Hpa1 harpin needs nitroxyl terminus to promote vegetative growth and leaf photosynthesis in Arabidopsis

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Hpa1 is a harpin protein produced by *Xanthomonas oryzae*, an important bacterial pathogen of rice, and has the growth-promoting activity in plants. To understand the molecular basis for the function of Hpa1, we generated an inactive variant protein, Hpa1ΔNT, by deleting the nitroxyl-terminal region of the Hpa1 sequence and compared Hpa1ΔNT with the full-length protein in terms of the effects on vegetative growth and related physiological responses in *Arabidopsis*. When Hpa1 was applied to plants, it acted to enhance the vegetative growth but did not affect the floral development. Enhanced plant growth was accompanied by induced expression of growth-promoting genes in plant leaves. The growth-promoting activity of Hpa1 was further correlated with a physiological consequence shown as promoted leaf photosynthesis as a result of facilitated CO_2 conduction through leaf stomata and mesophyll cells. On the contrary, plant growth, growth-promoting gene expression, and the physiological consequence changed little in response to the Hpa1ΔNT treatment. These analyses suggest that Hpa1 requires the nitroxyl-terminus to facilitate CO_2 transport inside leaf cells and promote leaf photosynthesis and vegetative growth of the plant.

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1. Introduction

Harpins are multifunctional proteins produced virtually by every species of Gram-negative plant pathogenic bacteria (He 1998; Kim and Beer 2000; Alfano and Collmer 1997; Chen *et al.* 2008a). After application to plants or expression in transgenic plants, harpins induce reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) in particular as well as the hypersensitive reaction (HR) (Peng *et al.* 2003; Liu *et al.* 2006; Wang *et al.* 2008; Sang *et al.* 2012), defense responses to microbial pathogens and herbivorous insects (Dong *et al.* 1999, 2004; Peng *et al.* 2004; Chen *et al.* 2008a, b; Sang *et al.* 2012), drought tolerance (Dong *et al.* 2005; Zhang *et al.* 2007, 2011), and plant growth enhancements (Dong *et al.* 2005; Ren *et al.* 2006a, b; Wu *et al.* 2007; Chen *et al.* 2008a, b; Ren *et al.* 2008). One such harpin is Hpa1 produced by *Xanthomonas oryzae*, an important bacterial pathogen of rice (Zhu *et al.* 2000; Peng *et al.* 2004), and this harpin has been demonstrated to function similarly to its orthologs from other bacterial species and cause a similar set of multifaceted effects in a variety of plants (Zhu *et al.* 2000; Peng *et al.* 2004; Liu et al. 2006; Ren et al. 2006a,b; Wu *et al.* 2007; Chen *et al.* 2008a; Shao *et al.* 2008; Wang *et al.* 2008; Miao *et al.* 2010a, b; Zhang *et al.* 2011; Sang *et al.* 2012). In particular, Hpa1 enhances plant growth in association with induced expression of growth-promoting genes, such as *EXP* genes encoding expansin proteins (Dong *et al.* 2004; Liu *et al.* 2006; Ren *et al.* 2006a, b; Chen *et al.* 2008a, b; Wu *et al.* 2007). The nitroxyl-terminal (N-terminal) region of Hpa1 has been shown to be responsible for its role in eliciting the HR in tobacco (Wang *et al.* 2008), but it is unknown whether the N-terminus is also required for Hpa1 to enhance plant growth. It is also not known what physiological consequences associate with the promoting effect of Hpa1 on plant growth.

To correlate Hpa1-induced growth enhancement with a physiological process in plants, we analysed the effect of Hpa1 on carbon dioxide (CO_2) conduction by leaf stomata and mesophyll cells and assimilation by photosynthesis.

Keywords. *Arabidopsis*; CO_2 transport; Hpa1; photosynthesis; plant growth enhancement; *Xanthomonas oryzae*

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Transport of CO_2 to the photosynthesis sites within chloroplasts must overcome a series of resistances, especially the barrier from the plasma membrane (PM) (Evans *et al.* 2009; de Groot and Hub 2011; Flexas *et al.* 2012). The conduction through mesophyll cells takes three steps: transport via the PM, diffusion in the cytosol, and penetration of the chloroplast envelope for targeting to the photosynthesis location (Evans *et al.* 2009). The first step determines the concentration of CO_2 transported into mesophyll cells and has a consequent effect on the CO_2 concentrating mechanism that increases the concentration of CO_2 available for assimilation by photosynthesis (Zabaleta *et al.* 2012). Particular isoforms of the PM intrinsic protein (PIP) family, one of five aquaporin protein families in plants (Maurel 2007), act as a key means for the PM permeability to CO_2 (Heckwolf *et al.* 2011; Flexas *et al.* 2012; Kaldenhoff 2012; Uehlein *et al.* 2012) on one hand. On the other hand, PIPs regulate cellular responses to environmental cues, such as hydrostatic pressure (Postaire *et al.* 2010), low temperature (Lee *et al.* 2012), and drought, salinity or wounding stress (Ayadi *et al.* 2011; Bae *et al.* 2011; Zawoznik *et al.* 2011). Moreover, PIPs are implicated in plant interactions with insects (Wei *et al.* 1992), a mycorrhizal fungus (Ruiz-Lozano *et al.* 2009), and a fungal pathogen (Jacobi *et al.* 2010). These findings suggest that PIPs sense abiotic or biotic signals and thus extend their physiological roles beyond substrate transports. Hpa1 and other harpins represent a special type of biotic signals, and they are recognized by plant receptors (Ausubel 2005; Torres 2010) to cause physiological responses, which at least partially contribute to plant growth (Dong *et al.* 2004, 2005; Oh and Beer 2007). However, there is as yet no study to correlate particular physiological responses with plant growth in response to a harpin protein.

In this study, we compared the full-length Hpa1 and its N-terminus–deleted variant Hpa1ΔNT in terms of the effects on vegetative growth and related responses in *Arabidopsis*. We show that the N-terminal region is required for Hpa1 to enhance vegetative growth in correlation with the promoting effects on leaf net photosynthesis and the expression of growth-promoting genes in the plant. We present evidence that leaf photosynthesis is promoted as a result of facilitated CO_2 conduction through leaf stomata and mesophyll cells in response to Hpa1 but not Hpa1ΔNT.

2. Materials and methods

2.1 *Protein production and bioactivity tests*

Genes used in Hpa1 and Hpa1ΔNT production were cloned by polymerase chain reaction (PCR) with specific primers designed to contain proper restriction sites (table 1) for subsequent recombination. Full length *hpa1* gene and the truncated version *Hpa1Δ159* with a deletion of 5′-terminal 159 nucleotides were cloned from the *X. oryzae* pv. *oryzae* strain PXO99. The *GST* gene was cloned from the pET-41a(+) vector (Dualsystems Biotech. Inc., San Francisco, CA, USA) with a 3′-terminal addition of the $His_{(6)}$ code provided by primers. Sequences of PCR products were confirmed by sequencing after cloning into the pMD19-T vector (Takara-Clontech Bio Inc., Nanjing Branch, Nanjing, China). Then, the *GST-His* union was fused to the 5′-terminus of *hpa1* or *Hpa1Δ159* by cloning in the prokaryotic expression vector pET30a(+) (Novagen, Madison, WI, USA). Gene sequences and their proper orientation in the vector were confirmed. Recombinant pET30a(+) vectors with *GST:His:Hpa1* and *GST:His:Hpa1Δ159* inserts were transferred separately into cells of the *Escherichia coli* strain BL21, followed by culture of the BL21 conjugant to produce the GST-His-Hpa1 and GST-His-Hpa1ΔNT fusion proteins. Proteins were purified by nickel chromatography under elution with 100–300 mM imidazole at a 50 mM gradient (Chen *et al.* 2008a). The well-purified final fraction was made as aqueous solution and maintained at –65°C for use in further studies. The GST-His-Hpa1 and GST-His-Hpa1ΔNT fusion proteins were produced by prokaryotic expression in *Escherichia coli* (*E. coli*), purified by nickel chromatography (Chen *et al.* 2008a, b), and used in 10 μg/mL aqueous solutions amended with 0.03% (v/v) Silwet-77 as a surfactant. Pure water containing 0.03% Silwet-77 was used as a control in all experimental treatments. The bioactivity of proteins was analysed according to the induction of HR (Peng *et al.* 2003), reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) (Deng *et al.* 2011), resistance to the bacterial pathogen *Pseudomonas syringae* (Sang *et al.* 2012) and drought tolerance (Dong *et al.* 2005) in tobacco or *Arabidopsis*.

2.2 *Arabidopsis treatment and growth scoring*

Arabidopsis ecotype Col-0 was used in this study. Plants were grown in pots containing potting soils in an environmentally controlled chamber under 22°C and 200 $\mu E/m^2/s$ illumination for 10 and 15 days in long day (16-h photoperiod) and short day (12-h photoperiod), respectively, before treatment. Protein solutions and pure water (in the presence of 0.03% Silwet-77) was applied separately to plants by spraying over plant tops with an atomizer. The vegetative growth was monitored at a 10 day interval till flowering and quantified by fresh weight. The floral development were observed every day after treatment and scored as days to flower and rosette leaf number at the flowering day.

2.3 *Gene expression analyses*

The expression of growth-promoting genes *EIN5*, *EXP1*, and *EXP2* in *Arabidopsis* leaves was determined by reverse

Table 1. Information on genes tested in this study

Gene	Accession number	Primers	Product size (bp)	Tests
Actin2	AT3G18780	5′-AGATGATGCTCCCAGGGCTGTTTTTC-3′ 5′-AGGTTTCCATCTCCTGCTCGTAGTCA-3′	620	RT-PCR
		5′-GTCTCCTGCAAGTCGGGGA-3′, 5′-CTAG GGAAAACAGCACGG-3′	207	Real-time RT-PCR
hpa1	PXO_03392	5′-CG**GGATCC**ATGAATTCTTTGAACACAC AATT-3′ (*Bam*H I), 5′-**GTCGAC**CTGCATCG ATGCGCTGTCGC-3′ (*Sal* I)*	420	Cloning by PCR
Hpa1 Δ159		5′-CG**GGATCC**ATGTCGAGCAAAAATGCTG AGG-3′ (*Bam*H I), 5′-**GTCGAC**CTGCATCGA TGCGCTGTCGC-3′ (*Sal* I)*	261	Hpa1ΔNT Production
GST	JF275063	5′-GG**GGTACC**ATGTCCCCTATACTAGGTTA TTGGA-3′ (*K*pn I), 5′-CG**GGATCC**ATCCGAT TTTGGAGGATGGTCGCCA-3′ (*Bam*H I)	660	Fusion to Hpa1 and Hpa1ΔNT
EIN5	AT1G54490	5′-GTCGCTCTTCAGTATTACATCC-3′, 5′-TTA TCGTCTATCGGTTCAGGT-3′	791	RT-PCR
		5′-GAGCCCTTTAAACCTTTTGAC-3′, 5′-ACTG TTGCGTTGTTGTTCTTC-3′	267	Real-time RT-PCR
EXP1	AT1G69530	5′-ATTCTACGGTGGTGGTGATGC-3′, 5′-TTAC TCTGCCAGTTCTGTCCC-3′	525	RT-PCR
		5′-CATTGTCTCTAACAACGTTGCTGG-3′, 5′-GCA CCACTTCTTTTTAGGTTAACC-3′	135	Real-time RT-PCR
EXP2	AT5G05290	5′-CCATAAACTCCGACGACAACG-3′, 5′-GCTCA CAACAGTCCGACCATC-3′	641	RT-PCR
		5′-GCACCCTCAACTTCTGCTTG-3′, 5′-ACCCAC AAGCACCACCCATTG-3′	120	Real-time RT-PCR

*Restriction enzymes are indicated in brackets and their recognition bases are shown as bolded letters prefixed with protection bases.

transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR (Chen *et al.* 2008a) using specific primers (table 1). The constitutively expressed *Actin2* gene (Sun *et al.* 2010; Deng *et al.* 2011; Sang *et al.* 2012) was used as a reference. In real-time RT-PCR analyses, no template control was included to verify amounts of gene transcripts. The ratio between amounts of a growth-promoting gene and *Actin2* transcripts was used as a relative unit of the gene expression.

2.4 *Cell growth evaluation*

Fifteen-day-old seedlings grown in short day were treated by spraying with pure water in control and with a 10 μg/mL aqueous solution of Hpa1 or Hpa1ΔNT. Five days later, the first leaves were excised, discolored in a chloral hydrate solution (Peng *et al.* 2003), observed by microscopy, and photographed by the equipped camera. Number of epidermal cells was counted in Adobe Photoshop, and epidermal cell size was estimated with the Magic Wand and Edit Tools as described (Kwack *et al.* 2005). Protoplasts were isolated (Sang *et al.* 2012) from the first leaves of 15-day-old seedlings and incubated in a liquid medium (Sang *et al.* 2012) for 16 hours in the absence (control) and presence of 10 μg/mL Hpa1 or Hpa1ΔNT, followed by microscopy and photography.

2.5 *Gas exchange and chlorophyll fluorescence measurements*

All measurements were conducted on the third and fourth leaves of 30-day-old plants grown under the short day condition. Gas exchange measurements were carried out with the Li-6400XT portable photosynthesis system and equipped 6400-15 extended reach 1 cm chamber (Li-Cor Biosci., Lincoln, NE, USA). Detailed measurements were accomplished by following the manufacturer's instructions and experimental procedures described previously (Flexas *et al.* 2007; Heckwolf *et al.* 2011). Gas (water vapour and gaseous CO_2) concentrations at the inlet and outlet of the leaf chamber were monitored by the non-dispersive infrared gas analyser installed in the system. Instantaneous gas exchange measurements were performed several times during the experiments at saturating light [photosynthetically active photon flux density (PPFD) of 1500 μM/m^2/s] and the chamber CO_2 concentration of 400 μM/M air. Values of stomatal conductance (g_s) to water vapour, the CO_2 concentration in the substomatal cavity (C_i) and net photosynthesis (A_N) were

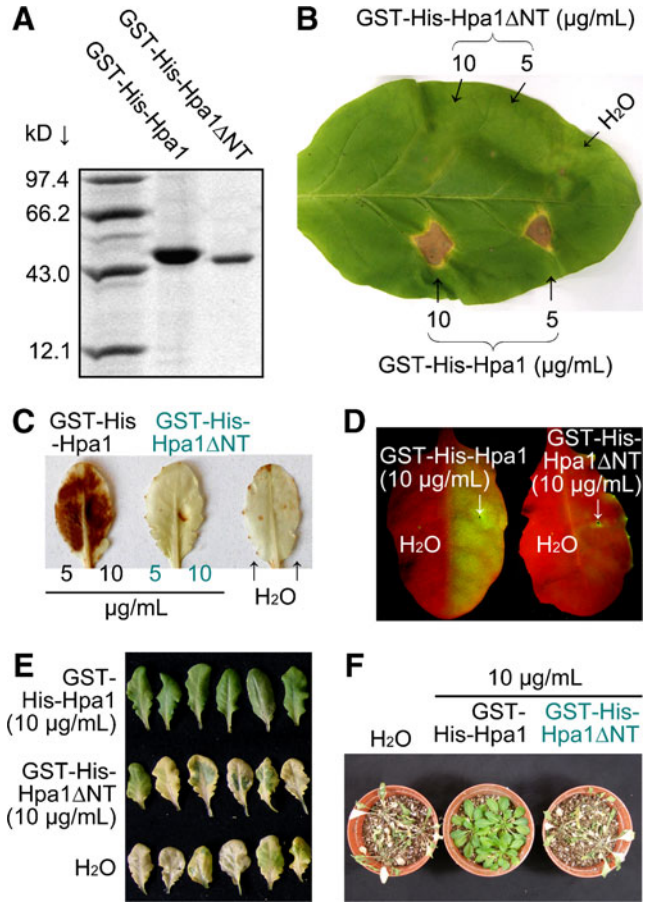

Figure 1. Deletion of N-terminus of Hpa1 cancels the bioactivity. (**A**) Protein electrophoresis. Hpa1ΔNT denotes the N-terminus–deleted version of Hpa1. The GST-His-Hpa1 and GST-His-Hpa1ΔNT fusion proteins from prokaryotic expression were purified by nickel chromatography before electrophoresis and bioactivity tests. (**B**) The induction of HR in leaves of tobacco (Xanthi NN). Tobacco was grown in a greenhouse for 30 days and the fourth leaves were infiltrated at the indicated sites with aqueous solutions of purified proteins and water as a control. HR was observed in Hpa1-infiltrated leaf areas within 12 h but photo was taken 5 days later. (**C**) H_2O_2 accumulation in Xanthi leaves. Leaves equivalent to those in (**B**) were sprayed with the protein solutions or water and 12 h later, H_2O_2 was visualized by staining leaves with a 0.5 mM aqueous solution of 3,3-diaminobenzidine. (**D**) Total ROS accumulation in *Arabidopsis* leaves. The fourth leaves of 30-day-old plants grown in short day were infiltrated with the protein solutions or water and 12 hours later, ROS was visualized by staining leaves with a 0.1 mM aqueous solution of 2,7-dichlorofluorescein diacetate. (**E**) Infection of *Arabidopsis* by the bacterial speck pathogen *P. syringae*. Plants grown as in (**D**) were treated with the protein solutions or water and 5 days later, inoculated with the bacterial suspension (2.5×10^7 colony formation unit per milliliter) by spraying over plant tops, respectively. Photos show leaves from single plant individuals 9 days after inoculation and represent 90 leaves from 15 plants tested in three experimental repetitions. (**F**) *Arabidopsis* response to drought stress. Ten-day-old plants were treated with the protein solutions or water by spraying over plant tops and were no longer watered since the treatment time. Photos were taken 15 days later.

recorded automatically by the S-501 digital monitor integrated into the system. The value of g_s to CO_2 was calculated as a quotient of the H_2O g_s value divided by 1.6 (H_2O/CO_2 conductance ratio) (Harley *et al.* 1992).

This conversion was used for the coessential comparison with the mesophyll CO_2 conductance (g_m), which was estimated by the formula $g_m = A_N \div [C_i - \Gamma^* \times (J_{flu} + 8 \times (A_N + R_l)] \div [J_{flu} - 4 \times (A_N + R_l)]$ (Pons *et al.* 2009), where R_l indicates respiration rate in light, Γ^* refers to CO_2 photocompensation point, and $\mathbf{J}_{flu}$ is photosynthetic electron transport rate. They were determined by combinative measurements of gas exchange and chlorophyll fluorescence. Values of R_l and Γ^* were obtained from A_N–C_i curves established by measuring both parameters at three different PPFD densities (50, 200, and 500 μM/m^2/s) with a range of CO_2 concentrations (0, 50, 75, 100, 150, 200, 400, 600, 800

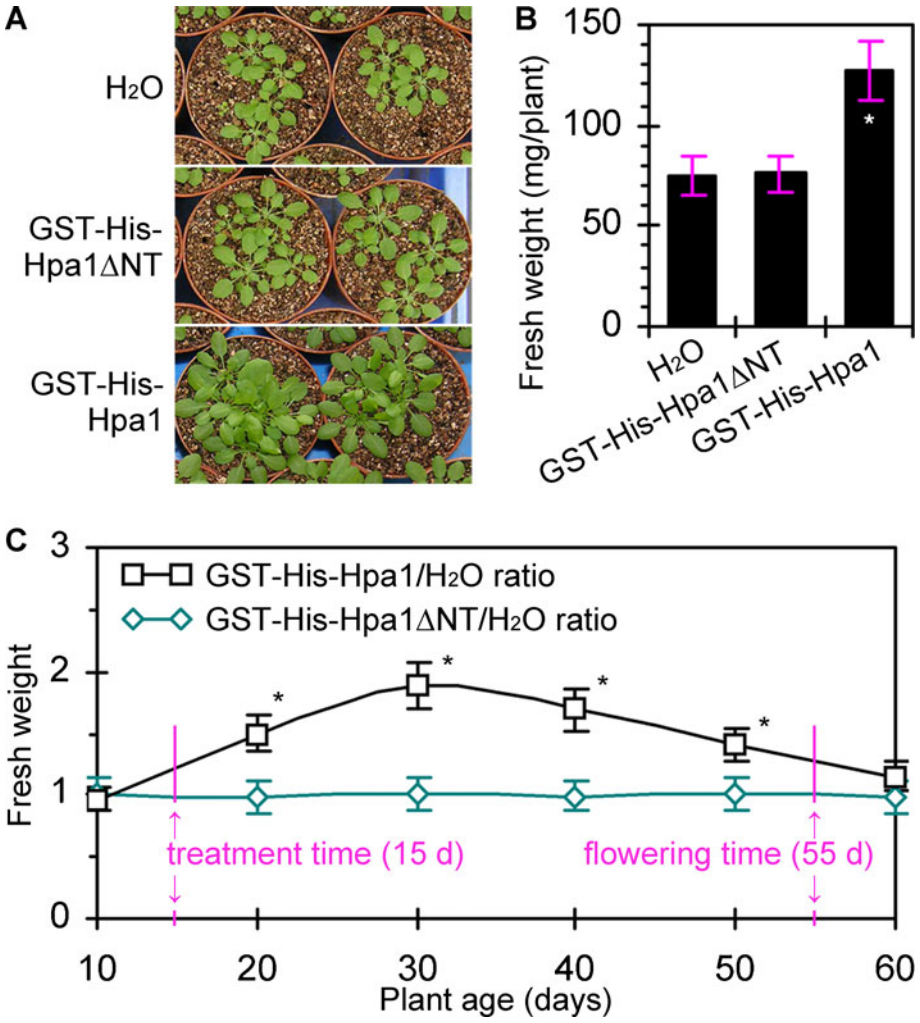

Figure 2. *Arabidopsis* growth in short day and following treatment with water, GST-His-Hpa1, or GST-His-Hpa1ΔNT. (**A**) Plants photographed after 40 day growth and 25 days after spraying treatments with aqueous solutions of purified proteins and water as a control. (**B**) Fresh weight of plants from (**A**) determined 20 days after treatment. (**C**) Time course of the effect of Hpa1 on the plant growth. Fresh plant weight was determined at a 10 day interval and at each time point, the ratio between treatments with GST-His-Hpa1 and water was scored in comparison to the ratio between treatments with GST-His-Hpa1ΔNT and water. (**B**) and (**C**) Data were provided as means ± standard deviation (SD) bars (n=5 repeats, 20 plants/repeat). One-way ANOVA along with the LSD method was used to test the significance (P<0.01), indicated by SD bars and asterisks, in differences between treatment groups of GST-His-Hpa1 and GST-His-Hpa1ΔNT or water.

and 1000 µM/M air) (Pons *et al.* 2009; Flexas *et al.* 2007, 2012). The value of $\mathbf{J}_{flu}$ was obtained by chlorophyll fluorescence measurement using the Multiple Excitation Wavelength Chlorophyll Fluorescence Analyzer (Heinz Walz GmbH, Eichenring, Germany). This analyser was operated under conditions of the leaf temperature kept at 25°C, the ambient CO_2 concentration adjusted to 400 µM/M air and the leaf-to-air vapour pressure deficit maintained at 1.2 kPa. Leaves were first adapted to dark for 20 minutes and then adjusted to PPFD 1500 µM/m^2/s, and chlorophyll fluorescence was measured on three 3 mm^2 circular bars located between leaf nervures in a single leaf.

2.6 *Data analysis*

All the experiments were carried out at least three times with similar results. Quantitative data were analysed by ANOVA and Fisher's Least Significant Difference (LSD) test.

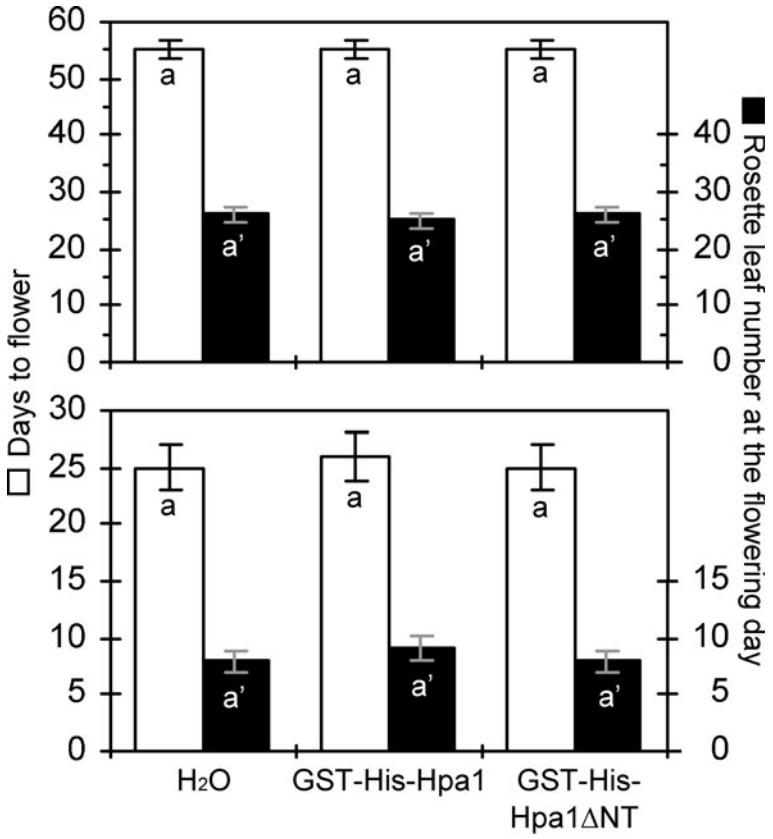

Figure 3. The *Arabidopsis* floral development following different treatments. Plants grown in short day (top bar graph) or long day (bottom bar graph) were treated with H_2O, GST-His-Hpa1, or GST-His-Hpa1ΔNT. Flowering time and rosette leaf number were scored as means ± SD bars (n=5 repeats, 30 plants/repeat). The same letters with bar graphs indicate insignificant differences according to one-way ANOVA and LSD test (P<0.01).

3. Results

3.1 *Deleting N-terminus of Hpa1 cancels its bioactivity*

X. oryzae pv. *oryzae* Hpa1 contains 140 amino acids and has a molecular weight of 15.6 kDa (Peng *et al.* 2004). The N-terminal region of Hpa1 has been shown to be required for the induction of HR in tobacco (Wang *et al.* 2008). To study whether N-terminus is also required for Hpa1 to enhance plant growth, we deleted the N-terminal 53 amino acid residues and generated a 13.4 kDa variant designated as Hpa1ΔNT. This variant and the full-length Hpa1 were respectively fused to the 26 kDa GST protein and additionally a 7 kDa His tag for protein purification. After expression in *E. coli*, the GST-His-Hpa1 and GST-His-Hpa1ΔNT fusion proteins were purified by nickel chromatography and resolved by electrophoresis. They gave molecular masses consistent with the prediction: 48.6 kDa for GST-His-Hpa1 and 46.4 kDa for GST-His-Hpa1ΔNT (figure 1A). In a bioactivity assay for purified proteins at concentrations (5 and 10 μg/mL) higher than the minimal functional dose determined previously (Peng *et al.* 2003; Liu *et al.* 2006), the GST-His-Hpa1 fusion protein was found to be active and GST-His-Hpa1ΔNT was inactive in inducing HR in tobacco (figure 1B). In contrast to Hpa1, moreover, Hpa1ΔNT failed to induce ROS or H_2O_2 and defense responses to *P. syringae* infection and drought stress (figure 1C–F). Clearly, deletion of N-terminus of Hpa1 cancels multiple effects of the protein in the plant.

3.2 *N-terminus is required for Hpa1 to enhance Arabidopsis growth*

Aqueous solutions of purified proteins were applied by spraying to 15-day-old *Arabidopsis* plants grown in short

day. Plants with GST-His-Hpa1 treatment were visually larger than the water spray control at 25 days after spray but GST-His-Hpa1ΔNT spray did not appear to have any effect as compared to water spray control (figure 2A). Plant growth was quantified by fresh weight. The GST-His-Hpa1 treatment caused a significant (P<0.01) increase (by 41%) in fresh plant weight while plant weight changed little in the treatment with GST-His-Hpa1ΔNT (figure 2B). The best growth-promoting effect of Hpa1 was observed in plants 30–40 days after sowing and 15–20 days after treatment (figure 2C). Under the short day condition, however, flowering time was similar in plants treated with GST-His-Hpa1, GST-His-Hpa1ΔNT, or water (figure 3). Growth enhancement by GST-His-Hpa1 was not evident after flowering (figure 2C). When plants were grown in long day (16 h photoperiod), GST-His-Hpa1 was also able to enhance growth but did not affect the floral development (figure 3). Therefore, the Hpa1 treatment enhances the vegetative growth without affecting the floral development and the N-terminal region in the Hpa1 sequence is critical for plant growth enhancement.

3.3 *Hpa1, but not the variant, induces expression of growth-promoting genes and promotes cell growth*

In response to harpins, plant growth is regulated by the ethylene signaling pathway that essentially requires the regulatory gene *EIN5* (Dong *et al.* 2004; Ren *et al.* 2008) and subsequently activates ethylene-responsive expansin genes *EXP1* and *EXP2* (Chen *et al.* 2008a, b; Ren *et al.* 2006a, b). Hundreds of *EXP*s have been identified in plants (http://www.bio.psu.edu/expansins/other_species.htm), and both *EXP1* and *EXP2* are widely characterized as required for plant growth (Cox *et al.* 2004; Sloan *et al.* 2009; Wang *et al.* 2011). Induced expression of *EIN5*, *EXP1*, and *EXP2* contributes to harpin-enhanced plant growth (Chen *et al.* 2008a, b; Ren *et al.* 2006a, b). In *Arabidopsis* plants treated with Hpa1, *EIN5*, *EXP1*, and *EXP2* were highly expressed in contrast to basal levels of expression in plants treated with water in control (figure 4A). Quantitative analyses indicated that the GST-His-Hpa1 treatment caused more than 8-fold increases in expression levels of *EIN5*, *EXP1*, and *EXP2* as compared to basal expression levels detected in the water treatment control (figure 4B). On the contrary, the GST-His-Hpa1ΔNT treatment caused little changes in gene expression extents (figure 4A–B). The association between levels of gene expression and extent of cell growth was analysed (figure 4C). The GST-His-Hpa1 treatment resulted in smaller number and larger average size of leaf epidermal cells while the treatment caused increases in both the number and size of leaf protoplasts, in contrast to little changes in the control or GST-His-Hpa1ΔNT treatment group (table 2). These analyses suggest that the N-terminal region is critical for Hpa1 to induce expression of growth-promoting genes and promote cell growth in the plant.

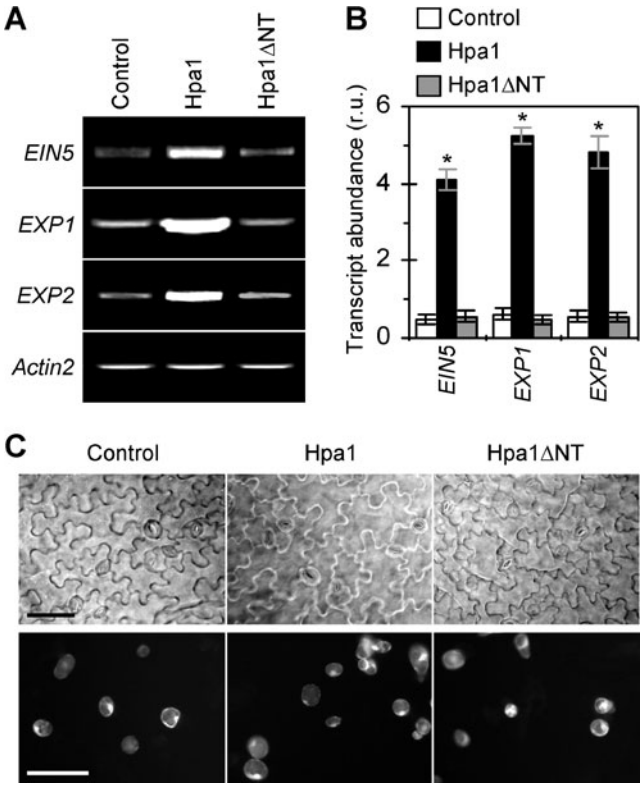

Figure 4. Expression of growth-promoting genes and variations of cell growth in differently treated *Arabidopsis* plants grown in short day. (**A**) and (**B**) Fifteen-day-old seedlings were treated and gene expression in the first leaves was determined 24 h later. (**A**) RT-PCR analyses using the stably expressed *Actin2* gene as a reference. (**B**) Real-time RT-PCR analyses. The ratio between amounts of a growth-promoting gene and *Actin2* transcripts was used as a relative unit (r.u.) of the gene expression. Data were given as means ± SD bars (n=3 repeats, 10 plants/repeat). Asterisks indicate significant differences in expression levels of the corresponding genes according to ANOVA and LSD (P<0.01) tests. (**C**) Photos showing leaf epidermal cells (top panel) and leaf protoplasts (lower panel). In the top panel, 15-day-old seedlings were treated and the first leaves were observed 5 days later. In lower panel, protoplasts were isolated from the first leaves of 15-day-old seedlings, incubated at 10 cells/mL in the absence (control) and presence of GST-His-Hpa1 (Hpa1) or GST-His-Hpa1ΔNT (Hpa1ΔNT). Sixteen hours later, a protoplast suspension of 5 μL was observed in each experimental group.

Table 2. Number and size of leaf epidermal cells and leaf protoplasts

Experimental treatment groups	Epidermal cells [a]		Protoplasts [b]	
	Number/leaf [c]	Size (μm^2)[c]	Number/mL [c]	Size (μm^2) [c]
H_2O (control)	1295±113	3981±226	1.58×10^6±15676	890±49
GST-His-Hpa1ΔNT	1278±116	3877±218	1.72×10^6±21430	841±64
GST-His-Hpa1	1042±87*	4790±304*	5.86×10^6±87654*	1024±56*

[a] Fifteen-day-old seedlings were treated and the first leaves were observed 5 days later.

[b] Protoplasts isolated from the first leaves of 15-day-old seedlings were incubated at 10 cells/mL in the absence (control) and presence of 10 μg/mL GST-His-Hpa1 or GST-His-Hpa1ΔNT. Sixteen hours later, a protoplast suspension of 5 μL was observed in each experimental group.

[c] The indicated values are means ± SD (n=3 repeats, 5 plants/repeat). Asterisks indicate significant different by one-way ANOVA and LSD (P<0.05).

3.4 *N-Terminus is required for Hpa1 to promote leaf CO_2 transport and photosynthesis*

Plant growth fundamentally relies on CO_2 conduction through leaf stomata and mesophyll cells and assimilation by photosynthesis (Taiz and Zeiger 2010). To test if Hpa1 may affect this physiological process with requirement for its N-terminus, we studied leaf stomatal and mesophyll conductance to CO_2 and leaf photosynthesis in plants treated with water, GST-His-Hpa1, or GST-His-Hpa1ΔNT. Measurements were performed with the third and forth leaves 15 days after treatment when the best growth-promoting effect of Hpa1 was observed (figure 2C). Leaf stomatal CO_2 conductance (g_s) and leaf net photosynthesis rate (A_N) were directly monitored by a portable photosynthesis system. As shown in figure 5A, values of g_s were similar in plants treated with water or GST-His-Hpa1ΔNT but were significantly (P<0.01) elevated in GST-His-Hpa1–treated plants. Mesophyll CO_2 conductance (g_m) was determined by combinative use of the portable photosynthesis system and a chlorophyll fluorescence analyser (figure 5B). Like g_s, g_m rates were also similar in plants treated with water or GST-His-Hpa1ΔNT but were significantly (P<0.01) elevated in GST-His-Hpa1–treated plants (figure 5C). Levels of g_s and g_m were correlated with net photosynthesis rates, significantly (P<0.01) greater in plants treated with GST-His-Hpa1, than those in controls (figure 5D). These analyses suggest that the Hpa1 treatment promotes CO_2 transport inside leaves and leaf photosynthesis, and this physiological consequence requires the presence of N-terminus in the Hpa1 sequence.

4. Discussion

In this study, analyses of phenotypic and molecular effects of Hpa1 in contrast to the N-terminus–deleted variant support the conclusion that the N-terminal region of Hpa1 is critical for the protein to induce multiple effects, and especially to enhance the vegetative growth (but not the floral development) and expression of growth-promoting genes in the plant. Furthermore, our results correlate the growth enhancement activity of Hpa1 with a physiological consequence, i.e. facilitating CO_2 transport inside leaves and thus promoting leaf photosynthesis, and this effect also requires the presence of N-terminus in the Hpa1 sequence.

On the basis of the significant role of CO_2 transport across the PM in mesophyll CO_2 conduction (Heckwolf *et al.* 2011; Flexas *et al.* 2012; Kaldenhoff, 2012; Uehlein *et al.* 2012), the phenotypic-physiological relevance for the Hpa1 activity may shed a light on further characterization for whether and how Hpa1 is recognized by a plant PM protein and for whether a molecular interaction is involved in the function of Hpa1. Hpa1 can localize to plant PMs whenever it is applied to plants or produced in transgenic plants (Sang *et al.* 2012). However, whether any PM protein plays a role in the growth-enhancing activity of Hpa1 is unknown. Currently, the only report regarding how harpins enhance plant growth is provided by a study on the HrpN harpin secreted by *Erwinia amylovora*, the bacterial pathogen that causes fire blight in rosaceous plants (Wei *et al.* 1992; Oh and Beer 2007).

HrpN-interacting protein (HIP) orthologs have been identified in *Arabidopsis*, apple, and rice (Oh and Beer, 2007). These HIP orthologs are all PM-integral, contain a single transmembrane domain (Oh and Beer, 2007), and thus do not belong to PIPs, which have six transmembrane α-helices (Gomes *et al.* 2009). Intriguingly, HIP is a negative regulator of plant growth but is involved in HrpN-induced plant growth enhancement. HrpN enhances vegetative growth of wild-type plants but not *HIP*-knockout mutants, suggesting that the growth enhancement effect of HrpN requires the

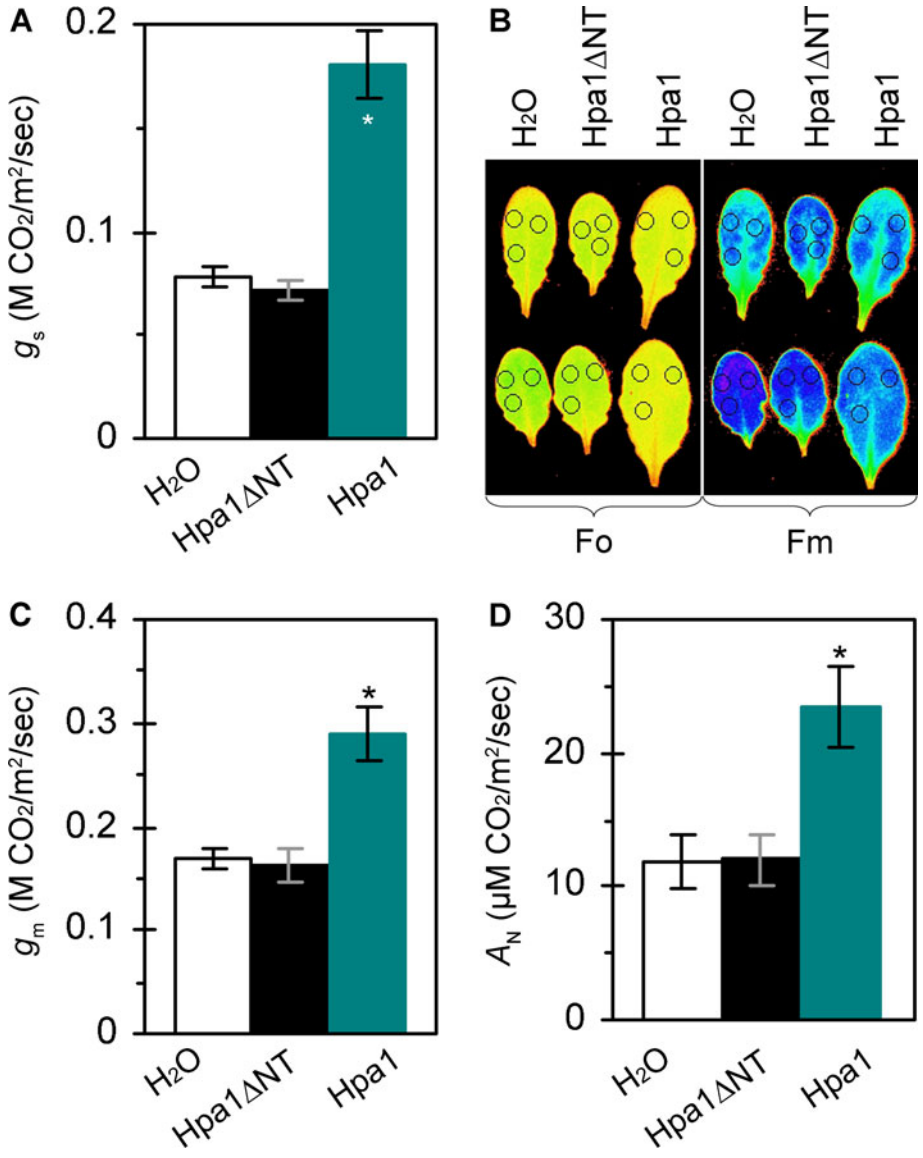

Figure 5. Leaf cell conductance to CO_2 and leaf net photosynthesis rate in differently treated *Arabidopsis* plants. (**A**) Stomatal CO_2 conductance monitored with a portable photosynthesis system. (**B**) Optimized original fluorescence yield (Fo) and maximal fluorescence yield (Fm) imaging for chloroplast fluorescence analysis used for determining mesophyll CO_2 conductance. (**C**) Mesophyll CO_2 conductance determined by combinative use of the portable photosynthesis system and a chloroplast fluorescence analyser. (**D**) Leaf net photosynthesis rates monitored with the portable photosynthesis system. In (**A**), (**C**) and (**D**), values shown are means ± SD bars (n=3 repeats, 20 plants/repeat). Asterisks indicate significant differences according to one-way ANOVA and LSD (P<0.01) tests.

presence of *HIP*. It is proposed that binding of HrpN may intercept HIP and thus inhibits its negative regulatory function, resulting in better growth of plants (Oh and Beer 2007). Whether this regulatory model is suited to Hpa1 remains to be studied.

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