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Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in *Nicotiana benthamiana*

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Abstract Salicylic acid (SA) is an important signal involved in the activation of defence responses against abiotic and biotic stress. In tobacco, benzoic acid or glucosyl benzoate were proposed to be precursors of SA. This is in sharp contrast with studies in *Arabidopsis thaliana*, where SA derives from isochorismate. We have determined the importance of isochorismate for SA biosynthesis in *Nicotiana benthamiana* using virus-induced gene silencing of the isochorismate synthase (*ICS*) gene. Plants with silenced *ICS* expression do not accumulate SA after exposure to UV or to pathogen stress. Plants with silenced *ICS* expression also exhibit strongly decreased levels of phylloquinone, a product of isochorismate. Our data provide evidence for an isochorismate-derived synthesis of SA in *N. benthamiana*

Keywords: Isochorismate synthase; Pathogen stress; Phylloquinone; Salicylic acid; UV stress; *Nicotiana*

1. Introduction

The phytohormone salicylic acid (SA) has been associated with various physiological processes including flowering, thermogenesis, stomatal closure and responses to biotic and abiotic stress (reviewed in [1]). It has been most intensely studied in relation to the response of plants to pathogens where it acts as a signalling molecule in the activation of defence responses against microbial pathogens (review in [2]). The importance of SA in the induction of defence responses has been well documented by many studies with mutants and transgenic plants that exhibit altered levels of SA (reviewed in [3]). SA is integrated in a signalling network together with two other phytohormones, jasmonic acid and ethylene. This network exhibits the typical hallmarks found in integrated systems including negative and positive crosstalk interactions and specific responses to combinations of stimuli [4].

Based on previous studies, SA is synthesized from phenylalanine and, depending on the plant species, either from free benzoic acid, benzoyl glucose or *o*-coumaric acid as direct precursors [5]. Most of these findings were supported by results obtained in tobacco, rice, cucumber and potato that showed an accumulation of radiolabelled ^{14}C-SA in tissues incubated with ^{14}C-phenylalanine [5]. Suppression of the expression of one phenylalanine ammonia-lyase (PAL) gene in transgenic tobacco leads to a decrease in SA accumulation [6]. The identification of SA-deficient *Arabidopsis thaliana* mutant *sid2* [7] and the subsequent localization of this mutation in a gene encoding a functional isochorismate synthase (ICS) shed some new light on the biosynthetic pathway of SA [8,9]. Only minimal levels of SA could be detected in *sid2* mutants after infection, UV or ozone exposure [7] providing a strong support for isochorismate as a precursor of stress-induced SA accumulation in *A. thaliana*. The biosynthetic pathway for SA in *A. thaliana* is therefore related to that described in bacteria where SA is synthesized from chorismate via the rate-limiting enzyme ICS and isochorismate pyruvate lyase (IPL) (reviewed in [10]). An increase in SA levels and heightened resistance to tobacco mosaic virus was shown to take place in transgenic tobacco plants transformed with bacterial ICS and IPL, indicating that this pathway can operate in plants [11]. The discrepancy in the biosynthetic pathway of SA proposed for *A. thaliana* and other plants was often assumed to reflect differences among species. To clarify the biosynthesis of SA in tobacco we have cloned the *ICS* gene from *Nicotiana benthamiana* (*NbICS*) and used virus-induced gene silencing (VIGS) to suppress the expression of *NbICS* in *N. benthamiana*. This led to an effective disruption of the accumulation of SA and of phylloquinone (PHQ) in plants undergoing a biotic and abiotic stress indicating that the ICS enzyme is also required for SA biosynthesis in *N. benthamiana*.

2. Materials and methods

2.1. Plant growth

N. benthamiana plants were grown in pots in potting soil at 25 °C in a growth chamber under 16 h light/8 h dark cycle.

2.2. Plasmid constructions

pTRV1 and pTRV2 VIGS vectors and derivatives have been described previously [12]. pTRV2-attB2-NbICS-attB1 harboured a 700 bp fragment of *N. benthamiana ICS* cDNA containing the *attB1* and *attB2* sequences and was obtained by polymerase chain reaction (PCR) using the primers 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG CTG CAA CTA TTG CAT GGG-3′ and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CCC ACA AAC TGC TGG AGT AGG-3′. This *NbICS* PCR product was recombined into pDONR-207 (Invitrogen, Basel, Switzerland) containing the *attP1* and *attP2* recombination sites using the BP CLONASE, the pTRV2-attR1-attR2 destination vector and the LR CLONASE. The product pTRV2-attB2-NbICS-attB1 was transformed into DH10B cells and selected on kanamycin-containing LB

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plates. pTRV2-attB2-AtPDS-attB1 contained a 442 bp fragment corresponding to the eleventh intron of the *A. thaliana* phytoene desaturase gene (*PDS*) (At4g14210) was amplified from genomic DNA with the primers: 5′-CAC CTA TAG AAT TCA AAG GTA CTT TGA TTG GTC-3′ and 5′-TAT AAA GCT TAG CTA TCT GGA GGAAGA C-3′. This PCR product was cloned into the pENTR vector using the TOPO cloning kit (Invitrogen) and pTRV2-attR1-attR2 as destination vector and the LR CLONASE. The final plasmid pTRV2-attB2-AtPDS-attB1 was transformed into DH10B cells and selected on kanamycin-containing LB plates.

For the VIGS assay, pTRV1 or pTRV2 and their derivatives were introduced into *Agrobacterium* strain GV3101 by electroporation. A 3-ml culture was grown overnight at 28 °C in 50 mg/l rifampicin and 50 mg/l kanamycin. *Agrobacterium* cells were harvested and resuspended in infiltration media (10 mM $MgCl_2$, 10 mM MES, 150 μM acetosyringone), adjusted to an OD of 0.4 and left at room temperature for 2 h. For VIGS, transformed *Agrobacterium* was injected into the two primary leaves of a plant at the four-leaves stage using a needle-less 1 ml syringe. Plants infiltrated with the co-culture of *Agrobacterium* containing pTRV1 and pTRV2-attB2-NbICS-attB1 plasmids were named *TRV-NbICS* plants. Plants infiltrated with the co-culture of *Agrobacterium* containing pTRV1 and pTRV2-attB2-AtPDS-attB1 plasmids were named TRV-INTRON plants. The effectiveness of VIGS was tested using the PDS gene as described in [13].

2.3. Southern blot

Genomic DNA of leaves was isolated using cetyldimethylethylammonium bromide and was digested with BglII and HindIII, separated and blotted according to Sambrook et al. [14] A PCR-amplified *NbICS* fragment was radiolabelled with P^{32}-dCTP using the prime-a-gene-labelling kit (Promega, Basel, Switzerland) was used as the probe for hybridization.

2.4. Race PCR

The full-length *NbICS* cDNA sequence was isolated using a RLM-RACE cDNA amplification kit (Ambion, Austin, USA), following the manufacturer's instructions. Degenerated primers were first designed based on an alignment of the three ICS genes of *A. thaliana*, *Catharanthus roseus* and *Lycopersicon pennelii*. Using these primers a sequence of *NbICS* was obtained corresponding to that of *NtICS*. Other primers were then designed using the *NbICS* sequence to perform the RACE. For the 5′cDNA-end amplification, the primers used were 5′ RACE inner: 5′-ACG GAG GAA ACC TGC CAT ATT G-3′ and 5′ RACE outer: 5′-TGC GAG TAG ATG TGA ACG AGA T-3′. For the 3′cDNA-end amplification the primers used were 3′ RACE outer: 5′-TTG CTC GTA GCT CCA GAG TTG T-3′ and 3′ RACE inner: 5′-CCA GTC AGC GGC ATT CAT T-3′.

2.5. RNA isolation and semi-quantitative RT-PCR analysis

Total RNA was extracted from control, silenced and non-silenced *N. benthamiana* plants (two leaves per plant) by using the phenol–guanidine isothiocyanate procedure (Trizol; Invitrogen). First strand cDNA was synthesized using 1 μg of total RNA, oligo d(T) primer and omniscript reverse transcriptase (Qiagen, Hombrechtikon, Switzerland). Fragments of *ICS* cDNAs from *N. benthamiana* were amplified by PCR from 2-μl aliquots of the cDNA reaction mixture with primers: 5′-ATT TCA TGG TCC CTC AGG TTG-3′ and 5′-TTC CTC GGT CAA ACA TTT CG-3′. As a control, fragments of EF1α were amplified using primers: 5′-TGG TGT CCT CAA GCC TGG TAT GGT TGT-3′ and 5′-ACG CTT GAG ATC CTT AAC CGC AAC ATT CTT-3′. Aliquots of 10 μl were taken every 10 PCR cycles until 50 cycles.

2.6. SA analysis

Leaf material of about 400 mg was harvested on ice and SA was extracted and quantified as previously described [15].

2.7. UVc light exposure

Plants were exposed to UVc light (254 nm, 16 W) during 1 h at a 50 cm distance from the lamp (CAMAG, Muttenz, Switzerland) to induce the production of SA. SA was measured at the end of the treatment and three days later.

2.8. Inoculation with Pseudomonas syringae pv. tomato (Pto) DC3000

Pto DC3000 was plated onto fresh KB medium [16] with the appropriate antibiotics. After growth for 24 h at 25 °C, 10 ml of 10 mm $MgCl_2$ were added to the plate. Ten minutes later, the bacterial suspension was washed out of the plates with a pipette, the samples adjusted to an $OD_{600} = 0.05$ and Silwet L-77 was added to a final concentration of 200 μl l^{-1}. Several 6 μl droplets of the bacterial suspension were deposited on each leaf.

2.9. PHQ analysis

Ground leaves (500 mg) were mixed with two volumes of *n*-heptane at 20 °C overnight. After solvent evaporation, extracts were resuspended in 300 μl MeOH for HPLC analysis using a Waters 2695 HPLC system (Milford, MA, USA) equipped with a photodiode array detector coupled to a Bruker Daltonics Esquire HCT ESI/MS spectrometer (Bremen, Germany). Separation was performed on a Macherey-Nagel (Düren, Germany) Nucleodur C18 pyramid 5μ column (125 × 3 mm i.d). Gradient elution was performed using a water MeOH gradient starting at 90% of MeOH for 4 min, to reach 100% in 10 min, MeOH was then maintained at 100% for another 20 min. The flow rate was set to 0.2 ml min^{-1}, column temperature at 30 °C and detection at 274 nm. For negative ionization mode, the pressure of the nebulizer (N_2) was set at 50 psi, dry gas flow at 9 ml min^{-1} and the temperature of the drying gas (N_2) at 32 °C. The voltage of the skimmer lens and the entrance lens in the ion source were automatically optimized by direct inlet of a solution of standard PHQ (Sigma–Aldrich, Buchs, Switzerland) at a concentration of 0.001 mg ml^{-1} in MeOH. PHQ was detected on the basis of UV absorption and the *m/z* of the deprotonated ion $[M-H]^-$ *m/z* 449 in comparison with the standard solution.

3. Results

3.1. NbICS sequence analysis

In order to clone the *N. benthamiana ICS* gene, total RNA was isolated from UVc exposed leaves and reverse-transcribed into cDNA. A full-length cDNA designed as *NbICS*was isolated using RACE and a sequence of 1296 bp long was obtained (available under genBank accession number EU257505). It encodes a protein of 431 amino acids which contains a chorismate-binding domain and a putative plastid-targeting sequence at the N-terminal end. This part of the protein showed 96%, 89%, 87%, 57% and 60% identity with the ICS proteins of *N. tabaccum* (NtICS), *Capsicum annum* (CaICS), *C. roseus* (CaICS) and *A. thaliana* (AtICS1 and AtICS2), respectively. The chorismate-binding domain is almost identical for all the ICS considered here (Fig. 1A). Southern blot analysis of genomic DNA of *N. benthamiana* digested with BglII and HindIII revealed two distinct bands indicating the presence of two copies of the *ICS* gene in the amphidiploïd *N. benthamiana* genome (Fig. 1B).

3.2. VIGS of NbICS

VIGS was performed on *N. benthamiana* plants for transient suppression of *NbICS* transcripts. A DNA fragment of 700 bp from the coding region of *NbICS* was cloned into a tobacco rattle virus (TRV)-based vector system. Inoculation of *N. benthamiana* with both vectors leads to the silencing of the transcripts of the endogenous *ICS* gene. These plants were referred to as TRV-*NbICS*. A DNA fragment corresponding to an intron of the *PDS* gene from *A. thaliana* was isolated and cloned into the VIGS vectors as a control for VIGS in *N. benthamiana*. Such plants were termed TRV-*INTRON* plants. To verify the efficiency of the gene silencing mediated by VIGS, semi-quantitative RT-PCR was performed, and the level of *NbICS* transcripts in WT, TRV-*INTRON* and TRV-

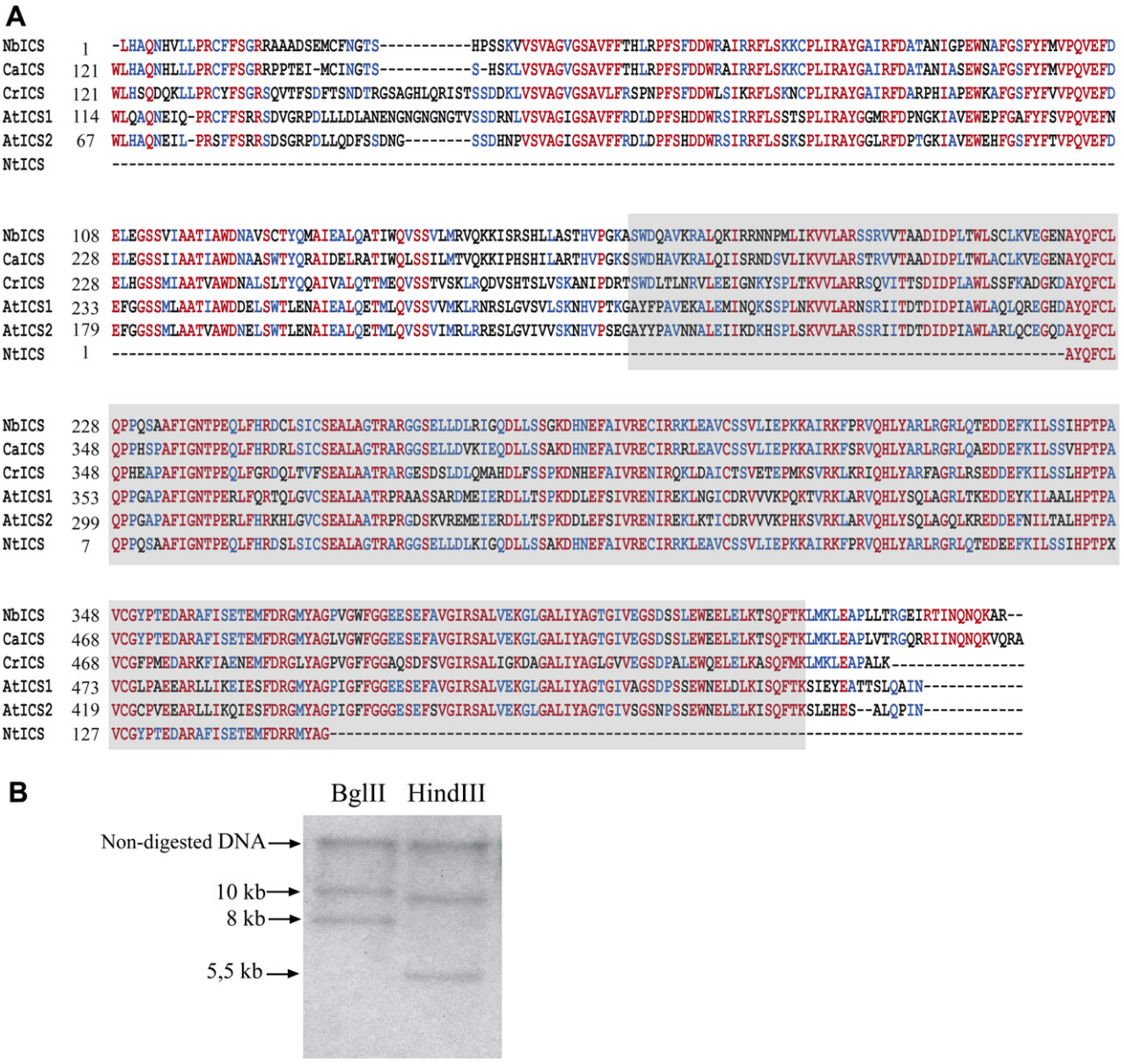

Fig. 1. (A) Clustal alignment of amino acids translated from cDNAs of selected isochorismate synthases. *CaICS*, *Capsicum annuum* (AY743431); *NtICS*, *Nicotiana tabacum* (AB182580); *CrICS*, *Catharantus roseus* (CAA06837); *AtICS1*, *A.thaliana* (AY056055); *AtICS2*, *A. thaliana* (AAF27094). Color codes: red: identity >90%; blue: >50%, the chorismate-binding domain is shaded in grey. (B) Southern blot analysis of genomic DNA extracted from *N. benthamiana* using a probe that includes parts of the chorismate-binding domain.

NbICS plants was determined (Fig. 2). The amount of transcripts of the housekeeping gene *EF*1α was used to estimate if the same quantity of cDNA of each plant had been used.

WT and TRV-*INTRON* plants showed the same quantity of *ICS* transcript the expression of which is lower than the *EF*1α transcripts. In TRV-*NbICS* plants, a band corresponding to the *ICS* transcripts could be distinguished after 50 PCR cycles in comparison to 30 cycles in WT and TRV-*INTRON*plants. This difference indicates a reduction in *ICS* close to an almost complete absence of *ICS* transcript in the TRV-*NbICS* plants and is a demonstration of effective silencing by VIGS.

3.3. Effect of ICS silencing on the content of PHQ

PHQ acts as a photosystem I (PSI) cofactor and is an essential component of the photosynthetic electron transfer chain. In *Arabidopsis*, ICS was shown to be required for synthesis of PHQ [17] and we verified whether the same process occured in

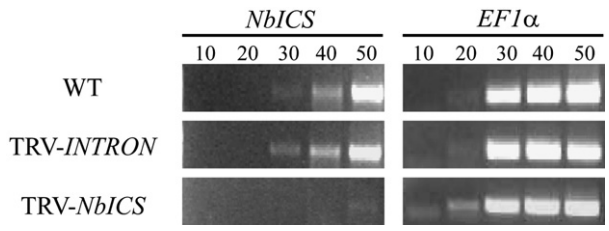

Fig. 2. RT-PCR analysis showing the effect of VIGS on transcription of *NbICS* and *EF*1α (from *N. benthamiana*). The first strand cDNA used for PCR was generated from total RNA isolated from upper leaves of WT, TRV-*INTRON* and TRV-*ICS* plants (the experiment has been repeated three times, one typical result is presented).

N. benthamiana. PHQ eluted at a retention time (RT) of 21.6 min (Fig. 3) when separated by HPLC. Mass spectrometry analysis of this fraction eluting at RT = 21.6 min presented a mass-to-charge ratio (*m*/*z*) of 449.2. In the chromatograms of WT and TRV-*INTRON* leaves, a peak was observed, eluting at 21.6. This fraction with a *m*/*z* ratio of 449.4 (respectively, 449.3) confirms the presence of PHQ in these plants. At the same RT, the chromatogrammes obtained from extracts of TRV-*NbICS* leaves showed a complete absence of PHQ. No specific peaks corresponding to PHQ could be identified in the mass spectrometry analysis of these plant samples. TRV-*NbICS* leaves were a paler shade of green than the two other plants and this phenotypic difference might be explained by the quantitative variation in the content of PHQ.

3.4. Effect of ICS suppression on SA accumulated after UVc light exposure

UVc photons are highly energetic and trigger biochemical responses that overlap in part with those induced by necrotizing pathogens [18]. For instance, exposure of tobacco leaves to UVc light induces the accumulation of SA. We have used UVc irradiation as an abiotic stress to increase SA levels in VIGS-plants. Without UVc exposure, WT, TRV-*INTRON* and TRV-*NbICS* plants all presented a low level of SA (Fig. 4A). Three days after UVc light treatment, the level of total SA in WT and TRV-*INTRON* plants had increased more than 10-fold, in accordance with values reported by Yalpani et al. [18] in *Nicotiana tabacum* L. cv. *Xanthi* nc. However, the absolute amount of SA measured in *N. benthamiana* was lower than in *N. tabacum*, that might indicate a weaker ability to produce SA in *N. benthamiana* or it might reflect a weaker stimulation by the UVc treatment than in the study cited above. Exposure of TRV-*NbICS* plants to UVc led to only a fourfold increase in SA, indicating a deficiency in SA production. In all experiments, a very low level of free compared to bound SA was observed (data not shown).

3.5. Effect of ICS suppression on SA accumulated after *P. syringae pv. tomato* (*Pto*) DC3000 infection

We have infiltrated the bacteria *Pto* DC3000 into *N. benthamiana* leaves as a biotic stress to induce SA (Fig. 4B). Two

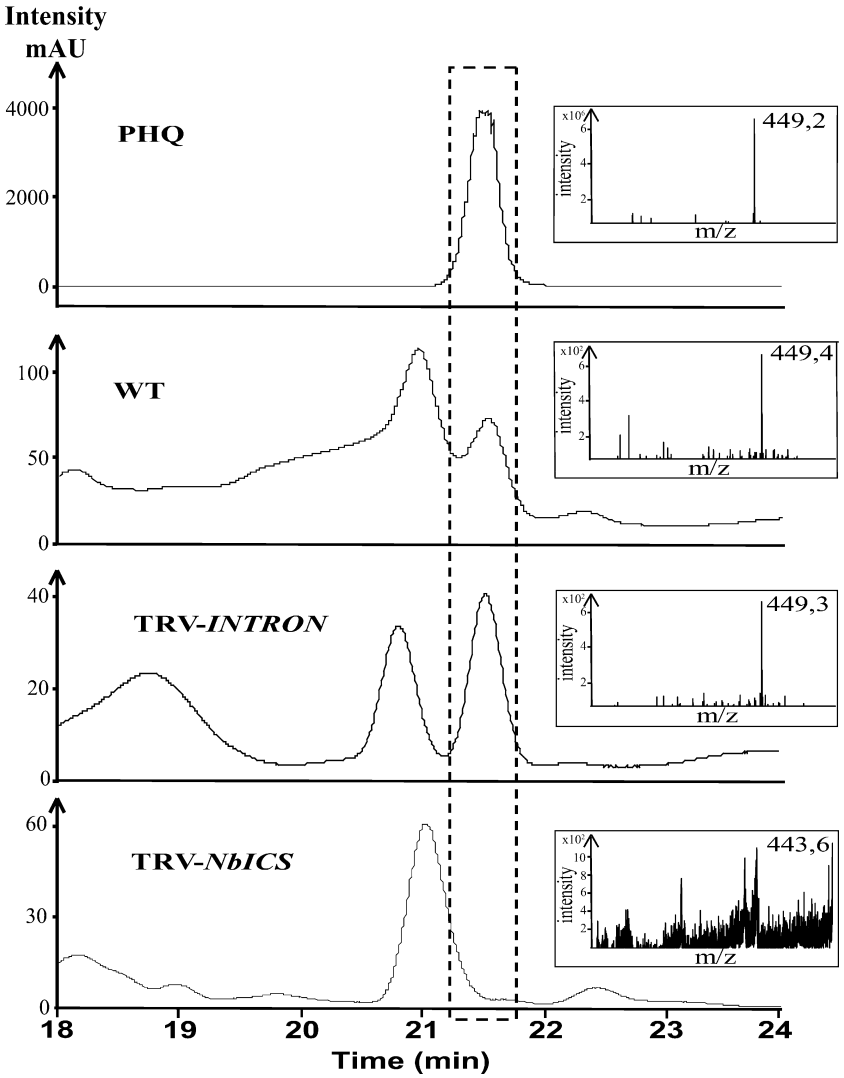

Fig. 3. HPLC elution profiles of leaf extracts of WT, TRV-*INTRON* and TRV-*NbICS* as well as a standard PHQ solution. Mass spectra of the peaks at the position of PHQ (dashed rectangle) are added on the right for each sample.

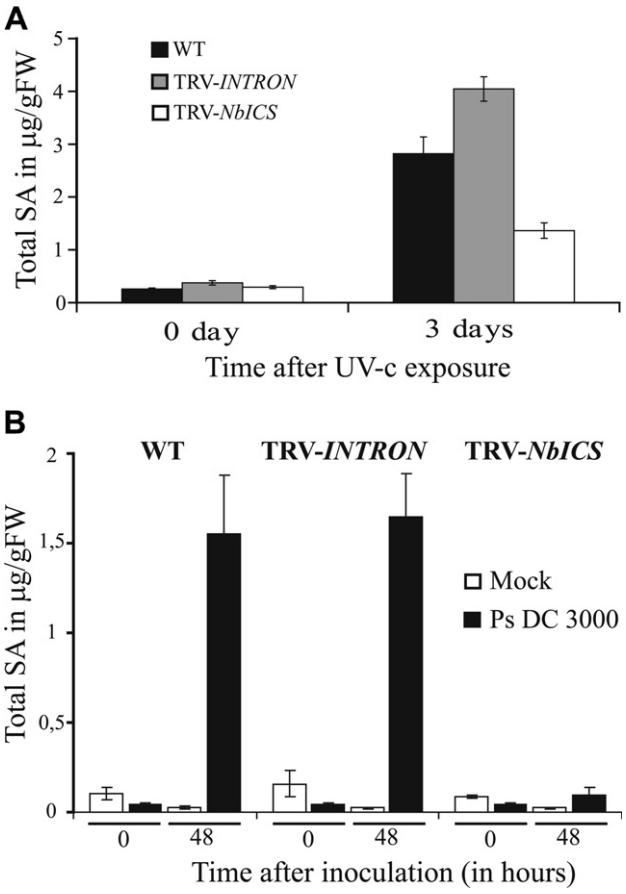

Fig. 4. Total SA accumulation in leaves (1–3) of WT, TRV- *INTRON* and TRV-*NbICS* plants: (A) after UVc light exposure for 1 h ($n = 5$; ±S.D.), (B) after mock-inoculation or inoculation with *Ps* DC3000 (black) ($n = 5$; ±S.D.).

days after inoculation, TRV-*NbICS* plants failed to accumulate SA, in contrast to WT and TRV-*INTRON* plants where the level of SA strongly increased. No accumulation of SA could be detected in WT, TRV-*INTRON*, TRV-*NbICS* plants after a mock infiltration (Fig. 4B).

4. Discussion

In the earlier studies based to a large extend on radiolabelling, the precursor for SA was postulated to derive from phenylalanine [5]. Recently, genetic and biochemical evidence in *A. thaliana* demonstrated that SA induced by pathogens or abiotic stress derives from the conversion of isochorismate by ICS [7,9]. The sequence of several genes encoding ICS has been described in *C. roseus* [19], *Capsicum annuum* (accession number AY743431), tobacco [20] as well as in the bacteria *Pseudomonas aeruginosa* and *Escherichia coli* [21]. In this article, we describe the sequence of an ICS in *N. benthamiana*. The chorismate-binding domain shows a strong conservation with other published sequences and was used as a target for VIGS. Suppression of *ICS* expression by VIGS in *N. benthamiana* led to substantial decrease in SA accumulation after UVc treatment and to an almost complete lack of SA increase after inoculation with *Pto* DC3000. These results support a biosynthetic pathway for SA derived from isochorismate and depending on ICS like that described in *Arabidopsis* [9]. PHQ derives from isochorismate via ICS [17,22] and did not accumulate in VIGS-plant providing additional experimental support for an efficient suppression of ICS.

After UVc exposure, ICS-suppressed plants still accumulated a residual level of SA above the level observed in untreated plants. An incomplete silencing of *NbICS* transcripts is unlikely but cannot be ruled out, although VIGS resulted in a strong suppression of ICS (Fig. 2). Alternatively, the residual SA could be explained by the action of another ICS. However, gene silencing was targeted at the conserved chorismate-binding domain (Fig. 1A), so we might reasonably exclude this possibility. The residual SA level could be produced from phenylalanine by PAL [23,24]. While the PAL pathway did not compensate the lack of SA due to the suppression of *NbICS* in plants undergoing biotic stress (Fig. 4) the possibility that some SA accumulation is derived from the PAL pathway in response to abiotic stress in *N. benthamiana* remains a possible explanation.

Our results supporting isochorismate-derived SA production in *N. benthamiana* are in contrast with several other reports in tobacco indicating an exclusive biosynthetic route via phenylalanine in tobacco [20,23,24]. These studies demonstrated the conversion of benzoic acid or benzoyl glucose to SA using radiolabelled precursors. While such results certainly reflect a possible source of SA, they do not exclude another pathway of SA production, and are likely to represent only a fraction of total SA produced. A comparison of the specific radioactivities between samples from control and SA-inducing treatments after radiolabelling with a precursor would be needed to answer this question. For example, a decrease in specific radioactivity in the treated sample compared to the control reflects an increase in unlabelled SA and the existence of another source of SA besides the radiolabelled precursor. Unfortunately, such data are not available for SA biosynthesis in plants. Previous studies on tobacco epigenetically suppressed in PAL expression showed a decrease in free SA, associated with a decrease in systemic acquired resistance to tobacco mosaic virus [6]. However, the downregulation of PAL may influence the flux through the isochorismate pathways, as discussed in [25]. In fact, these authors observed an overaccumulation of chlorogenic acid but not of SA in transgenic tobacco over-expressing *PAL* and there was no clear correlation between the expression of the PAL gene and the accumulation of SA [25]. In *N. tabacum* plants fumigated with ozone, SA was proposed to be made exclusively via the PAL pathway [20]. This conclusion was based on data showing a weak induction of *ICS* gene expression and undetectable ICS enzyme activity. However, these observations do not allow a definite conclusion regarding the origin of SA.

Here, we have shown that suppression of *ICS* expression by VIGS leads to a strong suppression of SA accumulation in *N. benthamiana* in response to abiotic and pathogen stress demonstrating that the isochorismate pathway for SA biosynthesis is also active at least in one *Nicotiana* species.

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