a significantly lower mean score of 1.6 ± 0.2 on a 0–6 scale ($P < 0.05$, Tukey's HSD) (Fig. 2d). Based on its high disease severity and pronounced symptom development, *SV4129* was selected as the susceptible genetic background for subsequent *NaDl* transformation experiments.

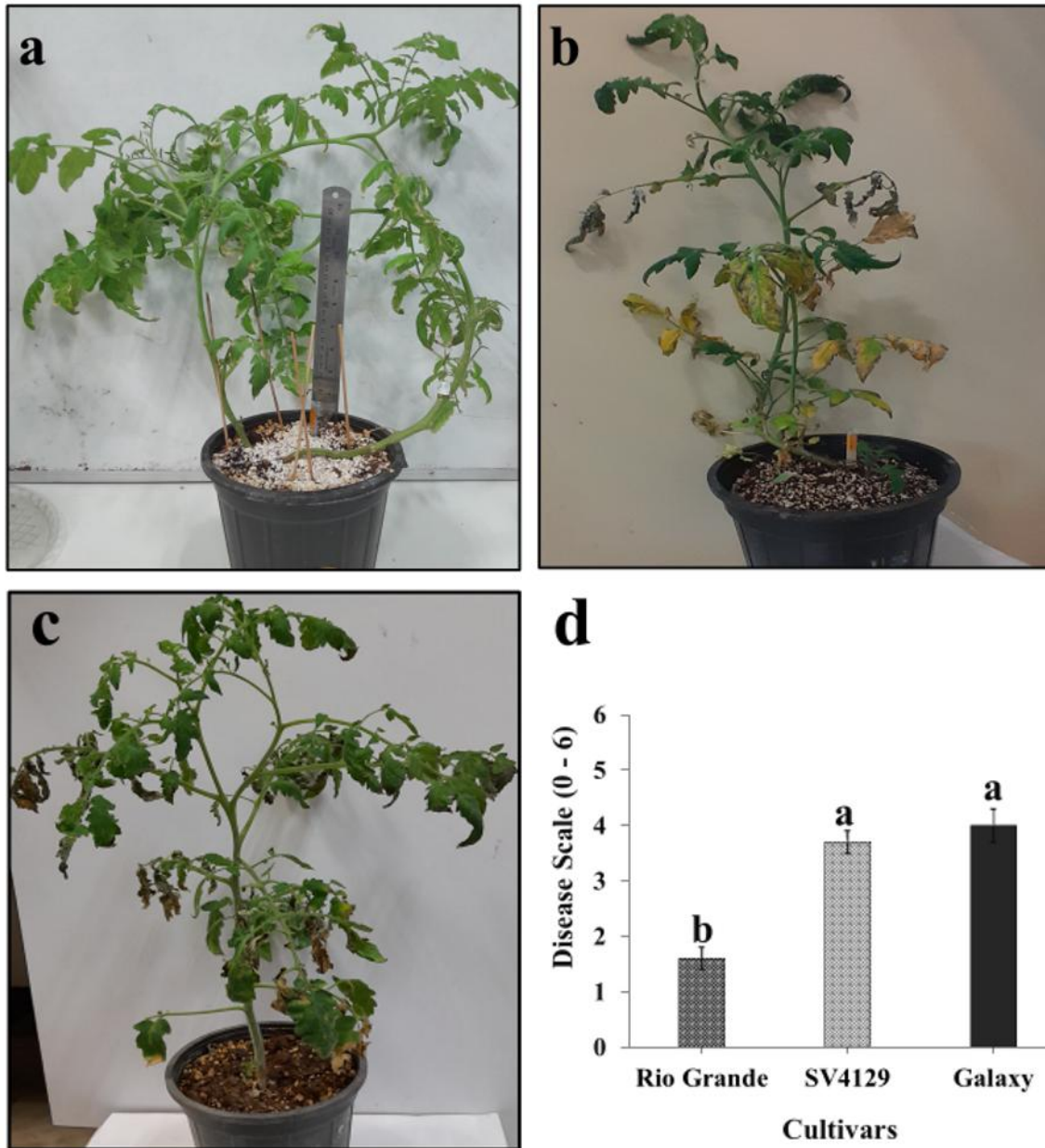


Fig. 2 Disease severity in three tomato cultivars at 28 days post-inoculation (dpi) with *Fol*. Representative plants are shown in panels (a–c): (a) *Rio Grande*, showing comparatively mild disease symptoms; (b) *Galaxy*; and (c) *SV4129*, with pronounced wilting and necrosis. Panel (d) shows mean disease severity scores on a 0–6 scale $\pm$ standard deviation (SD) for three biological replicates, with three independent plants per replicate. Different letters indicate significant differences among cultivars according to Tukey’s HSD test ($P < 0.05$)

Given its high disease severity (3.7 ± 0.2), reproducible symptom development, and clear contrast with the response of *Rio Grande*, *SV4129* was selected as a susceptible genetic background for *Agrobacterium*-mediated transformation and subsequent evaluation of *NaD1*-associated disease responses. Using a susceptible genetic background reduces the likelihood that pre-existing host tolerance will obscure differences associated with *NaD1* expression, thereby providing a stringent context for evaluating disease responses following transformation.

3.2. Molecular Verification of the pGSA1285:NaD1 Recombinant Construct in *A. tumefaciens* GV3101

Colony-PCR using *NaD1*-specific primers amplified the expected 106-bp fragment from *A. tumefaciens* GV3101 colonies harboring the pGSA1285:*NaD1* construct (Fig. 3), consistent with the presence of the target *NaD1* sequence in the bacterial transformants. The recombinant construct had been previously verified by sequencing (Royan *et al.* 2024). Together, these results verified the presence of the expected *NaD1* sequence in the *Agrobacterium* cultures used for subsequent plant transformation experiments.

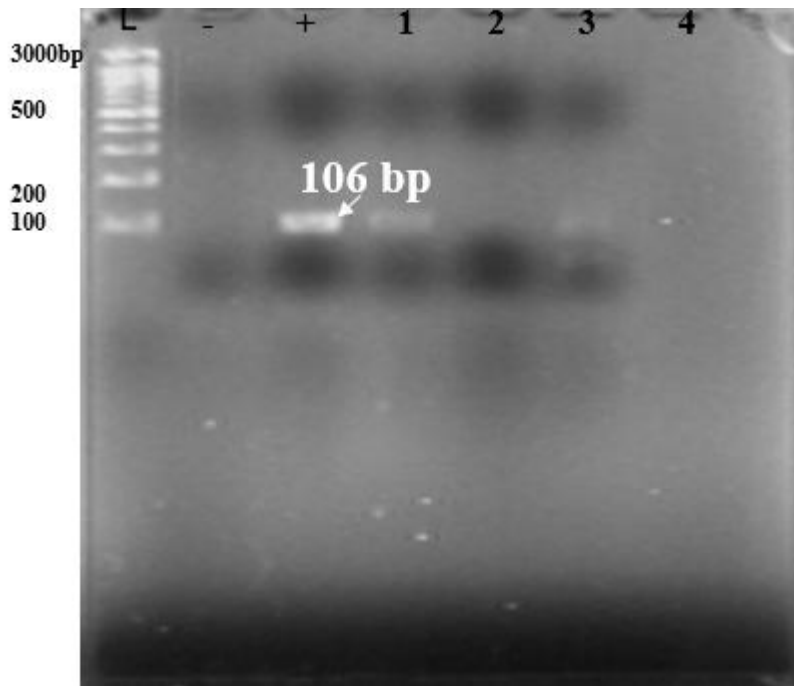


Fig. 3. Molecular verification of the pGSA1285:*NaD1* recombinant construct in *A. tumefaciens* GV3101 by colony-PCR. (a) Colony-PCR verification of pGSA1285:*NaD1* in *A. tumefaciens* GV3101, showing the expected 106-bp *NaD1* amplicon. L, DNA molecular weight marker; +, positive control; -, negative control; 1-4, colony-PCR products from colonies 1-4, respectively.

3.3. Molecular identification of putative *NaD1*-transformed tomato plants

Following floral-bud inoculation using the modified smearing procedure, eight putative T₁ transformants were identified by PCR screening. An expected 106-bp *NaD1* fragment (Fig.4a) was detected in all eight plants, whereas no corresponding product was detected in the untransformed wild-type control or the no-template control (Fig. 4b). The detection of the target sequence in all eight screened plants represents a high PCR-positive frequency and therefore warrants cautious interpretation. Because PCR amplification alone does not establish genomic integration or heritable insertion, the plants were considered putative transformants at this stage rather than confirmed stable transformants. The high frequency observed here may have been facilitated by the floral-bud transformation procedure used in this study, which combined direct inoculation of floral tissues with acetosyringone treatment, carboxymethyl cellulose, and vacuum-assisted infiltration. These conditions may have increased bacterial access to receptive floral tissues and thereby increased the probability of detecting PCR-positive plants. However, this interpretation remains provisional because the relative contribution of each transformation parameter was not experimentally dissected in the present study.

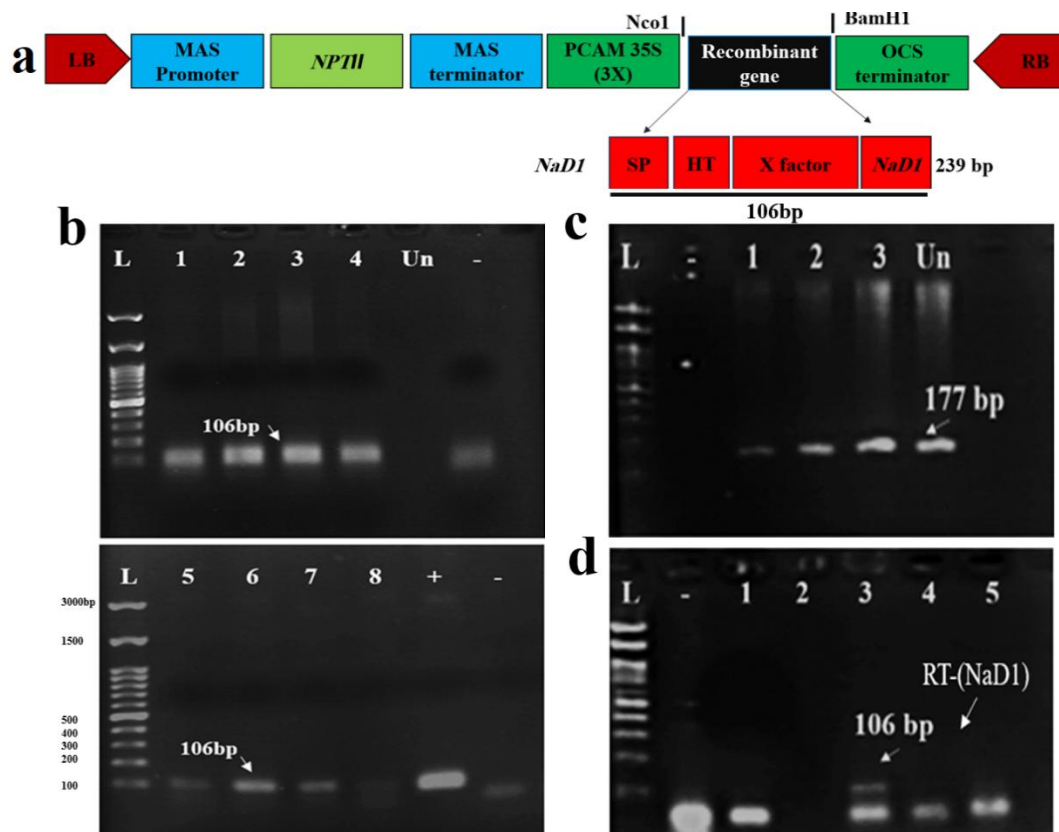


Fig. 4 Molecular characterization of putative *NaD1*-transformed T₁ tomato plants (a) Schematic representation of the T-DNA region of the pGSA1285-*NaD1* expression construct used for *Agrobacterium tumefaciens*-mediated transformation. The construct contains the right border (RB), mannopine synthase promoter (MAS promoter), *nptII* selectable marker, MAS terminator, 3×CaMV35S promoter, vicilin signal peptide (SP), His₆ tag, TEV/Xa protease cleavage region, *NaD1* coding sequence, octopine synthase terminator (OCS-ter), and left border (LB). (b) Genomic DNA PCR analysis of putative transformed plants using *NaD1*-specific primers to amplify the expected 106-bp fragment. Individual plant samples are indicated by their corresponding sample numbers. (c) Endpoint RT-PCR analysis using *elf1a*-specific primers, generating the expected 177-bp fragment, as an internal reference for the cDNA samples analyzed in panels (c) and (d). (d) Endpoint RT-PCR analysis of *NaD1* transcripts using *NaD1*-specific primers, generating the expected 106-bp fragment. M, DNA molecular weight marker; Un, untransformed wild-type control; +, positive control; -, negative control; NTC, no-template control. PCR products were separated by electrophoresis on 2% agarose gels and visualized under UV illumination

3.4. Assessment of Residual *Agrobacterium* in PCR-Positive Plants

To assess whether residual *Agrobacterium* was detectable in PCR-positive plants, the samples were examined using both molecular and culture-based assays. PCR with *virD2*-specific primers produced no detectable 441-bp amplicon in the plant samples, whereas the *Agrobacterium* positive control yielded the expected 441-bp product (Fig. 5a). In parallel, plant tissues were cultured on selective medium, and no *Agrobacterium*-like colonies were detected after 120 h of incubation at 28 °C (Fig. 5b). Together, the absence of a detectable *virD2* signal and the failure to recover culturable bacteria indicated that *Agrobacterium*-associated DNA and culturable bacterial cells were not detected in the sampled tissues under the conditions tested. However, these results do not exclude the possibility of bacterial persistence in unsampled tissues or at other time points, given the potential spatial and temporal variation in bacterial distribution within plant tissues.

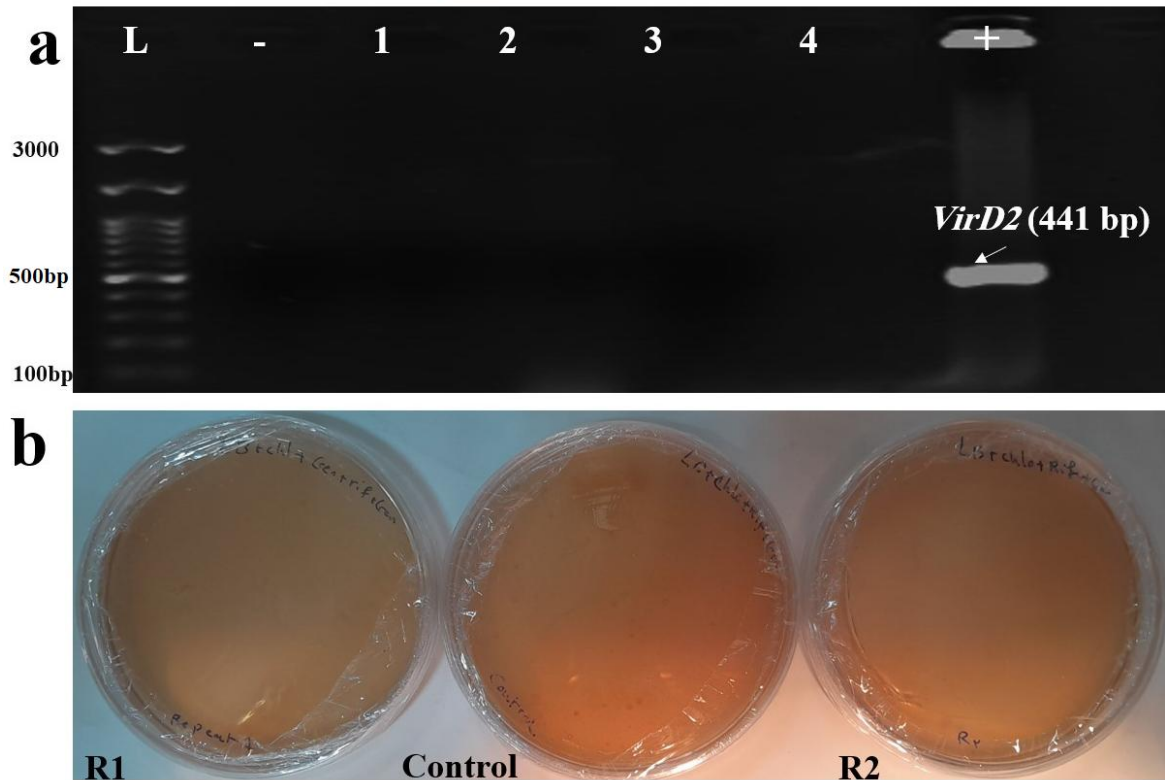


Fig. 5 Molecular and culture-based assessment of detectable residual *A. tumefaciens* in putative *NaD1*-transformed T0 tomato plants. (a) PCR analysis of plant genomic DNA using *virD2*-specific primers, which amplify a 441-bp fragment corresponding to the *virD2* sequence of *A. tumefaciens*. Genomic DNA from the analyzed plant samples was compared with the *Agrobacterium* positive control and the corresponding negative control. (b) Culture-based assay for detection of culturable *Agrobacterium* from plant tissue samples. Leaf and stem tissues collected from the analyzed plants were processed and cultured on selective medium as described in the Materials and Methods section. Representative culture plates corresponding to the analyzed samples and controls are shown. M, DNA molecular weight marker; +, *A. tumefaciens* positive control; -, negative control; WT, untransformed wild-type control; TSV4129-1 and TSV4129-2, selected T0 plants. PCR products in panel (a) were separated by electrophoresis on an agarose gel

3.5. Detection of *NaD1* transcripts in PCR-positive T₁ plants

Because genomic PCR establishes the presence of the target sequence but does not provide evidence of its transcriptional activity, expression of *NaD1* was subsequently assessed by endpoint RT-PCR. Four PCR-positive plants that were negative for *virD2* amplification and culturable *Agrobacterium* were selected for transcript analysis. Amplification of the *elf1a* reference transcript generated the expected 177-bp product in the analyzed samples, providing an internal control for the analyzed cDNA samples (Fig. 4c). A 106-bp *NaD1*-specific amplification product was detected in two of the four plants, designated TSV4129-1 and TSV4129-2, whereas no detectable *NaD1* amplification product was observed in the remaining two PCR-positive plants (Fig. 4d). Thus, the molecular screening revealed heterogeneity in *NaD1* transcript detectability among plants carrying the PCR-detectable target sequence.

The detection of *NaD1* transcripts in only two of the four PCR-positive T₁ plants, designated TSV4129-1 and TSV4129-2, indicates heterogeneity in *NaD1* transcript detectability among the analyzed transformation-derived plants. Such variation among plants carrying the same expression construct is well documented and may arise from differences in transgene integration site, transgene copy number, T-DNA structure, or the epigenetic state of the integration region (Matzke and Matzke 1998; De Neve *et al.* 1997). One possible explanation is that the expressing plants retain a transcriptionally competent *NaD1*-containing locus, whereas the non-expressing plants may differ in T-DNA copy number, structural organization, integrity, or regulatory state. Insertion of multiple, truncated, or rearranged T-DNA copies can occur during plant transformation and may influence transgene expression and stability (Mason *et al.* 2002; De Neve *et al.* 1997). However, the absence of a detectable *NaD1* transcript alone does not

establish T-DNA truncation or rearrangement. Distinguishing structural differences in the transgene locus from transcriptional or post-transcriptional silencing would require additional molecular analyses, such as PCR spanning different regions of the T-DNA, analysis of the left and right T-DNA borders, and determination of transgene copy number by approaches such as qPCR, ddPCR, or Southern blotting.

An additional observation was the detection of *NaDI* transcripts in leaves collected distal to the original floral inoculation site. This finding raises the possibility that the *NaDI*-associated signal was detectable beyond the tissue initially exposed to *Agrobacterium*. However, the observation should not be interpreted as direct evidence of systemic movement of *NaDI* RNA, T-DNA, or transformed cells. Detection of a transcript in a distal leaf by endpoint RT-PCR does not distinguish between local transcription from a transgene already present in those cells, developmental distribution of transformed cell lineages, movement of RNA or another nucleic-acid-derived signal, or other mechanisms that could produce a distal PCR signal.

Systemic trafficking of RNA molecules is nevertheless a biologically established phenomenon in plants. Mobile RNAs and RNA-derived silencing signals can move between cells and over long distances through the vascular system, with the phloem representing an important route for systemic RNA transport (Voinnet and Baulcombe 1997; Melnyk *et al.* 2011; Liu and Chen 2018). In particular, studies of systemic RNA silencing have demonstrated that sequence-specific signals generated locally can spread to distal tissues, and experimental evidence supports a role for plasmodesmata and the phloem in this process. These observations provide a biological framework in which nucleic-acid-derived signals can occur beyond their site of initial production, but they do not establish that an intact *NaDI* transcript follows the same route in the present transformation system.

The possibility of systemic distribution must also be considered in the context of *Agrobacterium* biology. Systemic movement of *Agrobacterium* itself has been experimentally documented. Cubero *et al.* (2006) detected pathogenic *Agrobacterium* by isolation and PCR in tomato tissues distant from primary tumor sites and also demonstrated bacterial movement in symptomless stem tissues of several plant species (Cubero *et al.* 2006). Earlier work by Jones and Raju (1988) similarly reported recovery of *A. tumefaciens* from apparently healthy stem tissues distal to developing galls in chrysanthemum (Jones and Raju 1988). These observations are relevant because they demonstrate that bacterial distribution within plants can extend beyond the original inoculation site. However, in the present study, the negative *virD2* PCR and culture assays reduce the likelihood that the distal *NaDI* signal was attributable to readily detectable residual *Agrobacterium* in the sampled tissues. Importantly, the cited studies concern bacterial movement and therefore cannot be used as evidence that *NaDI* RNA or an intact non-viral T-DNA cassette moved systemically in the present experiment.

The CaMV 35S promoter is a widely used constitutive promoter for transgene expression in plants and has been shown to drive expression of heterologous genes across diverse plant systems (Benfey and Chua 1990; Amack and Antunes 2020). Therefore, the presence of the 3×CaMV35S promoter provides a plausible basis for transcription of the introduced *NaDI* cassette in tissues in which the transgene is transcriptionally active. However, whether transcript abundance contributed to the detection of *NaDI* in distal leaves cannot be determined from the qualitative endpoint RT-PCR data. Consequently, the possibility that higher transgene activity increased the probability of detecting *NaDI* RNA in distal tissues remains a hypothesis rather than a demonstrated mechanism.

Taken together, the molecular analyses demonstrate that *NaDI*-specific sequences were PCR-detectable in eight screened plants and that *NaDI* transcripts were detectable by endpoint RT-PCR in two of four PCR-positive, *virD2*-negative plants selected for transcript analysis. The absence of detectable *virD2* amplification and culturable *Agrobacterium* in the sampled tissues strengthens the interpretation that the detected *NaDI* transcripts were not readily attributable to residual bacterial contamination under the conditions tested. The detection of *NaDI* transcripts in distal leaves is consistent with the possibility of transgene activity beyond the initially treated floral tissue, but the present experimental design does not establish whether this distal signal resulted from systemic RNA movement, local transcription, developmental distribution of transformed cells, or another mechanism. Direct discrimination among these possibilities would require spatially resolved and quantitative analyses, such as qRT-PCR of proximal and distal tissues, detection of *NaDI* DNA and RNA along defined tissue gradients, and, where feasible, analysis of vascular/phloem-associated material.

3.6. Phenotypic evaluation of *NaDI*-transcript-positive plants reveals reduced *Fusarium* wilt severity

To investigate the functional efficacy of the *NaD1* transgene, disease severity and vascular browning were assessed in both *NaD1*-transcript-positive T₁ plants (*TSV4129-1* and *TSV4129-2*) and compared with the wild-type *SV4129*. While wild-type plants developed severe wilting and necrosis, both transgenic events exhibited significantly reduced symptoms. Notably, *TSV4129-2* showed the mildest visible disease symptoms, maintaining vigorous growth with only minimal chlorosis on lower leaves (Fig. 6a), whereas *TSV4129-1* exhibited an intermediate phenotype, with delayed wilting relative to the wild type.

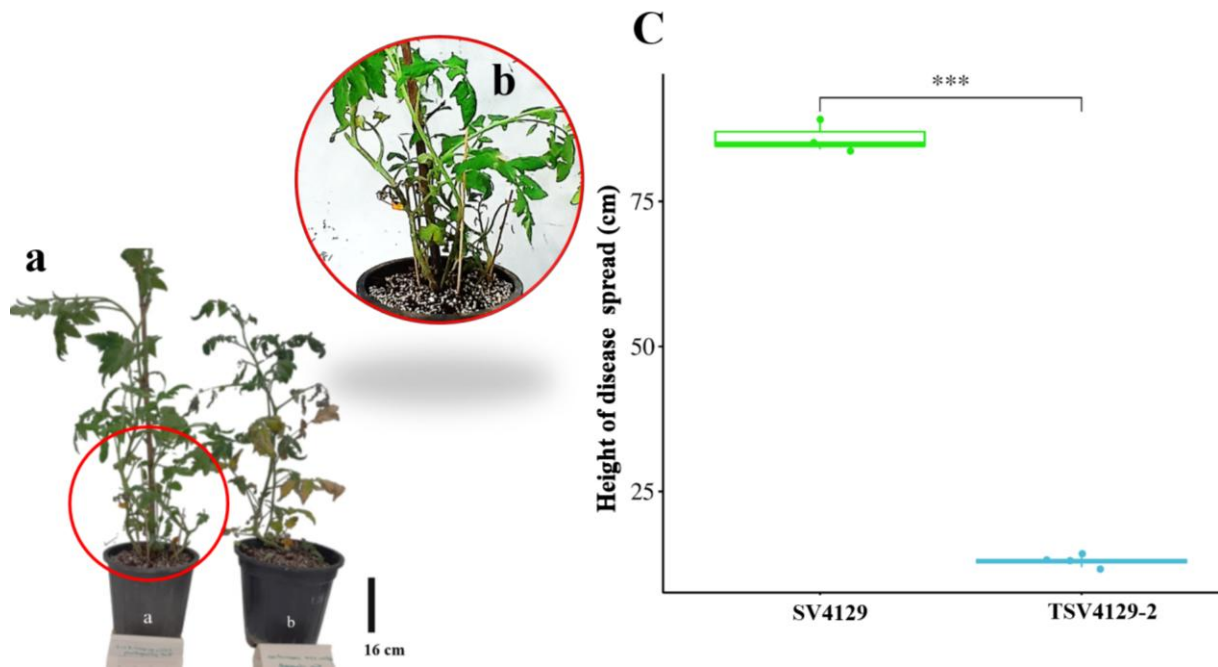


Fig. 6 Phenotypic comparison and disease progression of *NaD1*-transcript-positive T0 and wild-type tomato plants following inoculation with *Fol* (a) Representative plants at 28 dpi showing the contrasting disease phenotypes of the *NaD1*-transcript-positive T0 plant *TSV4129-2* (a) and wild-type *SV4129* (b). (b) Magnified view of the stem of *TSV4129-2* showing the limited extent of internal vascular browning. The scale bar represents 16 cm. (c) Quantification of extent of internal vascular browning measured as the height of disease spread (cm). The box plot illustrates the extent of internal vascular browning in both transgenic events compared with the greater extent of vascular browning observed in the wild-type (*SV4129*). The central line in each box represents the median, while the box boundaries represent the 25th and 75th percentiles from $n = 3$ biological replicates. The asterisks indicate significant differences based on Student's *t*-test (** $P < 0.01$, * $P < 0.05$)

Quantification of internal vascular browning confirmed this pattern. In wild-type plants, the visible vascular browning extended extensively along the stem; in *TSV4129-1*, it was limited to less than 24 cm from the base; and in *TSV4129-2*, it was confined to less than 15 cm from the base ($P < 0.01$ for both comparisons with wild type; $P < 0.05$ between events) (Fig. 6c).

3.7. *NaD1* Expression Is Associated with Reduced Fusarium Wilt Severity and Disease Progression

To investigate whether detectable *NaD1* transcripts were associated with reduced Fusarium wilt development, the two *NaD1*-transcript-positive plants, *TSV4129-1* and *TSV4129-2*, were evaluated following inoculation with *Fol*, using non-transformed *SV4129* as the susceptible reference. At 28 days post-inoculation (dpi), *SV4129* plants exhibited pronounced wilt, chlorosis, and necrosis, whereas both *NaD1*-transcript-positive plants showed substantially milder disease symptoms (Fig. 6a). *TSV4129-2* maintained comparatively vigorous growth, with symptoms largely restricted to older leaves, while *TSV4129-1* displayed an intermediate phenotype. These differences were consistent with the quantitative assessment of disease progression described below.

Internal vascular browning was also observed following *Fol* inoculation (Fig. 6a,b and c). In *SV4129*, browning extended extensively along the stem, whereas the height of visible internal vascular browning was less than 24 cm in *TSV4129-1* and less than 15 cm in *TSV4129-2*. Because vascular browning represents a disease-associated symptom rather than a direct measure of fungal biomass or vascular fungal colonization, these observations are interpreted here as an indicator of the extent of vascular symptom development rather than evidence of pathogen containment or restricted systemic colonization. The progressively smaller extent of vascular browning in the *NaDI*-transcript-positive plants was nevertheless consistent with their lower external disease severity.

Disease progression was further quantified at 11, 18, 22, and 28 dpi using a repeated-measures linear mixed-effects model (Fig. 7a). Plant line, time, and the plant line $\times$ time interaction was included as fixed effects, while replicate was treated as a random effect. An AR(1) covariance structure provided a better fit than a compound-symmetry structure (AIC = 56.8 versus 60.6). Disease severity increased progressively in *SV4129*, from 50% at 11 dpi to 89% at 28 dpi, whereas *TSV4129-1* increased from 11% to 32% over the same period. In contrast, *TSV4129-2* showed only a small increase, from 5% at 11 dpi to 6% at 28 dpi.

The model-adjusted least-squares means indicated significant differences among the three plant lines at every assessment date. At 28 dpi, disease severity was 89% in *SV4129*, 32% in *TSV4129-1*, and 6% in *TSV4129-2*, corresponding to reductions of 57 and 83 percentage points relative to *SV4129*, respectively. Tukey–Kramer-adjusted pairwise comparisons indicated significant differences among all three plant lines at each time point ($P < 0.0001$). Within-line temporal comparisons further showed that disease severity increased significantly between all assessed time points in *SV4129* and *TSV4129-1* ($P < 0.0001$ for all comparisons). In *TSV4129-2*, severity at 11 dpi differed significantly from that at 18, 22, and 28 dpi ($P < 0.0001$, $P = 0.0029$, and $P = 0.0234$, respectively), whereas no significant differences were detected among 18, 22, and 28 dpi ($P = 1.0000$). Thus, the disease trajectory of *TSV4129-2* was characterized by an early low level of symptom development followed by an apparent plateau after 18 dpi, in contrast to the continued progression observed in *SV4129* and *TSV4129-1*.

The plant line $\times$ time interaction was highly significant ($F = 3728.55$, $P < 0.0001$), together with significant main effects of plant line ($F = 3358.03$, $P < 0.0001$) and time ($F = 7032.71$, $P < 0.0001$). The significant interaction indicates that disease trajectories differed among the plant lines rather than simply reflecting a constant difference in disease severity throughout the experiment. These longitudinal results therefore provide evidence that the presence of detectable *NaDI* transcripts was associated with both lower disease severity and slower disease progression under the conditions tested.

The cumulative effect of disease development was assessed using the area under the disease progress curve (AUDPC) (Fig. 7b). Mean AUDPC values were 1554.5 ± 18.37 for *SV4129*, 367.5 ± 10.41 for *TSV4129-1*, and 126.0 ± 9.19 for *TSV4129-2*. Relative to *SV4129*, AUDPC was reduced by 76.4% in *TSV4129-1* and by 91.9% in *TSV4129-2*. Conversely, the AUDPC of *SV4129* was approximately 4.2-fold and 12.3-fold greater than that of *TSV4129-1* and *TSV4129-2*, respectively. The assumptions for one-way ANOVA were not violated, as indicated by the Shapiro–Wilk test ($W = 0.9266$, $P = 0.3454$) and Levene’s test ($F_{2,9} = 1.32$, $P = 0.3134$). One-way ANOVA revealed a highly significant effect of plant line on AUDPC ($F_{2,9} = 3307.78$, $P < 0.0001$), and Tukey’s HSD test identified significant differences among all three plant lines ($P < 0.05$).

The agreement between the longitudinal disease analysis and the AUDPC analysis strengthens the interpretation that the *NaDI*-transcript-positive plants experienced substantially less disease development over the experimental period. In particular, the markedly lower AUDPC of *TSV4129-2* was consistent with the near-plateau in disease severity observed after 18 dpi, whereas the continued increase in disease severity in *SV4129* resulted in a substantially greater cumulative disease burden.

The two *NaDI*-transcript-positive plants also differed in the apparent intensity of the endpoint RT-PCR signal. The *NaDI* amplicon was more pronounced in *TSV4129-2* than in *TSV4129-1*, coinciding with the substantially lower disease severity and AUDPC observed in *TSV4129-2*. This pattern is biologically consistent with the possibility that differences in *NaDI* transcript abundance or persistence may contribute to variation in disease phenotype. However, this observation should be considered descriptive rather than a quantitative expression–resistance relationship, because endpoint RT-PCR was not used to quantify transcript abundance and only two *NaDI*-transcript-positive plants were available for comparison. Other factors, including genomic context, transcript stability, or plant-to-plant variation, could also contribute to the phenotypic differences between the two plants.

The observed disease reduction is biologically compatible with the previously characterized antifungal activity of *NaDI*. The antifungal activity of *NaDI* has been linked to interactions with fungal hyphae. In *F. oxysporum* f. sp. *vasinfectum*, *NaDI* bound to the fungal cell wall, induced plasma-membrane permeabilization, and was subsequently detected within the cytoplasmic contents of treated hyphae, where its accumulation was associated with cytoplasmic granulation and cell death (Van Der Weerden *et al.* 2008). These findings provide a mechanistic context for the antifungal activity of *NaDI* observed in the present study; however, whether similar membrane-disruptive and intracellular effects occur in *F. oxysporum* f. sp. *lycopersici* in planta remains to be established. The same study also reported an IC_{50} of approximately 1.0 μ M against *F. oxysporum* f. sp. *vasinfectum* *in vitro*; however, this value should not be directly extrapolated to the *Fol* isolate used in the present study because it was obtained under different experimental conditions and with a different forma specialis. Subsequent studies provided additional mechanistic evidence for *NaDI*-mediated membrane disruption. Phosphoinositide-mediated oligomerization of *NaDI* is associated with membrane disruption (Poon *et al.* 2014), while Payne *et al.* (2016) demonstrated a specific interaction between *NaDI* and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) that introduces membrane disorder (Payne *et al.* 2016). In addition, dimerization of *NaDI* enhances its antifungal activity (Lay *et al.* 2012).

Together, these studies provide a plausible mechanistic context for the reduced disease development observed in the present *NaDI*-transcript-positive plants: if *NaDI* protein accumulated to biologically active levels, its established ability to interact with fungal membranes could contribute to suppression of fungal growth or viability. Nevertheless, the present experiment did not directly measure *NaDI* protein accumulation, fungal biomass, fungal viability, or *NaDI*-mediated membrane disruption *in planta*. Therefore, the current data support an association between detectable *NaDI* transcripts and reduced *Fol* disease development but do not establish the molecular mechanism responsible for the observed phenotype.

The reduction in internal vascular browning provides an additional symptom-level observation consistent with reduced disease development. However, because the extent of vascular browning was assessed visually rather than by direct quantification of fungal biomass or viable fungal propagules, these observations cannot establish reduced fungal accumulation within infected tissues. Nevertheless, the substantially shorter extent of vascular browning in the *NaDI*-transcript-positive plants may reflect reduced vascular disease development and could be consistent with lower fungal accumulation, a possibility that should be tested by direct pathogen quantification.

The persistence of the reduced disease phenotype throughout the 28-day assessment period is also notable, particularly in *TSV4129-2*, in which disease severity remained approximately 6% after 18 dpi. However, this observation demonstrates persistence only within the experimental period and should not be interpreted as evidence of heritable stability or durable resistance across generations or environments. Such conclusions would require evaluation of additional independent plants and progeny across generations and environmental conditions.

Several limitations should therefore be considered when interpreting these findings. First, only two plants showed detectable *NaDI* transcripts by endpoint RT-PCR, limiting the extent to which variation in transcript signal can be related to disease resistance. Second, *NaDI* protein accumulation was not quantified, and therefore transcript detection cannot be assumed to reflect the amount of biologically active peptide. Third, fungal biomass and viability were not directly measured, preventing a direct assessment of whether the reduced disease symptoms were accompanied by reduced fungal accumulation or survival. Future studies incorporating quantitative RT-PCR, direct measurement of *NaDI* protein accumulation, and quantitative assays of fungal biomass or viability would help resolve these relationships.

Overall, the phenotypic, longitudinal, and AUDPC data consistently indicate that the two *NaDI*-transcript-positive plants developed substantially less *Fol*-associated disease than the susceptible *SV4129* reference under the conditions tested. The stronger endpoint RT-PCR signal and more pronounced disease restriction observed in *TSV4129-2* are consistent with, but do not establish, a relationship between *NaDI* expression and resistance intensity. The known membrane-active antifungal properties of *NaDI* provide a biologically plausible context for these observations, while the absence of direct measurements of *NaDI* protein and fungal accumulation limits mechanistic interpretation. Thus, the present findings support an association between detectable *NaDI* transcripts and reduced *Fol* disease progression and provide a basis for further molecular and phenotypic characterization of *NaDI*-mediated resistance.

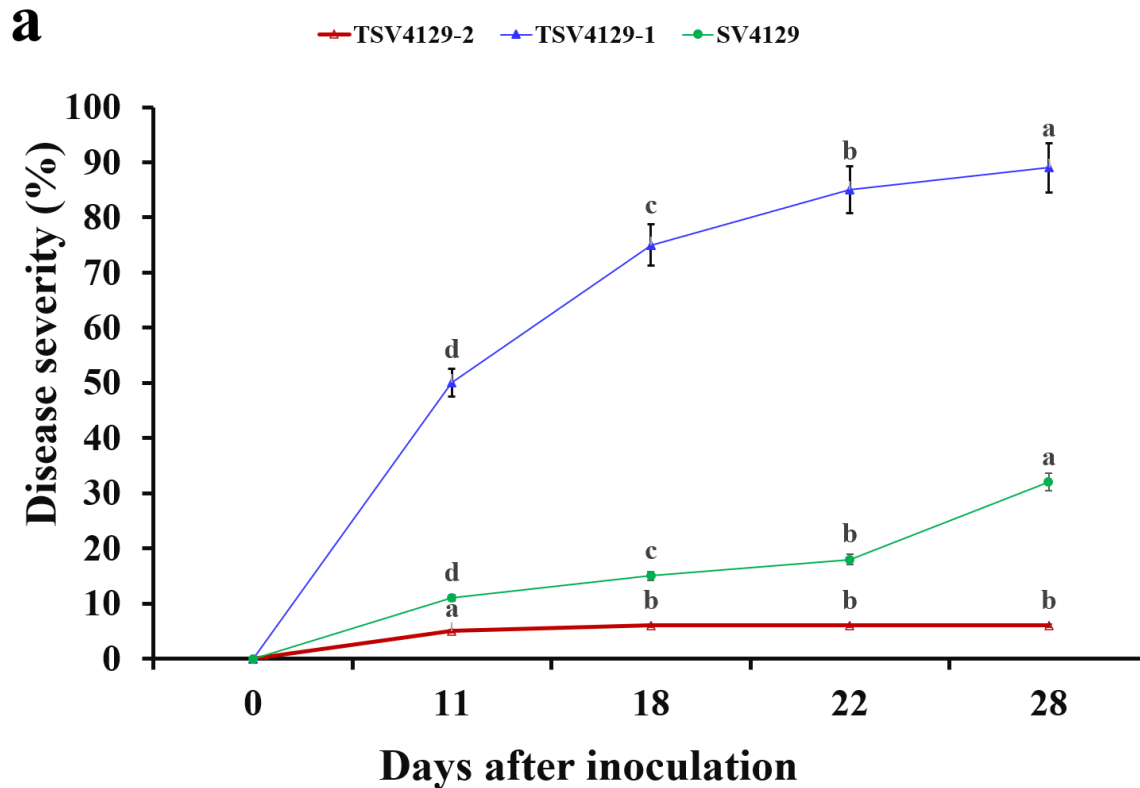
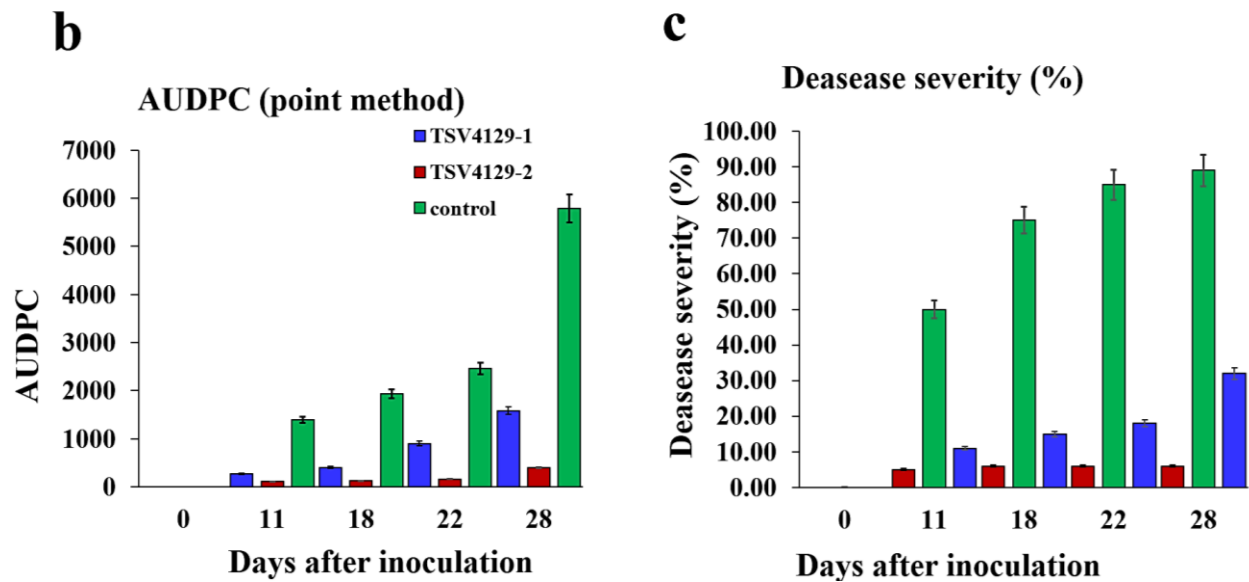


Fig. 7 Disease progression and AUDPC in wild-type and *NaD1*-transcript-positive plant lines. (a) Disease severity at 11, 18, 22, and 28 days post-inoculation in *SV4129*, *TSV4129-1*, and *TSV4129-2*. Values represent model-adjusted LS-means $\pm$ standard error obtained from a repeated-measures linear mixed-effects model with an AR(1) covariance structure. (b) AUDPC values calculated for individual plants using the trapezoidal method. Values are presented as means $\pm$ standard error. Different letters indicate significant differences among plant lines according to Tukey's HSD test at $\alpha = 0.05$.

3.8. Detectable *NaD1* Transcript Signal Is Associated with Event-Specific Disease Restriction

The pronounced and sustained suppression of disease progression in *TSV4129-2* (Fig. 6) prompted examination of whether the contrasting resistance phenotypes of the the two *NaD1*-transcript-positive T₁ plants were accompanied

by differences in detectable *NaDl* transcript levels. Endpoint RT-PCR followed by densitometric analysis revealed differences in the relative *NaDl* amplification signal between *TSV4129-1* and *TSV4129-2* (Fig. 8). Densitometric analysis of the *NaDl* amplification products, normalized to the *elf1a* reference gene, showed a significantly higher relative signal in *TSV4129-2* (95.7 ± 5.1) than in *TSV4129-1* (33.3 ± 3.1) (three technical densitometric measurements per sample; two-sample t-test assuming equal variances, $t(4) = -18.08$, $P < 0.0001$; Fig. 8). Thus, the two events differed in both the relative *NaDl* RT-PCR signal and the severity of Fusarium wilt symptoms.

This difference was concordant with the disease phenotypes observed in the two events. *TSV4129-2*, which exhibited the higher relative *NaDl* signal, had an AUDPC of 126.0 compared with 367.5 in *TSV4129-1*, corresponding to an approximately 2.92-fold lower cumulative disease burden. At 28 dpi, disease severity was 6% in *TSV4129-2* compared with 32% in *TSV4129-1* and 89% in the susceptible *SV4129* reference (Fig. 7). The correspondence between the higher relative *NaDl* signal and the lower disease burden in *TSV4129-2* is consistent with the possibility that a stronger *NaDl* RT-PCR amplification signal is associated with variation in disease restriction between these events.

However, the present data do not establish a quantitative or causal relationship between the relative *NaDl* RT-PCR amplification signal and resistance intensity. Densitometric analysis of endpoint RT-PCR products provides a relative measure of amplification signal but does not have the quantitative dynamic range or expression-level resolution of qRT-PCR. In addition, the comparison involved only the two *NaDl*-transcript-positive T_1 plants. Therefore, the observed difference may reflect variation in *NaDl* expression, but event-specific factors such as genomic insertion context, transcript stability, or other plant-level differences cannot be excluded. Quantitative analysis of *NaDl* transcripts across a larger number of independent events, together with measurement of *NaDl* protein accumulation, would be required to determine whether transcript or protein abundance is consistently associated with resistance intensity.

Notably, the event with the higher relative *NaDl* signal did not exhibit complete resistance. Although *TSV4129-2* maintained approximately 6% disease severity at 28 dpi, residual disease symptoms remained detectable. This finding indicates that detectable *NaDl* expression was associated with substantial disease restriction under the experimental conditions but does not support interpretation as complete immunity. Because fungal biomass and viability were not directly quantified, the present data cannot determine whether the remaining disease symptoms resulted from incomplete suppression of fungal growth, variation in host responses, or other components of the host–pathogen interaction.

The observed event-specific phenotypes are biologically consistent with the established antifungal activity of *NaDl*. van der Weerden *et al.* (2008) reported that *NaDl* interacts with fungal hyphae and is associated with plasma-membrane permeabilization and entry into the cytoplasm of *F. oxysporum* f. sp. *vasinfectum*. An *in vitro* IC_{50} of approximately 1.0 μ M was reported against this forma specialis (Van Der Weerden *et al.* 2008). This previous observation provides mechanistic context for the disease restriction observed here, but the reported IC_{50} cannot be directly extrapolated to the *Fol* isolate used in the present study or to *NaDl* concentrations within tomato tissues.

Taken together, the two *NaDl*-transcript-positive T_1 plants displayed distinct disease phenotypes that were accompanied by differences in relative *NaDl* RT-PCR signal intensity. The higher relative signal in *TSV4129-2* coincided with its lower disease severity and AUDPC, whereas *TSV4129-1* showed a lower relative signal and an intermediate disease phenotype. These findings support an association between the detectable *NaDl* RT-PCR amplification signal and event-specific variation in disease restriction, while the limited number of events and the absence of direct measurements of *NaDl* protein accumulation and fungal biomass preclude a causal or quantitative conclusion.

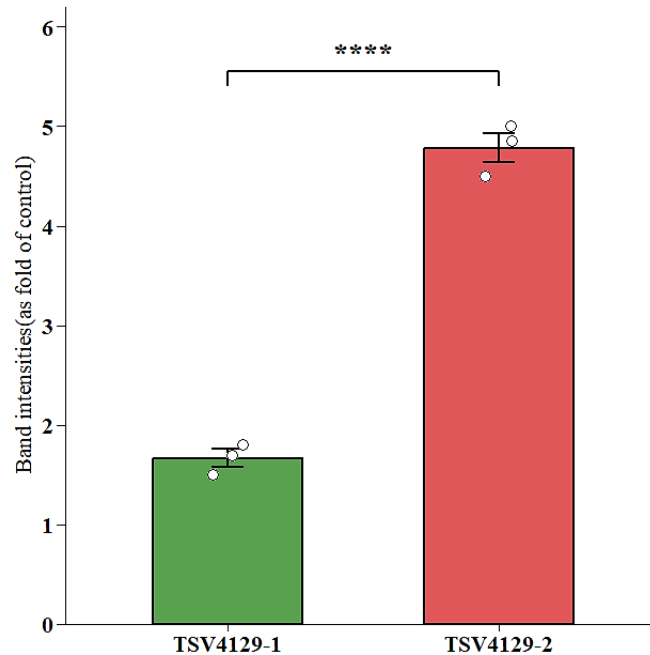
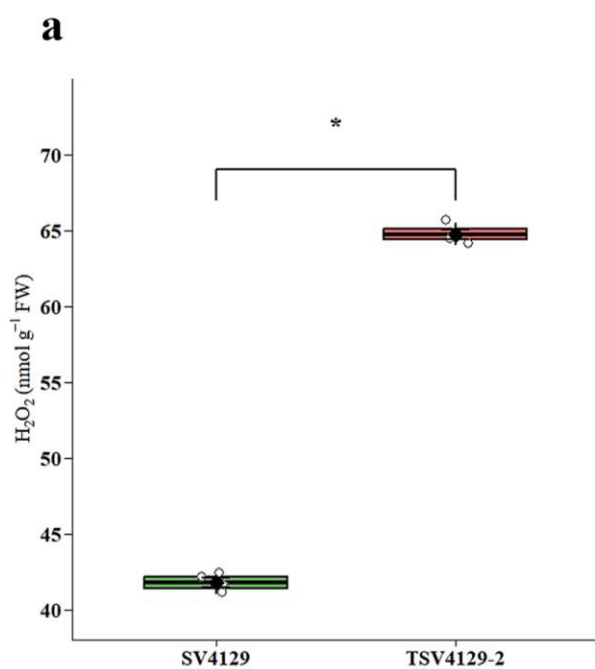
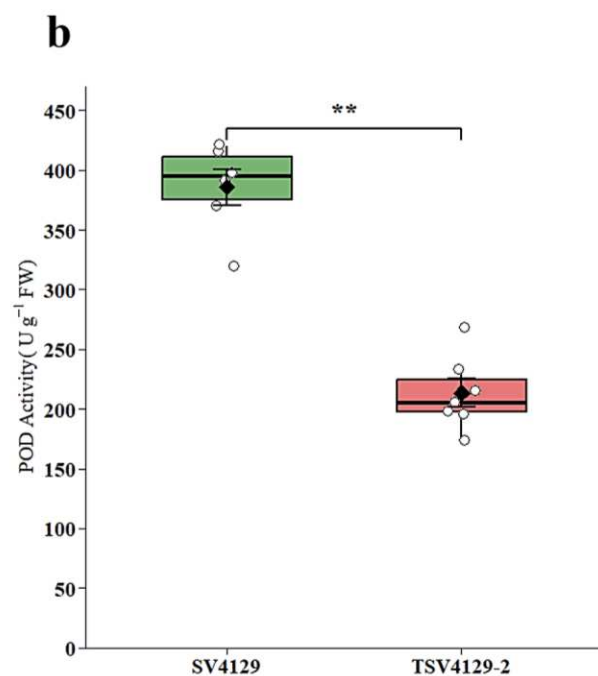
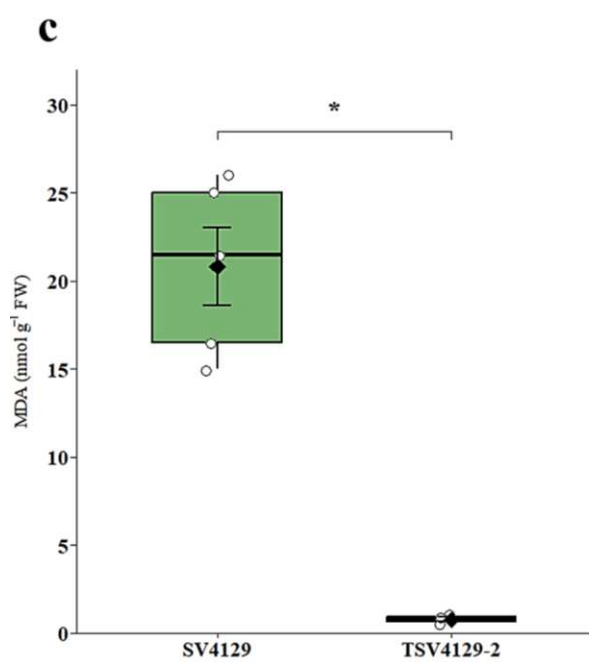
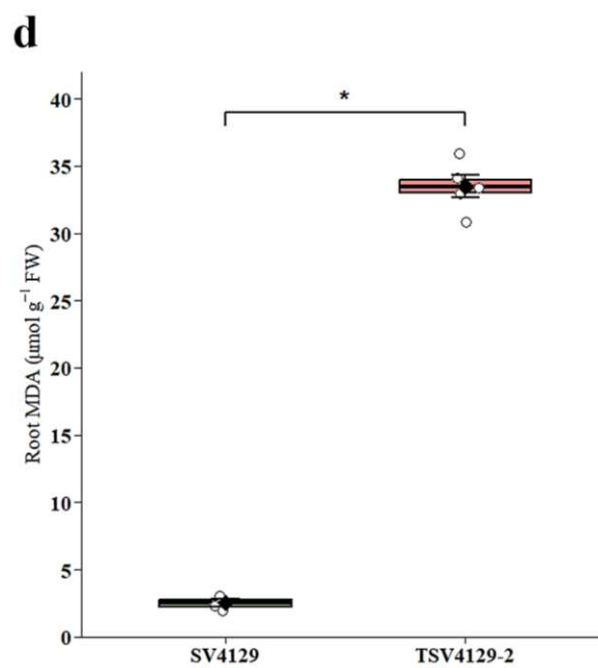


Fig. 8 Relative densitometric analysis of *NaD1* RT-PCR amplification signals in two independent transgenic events. *NaD1* amplification-band intensities were quantified using ImageJ and normalized to the corresponding *elf1a* reference signal. Data are presented as mean $\pm$ SE from three technical densitometric measurements per sample. The measured densitometric values differed significantly between the two samples based on a two-sample *t*-test assuming equal variances ($t(4) = -18.08$, $P < 0.0001$). *TSV4129-2* showed a higher relative *NaD1* amplification signal than *TSV4129-1*.

3.9. Tissue-differentiated biochemical responses associated with reduced disease in *NaD1*-transcript-positive plants

The pronounced reduction in disease development observed in *TSV4129-2* prompted an examination of whether the reduced disease phenotype was accompanied by distinct physiological and biochemical responses. Because plant responses to vascular pathogens may differ between tissues, H_2O_2 , POD activity, MDA, and total phenolic content were assessed in leaves and roots of *TSV4129-2* and wild-type *SV4129* plants at 28 days post-inoculation (dpi) (Fig. 9). The resulting biochemical profile revealed a clear tissue-dependent pattern, with the roots and leaves showing different combinations of oxidative, phenolic, and membrane-associated responses. This pattern raises the possibility that the reduced disease phenotype was associated not with uniform activation of defense metabolism throughout the plant, but with differentiated biochemical responses between tissues.



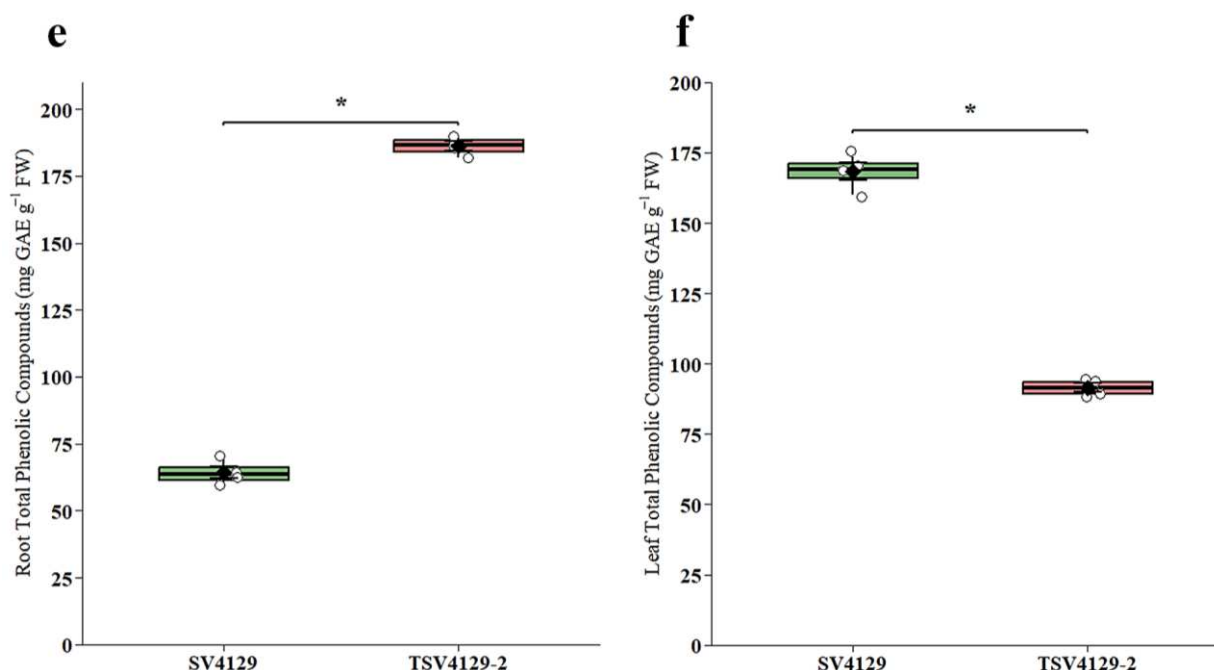


Fig. 9 Biochemical parameters in leaves and roots of transgenic *TSV4129-2* and wild-type *SV4129* plants at 28 days post-inoculation with *Fol.* (a) H₂O₂ content in leaves (nmol/g fresh weight). (b) POD activity in leaves (μmol/min/mg protein). (c) MDA content in leaves (nmol/g fresh weight). (d) MDA content in roots (nmol/g fresh weight). (e and f) Total phenolic content in leaves and roots (mg gallic acid equivalents/g fresh weight). Data are presented as box plots with individual data points, mean (black diamond), and standard error of the mean (SE). The number of replicates for each parameter corresponds to the respective experiment. Differences between the two genotypes were evaluated using the Wilcoxon rank-sum (Mann–Whitney U) test with an exact two-sided P value. Asterisks indicate statistical significance: P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***).

In leaves, *TSV4129-2* accumulated significantly more H₂O₂ than wild-type *SV4129*, reaching 64.75 ± 0.32 and 41.75 ± 0.32 nmol g⁻¹ FW, respectively (exact two-sided Mann–Whitney U/Wilcoxon $P = 0.0286$; Fig. 9a). H₂O₂ has a context-dependent role in plant–pathogen interactions, functioning not only as a reactive molecule that can contribute to oxidative injury but also as a signaling component involved in stress responses (Mittler *et al.* 2022). Depending on its concentration, localization, and timing, H₂O₂ can therefore contribute to the coordination or priming of defense responses rather than simply representing a product of oxidative damage. Therefore, the higher H₂O₂ concentration detected in *TSV4129-2* should not, in isolation, be interpreted as evidence of increased oxidative damage.

The biological significance of this difference becomes clearer when H₂O₂ is considered together with other indicators of cellular status. MDA is commonly used as an indicator of lipid peroxidation (Khoubnasabjafari *et al.*, 2015). Foliar MDA concentration was markedly lower in *TSV4129-2*, decreasing to 0.77 ± 0.15 nmol g⁻¹ FW compared with 20.80 ± 2.21 nmol g⁻¹ FW in *SV4129* (exact two-sided Wilcoxon $P = 0.0357$; Fig. 9c). Thus, the higher H₂O₂ concentration in *TSV4129-2* was not accompanied by greater foliar lipid peroxidation. Electrolyte leakage is also widely used as an indicator of membrane disruption or loss of membrane integrity (Tovar-Rosales *et al.* 2024). Electrolyte leakage was markedly lower in *TSV4129-2* than in *SV4129* ($3.54 \pm 0.29\%$ versus $50.75 \pm 0.48\%$, respectively; $P = 0.0286$), providing a complementary indicator of membrane-associated cellular status. Taken together, the concurrent elevation of H₂O₂ and reduction of MDA and electrolyte leakage are consistent with a distinct foliar redox state accompanied by comparatively preserved cellular integrity rather than generalized oxidative injury.

This distinction is important because ROS accumulation and oxidative damage are related but not synonymous processes (Schieber and Chandel 2014). The biological effects of H₂O₂ depend on its concentration, localization, temporal dynamics, and interaction with ROS-scavenging and signaling systems (Mittler *et al.*, 2022). The higher H₂O₂ level in *TSV4129-2*, therefore, may represent a component of an altered redox-signaling state associated with infection, rather than a direct indicator of oxidative injury. The present measurements nevertheless do not establish whether the higher H₂O₂ concentration resulted from enhanced production, reduced turnover, altered intracellular distribution, or a combination of these processes. Likewise, because ROS localization, production kinetics, and the

capacity of the broader antioxidant system were not directly assessed, the present data cannot establish H₂O₂ as a causal determinant of the reduced disease phenotype.

Previous work provides relevant context for the observed association. Exogenous H₂O₂ application has been reported to reduce the development of *Fol* in tomato and improve disease-related and growth parameters (Nawar *et al.* 2019). Such findings are consistent with the possibility that H₂O₂-associated redox changes can influence plant responses to *F. oxysporum*, including through modulation of defense-related processes. However, exogenous H₂O₂ treatment is mechanistically distinct from the endogenous increase detected in *TSV4129-2* and therefore does not demonstrate that elevated H₂O₂ itself confers resistance in the present system. Rather, the present findings suggest that altered H₂O₂ status forms part of the biochemical state associated with the reduced disease phenotype.

The H₂O₂ response can also be considered in relation to the known antifungal properties of *NaD1*. *NaD1* has been shown to interact with and enter *F. oxysporum* hyphae, supporting a direct pathogen-targeted component of its antifungal activity (Van Der Weerden *et al.* 2008). The coexistence of *NaD1* expression, reduced disease development, and altered foliar H₂O₂ levels in *TSV4129-2* is therefore biologically consistent with the possibility that both direct antifungal activity and host-associated redox responses may have been associated with the observed phenotype. However, these observations do not establish a mechanistic link between *NaD1* activity and H₂O₂ accumulation in the plant. Because the cellular source, localization, and temporal dynamics of H₂O₂ were not determined, the present data cannot distinguish between altered host redox signaling, changes associated with pathogen perception, or other processes contributing to the observed H₂O₂ accumulation. Accordingly, the increase in H₂O₂ is best interpreted as a biochemical feature associated with the *NaD1*-expressing resistant phenotype rather than as evidence of a specific *NaD1*–H₂O₂ causal pathway.

POD activity showed a distinct pattern that further differentiated the biochemical status of the aerial tissues. Foliar POD activity was significantly lower in *TSV4129-2* than in wild-type *SV4129*, with values of 213.57 ± 11.69 and 385.83 ± 15.08 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein, respectively ($P = 0.0012$; Fig. 9b). Notably, the higher POD activity in susceptible *SV4129* plants occurred in the context of substantially greater disease development and markedly higher foliar MDA and electrolyte leakage. Increased POD activity can occur as part of a plant antioxidant response to pathogen-associated or oxidative stress (Devi and Kumar 2023). This pattern may indicate that the elevated POD activity in *SV4129* represented part of a stress-associated antioxidant response elicited under greater disease pressure, rather than being indicative of more effective resistance.

In contrast, the markedly lower POD activity in *TSV4129-2* occurred together with lower MDA and electrolyte leakage despite the higher H₂O₂ concentration. Thus, the reduced disease phenotype was not accompanied by generalized activation of foliar POD. Instead, the lower POD activity in *TSV4129-2* was associated with comparatively low levels of lipid peroxidation and membrane leakage, suggesting a different biochemical state in the aerial tissues. Antioxidant enzyme activation should not necessarily be interpreted as evidence of stronger defense, as enzyme activity may instead reflect the intensity, timing, and duration of stress imposed on the tissue (Singh *et al.* 2017). The substantially greater disease burden in *SV4129*, together with its elevated POD activity, MDA, and electrolyte leakage, is consistent with a more pronounced disease-associated stress response.

This interpretation should nevertheless be considered cautiously. POD represents only one component of the antioxidant network, which comprises multiple enzymatic and non-enzymatic ROS-scavenging systems (Mohammadi *et al.* 2021), and its activity can vary according to the timing, localization, and severity of the stress response. The present data therefore do not establish that reduced POD activity contributed directly to resistance or that the susceptible plants experienced ineffective POD-mediated defense. Rather, the contrasting POD profiles, when considered together with MDA and electrolyte leakage, indicate that POD activation was associated with the different biochemical states of the two genotypes and may have reflected differences in disease-associated stress intensity.

The biochemical response in roots further emphasized the tissue-dependent nature of the *TSV4129-2* phenotype. Root MDA concentration was significantly higher in *TSV4129-2* than in *SV4129* (33.50 ± 0.81 versus 2.50 ± 0.29 nmol g^{-1} FW, respectively; $P = 0.0357$; Fig. 9d). In parallel, total phenolic content in root was markedly higher in *TSV4129-2*, reaching 186.25 ± 1.75 mg GAE g^{-1} FW compared with 64.25 ± 2.17 mg GAE g^{-1} FW in *SV4129* ($P = 0.0286$; Fig. 9e). The concurrent increase in root phenolics and MDA is consistent with a distinct biochemical state in the roots, which represent the primary tissue exposed to the soil-borne pathogen. The greater accumulation of

phenolics in the roots may therefore reflect an enhanced local biochemical response at the tissue directly exposed to *F. oxysporum*, although the present data do not establish whether this response resulted from direct *NaDl* activity, pathogen perception, or downstream host signaling.

Phenolic compounds can participate in several aspects of plant defense, including antioxidant activity, cell-wall reinforcement, and interactions with pathogen development (Xu and Wang 2025). Their accumulation in roots may consequently provide a biochemical context for the reduced disease development observed in *TSV4129-2*, particularly given the importance of roots as the initial site of contact with this soil-borne pathogen. However, because the individual phenolic compounds were not identified and pathogen growth within the roots was not directly quantified, the present data do not establish that increased phenolic accumulation directly restricted pathogen development.

Notably, the direction of the MDA response differed between tissues. Whereas root MDA increased in *TSV4129-2*, foliar MDA decreased markedly. This contrast does not support a uniform whole-plant change in oxidative status and is instead consistent with a spatially differentiated biochemical response. The simultaneous increase in root phenolics and MDA, together with the contrasting foliar pattern of increased H₂O₂ but reduced MDA and electrolyte leakage, suggests that the biochemical response associated with *NaDl* expression was spatially differentiated rather than uniformly activated throughout the plant. Such tissue-dependent partitioning may be particularly relevant for a vascular pathogen, in which the roots constitute the initial site of pathogen encounter whereas the leaves reflect a more distal physiological state. Nevertheless, the mechanisms underlying this spatial differentiation remain unresolved.

The lower foliar phenolic content observed in *TSV4129-2* should likewise be interpreted within this tissue-dependent framework. Although leaf phenolic content was significantly lower in *TSV4129-2* than in *SV4129* (91.50 ± 1.55 versus 168.25 ± 3.12 mg GAE g⁻¹ FW, respectively; $P = 0.0286$; Fig. 9f), this reduction should not be interpreted as evidence of weakened defense. Total phenolic content represents a broad pool of chemically diverse compounds with potentially different biological functions (Lattanzio 2013). Its abundance may be influenced by disease progression and the physiological state of the tissue. In the present study, the lower foliar phenolic content occurred together with lower MDA and electrolyte leakage, suggesting that the aerial tissues of *TSV4129-2* experienced a biochemical state distinct from that of the highly affected wild-type plants rather than simply displaying uniformly enhanced or reduced defense metabolism.

When considered together, the biochemical measurements indicate that the *NaDl*-associated phenotype was accompanied by different responses in roots and leaves. Roots exhibited increased phenolic accumulation together with higher MDA, whereas leaves showed elevated H₂O₂ together with reduced MDA, POD activity, and electrolyte leakage. Rather than indicating a generalized increase in all measured defense-related parameters, these contrasting patterns point toward a tissue-differentiated biochemical response associated with the reduced disease phenotype. In the roots, increased phenolic accumulation occurred at the primary site of pathogen exposure, whereas the leaves exhibited a higher H₂O₂ level without the accompanying increase in MDA or electrolyte leakage that characterized the susceptible plants. The contrasting POD profiles are particularly informative when considered alongside disease severity: the susceptible plants, which experienced substantially greater disease development, showed higher POD activity together with greater indicators of lipid peroxidation and membrane-associated damage, whereas the less-affected *TSV4129-2* plants showed lower POD activity together with lower MDA and electrolyte leakage. This pattern is consistent with POD activation being associated, at least in part, with the intensity of disease-associated stress rather than serving as a direct indicator of resistance capacity. The higher chlorophyll content observed in *TSV4129-2* was also consistent with comparatively preserved physiological status of the leaves under infection. Importantly, the higher foliar H₂O₂ occurred in the absence of increased MDA or electrolyte leakage, indicating that H₂O₂ accumulation and lipid/membrane damage were not coupled in the same manner in the two genotypes. Overall, the data indicate that the *NaDl*-associated disease phenotype coincided with an organ-dependent biochemical state characterized by distinct root responses and comparatively preserved foliar cellular integrity.

3.10. Photosynthetic pigment preservation and membrane integrity in aerial tissues of *TSV4129-2*

To determine whether the reduced disease severity of *TSV4129-2* was accompanied by preservation of physiological status in aerial tissues, photosynthetic pigments and electrolyte leakage were assessed in leaves of *TSV4129-2* and wild-type *SV4129* at 27 days post-inoculation (dpi) (Fig. 10).

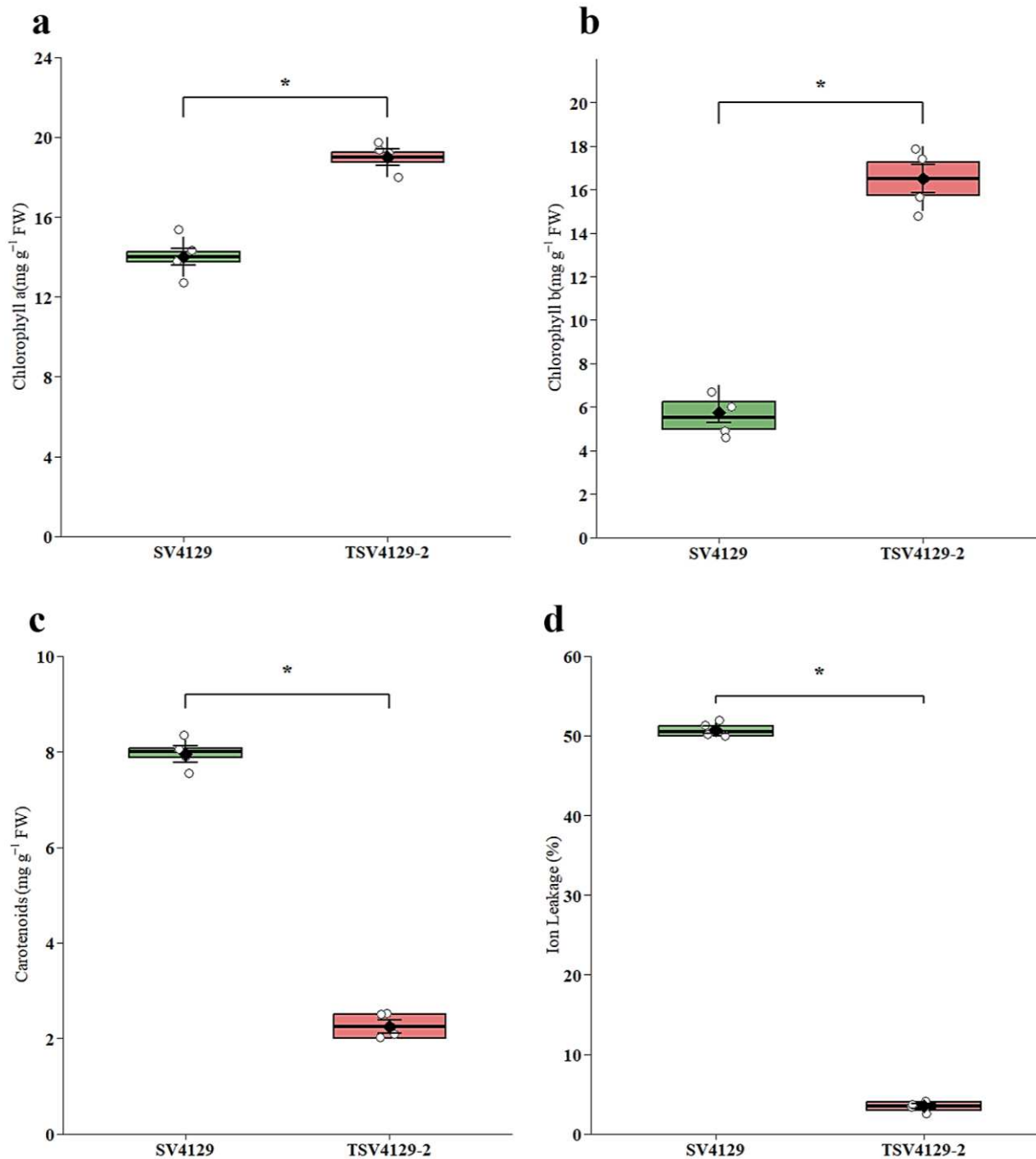


Fig. 10 Photosynthetic pigments and electrolyte leakage in leaves of transgenic *TSV4129-2* and wild-type *SV4129* at 27 days post-inoculation with *Fol* (a) Chlorophyll a content (mg g⁻¹ FW). (b) Chlorophyll b content (mg g⁻¹ FW). (c) Total carotenoid content (mg g⁻¹ FW). (d) Electrolyte leakage (%). Data are presented as mean $\pm$ SD; sample size and technical replication are indicated in the corresponding experimental descriptions.

Differences between *TSV4129-2* and wild-type *SV4129* were evaluated using the Wilcoxon rank-sum (Mann–Whitney U) test. Asterisks indicate statistical significance: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0$.

chlorophyll a (19.00 ± 0.41 vs. 14.00 ± 0.41 mg g⁻¹ FW; Wilcoxon rank-sum test, $P = 0.0286$) and chlorophyll b (16.50 ± 0.65 vs. 5.75 ± 0.48 mg g⁻¹ FW; $P = 0.0286$) than wild-type *SV4129* (Fig. 10a,b). The preservation of both major chlorophyll fractions, together with the substantially lower disease severity observed in *TSV4129-2*, is consistent with comparatively better maintenance of foliar physiological status under *Fol* infection. Chlorophyll loss during pathogen stress can be associated with alterations in chlorophyll metabolism and oxidative damage to chloroplast-associated structures (Kabashnikova *et al.*, 2020). However, because chlorophyll degradation pathways and chloroplast oxidative damage were not directly assessed in the present study, the observed pigment preservation should not be interpreted as direct evidence that these processes were suppressed. Rather, maintenance of chlorophyll a and b provides a physiological correlate of the comparatively limited disease-associated deterioration observed in *TSV4129-2*.

In contrast to chlorophylls, total carotenoid content was significantly lower in *TSV4129-2* than in *SV4129* (2.25 ± 0.10 vs. 7.95 ± 0.21 mg g⁻¹ FW, respectively; $P = 0.0286$; Fig. 10c). This reduction should not be interpreted in isolation as evidence of lower photooxidative stress, because carotenoids have diverse roles in photoprotection and pigment metabolism and their abundance may change in response to stress. Notably, the lower carotenoid content in *TSV4129-2* occurred together with higher chlorophyll a and b levels and was not accompanied by a generalized depletion of foliar pigments. Thus, the observed pattern is more consistent with a differentiated adjustment of foliar pigment composition than with generalized pigment deterioration.

Membrane-associated damage also differed markedly between the two genotypes. Electrolyte leakage was substantially lower in *TSV4129-2* than in *SV4129* ($3.54 \pm 0.29\%$ vs. $50.75 \pm 0.48\%$, respectively; $P = 0.0286$; Fig. 10d). Lower electrolyte leakage is consistent with better preservation of membrane integrity and lower nonspecific leakage of cellular electrolytes under *Fol* infection. This observation is further supported by the markedly lower foliar MDA content previously detected in *TSV4129-2* (Fig. 9c), providing two complementary indicators of comparatively limited membrane-associated damage. Because electrolyte leakage and MDA reflect related but distinct aspects of cellular injury, their concordant reduction strengthens the interpretation that foliar cellular integrity was comparatively preserved in *TSV4129-2*. Nevertheless, the underlying biochemical processes responsible for this phenotype were not directly resolved in the present study.

The contrasting biochemical profiles observed in leaves and roots further indicate that the physiological response was tissue dependent. In leaves of *TSV4129-2*, higher H₂O₂ content was accompanied by lower MDA and POD activity relative to *SV4129*, whereas roots showed higher MDA and total phenolic content (Fig. 9). This tissue-specific pattern suggests that the biochemical state associated with reduced disease development was not characterized by uniform changes in oxidative and phenolic markers throughout the plant. Instead, distinct biochemical adjustments may have occurred in aerial and below-ground tissues. The higher foliar H₂O₂ content should therefore not be interpreted simply as evidence of oxidative damage, particularly given the concurrent lower MDA and electrolyte leakage. Likewise, the lower POD activity in *TSV4129-2* should not necessarily be interpreted as weaker antioxidant defense, because antioxidant enzyme activity can reflect the intensity, timing, and duration of stress experienced by a tissue (Rajput *et al.* 2021).

The preservation of foliar chlorophyll and membrane integrity observed in *TSV4129-2* is also broadly consistent with previous reports describing physiological effects of plant defensins under stress. For example, *NaDl*-expressing tobacco plants exposed to drought maintained higher chlorophyll content and accumulated lower carotenoid levels than their wild-type counterparts (Royan *et al.* 2024), while expression of the chickpea defensin Ca-AFP in *Arabidopsis* was associated with improved chlorophyll retention under water-deficit conditions (Kumar *et al.* 2019). These studies involved different plant species, defensins, and stress conditions and therefore do not establish that *NaDl* directly regulates chlorophyll metabolism in the present tomato–*Fol* pathosystem. Rather, they provide contextual support for the possibility that defensin-associated responses may coincide with maintenance of foliar physiological status across distinct stress contexts.

The contrasting responses between roots and leaves may also be considered in the context of source–sink relationships during root-associated pathogen stress. Leaves generally function as major source tissues, whereas

infected roots can represent strong sink tissues, potentially altering the distribution and utilization of assimilates during disease development (Pucillo and Vitale, 2020). Such changes could contribute to tissue-specific metabolic responses; however, carbon assimilation, transport, and metabolic fluxes were not measured here, and therefore this explanation remains a working hypothesis rather than a demonstrated mechanism.

Overall, *TSV4129-2* exhibited a coordinated pattern of higher chlorophyll a and b, lower electrolyte leakage, and lower carotenoid abundance than wild-type *SV4129* under *Fol* infection. The higher chlorophyll content and markedly reduced electrolyte leakage, together with the previously observed lower foliar MDA, are consistent with comparatively preserved pigment and membrane status in aerial tissues of *TSV4129-2*. When considered alongside the root-associated changes in phenolics and MDA and the altered foliar H₂O₂ and POD profiles, these findings support an organ-dependent biochemical response rather than a uniform whole-plant stress response. Collectively, the data strengthen the association between detectable *NaDI* transcript signal and preservation of aerial-tissue physiological status during *Fol* infection, while causal relationships among root biochemical responses, foliar redox regulation, pigment metabolism, and membrane integrity remain to be resolved.

3.11. Conclusion

This study provides evidence that a detectable *NaDI* transcript signal was associated with substantial restriction of Fusarium wilt development in tomato. In the susceptible cultivar *SV4129*, plants with a detectable *NaDI* transcript signal exhibited markedly reduced disease severity and disease progression, accompanied by comparatively preserved foliar chlorophyll content and membrane integrity. These disease-associated differences were further accompanied by distinct biochemical profiles in leaves and roots, characterized by altered H₂O₂, MDA, POD activity, and total phenolic content, indicating that the physiological response associated with reduced disease development was organ dependent rather than uniform throughout the plant. Taken together, the findings link detectable *NaDI* transcript signal with both restriction of disease development and preservation of key aspects of foliar physiological status, while highlighting tissue-specific biochemical responses during *Fol* infection. Although these results support a role for *NaDI*-associated responses in the observed disease phenotype, they do not establish the underlying molecular mechanism. Direct assessment of *NaDI* protein accumulation, fungal biomass, and associated molecular responses will therefore be important to determine how *NaDI* contributes to restriction of Fusarium wilt and to clarify the relationship between pathogen limitation and host physiological adjustment.

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Author Contributions

RJ N did research experiments, data analysis and wrote the first draft of the manuscript. **RSH** conducted and supervised the whole research project and revised the manuscript. **VM** and **SR** cooperate as advisors in different parts of the work, respectively. All authors have read and approved the manuscript.

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1028 Data Availability Statement

1029 The datasets generated and/or analyzed during the current study are available from the corresponding author on
1030 reasonable request.

1031 Competing Interests

1032 The authors have no relevant financial or non-financial interests to disclose.

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