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Salicylic acid and its location in response to biotic and abiotic stress

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Salicylic acid (SA) is an important signal involved in the activation of plant defence responses against abiotic and biotic stress. SA may derive from the phenylpropanoid pathway or via isochorismate synthase as demonstrated in *Nicotiana benthamiana*, tomato and *Arabidopsis thaliana*. The phenylpropanoid pathway as well as isochorismate synthase are localized in the chloroplasts but it remains unknown if the end product SA is in the same organelle. We have studied the localization of SA in *A. thaliana* using the salicylate hydroxylase (*NahG*) gene expressed with a chloroplast targeting sequence. Plants expressing NahG in the chloroplasts are unable to accumulate SA induced after pathogen or UV exposure. Our data infer that SA is initially located in the chloroplasts.

1. Introduction

The phytohormone SA is involved in various physiological responses such as thermogenesis, stomatal closure or flowering [1]. Importantly it was discovered to play a role as signal in the activation of defence responses against microbial pathogens [2]. SA is induced after infection by many pathogens or abiotic stress such as UV or ozone exposure and the biological relevance of SA has been extensively documented in a number of mutants or in transgenic plants with altered levels of SA [3]. SA operates along with other phytohormones, such as jasmonic acid or ethylene and is part of a signalling network with crosstalks and synergisms that will determine an optimal response to a single or combination of invaders under given environmental conditions [4–7].

SA can be synthesized from phenylalanine and converted to SA either from free benzoic acid, benzoyl glucose or *o*-coumaric acid as precursors depending on the plant species [8]. The SA induction deficient *Arabidopsis thaliana* mutant *sid2* [9] is impaired in isochorismate synthase (*ICS*) [10] providing a strong support that SA is made from isochorismate as is known for bacteria [11]. Isochorismate as a precursor for SA has also been documented in *Nicotiana benthamiana* and tomato [12,13]. Two forms of isochorismate synthase, *ICS1* and *ICS2*, were described in *A. thaliana* and only residual levels of SA could be detected in *ics1*/*ics2* double mutants indicating another possible pathway for SA accumulation [8]. The localization of the chorismate pathway of *ICS1* and *ICS2* in the chloroplasts suggests that SA is mostly made in this organelle [8]. However, the last step in the biosynthesis of SA has not been described so far. For instance, no close homologue of the bacterial isochorismate pyruvate lyase that converts isochorismate to SA has been reported in plants. The possibility that the chloroplasts are indeed the centre of production of SA as it is for many other hormones is intriguing, and leads to interesting evolutionary considerations. At the cellular level, it raises the question of the efflux of SA from the chloroplasts. SA can also be relocalized from the cytoplasm in the form of SA 2-*O*-β-D-glucoside into vacuoles where it accumulates in this form [14,15].

The localization of the last step of SA biosynthesis remains thus an open question. Here, we have addressed this question using plants over-expressing the SA hydroxylase gene (*NahG*) from *Pseudomonas putida* under the control of a chloroplast-targeting sequence. Plants expressing a functional NahG protein in the chloroplasts are unable to accumulate SA after exposure to biotic or abiotic stress confirming that SA is located in the chloroplasts and making it unlikely that the bulk of SA biosynthesis takes place outside the chloroplasts.

2. Materials and methods

2.1. Plant maintenance

A. thaliana ecotype Columbia-0 (Col-0) wild-type plants were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK), transgenic *A. thaliana* seeds over-expressing the salicylate hydroxylase *NahG* gene were obtained from J. Ryals

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[16]. *A. thaliana* seeds were grown on a pasteurized soil mix of humus and perlite (3:1). Seeds were kept at 4 °C for two days and then transferred to the growth chamber. Plants were grown in a 12 h light/12 h dark cycle with 60–70% of relative humidity, with a day temperature of 20–22 °C and a night temperature of 16-18 °C. Flowering *A. thaliana* plants were grown a 16 h/8 h light-dark cycle.

2.2. Plant transformations: RNA extraction, cDNA constructions and transformations

Total RNA was prepared using Trizol reagent® following instructions of manufacturer (Invitrogen, Karlsruhe, Germany). Total RNA (1 μg) was retrotranscribed into cDNA using the kit Omniscript (Qiagen, Venlo, The Netherlands). The ribulose bisphosphate carboxylase small subunit (*RbcS*) transit peptide signal (222 bp) was obtained from reverse transcription of the gene of *A. thaliana* (TAIR ID: AT1G67090) and *NahG* cDNA from the *P. putida*, was provided by J. Draper, University Leicester, England [17]. The *RbcS* and *NahG* cDNA were amplified with Phusion enzyme (Finnzymes, Finland) using primers RbcS-fw 5′-ATGGCTTCCTC-TATGCTCTTCC; RbcS-rev 5′-AGGAAGGTAAGAGAGAGTCTCAAACTT C and NahG-fw 5′- ATGAAAAACAATAAACTTGGCTTGC; NahG-rev 5′-CCCTTGACGTAGCGCACC. *RbcS-NahG* cDNA were transcriptionally fused from the mix PCR product of *RbcS* and *NahG* and amplified by using primers RbcS-fw and NahG-rev.

GFP fusions were obtained using Gateway technology (Invitrogen, Karlsruhe, Germany). *RbcS*, *NahG* and *RbcS-NahG* cDNA were cloned into the entry vector pENTR-D-Topo. Entry clones were recombined with pB7FWG2 vector [18], generating cDNA-*GFP* fusions driven by the cauliflower CaMV 35S promoter. The pB7F2 (*35S::GFP*) control vector was generated by digestion of pB7FWG2 with *Eco*RV followed by self ligation, thus removing the ccdB cassette and placing *GFP* directly under the control of the CaMV 35S promoter. The vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation using the Gene pulser II system (Biorad, München, Germany). Bacteria were grown in LB medium containing 50 μg/μl of spectinomycin and 50 μg/μl of rifampicin during 2 days at 28 °C. After washing twice by adding 1/2 MS, the bacteria were resuspended in 5% of sucrose and diluted to an OD_{600} of 0.8. The surfactant Silwet L-77 0.05% (Loveland Industries, Loveland, USA) was added as well as 200 μM acetosyringone to induce bacterial virulence [19]. Flowering *A. thaliana* plants were used for *A. tumefaciens*-mediated plant transformation using the floral dip method [20]. The transformed plants were selected by using the herbicide Basta (Bayer CropScience, Monheim am Rhein, Germany). For all the experiments transgenic *A. thaliana* T2 plants were used.

2.3. Real-time RT-PCR

Expression of *NahG* and *GFP* were determined using a Real-time PCR, performed using Sensimix™ SYBR Green Kit (Bioline, London, UK). Gene expression values were normalized to expression of the plant gene At4g26410 (TAIR ID), previously described as a stable reference gene [21]. The primers used were NahG-fw; NahG-rev, and GFP-fw 5′-CACATGAAGCAGCACGACTT; GFP-rev 5′-GAAGTTCACCTTGATGCCGT.

2.4. Subcellular localization

The transformed T2 plants were analyzed by using a DM6000 automated microscope and the TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). For imaging GFP and chlorophyll, excitation was at 488 and 514 nm, respectively, and emissions were collected with a 495- to 511-nm band-pass filter and a 625- to 745-nm band-pass filter, respectively. Z-stack imaging was performed on 20 μm thick, and each optical section was of 1 μm. The fluorescence was analyzed by using Leica Confocal Software version 2.61.

2.5. Quantification of SA

Leaf material (approximately 200 mg) was collected, frozen in liquid nitrogen and homogenized 1 min with a Polytron (Kinematica, New York, USA) in 2 ml of 70% ethanol (v/v) containing 200 ng of the internal standard *ortho*-anisic acid. After centrifugation (10 min, 10 000 g), the supernatant was collected into a new tube. The remaining pellet was homogenized 1 min with 2 ml of 90% v/v methanol. After centrifugation (10 min, 10 000 g), both supernatants were pooled and evaporated under vacuum. Five hundred microliters of 5% trichloroacetic acid was added to the remaining aqueous solution (approximately 400 μl). After centrifugation (10 min, 10 000 g), the supernatant was transferred to a new tube and 500 μl of ethyl acetate/cyclohexane (1:1) was added and mixed with a Vortex. After centrifugation at full speed in a labtop centrifuge, the upper organic phase was collected. This step was repeated once and the final aqueous phase was kept. The pooled organic phase that contains the free phenols was evaporated to dryness (Speed Vac, Buchler). To measure conjugated SA, the remaining aqueous phase was submitted to acid hydrolysis in the presence of 4 N HCl at 80 °C for 1 h. The aqueous phase was cooled down, and after centrifugation at full speed in a labtop centrifuge, 100 ng of internal standard *ortho*-anisic acid was added. The liberated phenols were extracted twice into ethyl acetate/cyclohexane as described above and organic phases were pooled and evaporated. For metabolite quantification, organic phases were resuspended in 200 μl of HPLC starting solvent (methanol 40%, water 60%, acetic acid 1%) and loaded on a reverse-phase HPLC column (25 cm × 4.6 mm, 5 μm Supelco discovery® C18, Bellefonte, PA, USA). The amount of each metabolite was calculated in ng mg^{-1} fresh weight (FW) with reference to the amount of internal standard.

2.6. Extraction of chloroplasts

Chloroplasts were isolated from 4-week-old *A. thaliana* plants following the protocol previously described [22]. Intact chloroplasts were observed under the light microscope as round and bright green structures.

2.7. SDS–PAGE and protein blot analysis

Immunoblot analysis was performed essentially as earlier described [23] with the following modifications. Total protein was extracted by grinding plants and chloroplast suspension in 50 mM HEPES (pH 7.5), 5 mM EGTA, 5 mM EDTA, 1 mM sodium ortho vanadate, 50 mM glycerol 3-phosphate, 10 mM sodium fluoride, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM dithiothreitol (DTT). Protein concentration was determined according to Bradford (1976) [24] using the Bio-Rad (München, Germany) dye reagent and bovine serum albumin as standard. Proteins were resolved by discontinuous SDS–PAGE (on 12% gels). Proteins were transferred electrophoretically (at 100 V for 2 h) onto nitrocellulose membranes (Hybond-ECL). For detection of the green fluorescent protein (GFP) and histone H3, commercial polyclonal GFP and Histone H3-antibodies were used (GenScript, USA); both sera were used at a dilution of 1:1000. Specific proteins were detected using the alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins/HRP (DakoCytomation, Denmark) as recommended by the manufacturer. Coomassie blue (CB)-stained blot was used as loading control.

2.8. UVc light exposure

To induce the production of SA, 4-week-old *A. thaliana* plants were exposed to UVc light (254 nm) during 20 min at 30 cm distance from the lamp (CAMAG, Muttenz, Switzerland), as previously described [9]. SA was measured 24 h post UV induction.

2.9. Inoculation with Pseudomonas syringae pv. maculicola (Psm)

Infection with *Psm* ES432 has been previously described [22]. Briefly, *Psm* was grown overnight at 28 °C in KB medium [25] with the appropriate antibiotics. Cultures were pelleted and washed three times with 10 mM $MgCl_2$, re-suspended and diluted to OD 0.002. Four-week-old *A. thaliana* plants were infiltrated with *Psm* dilution and $MgCl_2$ as control, in the leave abaxial side with a needle-less syringe. SA content on the inoculated leaves was quantified 24 h post inoculation (hpi).

3. Results

3.1. Generation of A. thaliana plants over expressing the transcriptional fusion RbcS-NahG-GFP

The ribulose bisphosphate carboxylase small subunit (*RbcS*) transit peptide signal can efficiently target reporter fusions to the chloroplasts [26]. To study the localization of SA in *A. thaliana*, a transcriptional fusion was generated between *RbcS*, the *salicylate hydroxylase NahG* and the green fluorescent protein *GFP* (*RbcS–NahG-GFP*, Fig. 1A). This fusion was cloned in frame to the cauliflower mosaic virus CaMV 35S promoter into the binary vector pB7FWG2 (Fig. 1A). Additionally, the vector pB7F2 that carries the green fluorescent protein *GFP* driven by the same 35S promoter was used as a control (Fig. 1A).

Real-time-PCR gene expression analysis of *NahG* and *GFP* was performed for the WT untransformed plants, the line over-expressing the reporter gene *GFP* and two independent T2 transgenic *A. thaliana* lines over-expressing the fusion *RbcS-NahG-GFP* (Fig. 1B). As expected *NahG* was not expressed in the WT nor in *35S::GFP* line and was induced only in both transgenic lines carrying the fusion, however, different levels of induction were observed. A stronger expression was observed in line 2 than in line 11 (Fig. 1B). Furthermore, a strong induction of the reporter gene *GFP* was observed both in the line *35S::GFP* and in line 2, but only a modest induction was detected in line 11 (Fig. 1B). Nevertheless, the differences in the gene expression observed between the transgenic lines carrying the *RbcS-NahG-GFP* fusion resulted in similar accumulation of the fluorescent protein and SA (see below). These results indicate that expressing genes with the *RbcS* target peptide in stable transgenic *A. thaliana* plants did not interfere with the activity of the expressed protein.

3.2. Transcriptional fusion of the genes RbcS-NahG-GFP leads to a constitutive accumulation into the chloroplasts

In-vivo laser confocal microscopy was performed on green leaves and isolated chloroplasts of transgenic *A. thaliana* plants to determine the localization of the GFP fusion (Fig. 2A and B). In control transgenic lines (*35S::GFP*), green fluorescence was observed

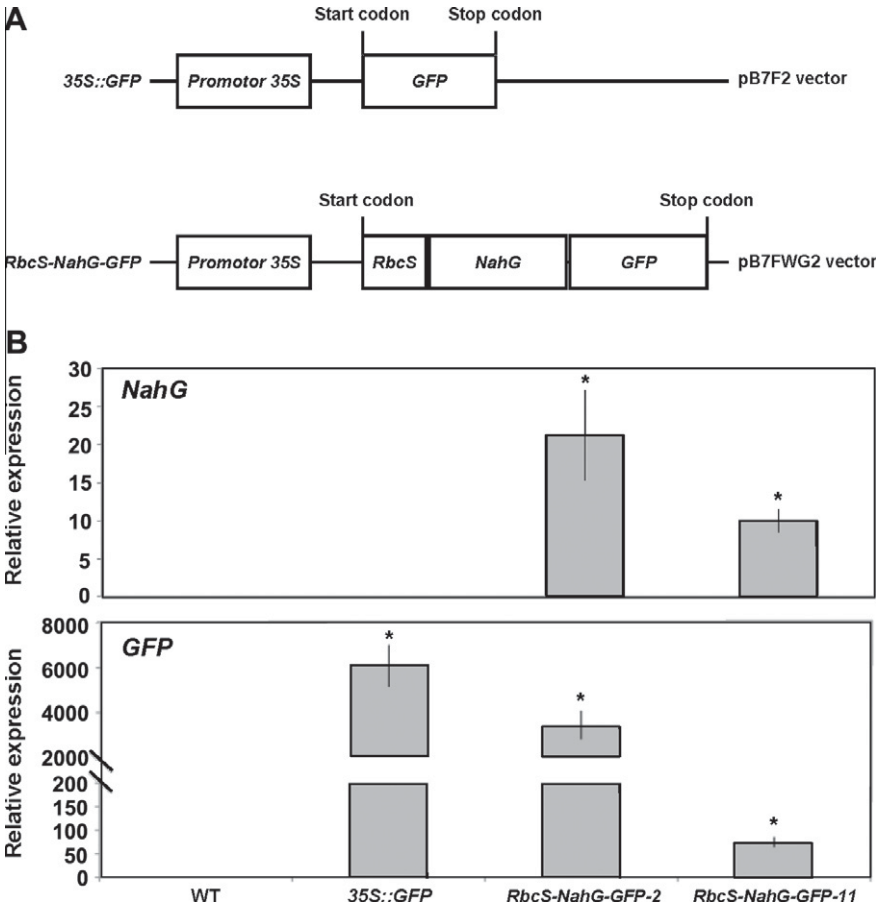

Fig. 1. Expression of the *NahG* and *GFP* genes in transgenic *A. thaliana* plants. (A) Schematic presentation of constructs used to generate stable *A. thaliana* transgenic plants. (B) Expression of *NahG* and *GFP* genes in WT and transgenic plants over-expressing *RbcS-NahG-GFP* and *GFP* determined by qRT-PCR (n=3; ±SD), asterisks indicate a significant difference in comparison to WT ($p<0.05$). Results of a typical experiment are shown (the experiments were carried out 3 times with similar results).

diffusely through the cytoplasm but rarely co-localized with the chloroplasts (Fig. 2A and B). However, green fluorescence co-localized with chlorophyll auto-fluorescence in lines over-expressing *RbcS-NahG-GFP* indicating that the fusion protein was accumulated in these plastids (Fig. 2A). Interestingly, the *RbcS-NahG-GFP* line 11 showed a more diffused green fluorescence through the chloroplast, whereas *RbcS-NahG-GFP* line 2 exhibited only fluorescent spots (Fig. 2A). To confirm the localization of the RbcS-NahG-GFP fusion, isolated chloroplasts from both lines were examined by confocal microscopy (Fig. 2B). Chloroplasts from *RbcS-NahG-GFP* line 2 showed green fluorescent spots clearly co-localized with chlorophyll autofluorescence (Fig. 2B). Similar observations were made for *RbcS-NahG-GFP* line 11, however the green fluorescence dispersed over larger areas and was more diffused (Fig. 2B).

To further confirm our observations, a protein blot analysis was performed using the total protein extracted from green leaves and from isolated chloroplast fractions of WT plants and in the two independent transgenic lines (Fig. 2C). Clearly, GFP was detected in the transformed lines but not in WT samples (Fig. 2C). A reduced amount of protein was detected in the chloroplast fractions compared to the protein extracted from the leaves (see loading controls). However, a strong level of the reporter protein was observed in the chloroplasts, indicating that GFP was concentrated in the isolated plastids (Fig. 2C). The ratio of the level of GFP to histone H3 can also be compared in leaves and chloroplasts and shows an enrichment of GFP in the chloroplast fraction accompanied with a decrease in histone H3. Indeed, the chloroplast preparation did not contain detectable traces of nuclear proteins as indicated by the use of a nuclear-specific histone H3 antibody (Fig. 2C).

These results indicate that the RbcS-NahG-GFP fusion has accumulated in the chloroplasts. The precise localization of this fusion product (inside or binding to the chloroplast envelope) was then studied using serial sections of isolated chloroplasts viewed by confocal microscopy (Fig. 2D). Green fluorescence was not detected on the surface sections, but only inside the transverse sections of chloroplasts, indicating that RbcS-NahG-GFP was localized inside the chloroplasts (Fig. 2D).

3.3. Plants expressing the NahG protein in the chloroplasts are unable to accumulate SA after exposure to biotic or abiotic stress

Transgenic plants over expressing the bacterial gene encoding the *NahG* gene have been shown to convert SA to catechol [16], hence only low levels of SA were detected after biotic or/and abiotic stimuli [3,16]. Our results are in agreement with these observations and exposure to UVc or inoculation with *Psm* of *35S::NahG* plants led to a substantial decrease in the level of total SA compared to untreated controls (Fig. 3A and B). Both *RbcS-NahG-GFP* lines showed a reduction of total SA level after these treatments compared to the untreated transgenic controls (Fig. 3A and B). The total level of SA was also higher in untreated WT plants compared to all transformed lines; but the mock-inoculated *RbcS-NahG-GFP* lines showed only an inhibition of approximately 50% compared to the WT plants (Fig. 3B). Nevertheless, a strong reduction of SA levels was observed in the *RbcS-NahG-GFP* lines 2 and 11 after *Psm* inoculation (Fig. 3B). Taken together, these results show that overexpression of the *NahG* gene in the chloroplasts of *A. thaliana* reduced the level of SA after exposure to abiotic and biotic stress.

4. Discussion

The objective of this study was to determine if SA is initially localized in chloroplasts of *A. thaliana* after exposure to biotic or abiotic stress. Only minimal amounts of SA are detected in transgenic plants over-expressing *NahG* in the cytoplasm indicat-

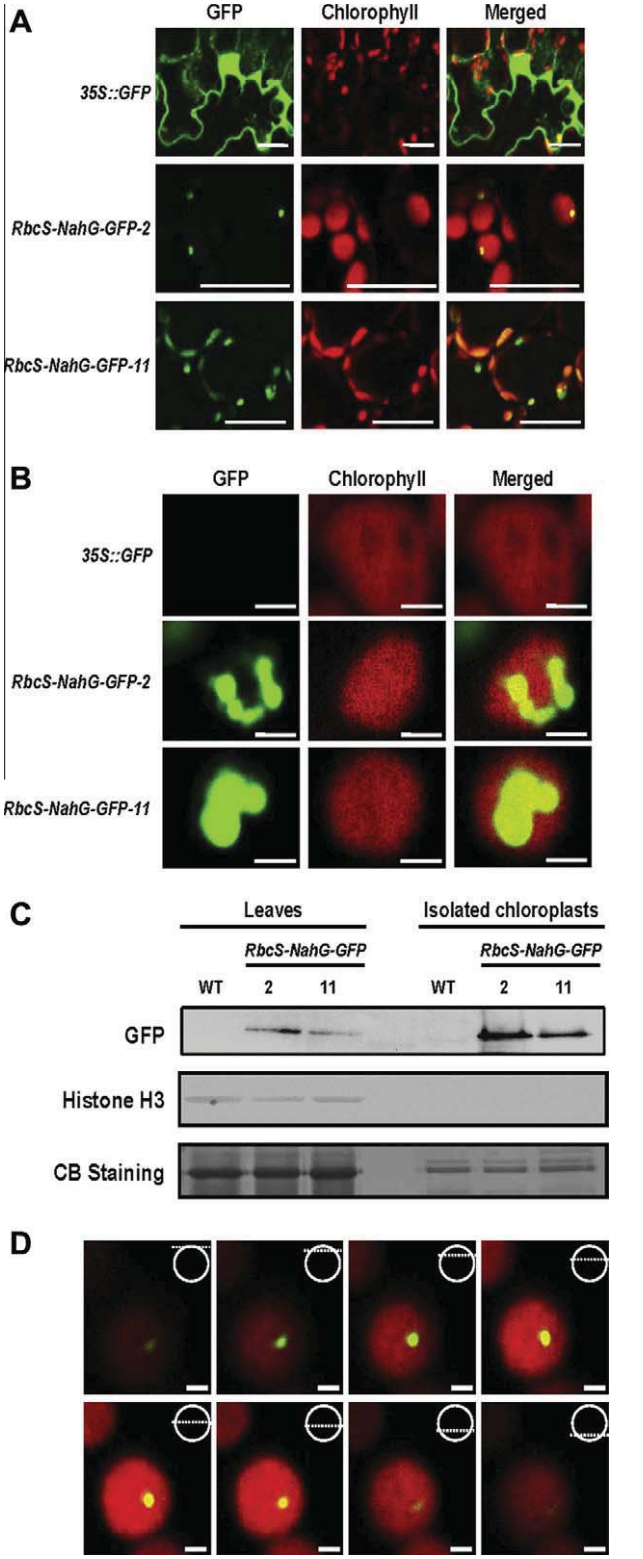

Fig. 2. Subcellular localization of NahG-GFP targeted with the RbcS peptide. GFP signal (green) and chlorophyll autofluorescence (red) were observed using confocal microscopy, overlay is shown in yellow. (A) GFP localization in vivo of *A. thaliana* leaves, bars = 30 μm. (B) GFP localization in isolated chloroplast, bars = 2 μm. (C) Immuno detection of GFP and the nuclear-localized protein Histone H3, in extracts of leaves and isolated chloroplasts. Specific proteins were detected with anti-GFP and anti-histone H3 antibodies, Coomassie blue (CB)-stained blot is shown as loading control. One of two independent experiments is shown. (D) Optical sections of confocal microscopy images of the green fluorescence in an isolated chloroplast, with increments of 1 μm. The positions of the sections are shown in the upper right of each picture, bars = 2 μm; representative images are shown.

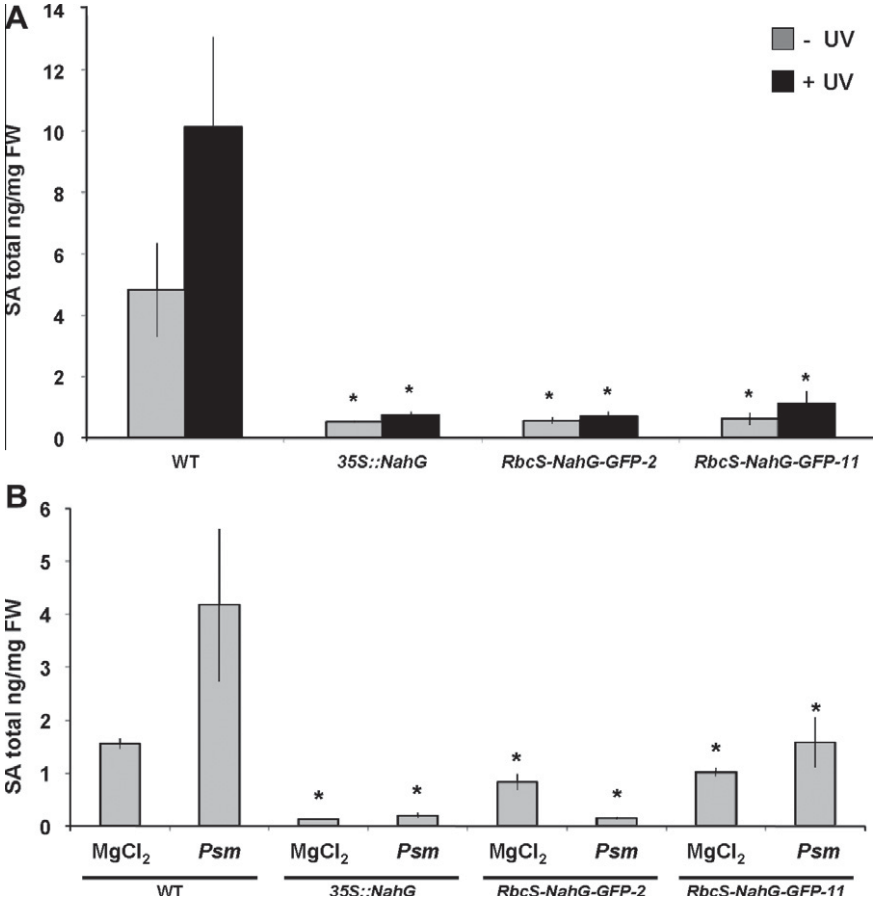

Fig. 3. Quantification of total SA in leaves of *A. thaliana* Col-0, *NahG* and *RbcS-NahG-GFP* plants. (A) Leaf tissue 24 h after UVc light treatment. (B) Leaf tissue after inoculation with *Psm*. The values represented are the average of three treated plants (±SD), asterisks indicate a significant difference in comparison to WT ($P < 0.01$). The experiment was repeated twice with similar results. FW, fresh weight.

ing that SA must occur in the same place [16,27]. We have used a similar approach to determine if SA is localized in the chloroplast. Several studies have reported the use of chloroplast transit peptides, such as *DCL* (defective chloroplasts and leaves) and *RbcS* (ribulose bisphosphate carboxylase (Rubisco) small subunit) to target the *GFP* reporter gene to the chloroplasts [26]. At least 73 amino residues of the N-terminal region of RbcS fused to GFP are required to direct the protein to the chloroplasts without affecting the efficiency [28,29]. Here we have used transgenic *A. thaliana* plants over-expressing a transcriptional fusion protein consisting of 74 amino acids of the N-terminal region of RbcS, NahG and GFP (RbcS-NahG-GFP, Fig. 1) to direct NahG to the chloroplast.

The GFP reporter protein can be monitored either in vivo by confocal microscopy or by immunoblot analysis [30]. The discrete spots of green fluorescence of GFP in *RbcS-NahG-GFP* lines were mostly observed to overlap with the position of chloroplasts in the transgenic lines of *A. thaliana* (Fig. 2A, B, D). The pattern of fluorescence observed in plants expressing *GFP* in the cytoplasm was not comparable to that observed in *RbcS-NahG-GFP* lines, making it unlikely that RbcS-NahG-GFP has remained in the cytoplasm (Fig. 2A). Consecutive optical sections (Z-stack, Fig. 2D) of isolated chloroplasts associated with green fluorescent spots showed that the fluorescent spots are localized inside the chloroplast rather than on the surface. Taken together, these results indicate that RbcS-NahG-GFP has been targeted to the chloroplasts. The images observed by confocal microscopy are in agreement with the results of the immunoblot detection where the fusion protein RbcS-NahG-GFP was observed in the isolated chloroplast fraction (Fig. 2C). Together with the microscopic observations these results confirm that the RbcS signal peptide can target the NahG-GFP fusion protein into the chloroplasts.

Transgenic *A. thaliana* over-expressing *NahG* plants exhibit reduced level of SA in response to pathogens [27]. Plants over-expressing *NahG* in the chloroplasts exhibited decreased levels of SA either after exposure to UVc light or inoculation with *P. syringae* (Fig. 3). The decreased levels in chloroplast–targeted *NahG* were in the same range as those observed in transgenic plants expressing *NahG* in the cytosol (Fig. 3). In the case of mock-inoculated ($MgCl_2$) treatment as well as for RbcS-NahG-GFP-11-inoculated plants the decrease in SA level was not as strong as that observed for cytoplasmic-targeted NahG. This less efficient action of the NahG gene product might perhaps suggest an additional minor site of production of SA. The interpretation that SA is initially in the cytoplasm or in another compartment and is then moved to the chloroplast cannot be dismissed. However, given the chloroplastic localization of ICS1 and ICS2 [8], a straightforward interpretation of the results presented here is that SA is initially localized in the chloroplasts, presumably prior its appearance in the cytoplasm. This observation opens the question how SA leaves the chloroplasts to its presumed site of action in the cytoplasm. One hypothesis is that the MATE (multidrug and toxin extrusion) *EDS5* transports SA across the chloroplast envelope [31]. This is supported by recent unpublished evidence showing that *EDS5* localized at the chloroplast (Garcion and Métraux, unpublished results). This observation now awaits further confirmation. It also remains to be explained why a mutation in the MATE transporter EDS5 putatively localized at the chloroplast leads to low SA levels.

Interestingly, the chloroplasts have evolved as the site of the biosynthesis of the precursors of many plant hormones (auxin, gibberellins, abscisic acid, jasmonic acid, brassinosteroids) as well as SA. The control of the biosynthesis of hormones in response to various stimuli such as development cues or various forms of stress poses the formidable problem of rapidly coordinating biochemical pathways between organelles. It implies therefore a well-defined communication between the site of perception of such stimuli and the chloroplasts. In particular, the chloroplasts appear to be closely involved in the coordination of defence responses [32]. Research in this field awaits now further attention.

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