

# Identification of an *Arabidopsis* Nodulin-Related Protein in Heat Stress

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We identified a Nodulin-related protein 1 (NRP1) encoded by At2g03440, which was previously reported to be RPS2 interacting protein in yeast-two-hybrid assay. Northern blotting showed that *AtNRP1* expression was suppressed by heat stress (42°C) and induced by low temperature (4°C) treatment. Strong GUS staining was observed in the sites of meristematic tissues of *pAtNRP1::GUS* transgenic plants, such as shoot apex and root tips, young leaf veins, stamens and stigmas of flowers, and abscission layers of young siliques. To study *AtNRP1* biological functions, we have characterized both loss-of-function T-DNA insertion and transgenic overexpression plants for *AtNRP1* in *Arabidopsis*. The T-DNA insertion mutants displayed no obvious difference as compared to wild-type *Arabidopsis* under heat stress, but the significant enhanced susceptibility to heat stress was revealed in two independent *AtNRP1*-overexpressing transgenic lines. Further study found that the decreased thermotolerance in *AtNRP1*-overexpressing lines accompanied significantly decreased accumulation of ABA after heat treatment, which was probably due to *AtNRP1* playing a role in negative-feedback regulation of the ABA synthesis pathway. These results support the viewpoint that the application of ABA inhibits nodulation and nodulin-related gene expression and threaten adverse ambient temperature can impact the nodulin-related gene expression.

## INTRODUCTION

Plants are exposed to various environmental stresses during their growth and development. Among these stresses, high temperature is one of the major problems that limit the growth and distribution of plants (Boyer, 1982). On the other hand, plants have evolved various mechanisms for adapting to the effects of heat shock (HS). Accumulation of heat shock proteins (HSPs), membrane compositional changes necessary for maintenance of functional integrity, and activation of oxidative defensive systems are involved in improving plant thermotolerance (Kaplan et al., 2004; Kotak et al., 2007b; Locato et al., 2008; Queitsch et al., 2000; Sung et al., 2001). Transcription activation of HSPs genes is regulated by heat shock transcrip-

tion factors (HSFs) through binding to heat shock promoter elements (HSEs) in the promoter regions of HSPs genes during heat stress (Baniwal et al., 2004; Yamamoto et al., 2005). In addition, the induction of abscisic acid (ABA), salicylic acid (SA), and calcium-based signaling pathways were reported to be involved in heat-stress adaptation (Clarke et al., 2004; Larkindale and Knight, 2002; Larkindale et al., 2005; Liu et al., 2008). Exogenous application of these signaling agents to plants can also result in some degree of enhanced thermotolerance (Larkindale and Knight, 2002). These multiple responses suggest that many alternative processes are involved in thermotolerance. Plants at various growth stages respond differently to heat stress, suggesting a link between development and thermotolerance (Hong et al., 2003).

The infection of the plant by rhizobia bacteria is a complex process which a number of plant genes take part in. Legumes form a specialized organ termed the nodule via the expression of their genes that encode proteins named 'nodulins' when the leguminous plants were elicited by the secretion of bacterial effectors called nod factors in their root hairs (Stougaard, 2000). For non-nodule plants, *Arabidopsis*, when the plant roots have been colonized by several specific strains of *Pseudomonas* spp., they would develop a protective defense response that is called rhizobacteria-mediated induced systemic resistance (Cartieaux et al., 2003). Recent study has indicated that abscisic acid (ABA) can not only inhibit Nod factor signal transduction and promote or suppress resistance against various pathogens (de Torres-Zabala et al., 2007; Ding et al., 2008; Mohr and Cahill, 2007), but also can break a new signaling pathway to heat stress (Larkindale and Huang, 2004; Larkindale et al., 2005). A transient peak in ABA levels was reported in response to HS in pea plants (Liu et al., 2006) and during recovery from HS treatments in creeping bentgrass (Larkindale and Huang, 2004). It has been reported that ABA induces thermotolerance in cell-suspension cultures of *Bromus inermis* Leys. (Robertson et al., 1994), and ABA pretreatment can increase cell viability and growth upon HS (Zhang and Ferevereiro, 2007). In addition, the overexpression of transcription factor ABF3 in ABA signaling induced high-temperature tolerance in transgenic *Arabidopsis* (Kim et al., 2004). These results suggested that ABA could also be involved in the response to heat stress. Moreover, genotypes with a putative high ABA

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level may be more tolerant to stresses (Xiong and Zhu, 2003). The *Arabidopsis* ABA signaling mutants *abscisic acid insensitive 1 (abi1)* and *abi2* showed reduced survival after HS, but the accumulation of HSPs was not affected in these mutants, and ABA does not appear to be required for HSP synthesis during HS (Larkindale et al., 2005). So it seems that ABA related heat tolerance may be independent on heat shock proteins synthesis pathway.

Nodulin-related protein 1, encoded by At2g03440, is a gene of unknown function encoding a putative mature protein of 187 amino acids. Bioinformatics analysis revealed that the amino acid sequence of the nodulin-related protein has some similarity to early nodulin 12B precursor (Bauer, 1994), and there are 41.3% identity (63.0% similarity) in proline-rich domain of the nodulin-related protein 1 and alfalfa early nodulin 12B precursor (see Supplementary Fig. 1), we designated the protein 'AtNRP1'. It has been demonstrated that AtNRP1 was an especially strong interactor of RPS2 (recognition of *P. syringae* strains) in yeast-two-hybrid assay (Quirino et al., 2004), and a recent study showed that AtNRP1 was strongly up-regulated in *Capsicum annuum* hypersensitive induced reaction gene (*CaHIR1*)-overexpressing transgenic *Arabidopsis*, and was also differentially induced at the transcriptional level by infection of *Pseudomonas syringae* pv. *tomato* DC3000 (Jung et al., 2008). However, the specific roles of AtNRP1 in *Arabidopsis* have not been determined so far.

In this study, we cloned AtNRP1 cDNA using a temperature screen. *AtNRP1* expression was significantly suppressed by heat stress and induced by low temperature treatment. Histochemical analysis in *pAtNRP1::GUS* transgenic plants showed that *AtNRP1* expressed mainly in the apical meristematic tissues. The *AtNRP1*-overexpressing transgenic plants showed decreased thermotolerance, but the expression of some known heat-induced genes, such as *HsfA2*, *MBF1c*, *Hsp101* and *Hsp70*, had no significant difference compared with wild-type plants under heat stress. Further study found that *AtNRP1* was an ABA regulated gene, and the ABA accumulation in *AtNRP1*-overexpressing transgenic plants was lower than that of WT and *nrp1* under heat stress. These results showed AtNRP1 might play a role in thermotolerance through the ABA synthesis pathway.

## MATERIALS AND METHODS

### Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Columbia was used as the WT for experiments in this study. The *Arabidopsis* seeds (WT, *nrp1*, and *AtNRP1*-overexpressing transgenic plants) were surface sterilized for 15 min in 20% (v/v) bleach, and rinsed twice with sterile distilled water. Seeds were grown on 1/2 Murashige and Skoog (MS) solid medium with 0.7% (w/v) agar and 1.5% sucrose. After 3 days stratification in darkness at 4°C, seeds were transferred to plant incubators with a 12 h-light (100  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) / 12 h-dark photoperiod at 22°C. The seedlings were either grown in axenic culture on 1/2 MS medium or transferred into soil in the greenhouse (22°C with a 12 h photoperiod) 7 d after germination on 1/2 MS medium.

### Isolation of T-DNA insertion mutants

The *AtNRP1* T-DNA insertion line (SALK\_111768), in the Col background, was obtained from the *Arabidopsis* Biological Resource Center (ABRC; Ohio State University, USA). The T-DNA insertion sites were confirmed by PCR using a T-DNA border primer (5'-AAACGTCGCAATGTGTAT-3') and the gene-specific primer (5'-ACGAGTCAGACAAGCTTGACA-3'). Plants homozygous for the T-DNA insertion were identified by

another PCR using the above gene-specific primer and gene-specific reverse primer (5'-TTTGCCAAAACCTAAAAATTA-TCA-3') and Northern blot analysis.

### Construction of *AtNRP1*-overexpressing transgenic plants

The full-length cDNA of *AtNRP1* (U22696) was obtained from ABRC and confirmed by sequence analysis. To generate the 35S::*AtNRP1* construction, the full-length cDNA fragment of *AtNRP1* was cloned into the BamHI and Sall sites of pOCA30 (Chen and Chen, 2002). The recombinant plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 and followed by transformation into wild-type *Arabidopsis* plants by the floral dip method (Clough and Bent, 1998). The seeds were collected from the *A. tumefaciens*-infected plants and selected on 1/2MS agar medium containing 50  $\mu\text{g ml}^{-1}$  kanamycin. Northern blot analysis was performed to further identify transgenic plants. T<sub>3</sub> homozygous *AtNRP1*-overexpressing transgenic plants were selected for further experiments.

### Heat treatments assays

Thermotolerance assays of seven-day-old normal-grown and 2.5-day-old dark-grown seedlings were performed as described by Larkindale et al. (2005) with minor modifications. Seven-day-old seedlings were exposed to a 45°C HS for 260 min after a pretreatment for 90 min at 38°C and 120 min at 22°C, and then returned to 22°C to recover for 7 d. Seedlings were photographed to record viability, and the seedlings that were still green and producing new leaves were scored as surviving. The survival rate was the ratio of surviving seedlings to total treated seedlings. For the hypocotyl elongation assay, 2.5-day-old dark-grown seedlings on vertical plates were heat-treated at 45°C for 180 or 220 min after a pretreatment for 90 min at 38°C and 120 min at 22°C. After an additional 2.5 days growing in the dark, seedlings were measured and photographed. In these assays, *nrp1* and *AtNRP1*-overexpressing transgenic plants were compared with WT plants growing on the same plate to ensure the compared plants received identical stress conditions. Three-week-old WT, *nrp1* and *AtNRP1*-overexpressing transgenic plants cultured in soil were heat treated at 42°C for 0 min, 30 min, or 90 min for northern blot analysis.

### Electrolyte leakage assays

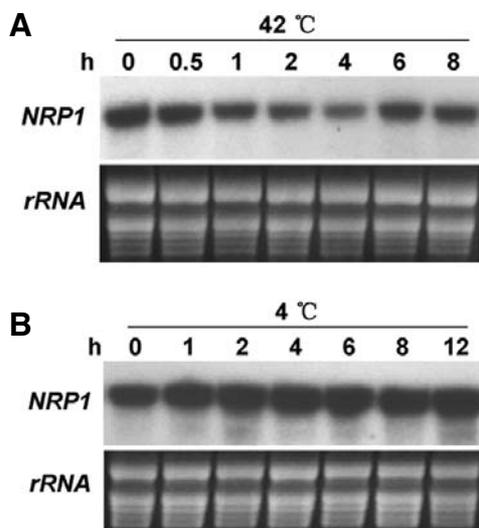
To explore the difference in thermotolerance of adult plants, leaves subjected to heat stress were assayed for electrolyte leakage according to methods of Clark et al. (2004). The plants were cultured in soil for 3 weeks, and the 3rd to 5th leaves were used for electrolyte leakage tests. The leaves were placed in glass tubes containing 10 ml distilled water and were incubated in the water bath at 42°C in the dark. Water conductance was measured at intervals during heat treatment and calculated per milligram FW.

### Quantification of ABA content

The ABA content was assayed using enzyme-linked immunosorbent assays (ELISA) as previously described (Yang et al., 2001). Three-week-old seedlings were treated at 38°C for 0.5, 1 and 2 h. Approximately 0.5 g tissue samples were frozen in liquid nitrogen and ground to a fine powder. Endogenous ABA was extracted and the content analyzed by ELISA.

### RNA isolation and gel blotting

Total RNA was extracted from three-week-old *Arabidopsis* plants using the TRIzol reagent (Invitrogen). For RNA gel blot analysis, total RNA (10  $\mu\text{g}$ ) was separated on 1.2% agarose-formaldehyde gels and blotted onto nylon membranes. Blots



**Fig. 1.** Expression analysis of *AtNRP1*. Expression of *AtNRP1* in response to (A) 42°C and (B) 4°C. Each lane was loaded with 10 µg total RNA prepared from 3-week-old plants treated with the various stresses as described in experimental procedures. Equal loadings were confirmed by Ribosomal RNA prestained gels with ethidium bromide. The 400 bp specific fragment of *AtNRP1* cDNA was used for the probe. The experiments were repeated at least three times with similar results. h, hours post-treatment.

were hybridized with  $\alpha$ -<sup>32</sup>P-dATP-labeled gene-specific probes corresponding to the following genes: *AtNRP1*, *HsfA2*, *MBF1c*, *Hsp70* and *Hsp101*, respectively, following standard procedures. Hybridization was performed in PerfectHyb plus hybridization buffer (Sigma-Aldrich) overnight at 68°C. The membrane was then washed according to the procedure described by Yu et al. (2001).

#### ***pAtNRP1::GUS* construction and GUS staining**

To clone the promoter of *AtNRP1*, a 1866-bp *AtNRP1* promoter region was amplified from *Arabidopsis* genomic DNA by PCR using the specific forward primer 5'-TTGCGGCCGCAATGAAAGATCCGAAAGTGTAATTT-3' and the specific reverse primer 5'-TTTCTAGAGTTTCTTTGTTTCAAAGGAAAAGTT-3', which contain a *NotI* and a *XbaI* restriction sites (underlined), respectively. The PCR fragment was digested with *NotI* and *XbaI*, and was firstly inserted in frame upstream of the glucuronidase (GUS) reporter gene in pJS131B vector. The recombinant plasmids were digested with *SmaI* and *BamHI*, and the resulting *AtNRP1* promoter-*GUS* fusion fragments were cloned into transformation vector pOCA28 (Du and Chen, 2000).

GUS staining was performed using the method of Sieburth and Meyerowitz (1997). Leaves or tissues were gently fixed by incubation in 90% acetone on ice for 20 min and were then transferred into staining solution that contains 50 mM sodium phosphate (pH 7.0), 0.5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 0.5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 0.1% v/v Triton X-100 and 2 mM X-gluc (Sigma-Aldrich), vacuum infiltrated for 5 min, and incubated in darkness at 37°C overnight. After staining, tissues were washed with 70% ethanol.

## **RESULTS**

### **Expression level of *AtNRP1* is regulated by temperature stress treatments**

In order to determine the biological function of *AtNRP1*, we inves-

tigated its expression pattern in response to different stress with northern blot analysis. Three-week-old seedlings were treated with either 42°C, 4°C, 300 mM NaCl or natural drought at indicated time. As shown in Fig. 1, expression of the *AtNRP1* gene was readily affected by temperature stress. The expression of *AtNRP1* decreased significantly and a minimum expression level was reached at 4 h after 42°C treatment, followed by a gradual recovery of transcription to the background level at 6 h (Fig. 1A). On the other hand, *AtNRP1* transcription was induced by low-temperature stress. A rapid accumulation of *AtNRP1* transcripts was observed 2 h after initiation of cold treatment, and these accumulated over a period of 12 h with a maximum level of transcripts attained after 4-6 hours (Fig. 1B). Expression of the *AtNRP1* gene was down-regulated slightly under NaCl stress and did not respond to dehydration stress (data not shown). These results suggest that *AtNRP1* is very sensitive to environmental stimuli, and it appears to be positively regulated by cold stress but negatively by heat stress. Collectively, these data indicate that *AtNRP1* may behave as a stress-responsive gene.

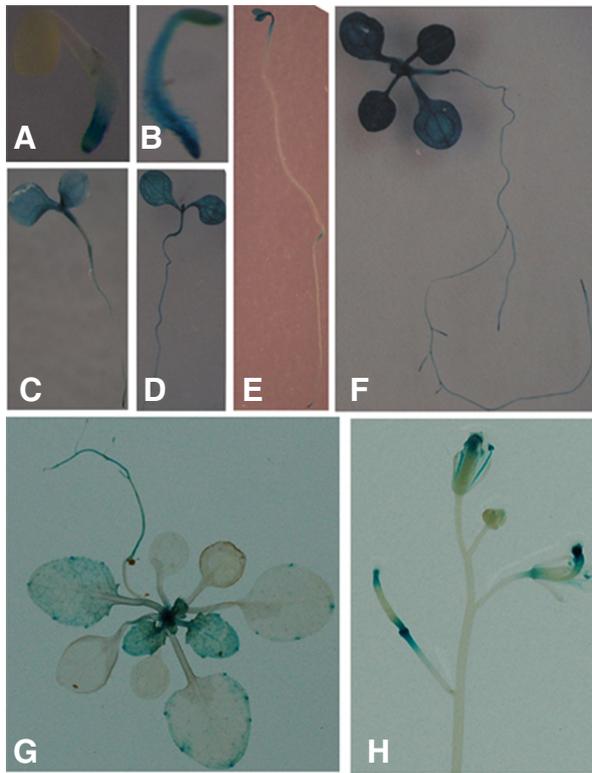
### **Developmental expression pattern directed by *pAtNRP1::GUS* fusion**

To study the expression pattern of *AtNRP1* in different developmental stages and different tissues, we analyzed transgenic *Arabidopsis* plants expressing a  $\beta$ -glucuronidase (GUS) reporter gene driven by *AtNRP1* promoter. GUS staining was observed in root tips of 1- and 2-day-old *pAtNRP1::GUS* transgenic seedlings after germination, whole plant of 3- and 5-day-old *pAtNRP1::GUS* transgenic seedlings grown in normal condition, root tips and junctions of roots and hypocotyls of 5-day-old transgenic seedlings grown in darkness (Figs. 2A-2E). With the rapid growth, GUS staining enhanced greatly in 10-day-old transgenic seedlings (Fig. 2F). While the expression of *AtNRP1* dramatically declined with further growth of three-week-old transgenic seedlings, GUS staining was only found in vasculature, hydathodes of adult leaves, the sites of meristematic tissues, such as vegetative shoot apex and root tips (Fig. 2G). *AtNRP1* also expressed in developing organs such as stamens and stigmas of flowers, and abscission layers of young siliques (Fig. 2H). Interestingly, the expression level of the *AtNRP1* gene in apical meristematic regions was much higher than that in other regions.

### **Overexpression of *AtNRP1* results in altered response to heat stress**

In order to further determine the function of *AtNRP1*, we identified a T-DNA insertion mutant for *AtNRP1*. The mutant (Salk\_111768) contains a T-DNA insertion in the exon region of *AtNRP1* (Fig. 3A), and the homozygous mutant plants were confirmed by PCR with gene-specific primers. Northern blot analysis used a DNA fragment that corresponds to the region downstream of the T-DNA insertion site in *AtNRP1* as the probe. The *AtNRP1* transcripts were detected in WT plants but not in *nrp1* under normal conditions (Fig. 3B). Mutant plants grow and develop normally, with no obvious differences in morphological or growth phenotype from that of WT plants (data not shown).

To characterize the effect of *AtNRP1* overexpression, *Arabidopsis* WT plants were transformed with a full-length *AtNRP1* cDNA driven by the CaMV 35S promoter. Overexpression of *AtNRP1* was confirmed by RNA gel blot analysis in the independent lines. Besides the level of *AtNRP1* expression, no other notably different morphological or growth phenotypes were observed between WT plants and the 35S::*AtNRP1* transgenic lines (data not shown). Two transgenic lines, line 3 and line 8,



**Fig. 2.** Developmentally-regulated GUS expression driven by *AtNRP1* promoters in transgenic *Arabidopsis*. (A) GUS staining of *pAtNRP1::GUS* transgenic seedling 1 DAG. (B-D) GUS staining of transgenic seedlings 2, 3 and 5 DAG, respectively. (E) GUS staining of dark-grown transgenic seedling 5 DAG. (F) GUS staining of transgenic seedling 10 DAG. (G) GUS staining of transgenic seedling 21 DAG. (H) GUS staining in inflorescence of transgenic seedling. DAG, days after germination.

which constitutively expressed *AtNRP1* at elevated levels (Fig. 3C), and an *AtNRP1* mutant line were chosen for further study.

$T_3$  homozygous *35S::AtNRP1* transgenic, WT and *nrp1* plants were then tested for differences in their response to heat stress. The seeds of the tested materials were grown on 1/2 MS medium plates. The *nrp1* and WT seedlings exhibited similar thermotolerance with survival rates of 73% and 68%, respectively (Figs. 4A and 4B). However, the *35S::AtNRP1* transgenic lines

showed severely impaired thermotolerance compared with the WT. After HS at 45°C, only 18% or 23% of the *AtNRP1*-overexpressing lines survived after 7 d recovery at 22°C, and thus about 80% of the *AtNRP1*-overexpressing seedlings died (Figs. 4A and 4B).

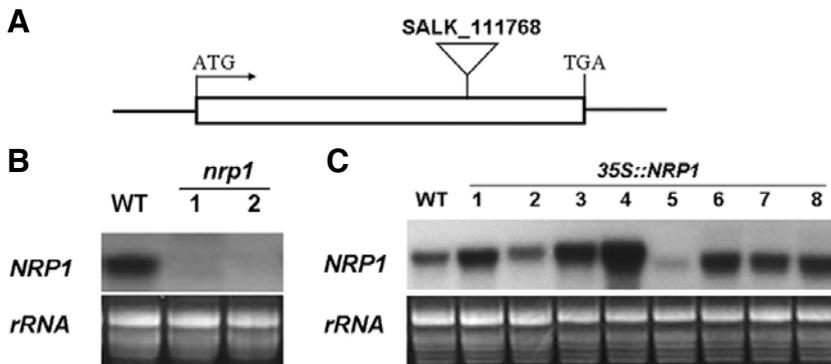
The 2.5-day-old dark-grown seedlings displayed a severe reduction in hypocotyl elongation under HS compared with the control treatment. Hypocotyl elongation of *AtNRP1*-overexpressing seedlings was nearly 70% lower compared with that recorded in the WT and *nrp1* seedlings (Figs. 4C and 4D). The WT and *nrp1* seedlings exhibited a marked delay in hypocotyl elongation to some extent after HS, but most of the seedlings continued to grow (Figs. 4C and 4D).

We then tested electrolyte leakage (EL) of 21-day-old plants during 42°C stress. No significant difference in relative electrolyte leakage was recorded among the tested leaves bathed at 42°C for 0 h or 1 h. However, the *AtNRP1*-overexpressing transgenic leaves showed almost 2-fold greater relative electrolyte leakage than that of the *nrp1* and WT leaves after bathing at 42°C for 3 h or 6 h (Fig. 4E). This result indicates that the reduced thermotolerance in adult *AtNRP1*-overexpressing plants is associated with increased relative electrolyte leakage. Compared with WT and *nrp1*, the *AtNRP1*-overexpressing lines exhibited thermosensitivity at different developmental stages.

#### ***AtNRP1*-mediated thermosensitivity is independent of HS-related regulon expression**

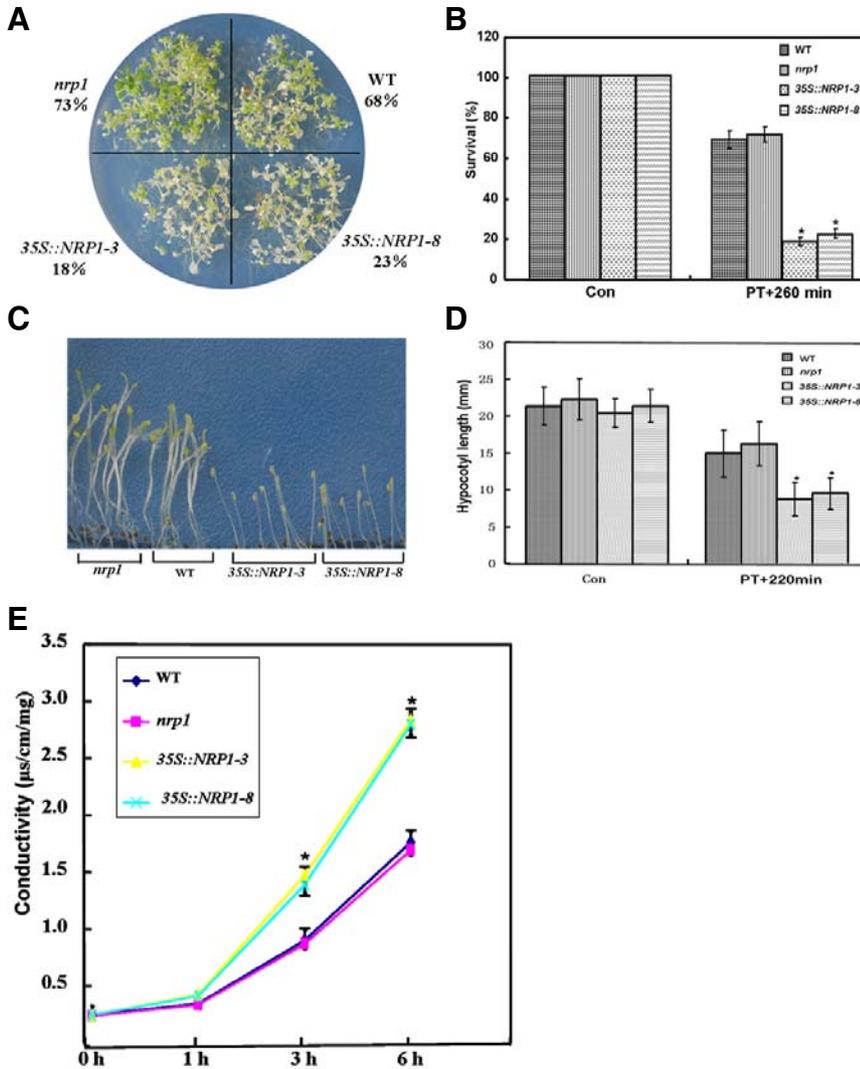
Many HSFs and HSPs have been shown to be important in resistance to heat stress (Baniwal et al., 2004). The different thermotolerance among *AtNRP1*-overexpressing lines, WT and *nrp1* led us to hypothesize that the biological function of *AtNRP1* may be dependent on heat-inducible genes in response to changing thermotolerance. To explore the molecular basis of the altered responses of the *AtNRP1*-overexpressing transgenic lines to heat stress, we compared the expression of *HsfA2*, *MBF1c*, *Hsp70* and *Hsp101* in these plants after heat stress.

First, expression of two heat stress-induced transcriptional regulator factors, *HsfA2* and *MBF1c*, were monitored. The transcript abundance for the two genes was similar in the *AtNRP1*-overexpressing transgenic lines compared with those of WT and *nrp1* in response to heat stress (Fig. 5), indicating that *AtNRP1* was not regulated by these two genes. Moreover, the expression of *Hsp70* and *Hsp101* were also not altered in WT, *nrp1* and *AtNRP1*-overexpressing plants after heat shock treatment (Fig. 5). These results indicate that the functions of *AtNRP1* in thermal adaptation may be independent of the HSF-controlled regulon.



**Fig. 3.** Characterization of the *AtNRP1* T-DNA insertion mutant and overexpressing transgenic plants. (A) Diagram of *AtNRP1* and its T-DNA insertion mutant (SALK\_111768). (B) Northern blot analysis of the *nrp1*. Each lane was loaded with 20  $\mu$ g RNA prepared from three-week-old wild-type plants (Col-0) or *nrp1*. The blot was probed with an *AtNRP1* DNA fragment corresponding to the region downstream of the T-DNA insertion in the mutant. Ribosomal RNA was used as a loading control. (C) *AtNRP1* expression in transgenic plants constitutively overexpressing *AtNRP1*. RNA samples (5  $\mu$ g) were prepared

from leaves of wild-type plants (Col-0) or eight independent transgenic *35S::AtNRP1* lines. Lines 3 and 8 expressed elevated levels of *AtNRP1*.  $T_3$  homozygous progeny plants of the two lines were used for further analyses.



**Fig. 4.** Altered responses of *35S::AtNRP1* plants to heat stress. (A) Seven-day-old seedlings of wild-type, *nrp1* and *35S::AtNRP1* lines grown on 1/2 MS solid medium were treated at 45°C for 260 min after a pretreatment for 90 min at 38°C and 120 min at 22°C. Seedlings were scored and photographed after recovery for an additional 7 days. (B) Percentage survival after heat treatment in the experiment summarized in (A). Data are presented as the mean  $\pm$  SD ( $n = 3$ ). Each replicate consisted of 50 seedlings. Data were analyzed using the Mann-Whitney *U* test; significant at \* $P < 0.05$ . (C) Hypocotyl elongation of 2.5-day-old seedlings. Seedlings were grown on vertical plates in the dark for 2.5 d and, following a pretreatment for 90 min at 38°C and 120 min at room temperature, and 220 min heat stress at 45°C, received a further 2.5 d recovery. (D) Hypocotyl length recorded after the final 2.5 days recovery in the experiment summarized in (C). Data are presented as the mean  $\pm$  SD ( $n = 3$ ). Each replicate consisted of 40 seedlings. Data were analyzed using the Mann-Whitney *U* test; significant at \* $P < 0.05$ . (E) Electrolyte leakage assay performed on 21-day-old seedlings grown in soil. The 3<sup>rd</sup>-5<sup>th</sup> leaves of WT, *nrp1* and *AtNRP1*-over-expressing lines were incubated in a 42°C water bath for 1, 3 or 6 h, and transferred to 22°C, when the bathing solution conductivity was monitored. Data are presented as the means of three experiments each with 10 plants per treatment. Bars represent the SD ( $n = 20$ ). Data were analyzed using the Mann-Whitney *U* test; significant at \* $P < 0.05$ .

### ABA content analysis

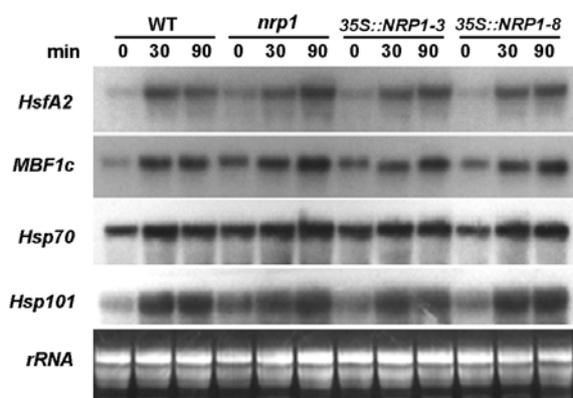
ABA can inhibit Nod factor signal transduction of resistance to various pathogens (de Torres-Zabala et al., 2007; Ding et al., 2008), and some studies have indicated that the ABA content was increased in response to heat stress (Kim et al., 2004; Zhang and Feveireiro, 2007). So we propose *AtNRP1* may function in thermotolerance through the ABA signaling pathway. One assumption is that the decreased heat tolerance of *AtNRP1*-overexpressing lines under heat stress resulted from alteration of endogenous ABA homeostasis. Quantification of ABA content in *AtNRP1*-overexpressing lines, WT and *nrp1* plants under heat stress showed that ABA content rapidly accumulated in all seedlings under heat stress after 30 min, and increased continuously for the duration of the heat-stress treatment, however, *AtNRP1*-overexpressing transgenic lines accumulated substantially lower amounts of ABA than WT and *nrp1* (Fig. 6A). We also analyzed *AtNRP1* expression in response to ABA treatment. As shown in Fig. 6B, *AtNRP1* expression decreased gradually after treatment with 100  $\mu\text{M}$  ABA. In contrast, no significant difference was observed in *AtNRP1* expression after treatment with water control. Northern blot analysis of *AtNRP1* expression in the ABA biosynthesis mutant *aba2* under heat stress showed that heat-stress-induced re-

pression of *AtNRP1* transcription was abolished, and the *aba2* have higher level of *AtNRP1* compared to WT under heat stress (Fig. 6C). These data indicate that *AtNRP1* gene may be regulated by the ABA synthesis pathway during heat stress.

### DISCUSSION

*AtNRP1* was an interacting protein of RPS2, and took part in response in restricting growth of DC3000 (*avrRpt2*<sup>+</sup>) (Quirino et al., 2004). *AtNRP1* was a nodulin-related protein, and regulated by temperature stress and ABA treatment (Figs. 1 and 6B). *AtNRP1* may play a role in tolerance to pathogens and the extreme temperatures.

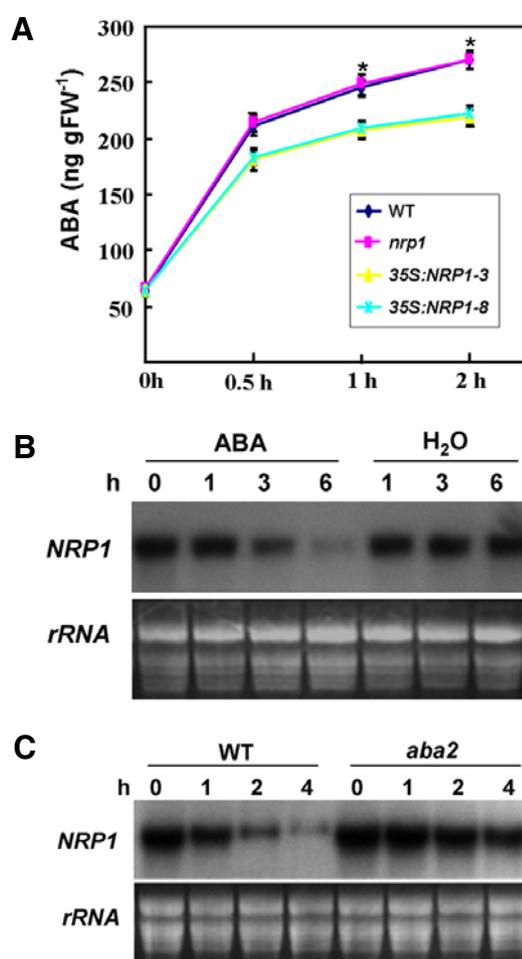
In the present study, we analyzed preliminarily the expression profile and GUS staining-histochemical location of *AtNRP1*. Northern blot showed that *AtNRP1* expression showed that *AtNRP1* was suppressed by heat treatment, however rapidly increased during cold stress (Fig. 1), suggesting it was ready to be affected by adverse ambient temperature. Extreme temperature is one of major factor which affect plant growth and development, and many genes expression had similar patterns in response to temperature factor, that is to say, the genes are induced or suppressed by high or low temperature stress (Fu et al.,



**Fig. 5.** Expression of heat-induced genes in three-week old WT, *nrp1* and *35S::AtNRP1* plants during heat treatment. Total RNA (10  $\mu$ g) was loaded into each lane and Ribosomal RNA was used as a control for RNA loading. The gene-specific fragments for *HsfA2*, *MBF1c*, *Hsp70*, and *Hsp101* were used for the probe. The experiments were repeated three times with similar results.

1998; Knight and Knight, 2001), however, *AtNRP1* was suppressed with heat treatment and induced during cold stress. Response mechanism of *AtNRP1* in high and low temperature stress may be different. Further characterization is still needed to determine the specific functions of *AtNRP1* in temperature stress. It is clear that genes affected by multiple types of stress are general and potentially confer tolerance to environmental stress (Aviezer-Hagai et al., 2007; Hazen et al., 2003). *AtNRP1* expression is affected by temperature factor (Fig. 1), suggesting that *AtNRP1* may play a role in resistance to environmental stress. GUS staining demonstrated that *AtNRP1* expressed in the sites of meristematic tissues such as the vegetative shoot apex and root tips, developing organs such as floral buds, stamens and pistils of young flowers, abscission layers of immature siliques and junctions of pedicels (Fig. 2). Considering the specificity of the expression, *AtNRP1* may play a certain function in development of *Arabidopsis* seedlings and floral organs. In addition, these tender tissues are sensitive to environmental factors and are readily to affect by stresses (Cazalé et al., 2009).

To elucidate the biological function of *AtNRP1*, *AtNRP1* null mutants and *AtNRP1*-overexpressing transgenic plants were generated (Fig. 3). Transgenic plants overexpressing *AtNRP1* exhibited less tolerance to high temperature stress than WT and *nrp1*. This reduced tolerance was illustrated by both the lower survival rate of seven-day-old seedlings and shorter hypocotyl elongation of 2.5-day-old dark-grown seedlings under heat stress (Figs. 4A and 4C). Electrolyte leakage assays also showed that the three-week-old overexpressing transgenic plants were thermosensitive compared with WT (Fig. 4E). Hypocotyl elongation, survival rate of seedling, and electrolyte leakage assay are effective parameters in analysis of heat tolerance (Clark et al., 2004; Hong and Vierling, 2000; Li et al., 2009). The *AtNRP1*-overexpressing transgenic plants showed decreased thermotolerance at different growth stages and the null mutation at the *AtNRP1* gene was the same as WT under heat stress, which indicated that the single-gene mutation of *AtNRP1* was insufficient to alter heat tolerance. There are many cases where a T-DNA KO line does not show any phenotype because of functional complementation of related genes (Cutler and McCourt, 2005; Higashi et al., 2008; Lee, et al., 2008). *AtNRP1* may have functional overlap with other heat-related genes, and these functional-overlap genes may compensate for



**Fig. 6.** Analysis of the molecular mechanisms of decreased thermotolerance in *35S::AtNRP1* plants. (A) ABA content in WT, *nrp1*, and *35S::AtNRP1* transgenic plants after heat treatment at 38°C for 0.5, 1 or 2 h. Three-week-old WT, *nrp1*, and *35S::AtNRP1* transgenic plants grown in soil were harvested after heat treatment for the indicated time periods. ABA content was determined by ELISA analysis. The results showed *35S::AtNRP1* overexpressing plants accumulated low levels of ABA with three independent experiments in the same condition. Data are presented as the mean  $\pm$  SD ( $n = 10$ ). Data were analyzed using the Mann-Whitney *U* test; significant at  $*P < 0.05$ . (B) Expression of *AtNRP1* in response to application ABA. (C) Expression of *AtNRP1* in WT and *aba2-3* during heat treatment. Total RNA (10  $\mu$ g) was loaded into each lane and Ribosomal RNA was used as the control for RNA loading. The gene-specific fragment of *AtNRP1* was used for the probe. The experiments were repeated three times with similar results.

the lack of function of *AtNRP1*, so there are no obvious phenotype differences between *nrp1* and WT under normal conditions or heat stress. Thus, we deduced that *AtNRP1* might play a negative role in thermotolerance.

This decreased thermotolerance exhibited by *AtNRP1*-overexpressing transgenic plants may be due to the decreased expression of heat-stress-inducible HSFs and HSPs genes. *AtHsfA2* is an important regulator of thermotolerance, and is required for extension of acquired thermotolerance in *Arabidopsis* (Chang et al., 2007). *AtHsp101* and *AtHsp70* are believed to play a pivotal role in thermotolerance in *Arabidopsis*

(Queitsch et al., 2000; Sung et al., 2001). The transcriptional coactivator MBF1c, is a key regulator of thermotolerance in *Arabidopsis thaliana*, and MBF1c is not required for the expression of transcripts encoding HsfA2 and different heat shock proteins in response to heat stress (Suzuki et al., 2008). Expression of *HsfA2*, *MBF1c*, *Hsp70* and *Hsp101* were characterized in among *AtNRP1*-overexpressing plants, *nrp1* and WT under heat stress. Northern blot analyses indicated that over-expressing of *AtNRP1* did not alter the expression of these known heat inducible genes. Expression levels of *HsFA2*, *MBF1c*, *HSP101* and *HSP70* are same among the over-expression, *nrp1* and WT under heat stress (Fig. 5). These results suggest that the heat sensitivity of *AtNRP1*-overexpressing plants is not conferred by these heat-induced genes, and other signaling pathways may be involved in the heat stress response of *AtNRP1*-overexpressing plants.

ABA acts through complex signalling networks to induce changes in multiple physiological processes. ABA levels increase when plants encounter adverse environmental conditions, and increased ABA content is beneficial for plants under environmental stress (Xiong and Zhu, 2003; Zhu, 2002). The accumulation of substantially amounts of ABA in *nrp1* and WT plants was higher than in *AtNRP1*-overexpressing transgenic lines (Fig. 6A), which was consistent with their relative thermotolerance phenotypes. The signals of environmental stresses trigger ABA synthesis, which in turn induces expression of various responsive genes. These downstream genes also appear to involve extensive feedback regulation of ABA accumulation (Uno et al., 2001; Verslues and Bray, 2006). The expression level of *AtNRP1* was higher in *aba2* than in wild-type under heat stress (Fig. 6C). Heat stress-induced inhibition of *AtNRP1* expression is likely to be mediated through the ABA synthesis pathway, and *AtNRP1* may affect feedback regulation of ABA accumulation in *AtNRP1*-overexpressing lines during heat stress. These results suggest that *AtNRP1* takes part in heat stress response in plants via regulation of the ABA synthesis pathway.

Although the molecular mechanisms underlying the roles of *AtNRP1* under heat stress conditions are not clearly understood, the alteration of ABA accumulation in 35S::*AtNRP1* plants compared with wild-type plants suggests a possible function of *AtNRP1* under heat-stress conditions. It appears that the change in ABA accumulation in *AtNRP1*-overexpressing plants partly contributes to the decreased heat-stress resistance of *AtNRP1* transgenic plants.

In conclusion, this paper presents these novel experimental data that increase our knowledge of the roles of *AtNRP1* in response to environmental stresses. Phenotypic analysis of *AtNRP1*-overexpressing transgenic plants and knockout mutants implies that *AtNRP1* may play a role as a negative regulator of seedling growth under heat stress. Whether the *AtNRP1*-overexpressing plants and/or the loss-of-function mutant plants exhibit altered responses to other abiotic stress conditions has not been determined. Future studies in this direction may reveal not only additional roles for *AtNRP1* in plant stress responses, but also possible interactions between their respective signaling pathways.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

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