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Downregulation of the Nb-SABP2 gene promotes transient recombinant protein accumulation in N. benthamiana

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Abstract

Delivery of plant virus-based vectors by *A. tumefaciens* into plant cells would be expected to trigger plant immune response against microbial pathogens, which has negative impact on recombinant protein accumulation. In this study we modulated *Nicotiana benthamiana* defence response by targeting endogenous *Nb-SABP2* gene with dsRNA. Suppression of the *Nb-SABP2* gene did not affect negatively plant morphology, promoted multiplication of viral component of transient vector delivery system and boosted recombinant protein expression. We demonstrated the utility of this approach for GFP and NGF proteins delivered by TMV- and PVX-based vectors. Compared to non-modified wild type plants in the best performing transgenic Nb-SABP2 RNAi line the recombinant protein accumulation was increased 2-3-folds. Our results further validate a core idea that attenuating host defence response to agroinfiltration can improve the efficiency of foreign protein production in *N. bethamiana*

Introduction

Transient expression in plants made a great progress on the road to commercial application (Lomonossoff and D'Aoust 2016; Akher et al 2025). It offers a unique combination of features including low capital costs compared to classical mammalian and bacterial manufacturing systems, high scalability, capacity to produce complex proteins and perform mammalian-like post-translational modifications and production speed (Nosaki et al 2021). The last characteristic, the high speed of target protein production, makes this technology very attractive for time sensitive scenarios such as pandemic and emergency infection disease outbreaks. The great potential of this technology has already been demonstrated for Ebola (The PREVAIL II Writing Group; Multi-National PREVAIL II Study Team 2016), seasonal influenza diseases (Ward et al 2021) and Covid-19 (England et al 2023).

In this method the expression construct harbouring the gene of interest is delivered into plant cells by *Rhizobium radiobacter* (formerly known as *Agrobacterium tumefaciens*) resulting in the production of recombinant protein within days (Fischer et al 1999; Kapila et al 1997). Over the past decades transient expression technology has improved significantly due to the optimization of both foreign gene delivery systems and host plant. In respect to foreign gene delivery method one of the key achievements is the development of high performing expression systems combining plant viruses with *A. tumefaciens* (Gleba et al 2014; Peyret and Lomonossoff 2015). The backbones of potexviruses, tobraviruses, tobamoviruses, geminiviruses and comoviruses have been used to design expression vectors for *A. tumefaciens*-mediated delivery (Shahgolzari et al 2026). Concerning the production host *N. benthamiana* is one of the most efficient plants for transient expression. Its popularity is attributed to a number of characteristics such as impaired RNA silencing system (Ying et al 2010; Ludman et al 2024), high amenability to genetic transformation, short life cycle, rapid growth, high leaf biomass production and non-food, non-feed status (Bally et al 2018).

Delivery of plant virus-based vectors by *A. tumefaciens* into plant cells would be expected to trigger plant immune response against microbial pathogens (Beritza et al 2024; Hamel et al 2026). Different

approaches can be taken to evade or reduce this defence reaction and support foreign protein production. For example, avoidance of plant defence response by the depletion of the *NbCORE* immune receptor recognizing cold shock protein of *A. tumefaciens* improved agroinfiltration productivity in *N. benthamiana* (Dodds et al 2023). Another study explored the intercellular trafficking system of cell-to-cell transmission of the tobacco mosaic virus (TMV)-based vector to boost recombinant protein accumulation in *N. benthamiana* (Wang et al 2023). A growing body of research investigating correlation between the post transcriptional gene silencing (PTGS) and transient expression efficiency revealed that the suppression of PTGS can have positive effect on recombinant protein yield. In particular, different components of RNA silencing network including RNA-dependent RNA polymerase 1 (RDR1) (Ramadan et al 2024), RNA-dependent RNA polymerase 6 (RDR6) (Ludman and Fátyol 2019; Matsuo and Atsumi 2019), Dicer-like 2 (DCL2), Dicer-like 4 (DCL4) (Katsarou et al 2019; Matsuo 2022), and Argonaut 2 (Ludman et al 2017) proteins were modified to optimize transient expression system.

Plants have developed an intricate system to protect themselves from the pathogen attack (Ngou et al 2022). One of the essential components of this system is a phenolic phytohormone salicylic acid (SA) that plays a central role in the activation of local and systemic acquired resistance (SAR) (Ding and Ding 2020; Spoel et al 2024). Endogenous SA levels in plant are regulated at both the biosynthesis and metabolic levels to enable precise adjustment of its accumulation, function, and/or mobility. During pathogen infection the methyl salicylate esterase (SABP2) acts as a high-affinity receptor for the conversion of biologically inactive methyl salicylate (MeSA), which is responsible for mobile SAR signal, to its active SA form leading to activation of the SA-dependent defence response (Forouhar et al., 2005; Park et al., 2007; Tripathi et al 2010). In line with this, the *Nb-SABP2* gene was upregulated in *N. benthamiana* plants agroinfiltrated with the TMV-based virus vector as a response against both components of hybrid gene delivery system, *A. tumefaciens* and virus vector (Kopertekh 2024).

In this study we attenuated plant immune response by targeting the *Nb-SABP2* gene with corresponding dsRNA. Transgenic lines with downregulated *Nb-SABP2* expression supported viral vector multiplication and subsequent recombinant protein accumulation. Moreover, downregulated plants displayed normal phenotype without growth penalties. These results indicate that modulation of *N. benthamiana* response to agroinfiltration might be useful for improving transient expression efficiency.

Materials and methods

DNA constructs

The construction of the pLH-*35S-SABP2-INT-SABP2*, pLH-*TMV-GFP*, pLH-*TMV-βNGF* and pLH-*PVX-βNGF* plasmids has been described previously (Kopertekh 2024). To transfer the *PVX-GFP* sequence into the binary expression vector the p*PVX-GFP* construct (Baulcombe et al 1995) was linearized with *Sph*I and, following removal of the single stranded termini with T4 polymerase, digested with *Ehe*I. The *Sph*I-*Ehe*I DNA fragment was ligated to *Stu*I restricted pLH-*Δbar-PacI* plasmid (Kopertekh et al 2004). The final

plasmid was designated as pLH-*PVX-GFP*. The schematic representation of the pLH-*35S-SABP2-INT-SABP2*, pLH-*TMV-GFP*, pLH-*PVX-GFP*, pLH-*TMV-βNGF* and pLH-*PVX-βNGF* constructs is shown in Fig. 1.

Generation of transgenic plants

The pLH-*35S-SABP2-INT-SABP2* plant transformation vector was introduced into Agrobacterium tumefaciens strain LBA4404 by the freeze-thaw method (Holsters et al 1978). Transformed colonies were selected on solid LB medium supplemented with 300 mg/L streptomycin (Duchefa, Haarlem, the Netherland), 100 mg/L spectinomycin (Duchefa, Haarlem, the Netherland), 50 mg/L rifampicin (Duchefa, Haarlem, the Netherland) and checked by PCR analysis. The transformation of *N. benthamiana* leaf explants was performed according to previously published protocol (Kopertekh, Reichardt 2021). Rooted plants were transferred to soil and grown in the greenhouse to set seeds. Explants and regenerants were cultivated in the controlled environment chamber or greenhouse at 24/22^{0}C day/night temperature with 16 day/8 night photoperiod.

Molecular characterisation of transgenic lines

To select transgenic plants the genomic DNA from leaf tissue of primary regenerants was extracted according to CTAB DNA extraction protocol (Dale and Ow1991) and subjected to PCR analysis. Two pairs of primers, bar-forw/bar-rev and Nb-SABP2-forw/ST-LS1-INT-rev, specific to *bar* and *RNAi* insertion sequences, respectively, were used in PCR reactions (Table S1). Amplification was carried out at 94°C for 5 min followed by 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and 10 min of a final elongation at 72^{O}C using the PTC-200 Peltier Thermal Cycler (Bio-Rad, Feldkirchen, Germany).

For qPCR analysis total RNA was isolated from *N. benthamiana* leaf tissue using RNA Tri-Liquid reagent (Bio&SELL, Feucht, Germany), and treated with DNAse I (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. One µg of RNA was subjected to cDNA synthesis with the random hexamer primer and Maxima Reverse Transcriptase following the protocol provided by supplier (Thermo Scientific, Waltham, USA). qPCR reactions were performed with Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, USA) in the Mastercycler® EP realplex (Eppendorf, Hamburg, Germany) system and standard conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s. For amplification of *βNGF* sequence the annealing temperature was increased to 59°C. Relative quantifications were performed based on the ΔCT method using cyclophilin (*cyp*) gene as an internal standard. Primer sequences used for qPCR are listed in Table S1.

The levels of the Nb-SABP2 transcripts in the pLH-*35S-SABP2-INT-SABP2* plants were estimated based on comparison of TN (treated/untreated) ratios between Nb-SABP2 RNAi and non-transgenic plants. To this end non-transgenic and T1 progeny plants of the Nb-SABP2 RNAi lines were agroinfiltrated with the pLH-*TMV-GFP* expression vector. Total RNA was extracted from leaf tissue of both TMV-GFP treated (T) and non-treated (N) plants at 4 days after agroinfiltration (dpi), converted to cDNA and investigated by qPCR analysis with the Nb-SABP2-forw and Nb-SABP2-rev primers. Following relative quantification of the *Nb-SABP2* gene, the TN ratio of relative Nb-SABP2 expression in agroinfiltrated plants to relative Nb-

SABP2 expression in non-agroinfiltrated plants was calculated. After comparison of TN ratios between transgenic and non-transgenic plants the transgenic lines with Nb-SABP2 expression ratio ≤ 2 were selected for subsequent experiments. The leaf tissue samples were collected from middle agroinfiltrated leaves of 3 plants.

Expression of the PVX, TMV, GFP and βNGF was estimated using PVX-forw/PVX-rev, TMV-forw/TMV-rev, gfp-forw/gfp-rev and βNGF-forw/βNGF-rev primers, respectively, according to the protocol described above. The agroinfiltrated leaves of control and Nb-SABP2 RNAi transgenic plants were collected at 4 dpi and 5 dpi for PVX- and TMV-based expression vectors, correspondingly. Overall, samples from 8–10 plants for each experimental variant were investigated. Each sample represents a pooled sample form three middle leaves of one plant. The number of samples is indicated as n in figure legends. The Mann-Whitney U test was applied to test if there were differences between wild type and Nb-SABP2 RNAi plants.

Phenotypic screening of transgenic lines

For phenotypic screening the seeds of T1 progeny of the pLH-*35S-SABP2-INT-SABP2* transgenic plants were surface-sterilised for 5 min with 70% ethanol, rinsed five times with sterile water and germinated on solid MS medium supplemented with 20 g/L sucrose, 0.5 g/L 2-(N-morpholino)ethanesulfonic acid (MES) and 5 mg/L phosphinothricin (PPT) (Roth, Karlsruhe, Germany). Non-transgenic *N. bethamiana* plants were germinated without selection pressure. After two weeks, seedlings of PPT resistant lines containing T-DNA of the pLH-*35S-SABP2-INT-SABP2* plasmid and control wild type plants were transferred into the greenhouse and cultivated at 24^{O}C with 16 day/8 night photoperiod. After four weeks wild type and transgenic plants were assessed for phenotypic parameters including plant height, number of main and secondary leaves, fresh weight of primary and secondary leaves and stem biomass. The experiment was repeated three times with 15 plants for each transgenic line and non-transgenic control for a total of 45 plants per genotype. Differences between mean values were determined by unpaired T-test using SigmaStat software. Significance level was set at $p < 0.05$.

To estimate plant leaf area all leaves of individual plant were collected at 4 weeks after planting and scanned. Leaf area of primary and secondary leaves was measured by ImageJ software (http://rsb.info.nih.gov/ij/) and summed up to the total leaf area and leaf area for each leaf group of single plant. The experiment was repeated four times. Each repeat included four plants per genotype. Non-transgenic *N. bethamiana* plants served as control. The data were statistically analysed by the unpaired T-test using SigmaStat software (SigmaStat; Systat Software, San Jose, CA, USA). Significance level was set at $p < 0.05$.

Plant agroinfiltration

The freeze–thaw method (Holsters et al 1978) was used for mobilizing pLH-*TMV-GFP*, pLH-*PVX-GFP*, pLH-*TMV-βNGF* and pLH-*PVX-βNGF* constructs into *A. tumefaciens* (strain C58C1). For agroinfiltration *A. tumefaciens* cultures carrying pLH-*TMV-GFP*, pLH-*PVX-GFP*, pLH-*TMV-βNGF* and pLH-*PVX-βNGF*

expression vectors were cultivated overnight in LB medium supplemented with 100 mg/L spectinomycin, 300 mg/L streptomycin, 50 mg/L rifampicin and 100 mg/L carbenicillin. Following centrifugation, the pellets were resuspended in agroinfiltration buffer (10 mM MES, 10 mM $MgCl_2$, 150 µM acetosyringone, pH 5.6) to final optical density OD600 of 0.1. Then the expanded leaves of 5-6-week-old *N. benthamiana* plants were infiltrated using a syringe without a needle. *N. benthamiana* plants were cultivated in the greenhouse at 24/22^{0}C day/night temperature with a 16 h light and 8 h dark photoperiod.

Measurement of GFP fluorescence

To estimate the suitability of the Nb-SABP2 silenced plants for transient recombinant protein production the wild type *N. benthamiana* plants and T1 progeny of transgenic lines were agroinfiltrated with pLH-*TMV-GFP*, pLH-*PVX-GFP* expression vectors. Leaf discs (30 mg) were taken at 5 and 7 dpi and homogenized in 700 µL of PBS buffer. The GFP signal was measured using an Infinite 200 Pro microplate reader (Tecan, Griesheim, Germany) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The background fluorescence (non-transgenic control and PBS buffer) was subtracted from the raw fluorescence reads. The experiment was performed four times with 5 plants per genotype in each repeat. Each sample for individual plant represents the polled sample from three middle agroinfiltrated leaves. A mixed linear model was fitted to conduct analysis of variance (ANOVA), to estimate least square means (lsmeans) and to calculate differences between the non-transgenic control and the transgenic lines (SAS 9.6; PROC MIXED; SAS Institute 2023).

Quantitative ELISA

The GFP and βNGF proteins were determined by specific ELISA. Plant samples were collected from the middle agroinfiltrated leaves at 5 and 7 dpi for PVX- and TMV agroinfiltrated leaves, respectively. The description of the GFP and βNGF quantification can be found in previous publications (Kopertekh and Reichardt 2021; Kopertekh 2024). GFP and βNGF accumulation was analysed in 10–15 biological and three technical replicates. The biological replicate represents a pooled sample from three middle leaves of one plant. The Mann-Whitney U test (significance level $p < 0.05$) was performed to test if there were differences in recombinant protein accumulation between agroinfiltrated wild type and Nb-SABP2 RNAi transgenic plants. SigmaStat software was used.

Results

Generation and verification of Nb-SABP2 RNAi transgenic lines

To modulate the immune response of *N. benthamiana* the *Nb-SABP2* gene was downregulated by stable integration of the pLH-*35S-SABP2-INT-SABP2* construct in plant genome. The T-DNA region of the pLH-*35S-SABP2-INT-SABP2* is depicted in Fig. 1A and contains the *bar* selectable marker and *Nb-SABP2* dsRNA expression units. In the *Nb-SABP2* dsRNA expression cassette the 230 bp sense and

antisense DNA fragments of the *Nb-SABP2* gene separated by the second intron of the potato *ST-LS1*gene were fused together. Both expression cassettes are controlled by the CaMV 35S promoter and terminator. The pLH-*35S-SABP2-INT-SABP2* binary vector was introduced into *N. benthamiana* by Agrobacterium-mediated transformation and primary regenerants were selected on phosphinothricin (ppt) containing medium. In total 88 plants were screened and subjected to PCR analysis using the bar-forw/bar-rev and Nb-SABP2-forw/ST-LS1-INT-rev primers that amplify the *bar* and *RNAi* insertion sequences, correspondingly. The expected PCR fragments of 401 and 440 bp for *bar* and *Nb-SABP2* dsRNA sequences, respectively, have been detected for 55 regenerants confirming their transgenic nature. The representative DNA gels for Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-79 lines are shown in Fig. 2A. PCR verification of remaining lines, which were involved in subsequent expression analysis at RNA level, is shown in Figure S1.

Primary regenerants confirmed as transgenic by PCR analysis were transferred to the greenhouse to set seeds. The T1 progeny of 35 transgenic lines was evaluated at RNA level using TN-ratio, which represents the ratio of *Nb-SABP2* expression between stress-induced (T) and non-induced (N) plants. TMV delivered by *A. tumefaciens* was selected as a stress trigger. For wild type and RNAi transgenic lines, the level of *Nb-SABP2* transcripts was determined by qPCR in non-treated plants and plants agroinfiltrated with the pLH-*TMV-GFP*. Then the ratio of the *Nb-SABP2* transcripts in virus infected to that in non-infected plants was calculated and compared to wild type control. The TN-rate for the *Nb-SABP2* mRNA in wild type *N. benthamiana* plants was increased more than 10-fold (12.9–17.1) indicating upregulation of the *Nb-SABP2* gene in treated plants (Fig. 2B). The investigated *Nb-SABP2* RNAi lines can be divided into three groups in respect to the values of TN-ratio. In the first group consisting of two lines, Nb-SABP2-23 and Nb-SABP2-26, the TN rates equal to control *N. benthamiana* plants were observed suggesting that the *Nb-SABP2* gene was not suppressed. Seventeen transgenic lines (Nb-SABP2-1, Nb-SABP2-2, Nb-SABP2-3, Nb-SABP2-5, Nb-SABP2-6, Nb-SABP2-13, Nb-SABP2-21, Nb-SABP2-24, Nb-SABP2-45, Nb-SABP2-51, Nb-SABP2-54, Nb-SABP2-56, Nb-SABP2-59, Nb-SABP2-61, Nb-SABP2-66, Nb-SABP2-76 and Nb-SABP2-78) showing TN ratios in the range of 2 to 11.2 were clustered into second group. The distinct TN ratios suggest different degree of the *Nb-SABP2* suppression in these genotypes. The third group with TN values for the *Nb-SABP2* gene ≤ 2 includes following 16 lines: Nb-SABP2-4, Nb-SABP2-9, Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-29, Nb-SABP2-35, Nb-SABP2-39, Nb-SABP2-41, Nb-SABP2-49, Nb-SABP2-50, Nb-SABP2-53, Nb-SABP2-70, Nb-SABP2-79, Nb-SABP2-81, Nb-SABP2-84 and Nb-SABP2-88. The observed TN rations in these transgenic lines indicate high degree of the *Nb-SABP2* suppression in TMV-GFP infected plants.

Generally, the expression analysis revealed that stable integration of the pLH-*35S-SABP2-INT-SABP2* construct into *N. benthamiana* genome resulted in suppression of the *Nb-SABP2* target gene in stress-induced plants in 33 lines out of 35 investigated. Based on molecular characterization 16 Nb-SABP2 RNAi lines with TN values for the *Nb-SABP2* gene ≤ 2 were selected for phenotypic analysis.

Phenotypic evaluation of the Nb-SABP2 RNAi transgenic lines

To perform phenotypic analysis the T1 seeds of the Nb-SABP2 RNAi transgenic lines were germinated in presence of selective agent, transmitted to the greenhouse and grown for 4 weeks. Six morphological characteristics including plant height, number of primary leaves, number of secondary leaves, primary leaf biomass, secondary leaf biomass and stem biomass were assessed. (Fig. 3, Figure S2). Among 16 examined Nb-SABP2 RNAi transgenic lines, we did not detect any morphologic aberrations. Combined evaluation of six parameters allowed us to select 4 transgenic lines, Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49, Nb-SABP2-79, that might be interesting for recombinant protein production. For T1 progeny plants the number of primary leaves per plant was significantly increased

by 106%, 116%, 105%, 115% for the Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-79 lines, respectively (Table S2). The same tendency was observed for the number of secondary leaves per plant. Compared to non-transgenic control plants the values for this characteristic were significantly increased by 123% for Nb-SABP2-16, 114% for Nb-SABP2-19, 116% for Nb-SABP2-49 and 108% for Nb-SABP2-79 (Table S2). Transgenic Nb-SABP2 lines also demonstrated an enhanced above ground biomass accumulation. Primary leaf biomass was 26, 30, 25 and 30% higher as that for non-transgenic plants for Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-79 lines, respectively. The Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-79 lines produced 33%, 34%, 43% and 44% more secondary leaf biomass as wild type *N. benthamiana* plants, respectively. The height of T1 progeny plants varied from 95.5% (Nb-SABP2-49) to 120% (Nb-SABP2-16) when compared to non-transgenic control (Fig. 3, Table S2).

Further investigation of the Nb-SABP2 RNAi lines revealed that the enhanced biomass accumulation was accompanied by the increased total leaf area per plant (Fig. 4). The quantification of leaf area for

primary stem leaves uncovered that the values of this phenotypic parameter were of 39%, 37%, 29% and 16% higher for the Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-79 lines, respectively in comparison to non-transgenic counterpart (Fig. 4A). For total area of secondary axillary leaves this enlargement was more obvious. The leaf area of secondary leaves was increased by 191% for the Nb-SABP2-16 line, 229% for the Nb-SABP2-19 line, 182% for the Nb-SABP2-49 line and 170% for the Nb-SABP2-79 line (Fig. 4B). In respect of combined surface area of both primary and secondary leaves per plant the investigated lines can be ranked in the following progressive order: Nb-SABP2-79 (122%), Nb-SABP2-49 (136%), Nb-SABP2-16 (145%), Nb-SABP2-19 (148%) (Fig. 4C).

Collectively, stable downregulation of the *Nb-SABP2* gene by dsRNA did not negatively affect plant growth and morphology.

Recombinant protein production in the Nb-SABP2 RNAi transgenic plants

To investigate the practical potential of the Nb-SABP2 RNAi transgenic lines for recombinant protein production the initial screening based on measurement of GFP fluorescence was carried out. The non-transgenic and T1 progeny plants of the Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-79 RNAi lines were agroinfiltrated with the pLH-*TMV-GFP*, pLH-*PVX-GFP* expression vectors. Leaf samples of

agroinfiltrated plants were collected at 5 and 7 dpi and subjected to GFP fluorescence analysis. Five days after agroinfiltration in pLH-*TMV-GFP* inoculated samples the GFP fluorescence was 2.4-2.6-fold higher in the Nb-SABP2 RNAi transgenic lines in comparison to non-transgenic control (Fig. 5).

The comparable trend was noted for the pLH-*TMV-GFP* agroinfiltrated leaves that were collected at

7 dpi. The GFP fluorescence intensity was increased by 1.6, 1.9, 2,0 and 1,7-folds for Nb-SABP2-79, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-16 genotypes, respectively. The suppression of the *Nb-SABP2* gene had positive impact on GFP fluorescence when *gfp* gene was delivered by the pLH-*PVX-GFP* vector. The quantification of GFP fluorescence in the pLH-*PVX-GFP* agroinfiltrated leaves collected at 5 dpi revealed 1.8-fold increase for the Nb-SABP2-79, Nb-SABP2-19 and 2-fold increase for the Nb-SABP2-16, Nb-SABP2-49 transgenic lines in comparison to control. The assessment of GFP fluorescence in PVX-GFP samples at 7 dpi showed 1.3, 1.6, 1,8 1nd 1.9-fold enhancement for lines Nb-SABP2-79, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-16, correspondingly. Therefore, downregulation of the *Nb-SABP2* gene by stable integration of the pLH-*35S-SABP2-INT-SABP2* construct increase GFP fluorescence intensity in case of both TMV- and PVX-based expression vectors at both sampling points.

To further test the usefulness of Nb-SABP2 downregulated plants in improving the accumulation of recombinant proteins, we focused on the Nb-SABP2-16 transgenic line. Two virus expression vectors, TMV- and PVX-based, harbouring *gfp* and *βNGF* genes encoding GFP and βNGF proteins, respectively,

were involved in the experiments. Consequently, the wild type and Nb-SABP2-16 transgenic plants were agroinfiltrated with four expression constructs, namely, pLH-*TMV-GFP*, pLH-*PVX-GFP*, pLH-*TMV-βNGF* and pLH-*PVX-βNGF* (Fig. 1B, C). The collected leaf probes were investigated at RNA and protein levels. In comparison to non-transgenic plants in the SABP2-16 plants higher amounts of TMV and GFP RNA were detected (Fig. 6A). Subsequent GFP protein quantification by ELISA revealed higher GFP levels in *N. benthamiana* plants with supressed *Nb-SABP2* gene. In wild type plants 86.8 ± 9.2 μg/g GFP of fresh-weight tissue were detected, whereas in transgenic line 215.1 ± 10.7 μg/g GFP of fresh-weight tissue were observed. Similar to the pLH-*TMV-GFP* expression vector in the pLH-*PVX-GFP* agroinfiltrated plants carrying Nb-SABP2 RNAi construct higher amounts of PVX and GFP RNA were found when compared to non-transgenic counterpart (Fig. 6B). The GFP protein accumulation for PVX-based vector was 72.3 ± 3.9 μg/g of fresh-weight tissue and 161.3 ± 5.2 μg/g of fresh-weight tissue for non-transgenic and transgenic plants, respectively.

Second recombinant protein which we tested is βNGF. For the pLH-*TMV-βNGF* expression vector increased amounts of TMV and βNGF RNA were detected in transgenic plants (Fig. 7A). Wild type *N. benthamiana* plants produced 22.6 ± 3.9 ng/g βNGF of fresh-weight tissue as was determined by ELISA. For transgenic plants this mean was 47.2 ± 8.1 ng/g of fresh-weight tissue. In the same way as for pLH-*TMV-βNGF* the enhanced accumulation of PVX RNA correlated with that for and βNGF RNA in pLH-*PVX-βNGF* treated plants of line Nb-SABP2-16 (Fig. 7B). In addition, accumulation of virus and target gene RNA was higher in Nb-SABP2-16 leaf samples in comparison to wild type plants. This enhanced RNA accumulation was accomplished by enhanced protein accumulation. ELISA detected 32.8 ± 6.3 ng/g

βNGF of fresh-weight tissue and 79.3 ± 9.1 ng/g βNGF of fresh-weight tissue for wild type and transgenic plants, respectively.

Two conclusions can be made based on our data. First, the RNAi-mediated suppression of the *Nb-SABP2* gene enhanced susceptibility to PVX and TMV, which was manifested by increased virus RNA accumulation. Second, increased accumulation of virus component of the transient vector deliver system correlated with higher amounts of GFP and βNGF recombinant proteins.

Discussion

Over the past decades significant efforts have been made to advance the efficiency of transient foreign protein production including the development of new *A. tumefaciens* strains, improved expression vectors and genetic components enhancing expression such as promoters, terminators and non-translated regions. Currently the impact of agroinfiltration on host plant is receiving considerable attention (Hamel 2026). Recent studies on molecular response of agroinfiltrated *N. benthamiana* plants expressing recombinant proteins disclosed extensive transcriptomic and metabolomic changes in plant cells (Drapal et al 2021; Hamel et al 2024; Hamel et al 2026). Particularly, agroinfiltration triggers a strong defence response associated with the lignification, terpene biosynthesis, oxidative stress and key stress hormone pathways (Hamel et al 2026; Hamel et al 2026a).

Consequently, attenuation of the host plant immune response by transient downregulation of the *Nb-SABP2* gene, which is involved in SA metabolism pathway, has positive impact on recombinant protein accumulation in *N. benthamiana* (Kopertekh 2024). Currently two widely used techniques to modify gene expression *in vivo* are CRISPR/Cas 9 and RNAi. CRISPR/Cas9-mediated gene editing allows knockout of target gene, whereas RNAi suppresses gene expression indirectly and the knockdown is in most cases only partial. The partial loss-of-function can be beneficial for modification of genes, which are important for plant physiology. Indeed, the DCL2/DCL4 double supressed RNAi transgenic *N. benthamiana* plants did not display significant morphological differences in comparison to non-transgenic counterparts (Matsuo and Matsumura 2017). In contrast, the DCL2/DCL4 knockout transgenic plants developed by CRISPR/Cas9 exhibited abnormal phenotype manifested by short height, large lateral buds, smaller elongated leaves and reduced fertility (Matsuo 2022). In another study the ΔRDR6 *N. benthamiana* plants edited by CRISPR/Cas9 system displayed developmental abnormalities such as reduced plants height, larger axillary buds, elongated leaves and sterility (Matsuo and Atsumi 2019). Conversely, the phenotype of the RDR6 RNAi transgenic lines was equivalent to that of wild type *N. benthamiana* plants (Schwach et al 2005; Vaistij et al 2009).

Considering facts that the plant phenotype determines the success of recombinant protein production and the *Nb-SABP2* gene is involved in several plant physiological processes we generate transgenic *N. benthamiana* plants in which the *Nb-SABP2* gene was target by dsRNA. In order to achieve this the pLH-*35S-SABP2-INT-SABP2* binary plant transformation vector was introduced in plant genome. The presence of transgene construct was confirmed by specific PCR in 55 independent primary regenerants out of 88

investigated. Then the functionality of introduced dsRNA expressing construct was examined in T1 progeny of 35 lines by qPCR analysis of the *Nb-SABP2* transcript abundance in stress-induced plants. This investigation revealed that the *Nb-SABP2* expression was reduced by RNA interference in the stress induced pLH-*35S-SABP2-INT-SABP2* transgenic lines with a range of target gene silencing efficiencies from non-detectable silencing in 2 lines to hight degree of silencing in 16 lines. Such variation is usual for RNAi technology and diverse frequences for silencing of an endogenous target have been already reported (Chuang and Meyerowitz 2000; McGinnis et al 2007). Many potential factors can contribute to RNAi effectiveness in a gene specific manner including sequence, expression pattern of target gene and copy number of dsRNA construct (Kerschen et al 2004).

After it was determined that stable integration of dsRNA expressing construct could suppress endogenous *Nb-SABP2* gene we estimated the phenotype of downregulated *N. benthamiana* mutants. The T1 progeny of the *Nb-SABP2* RNAi transgenic lines did not exhibit any obvious phenotypic aberrations. Similarly, the *Nicotiana tabacum* transgenic plants with suppressed *Nt-SABP2* gene were indistinguishable from that of non-transgenic control (Kumar and Klessig 2003). Furthermore, one of the recent publications describes the *N. benthamiana* mutants in which the *Nb-SABP2* gene was knockout by the CRISPR/Cas9 system. The authors did not disclose any morphological aberrations for these modified plants, although the detailed phenotypic analysis was not provided (Gong et al 2023).

Suppression of the *Nb-SABP2* gene via RNAi gene silencing compromised SA-mediated defence in *N. benthamiana* resulting in enhanced accumulation of the TMV and PVX RNA. Thus, our results further confirmed well documented involvement of the SABP2 in plant response against biotic stress. In particular, Kumar and Klessig (2023) demonstrated in their original work that the transgene downregulation of the *Nt-SABP2* gene in *N. tabacum* led to increased susceptibility to TMV. In a similar fashion higher amounts of TMV and PVX RNA were detected in *N. benthamiana* leaves in which the expression of the *Nb-SABP2* gene was reduced in a transient manner (Zhu et al 2014; Kopertekh 2024).

As showed in this study, manipulating plant defence response by RNAi-mediated suppression of the *Nb-SABP2* gene appears to have practical potential to facilitate foreign protein production using virus vectors. We demonstrated the usefulness of this approach for GFP and βNGF recombinant proteins and TMV and PVX expression vectors. Positive correlation between defence response suppression of host plant and accumulation of foreign proteins has been documented in several publications. In particular, the amounts of GFP and human acidic fibroblast growth factor (αFGF) were increased by 2.2 and 5-folds, respectively, in DLC2/DLC4 transgenic *N. benthamiana* plants developed by the RNAi technology (Katsarou et al 2019; Matsuo and Matsumura 2017). Similar 2.5-fold enhancement of GFP production was observed in the CRISPR/Cas9-edited loss-of-function RDR6 *N. benthamiana* mutants (Matsuo and Atsumi 2019). In another publication the beneficial effect of a semi-dominant negative gain-of-function mutant form of plasmodesmata located protein 5 (PDLP) from *Arabidopsis thaliana* on TMV-based vector movement and enhanced foreign protein production was demonstrated. Using this approach the accumulation of yellow fever virus envelope protein (YFE-1) and *Bacillus anthracis* protective antigen (PA83) was increase up to 60% (Wang et al 2023). Finally, CRISPR/Cas9-mediated editing of a glycine-

rich cell wall structural protein (NbGRP) from *N. benthamiana* confirmed as an antiviral host factor boosted GFP yield by 2-fold (Fang et al 2026).

In conclusion, our study provides experimental evidence that stable downregulation of the *Nb-SABP2* gene promoted multiplication of viral component of transient vector delivery system and boosted recombinant protein expression in *N. benthamiana*. We demonstrated usefulness of this approach for TMV- and PVX-based expression vectors. Although the utility of the Nb-SABP2 RNAi lines might be extended to other viral vector systems. We need to emphasise that the modified *N. benthamiana* plants developed in this study might be unsuitable to expression vectors harbouring strong suppressors of gene silencing. However, the commercial TMV-based transient expression systems such as GENEWARE® (Shivprasad et al 1999), magnICON® (Gleba et al 2007), TMV launch vector (Musiychuk et al 2007), TRBO (Lindbo 2007) do not include strong silencing suppressors.

Declarations

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Competing interests

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

Author Contribution

LK contributed to the study conception, analysis and interpretation of the results. HL performed statistical analysis of data. Both authors drafted the Manuscript and approved its submitted version.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

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Figures

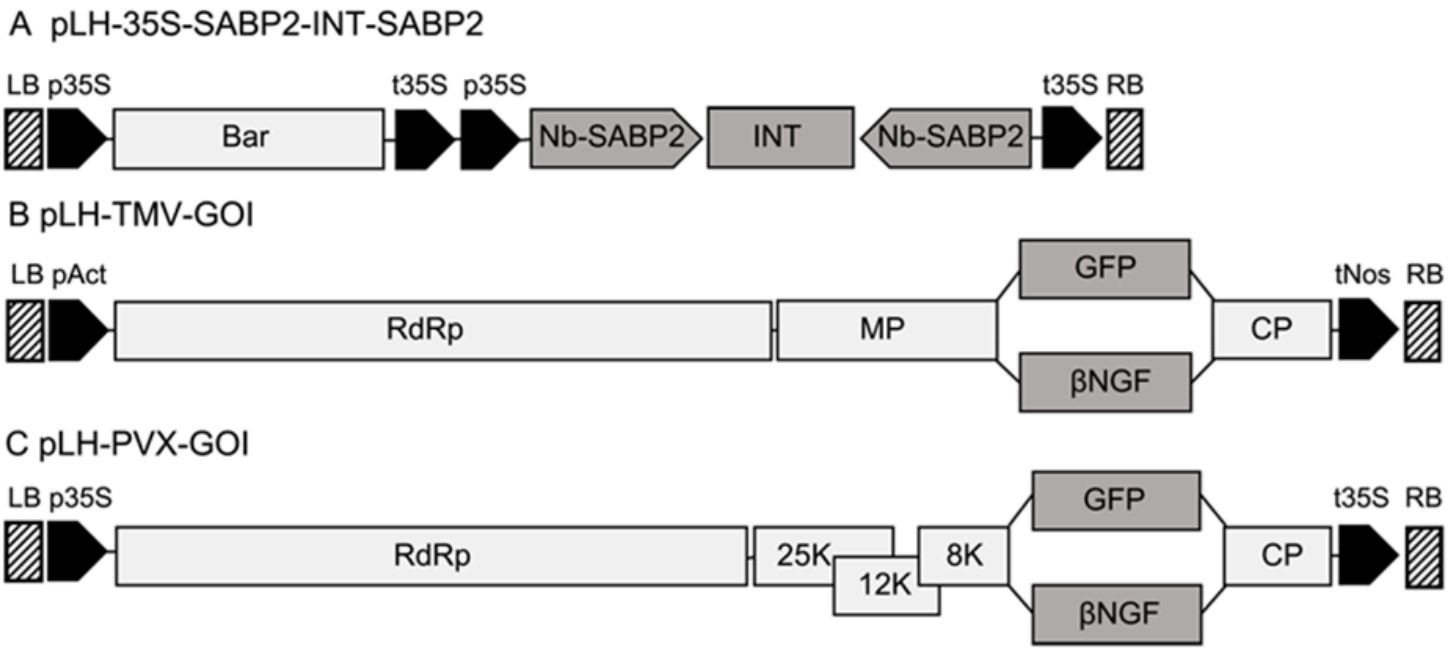


Figure 1

Schematic representation of the pLH-*35S-SABP2-INT-SABP2*, pLH-*TMV-GOI* and pLH-*PVX-GOI* expression vectors. (A) The pLH-*35S-SABP2-INT-SABP2*RNAi transformation construct. T-DNA region of the pLH-*35S-SABP2-INT-SABP2*includes two expressions units for *bar* selection marker gene and *Nb-SABP2*RNAi sequence. The genetic elements of the pLH-*35S-SABP2-INT-SABP2* are specified as follows: bar, *bar* gene; *Nb-SABP2*, the 230 bp fragment of the *Nb-SABP2* gene in sense and antisense orientation; INT, the second intron (IV2) of the potato *ST-LS1* gene. (B) The pLH-*TMV-GOI*expression vector. The genetic elements are as follows: RdRp, RNA-dependent RNA polymerase; MP, movement protein; βNGF, *βNGF* gene; GFP, *GFP* gene; CP, coat protein encoding sequence; (D) The pLH-*PVX-GOI* expression vector. The genetic elements are as follows: RdRp, RNA-dependent RNA polymerase; 25K, 12K, 8K, triple gene block; βNGF, *βNGF* gene; GFP, *GFP*gene; CP, coat protein encoding sequence. Following regulatory elements are included into expression vectors: pAct2, Act2 promoter from *A. thaliana*; tNos, nopaline synthase terminator, p35S, CaMV 35S promoter; t35S, CaMV 35S terminator. Left (LB), and right (RB) borders of T-DNA are also shown

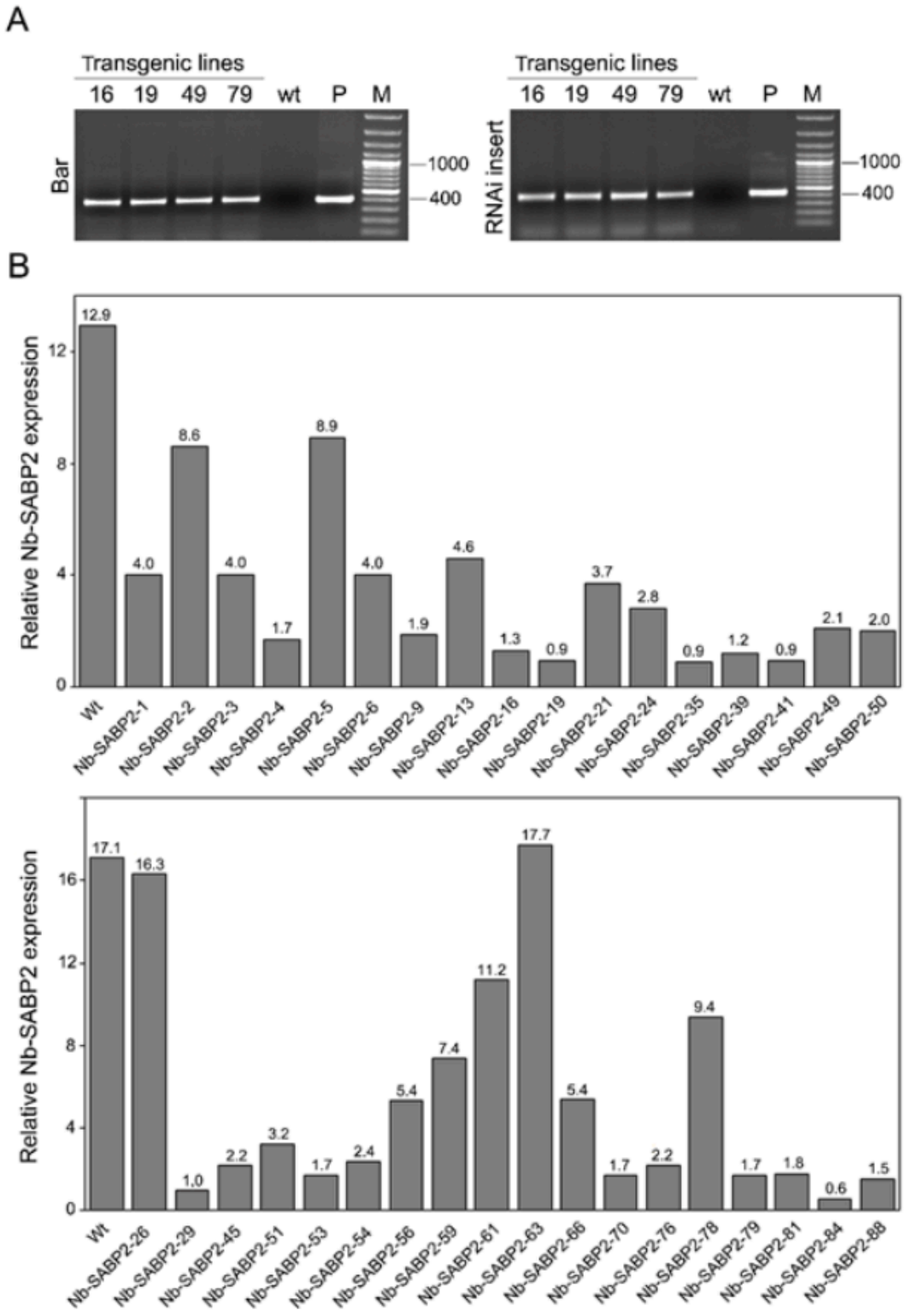


Figure 2

Molecular analysis of transgene integration. (A) PCR verification. Genomic DNA of primary regenerants (Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-79) was amplified using bar-forw/bar-rev and Nb-SABP2-forw/ST-LS1-INT-rev primers specific to *bar* and *RNAi* insertion sequences, respectively. Non-transgenic *N. bethamiana* (wt) represents negative control, whereas DNA of the pLH-*35S-SABP2-INT-SABP2* plasmid (P) was included as positive control. Molecular marker (M). (B) qPCR analysis. Total RNA

from the TMV-GFP infected and non-infected wild type and Nb-SABP2 RNAi transgenic plants was investigated by qPCR using the Nb-SABP2-forw/Nb-SABP2-rev primers as described in Materials and methods section. The TN-ratio, which represents the ratio of Nb-SABP2 expression between the pLH-*TMV-GFP* infected (T) and non-treated (N) samples, is shown.

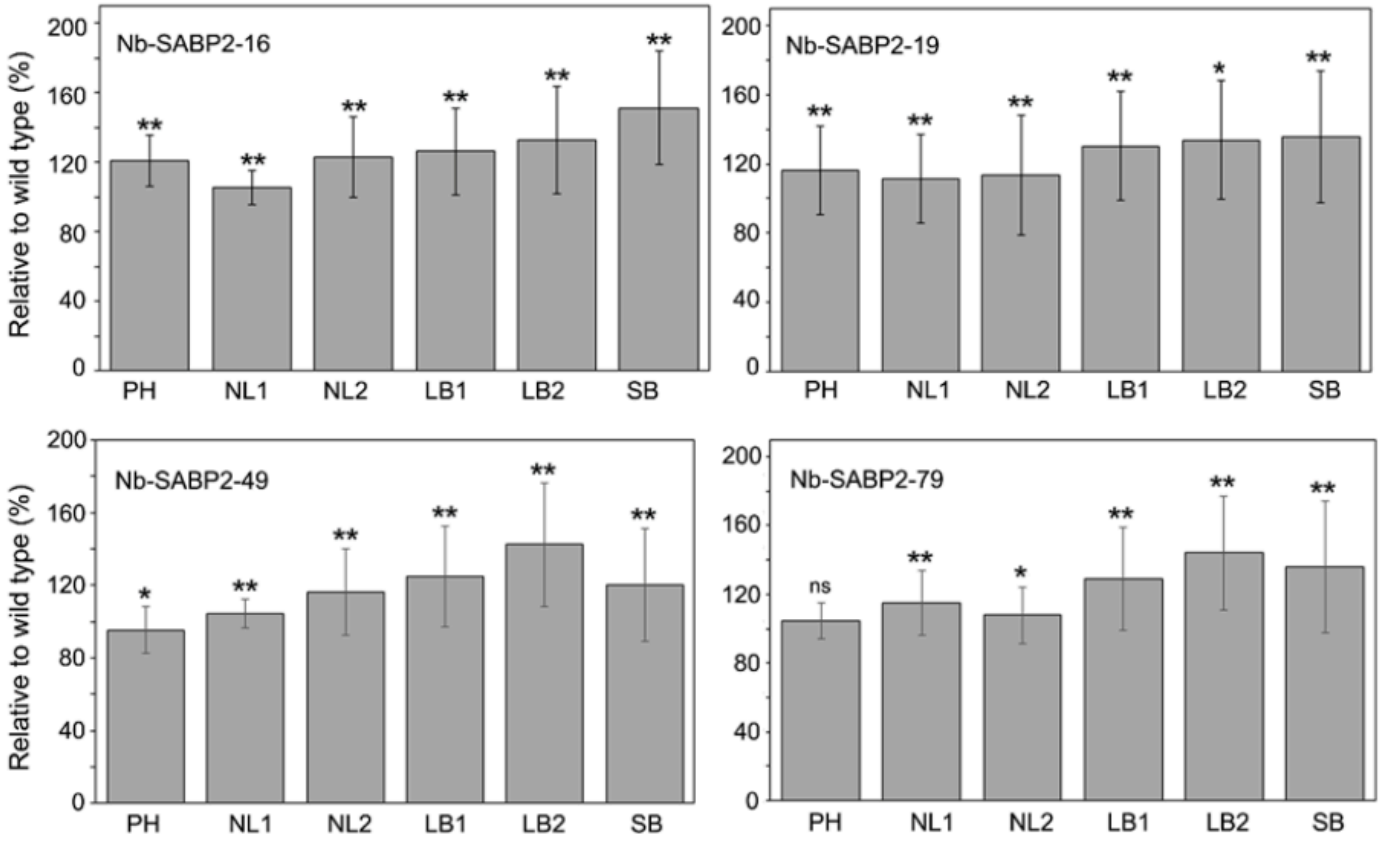


Figure 3

Phenotypic evaluation of Nb-SABP2 RNAi transgenic lines. The T1 progeny of the Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-79 transgenic lines was grown in the greenhouse for 4 weeks and scored for plant height (PH), number of primary leaves (NL1), number of secondary leaves (NL2), primary leaf biomass (LB1), secondary leaf biomass (LB2) and stem biomass (SB). The morphological parameters are expressed as precent relative to non-transgenic *N. benthamiana* plants. Values represent the means with standard deviation (n=45). Asterisks indicate significance as determined by unpaired T-test, with * and ** denoting $p<0.05$ and $p<0.01$, respectively. Not significant values are determined as ns.

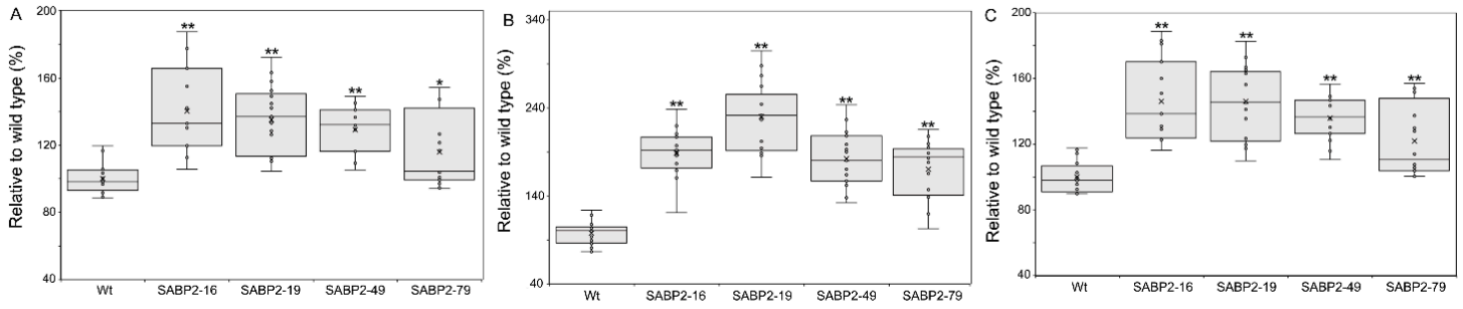


Figure 4

Leaf area of the Nb-SABP2 RNAi transgenic lines. The box plots represent area of primary (A), secondary leaves (B) and total leaf area (C) per plant. Values show the means with standard deviation (n=16). Asterisks indicate significance as determined by unpaired T-test, with * and ** denoting $p<0.05$ and $p<0.01$, respectively.

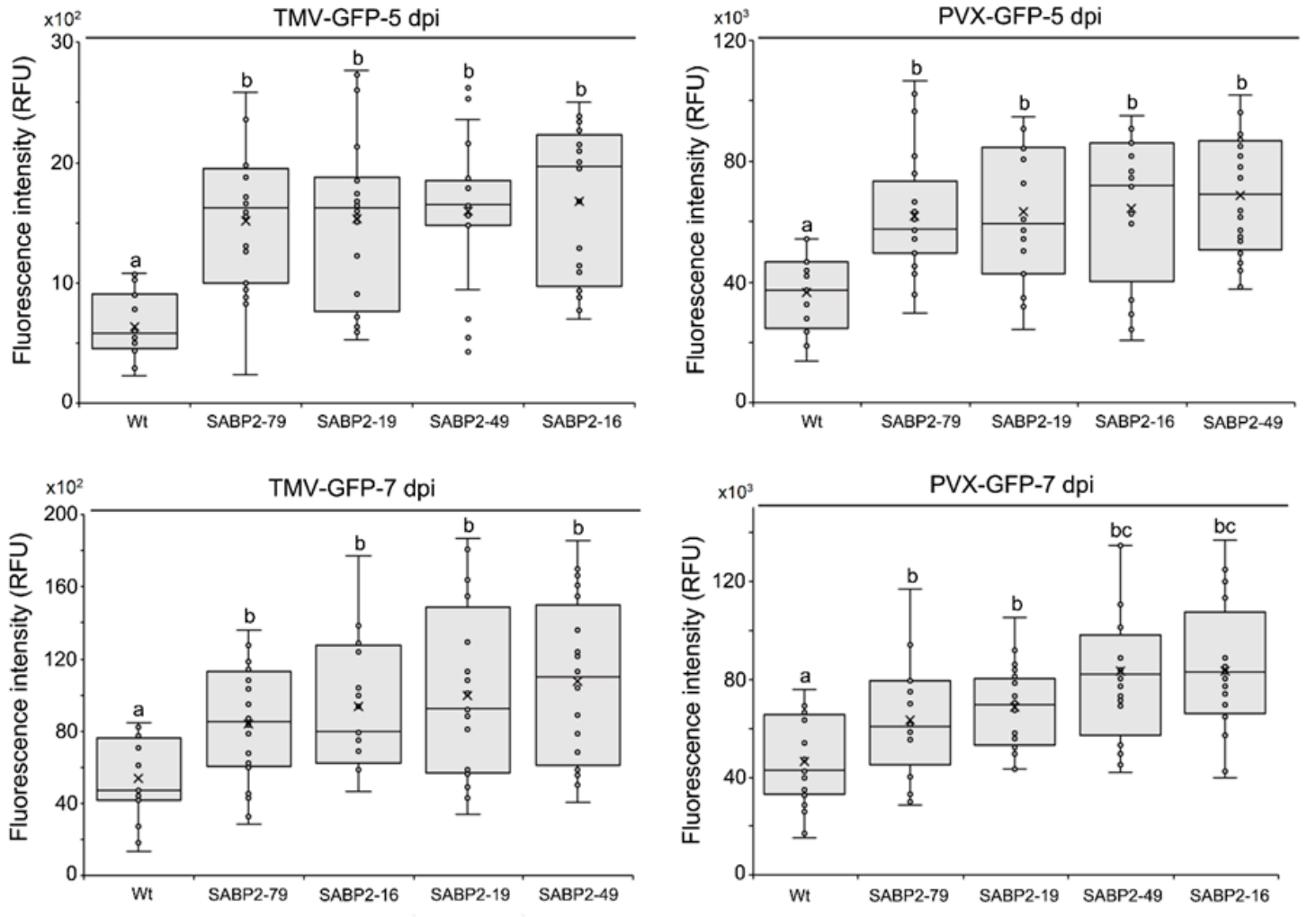


Figure 5

GFP fluorescence in Nb-SABP2 RNAi and wild type *N. benthamiana* plants. The non-transgenic and Nb-SABP2-16, Nb-SABP2-19, Nb-SABP2-49 and Nb-SABP2-79 RNAi transgenic lines were agroinfiltrated with pLH-*TMV-GFP* and pLH-*PVX-GFP*expression vectors. GFP fluorescence intensity was measured in agroinfiltrated leaves at 5 and 7 dpi. Box plots represent data from 4 independent experiments (n=20). Different letters indicate statistically significant differences ($p<0.05$). RFU, relative fluorescence units.

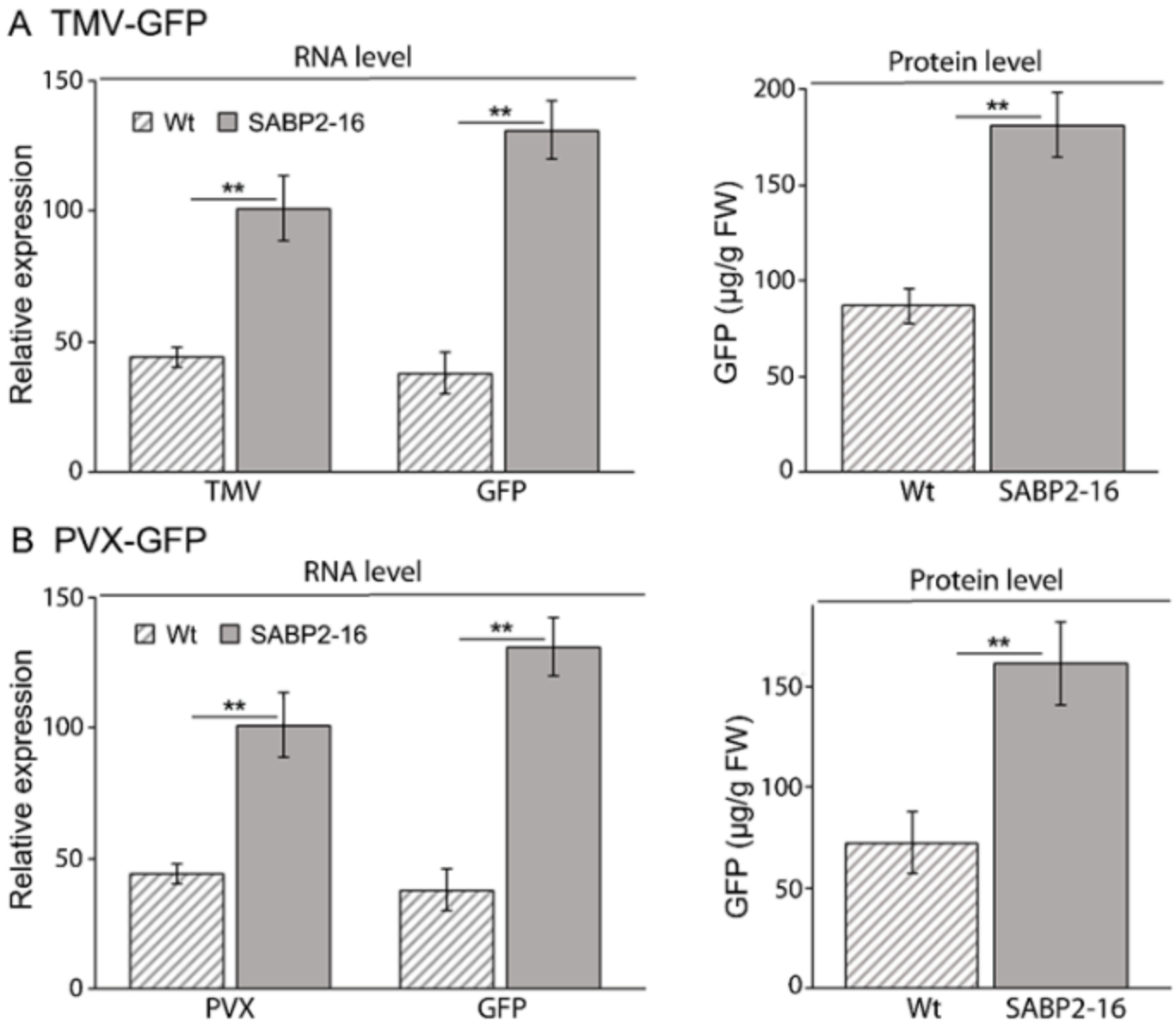


Figure 6

Transient production of GFP protein. The non-transgenic and transgenic plants with downregulated *Nb-SABP2* gene were agroinfiltrated with *A. tumefaciens*cultures carrying pLH-*TMV-GFP* (A) and pLH-*PVX-GFP* (B) constructs. The level of virus (TMV and PVX) and GFP RNA was detected in leaf samples by qPCR analysis at 4 and 5 dpi for PVX- and TMV-based expression vectors, correspondingly. To estimate the GFP protein accumulation level agroinfiltrated leaves were collected at 5 dpi for pLH-*PVX-GFP* and 7 dpi for pLH-*TMV-GFP*and subjected to specific ELISA. Values represent the means with standard

deviation (n=10-15). Asterisks indicate significance as determined by the Mann-Whitney test, with * and ** denoting p<0.05 and p<0.01, respectively.

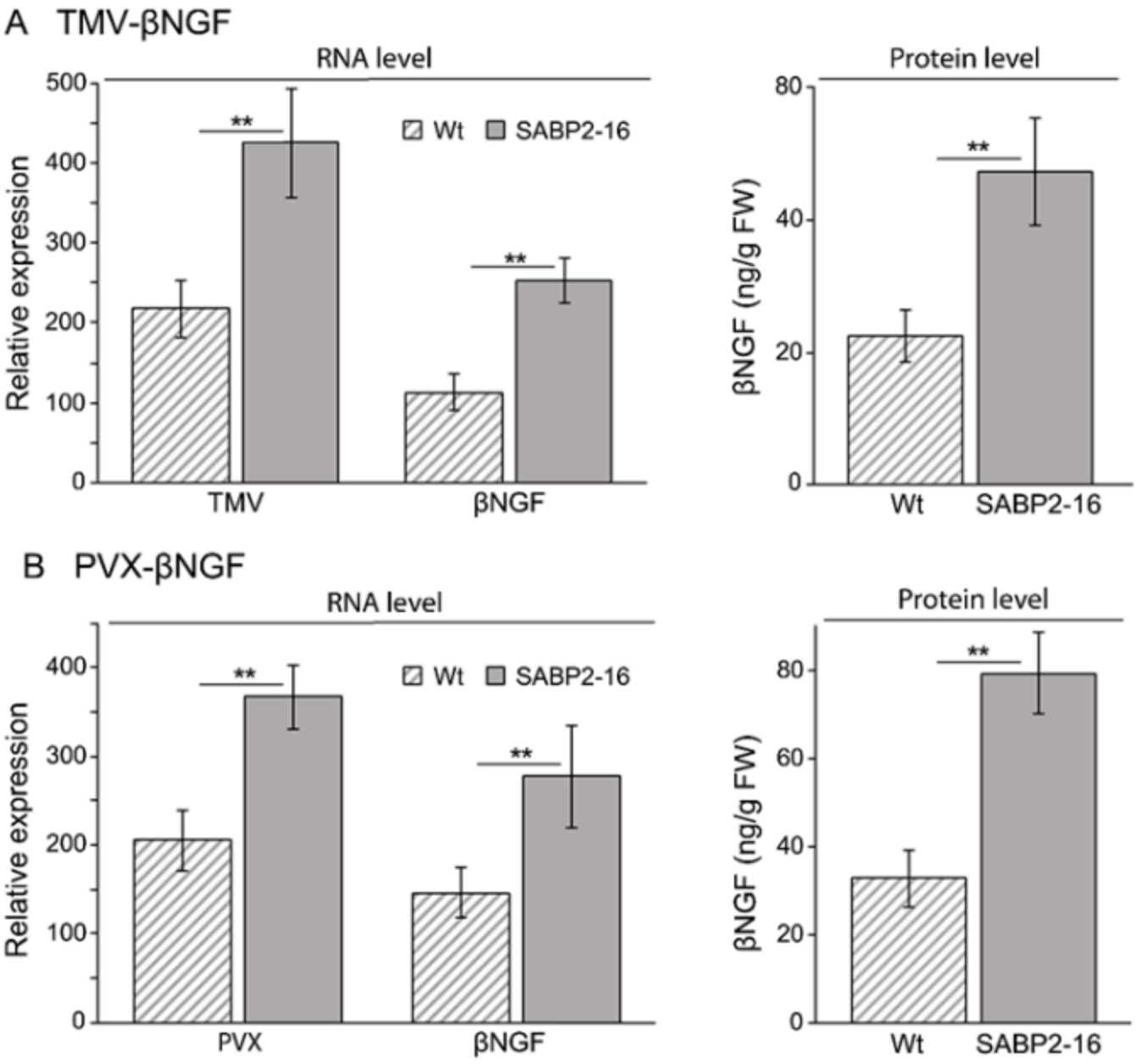


Figure 7

Transient production of βNGF recombinant protein. The non-transgenic and transgenic plants with downregulated *Nb-SABP2* gene were agroinfiltrated with *A. tumefaciens* cultures carrying pLH-TMV-*βNGF* (A) and pLH-PVX-*βNGF*(B) constructs. The level of virus (TMV and PVX) and βNGF RNA was detected in leaf samples by qPCR analysis at 4 and 5 dpi for PVX- and TMV-based expression vectors, correspondingly. To estimate the βNGF protein accumulation agroinfiltrated leaves were collected at 5 dpi for pLH-*PVX βNGF* and 7 dpi for pLH-TMV-*βNGF* and subjected to specific ELISA. Values represent

the means with standard deviation (n=10-15). Asterisks indicate significance as determined by the Mann-Whitney test, with * and ** denoting $p<0.05$ and $p<0.01$, respectively.

Supplementary Files

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- Supplementarymaterial.pdf