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RESEARCH PAPER

# Activation of the *Oryza sativa* non-symbiotic haemoglobin-2 promoter by the cytokinin-regulated transcription factor, ARR1

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## Abstract

**Using *in silico* methods, several putative phytohormone-responsive *cis*-elements in the *Oryza sativa* non-symbiotic haemoglobin (*NSHB) 1-4* and *Arabidopsis thaliana NSHB1-2* promoters have been identified. An *OsNSHB2* promoter::GUS reporter gene fusion shows tissue-specific expression in *A. thaliana*. GUS expression was observed in roots, the vasculature of young leaves, in flowers, and in the pedicel/stem junction. In transient assays, activity of the *OsNSHB2* promoter was significantly up-regulated in the presence of the cytokinin, 6-benzylaminopurine (BA). Deletion analyses indicated that the full-length promoter was required for maximal *trans*-activation in the presence of cytokinin. Mutation of the single cytokinin-regulated ARR1-binding element abolished promoter activation in response to cytokinin. Constitutive expression of ARR1 under the control of the 35S cauliflower mosaic virus promoter enhanced wild-type *OsNSHB2* promoter activity, but had no effect on the activity of the mutated promoter in the absence of cytokinin. However, overexpression of ARR1 in the presence of cytokinin resulted in super-activation of the wild-type promoter. The mutated promoter was only moderately activated in the presence of cytokinin and ARR1, indicating that the *OsNSHB2* promoter can be regulated by the ARR1 protein, but requires other cytokinin-induced factors for optimal activation. This is the first report that identifies a *trans*-acting factor involved in the activation of a *NSHB* gene.**

Key words: ARR proteins, cytokinins, gene regulation, non-symbiotic plant haemoglobin.

## Introduction

Cytokinins are hormones that have been implicated in numerous aspects of plant development, including but not limited to cell division, shoot initiation and growth, and leaf senescence (Mok and Mok, 2001). Cytokinins, as well as auxins, are able to reprogramme terminally differentiated leaf cells into stem cells and maintain indefinite shoot regeneration in tissue culture, suggesting that these hormones are master regulators of plant growth and development (Bolwell *et al.*, 1995; Hwang *et al.*, 2002; Mok and Mok, 2001).

Cytokinins are sensed by histidine protein kinase receptors (CRE1, AHK2, and AHK3) that transmit signals via histidine phosphotransfer proteins to nuclear response regulators (ARRs) to activate or repress transcription. The

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members of the ARR family are classified into three subgroups, the pseudo-response regulators that lack the critical Asp acceptor site, type-A, and type-B. Type-A and type-B are subgrouped based on the architecture of their polypeptides and the cytokinin-inducible expression patterns of their transcripts (Imamura *et al.*, 1999; Kiba *et al.*, 1999). The type-A ARRs are cytokinin primary response genes that contain only a receiver domain and function as repressors of cytokinin signalling (Hwang and Sheen, 2001). The type-B ARRs contain an N-terminal receiver domain, which acts as a repressor until cytokinin-induced phosphorylation liberates it to its active conformation, and a C-terminal output domain containing a Myb-DNA binding domain (Hwang *et al.*, 2001; Sakai *et al.*, 2001). The type-B ARRs regulate the type-A ARRs, by binding to their promoters and activating transcription. Genes other than type-A ARRs that are regulated by type-B ARRs are not well understood. Recent expression profiling of cytokinin action in *A. thaliana* has revealed a subset of genes whose expression is altered by cytokinin. This work, while limited to examining the genes represented on the approximately 8300-element Affymetrix GeneChip, also identified a sequence motif enriched in the upstream regions of cytokinin-up-regulated genes (Rashotte *et al.*, 2003).

Haemoglobins (HBs) are multifunctional proteins found in a wide variety of organisms (Kundu *et al.*, 2003). Documented functions of HBs include storage and transport of oxygen (Bulow *et al.*, 1999; Giardina *et al.*, 1995), nitric oxide detoxification (Bulow *et al.*, 1999; Wittenberg *et al.*, 2002), and facilitating oxygen diffusion in symbiotic tissues (Jacobsen-Lyon *et al.*, 1995). While the physiological functions for several HBs have been identified, the function(s) of ubiquitous plant non-symbiotic haemoglobins (NSHBs) are largely unknown (Kundu *et al.*, 2003). Plants contain three known types of HBs, the symbiotic (SYMHBs), which facilitate oxygen diffusion in the nitrogen-fixing nodule, truncated HBs of unknown function, and NSHBs. The X-ray crystallographic structures of the NSHBs are strikingly similar to the SYMHBs (Hargrove *et al.*, 2000), however, these proteins differ in their biochemical properties. The NSHBs are divided into two groups; class 1 exhibit very tight oxygen binding (Arrendondo-Peter *et al.*, 1997; Hargrove *et al.*, 2000) and class 2 exhibit oxygen binding kinetics more similar to the SYMHBs (Kundu *et al.*, 2003; Trevaskis *et al.*, 1997). Therefore, the regulation of the *NSHB* genes is currently being pursued to further understand their potentially different physiological roles.

NSHBs are encoded by multigene families, which suggest multiple or essential functions. Four *NSHB* isogenes are found in two clusters in the *Oryza sativa* genome: *OsNSHB1/3/4* and *OsNSHB2* (Lira-Ruan *et al.*, 2001), and *Arabidopsis thaliana* contains two *NSHB*s (Hunt *et al.*, 2002). Each gene has its own separate and distinct promoter, signifying potential differential expression of these genes. Overexpression of *AtNSHB1* (class 1) can protect *A. thaliana* plants from hypoxia and enhance seedling growth, whereas *AtNSHB2* (class 2) expression is enhanced by addition of exogenous cytokinins (Hunt *et al.*, 2002). The molecular mechanisms behind these physiological observations have yet to be elucidated.

Tissue expression patterns dictated by a number of dicotyledonous *NSHB* upstream regulatory regions (promoters) have been analysed by transgenic expression of promoter::reporter gene fusions. The activities of *NSHB* promoters of *Parasponia andersonii* and *Trema tomentosa* were localized to the root meristem and vascular cylinder of transgenic tobacco (Bogusz *et al.*, 1990). The *Casuarina glauca NSHB* promoter was primarily active in *L. corniculatus* roots, including tip meristems, the vascular stele, and the parenchyma internal to the endodermis (Jacobsen-Lyon *et al.*, 1995). *AtNSHB1* and *2* promoters are active in distinct tissues under normal conditions and respond differentially to specific environmental stimuli, such as hypoxia (*AtNSHB1*) and cytokinin treatment (*AtNSHB2*) (Hunt *et al.*, 2001). These results underscore the necessity for a detailed understanding of specific promoter regulation *in planta*. Such studies are critical in deciphering the functions of individual NSHB proteins *in vivo*.

Monocot NSHBs have been immunologically localized to differentiating tissues, such as the vasculature and root cap cells, and germinating seeds (Arechaga-Ocampo *et al.*, 2001; Lira-Ruan *et al.*, 2001; Ross *et al.*, 2001). However, expression of individual monocot *NSHB* genes (class 1) is not known. Based on the expression of individual dicot *NSHBs*, it is likely that monocot *NSHBs* are also differentially expressed.

In this work, a sequence-based approach has been taken to identify putative *cis*-elements that regulate the expression of the *OsNSHB1-4* and *AtNSHB1* and *2* genes. All of the known *Os* and *At NSHB* promoters contain *cis*-regulatory elements implicated in hormone responses. Moreover, the cytokinin-activated ARR1-binding-*cis*-regulatory element was prevalent. Deletion and site-directed mutational analyses of a predicted ARR1-binding *cis*-element in the *OsNSHB2* promoter confirmed its functionality in cytokinin response. Co-expression studies of promoter::*GUS* fusions with the transcription factor *ARR1* (D'Agostino *et al.*, 2000) up-regulated the *OsNSHB2* promoter in the absence of cytokinin. Therefore, *OsNSHB2* can be added to the growing list of genes (other than type-A ARRs) regulated by the type-B ARRs (Rashotte *et al.*, 2003).

## Materials and methods

### *Analysis of non-symbiotic haemoglobin promoters*

Promoter sequences for *Oryza sativa NSHB1*, *3* and *4*, and *NSHB2* (Accession nos. AF335504 and AF335503, respectively) and *Arabidopsis thaliana NSHB1* and *2* (Accession nos. U94998 and U94999 [PUBMED] and At2g16060 and At3g10520 [Arabidopsis database],

respectively) were analysed for known consensus *cis*-regulatory sequences utilizing the Signal Scan Search technology (Higo *et al.*, 1999), available at the PLACE website (http://www.dna.affrc.go.jp/htdocs/PLACE/). In addition, manual searches were performed for the sequence AGATT found in the promoter of ARR6 (Sakai *et al.*, 2001).

### *Cloning of the* O. sativa NSHB2 *5′-upstream regulatory sequence*

*O. sativa* var. Jackson genomic DNA was extracted from 5 g of plant tissue using previously described methods (Dellaporta *et al.*, 1983). The predicted 960 bp *OsNSHB2* promoter region was amplified using 1 nM of both a forward primer engineered with a *Hin*dIII restriction site (underlined): 5′-AAGCTTATGGGTCGTTCTCGAAGGCT-3′, and a reverse primer (Hb2Pro.RVS) engineered with an *Eco*RI restriction site (underlined): 5′-GAATTCTTAAGAGGTTGCTTC-CT-3′. PCR reactions were performed at an annealing temperature of 55 °C in a solution containing approximately 5 ng of *O. sativa* genomic DNA, 1 nM dNTPs, 1 mM $MgCl_2$, 1× PCR buffer (Invitrogen) and 1 unit of *Taq* DNA polymerase (Invitrogen) in a final volume of 25 µl. The 'full-length' promoter was denoted *HB2p*.

Deletions from the 5′ end of *HB2p* were generated by PCR. A truncated 652 bp promoter (*Δ960/652p*; Δ960 to 653) was amplified using a forward primer engineered with a *Hin*dIII site (5′-AAG-CTTCCCAGTTAACGAGGAAACGA-3′) and the Hb2Pro.RVS primer. The 272 bp promoter (*Δ960/272p*; Δ960 to 273) was amplified using a *Hin*dIII-engineered forward primer (5′-AAGCTT-GATATCCGCC-GTAGTCTCCA-3′) and the Hb2Pro.RVS primer. The predicted ARR1-binding element (AGATT; (Sakai *et al.*, 2001) of the wild-type *HB2p* was mutated to a *Bam*HI site (GGATCC) using the megaprimer method (Sarkar and Sommer, 1990), resulting in the *hb2p* mutant promoter. The primer used to mutate this site was 5′-GCTGCATCCGGCACAGGATCCTTCGGCTCGAATGGA-3′. The resulting promoters *HB2p*, *Δ960/652p*, *Δ960/272p*, and *hb2p* were cloned into PCR2.1-TOPO (Invitrogen), and the sequences were verified at the UNL-DNA Sequencing Facility.

### *Construction of* NSHB2 *promoter fusions to the* GUS *reporter gene*

*OsNSHB2* promoter constructs were ligated into the 5′- *Hin*dIII and 3′- *Eco*RI sites of the plasmid pPTN134 (obtained from the UNL-Plant Transformation Facility, http://www.biotech.unl.edu/) immediately upstream of the *Escherichia coli* β-glucuronidase gene (*GUS*). The resulting plasmids, denoted *HB2p*::*GUS*, *Δ960/652p*::*GUS*, *Δ960/272p*::*GUS*, and *hb2p*::*GUS*, were used for transient bombardment experiments. All plasmids were maintained in *E. coli* XL1-Blue cells (Stratagene).

### *Plant transformation of* A. thaliana *with the* HB2p::GUS *construct*

The *HB2p::GUS* construct was ligated into the binary vector pZP212 (Hajdukiewicz *et al.*, 1994) to generate plasmid pZP2P. pZP2P was introduced into *Agrobacterium tumefaciens* strain C58C1 (pMP90) (Koncz and Schell, 1986) using triparental mating (Ditta *et al.*, 1980) with the pRK2013 plasmid in helper *E. coli* HB101 (Horsch *et al.*, 1985). pZP2P was transformed into *A. thaliana* (Col-0) using the floral dip method (Bechtold *et al.*, 1993). Transformed plants were selected on MS plates supplemented with kanamycin containing 100 mg $l^{-1}$ cefotaxime in 4% (w/v) top agar. The $T_2$ and $T_3$ populations of *A. thaliana* were harvested and analysed for GUS activity by incubating plant organs in the GUS substrate 5-bromo-4-chloro-3-indoyl-β-glucuronic acid for 3 h at 37 °C and washing three times with 70% (v/v) ethanol. Light microscopy was performed on a Nikon SMZ800 microscope.

### *Detection of native* Hb2 *transcripts in* O. sativa *and* GUS *transcripts in stably transformed* A. thaliana *plants*

*O. sativa* var. Jackson and *A. thaliana* plants harbouring the *Hb2p*::GUS construct were sprayed in triplicate with approximately 5–10 ml of either $dH_2O$+0.05% Tween-20 or 3 mg $l^{-1}$ 6-benzylaminopurine+0.05% Tween-20 at 0 h and 1 h. Approximately 200 mg of tissue was harvested at 0, 1, and 2 h from each plant. Total RNA was isolated using Trizol (Invitrogen). A cDNA mix was obtained using reverse transcriptase (Ambion) and random hexamer primers from a starting concentration of 1 µg RNA. Samples were standardized using an 18S RNA primer pair (Ambion). Native *Hb2* transcripts in *O. sativa* were analysed using the specific oligonucleotide primers F: 5′-CCATGGCTCTCGTG-GAGGGAAACAACGGCGTGTCGGGGGGA-3′ and R: 5′-CAA-TTCTCACTCAGCAGGCTTCA-3′ to generate a 477 bp product. GUS transcripts in *A. thaliana* were analysed using the oligonucleotide primers F: 5′-CCACGCCGAACACCTGGGTGGACG-3′ and R: 5′-GAGAGGTTAAAGCCGACAGCTGC-3′ to generate a 546 bp product. PCR reactions were 95 °C 2 min, 35 cycles of 95 °C 30 s, 60 °C 30 s, 72 °C 2 min, and 72 °C for 5 min. Transcripts were analysed on a 1% agarose gel.

### *Cloning of the* A. thaliana *ARR1 cDNA*

The 1863 bp coding sequence of the *ARR1* gene was amplified from a reverse-transcribed cDNA mixture from various tissues of *A. thaliana* (Col-0) using 1 nM of a forward primer engineered with a *Nco*I restriction site (underlined): 5′-CCATGGCATTG-TCTCTGCTCCGGAAGAACAACATGG-3′ and a reverse primer engineered with a *Bam*HI site (underlined): 5′-GGATCCTCAAC-CGGAATGTTATCGATGGAGTATGCG-3′. PCR reactions were performed for 40 cycles at an annealing temperature of 58 °C in a solution containing approximately 5 ng of cDNA, 3% (v/v) DMSO, 1 nM dNTPs, 1 mM $MgCl_2$, 1× PCR buffer, and 1 unit of *Taq* Precision Plus polymerase (Stratagene) in a final volume of 25 µl. The amplified product was ligated into PCR2.1 TOPO (Invitrogen) and verified by DNA sequencing. The amplified *ARR1* gene was then ligated into the *Nco*I and *Bam*HI sites of pPTN134 to produce the *35S::ARR1* construct.

### *Transient transformation of* NSHB2 *promoter::*GUS *constructs*

The *35S*::*GUS* (positive control), *35S* alone (negative control), *HB2p*::*GUS*, *Δ960/652p*::*GUS*, *Δ960/272p*::*GUS*, or *hb2p*::*GUS* were introduced into sterilized tobacco (*Nicotinana tabacum* cv. Xanthii) leaf discs using microprojectile bombardment with the PDS-1000 He Biolistic Particle Delivery System (BioRad) at 1100 psi. Leaf discs had been previously incubated for 48 h on either MS agar (Murashige and Skoog, 1962) or MS agar containing 3 mg $l^{-1}$ 6-benzylaminopurine (BAP) (Sigma) under an 18/6 h light/dark period at 24 °C. Bombardments were performed in triplicate with 1.25 µg plasmid DNA per treatment coated on tungsten particles. The leaf discs were incubated under similar conditions as above and harvested 48 h following bombardment. Extracts of the discs were analysed using a quantitative GUS assay performed with 1 mM methylumbelliferyl glucuronide (MUG) as substrate (Jefferson, 1987). GUS activity was expressed as nmol of 4-methylumbelliferone (4-MU) released mg $protein^{-1}$ $min^{-1}$. Protein concentrations were determined using the BCA protein assay (Pierce) using bovine serum albumin as a standard.

Statistical analysis was conducted using the PROC-GLM program of SAS 8e. Analysis of variance was performed for each treatment. Means were separated at the 5% probability level with the LSD test when a significant *F* ratio occurred ($P \geqslant 0.05$). Significant differences are indicated by different letters above the bars.

## Results

### *Detection of consensus* cis-*element regulatory sequences in the upstream promoter regions of the* O. sativa *and* A. thaliana NSHB *genes*

The sequences of the *O. sativa* and *A. thaliana NSHB* promoters were analysed for potential consensus sequences using the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/), a collection of known plant regulatory promoter elements reported in the literature (Higo *et al.*, 1999). Because all known regulatory sequences are not found in the PLACE database, a pattern search was used to identify the cytokinin-responsive ARR1-binding element (AGATT; Sakai *et al.*, 2000). The *Os* and *At NSHB* promoters (approximately 1 kbp upstream of the translation start sites) contain a number of putative *cis*-elements known to confer regulation by plant hormones (Fig. 1). In particular, the cytokinin-responsive ARR1-binding element (Oka *et al.*, 2002; Sakai *et al.*, 2000) was found in most *NSHB* promoters analysed, and the consensus site determined by expression profiling (GATCTT; Rashotte *et al.*, 2003) was found in the *OsNSHB4* promoter. Each *NSHB* promoter also contains at least one W-box (TTTGACT) (Eulgem *et al.*, 2000), involved in response to plant defence signalling (Eulgem *et al.*, 1999; Yu *et al.*, 2001). A GCC-box (AGCCGCC) was only detected in *OsNSHB*2. This element is involved in an ethylene response to pathogens (Ohme-Takagi *et al.*, 2000) and abiotic stress (Fujimoto *et al.*, 2000). Each of the *OsNSHB* promoters, but not those from *A. thaliana* contain at least one ACG box (AACGTT), which bind members of the abscisic acid-responsive bZip family of transcriptional activators (Hobo *et al.*, 1999; Izawa *et al.*, 1994; Toyofuku *et al.*, 1998). The presence of these candidate promoter elements suggests that the *O. sativa* and *A. thaliana NSHB* genes may be hormonally regulated.

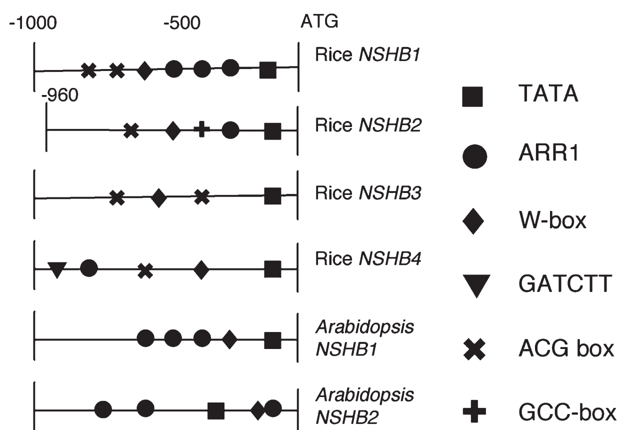


**Fig. 1.** The *O. sativa* and *A. thaliana* non-symbiotic haemoglobin genes possess a number of predicted *cis*-regulatory elements in their promoter regions. Shown are the predicted *cis*-regulatory regions upstream of the *OsNSHB1-4* and *AtNSHB1* and *2* coding sequences. Computational analyses of *O. sativa* and *A. thaliana* sequences were performed using analysis tools from the PLACE website (http://www.dna.affrc.go.jp/htdocs/PLACE/) and manual searches for *cis*-regulatory elements not present in the database. Black spot, ARR1 binding element; black square, TATA box; black cross, ACG-box; plus sign, GCC-box; black diamond, W-box; black inverted triangle, GATCTT.

### *Activity of the* O. sativa NSHB2 *gene promoter in transgenic* A. thaliana

The activity of a monocot (*O. sativa*) promoter in a dicot (*A. thaliana*) has been evaluated to address the conservation of *NSHB* promoter regulation *in planta*. The activities of the *AtNSHB1* and *2* promoters in transgenic *A. thaliana* tissues were generated using promoter::GUS fusions (Hunt *et al.*, 2001). Similarly, activity of the *OsNSHB2* gene promoter (*HB2p*) was analysed in transgenic *A. thaliana* using *GUS* as a reporter gene. The *OsNSHB2* promoter was chosen over *OsNSHB1*, *3*, and *4* based on its unique location on the *O. sativa* genome (Lira-Ruan *et al.*, 2001), and because it contains a single putative ARR1-binding element (Fig. 1), permitting evaluation of this single *cis*-element without interference from the other identical *cis*-acting elements.

In the $T_2$ and $T_3$ generations of plants harbouring the *OsHB2p::GUS* construct, light microscopy showed strong GUS staining in root vasculature, root cap cells, and root hairs throughout plant development (Fig. 2A–C). GUS activity was observed in the cotyledon and leaf vasculature during early development (Fig. 2D, E), but is absent in cauline leaves (Fig. 2F). In young flowers, GUS expression in the short style was observed prior to the opening of the bud (Fig. 2G), and throughout flower development (Fig. 2G–I). In inflorescences with open flowers, GUS staining was evident in the vasculature of the sepals, petals, and filaments (Fig. 2G, H). In addition, elevated GUS activity was detected in pollen throughout pollen maturation and anther dehiscence (Fig. 2H). GUS activity was also evident in pedicel abscission zones in late flower development through silique formation (Fig. 2I).

Using confocal laser-scanning microscopy to visualize *HB2p::GUS* expression, GUS activity was again evident in the abscission zone of the pedicel (Fig. 2J). Staining was also observed in the nectaries (Fig. 2K), and in the short style throughout development of the flower and maturation of the silique (Fig. 2L). Finally, in areas of the plant wounded during excision, GUS staining was pronounced, indicating that the *HB2p::GUS* was activated at wound sites (Fig. 2M). Expression of the *OSNSHB2* promoter overlaps with the activities of the promoters of both *AtNHSB1* and *2* (Hunt *et al.*, 2001). These data suggest that monocot and dicot *NSHB* regulation is similar and that molecular components necessary for expression of a monocot promoter are conserved in a dicot.

### *Activation of the* O. sativa NSHB2 *promoter by exogenous cytokinin application*

Putative ARR1 binding elements are found in all of the *NSHB* promoters analysed (one is located greater than 1

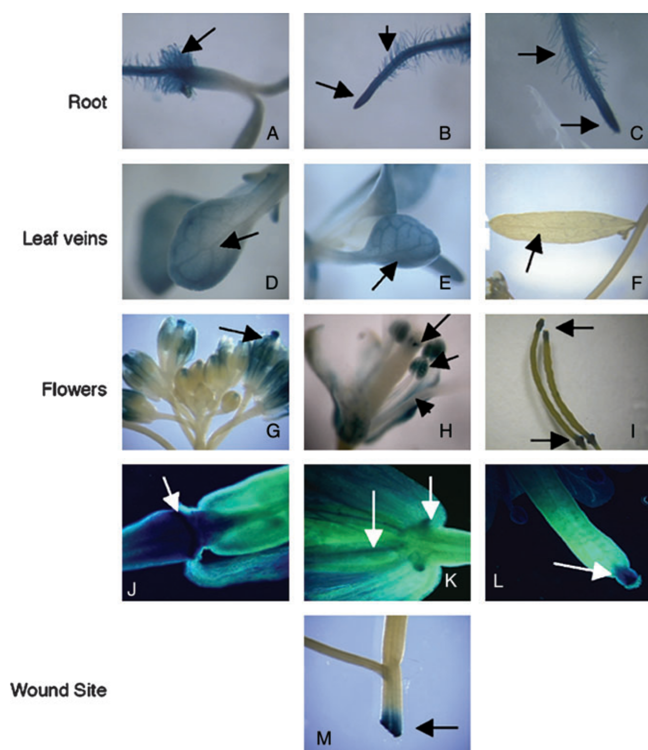


**Fig. 2.** The *OsNSHB2* promoter is active in *A. thaliana*. Expression of the *OsNSHB2* promoter::*GUS* construct in *A. thaliana* (Col-0) $T_2$ and $T_3$ plants was analysed by staining with GUS substrate (X-Gluc) and visualized using a light microscope (A–I) and a confocal laser scanning microscope (J–M). (A) Young roots; junction of root and shoot; (B) young root tips; (C) cauline-leaf stage root tips; (D) cotyledons; (E) primary leaves; (F) cauline leaves; (G) young inflorescences; (H) an inflorescence at anthesis; (I) maturing siliques; (J) mature floral organ abscission zone; (K) nectaries and anther vasculature; (L) stigma and style; and (M) stem wound site. Arrows indicate areas of significant GUS staining.

kbp upstream of the *OsNSHB3* translational start site; data not shown). The activation of a type-A cytokinin-responsive gene *ARR6* in *A. thaliana* occurs through the binding of the transcription factor ARR1 to the ARR1-binding elements found in the *ARR6* promoter. Three ARR1-binding elements exist in the 200 bp region upstream from the *ARR6* translation start site (Sakai *et al.*, 2001), however, it is not known if all the ARR1-binding elements are required or bind to the ARR1 protein during cytokinin activation of the *ARR6* gene. Only one putative ARR1-binding element was identified in the 960 bp *OsNSHB2* promoter. The presence of only one ARR1 binding element in the *OsNSHB2* promoter allowed it to be directly tested whether a single element was adequate for gene activation by ARR1 binding to its cognate site on the *OsNSHB2* promoter.

To analyse the *OsNSHB2* promoter response to cytokinin, *HB2p::GUS* was used in transient transformation assays via particle bombardment. Sterile tobacco leaf discs were placed on MS agar (control) or MS agar supplemented with 6-benzylaminopurine (BA). Leaf discs were individually bombarded with (i) the negative control plasmid, containing the CAMV *35S* promoter, but no *GUS* gene; (ii) a positive control containing *35S*::*GUS*; or (iii) a plasmid containing the *HB2p*::*GUS* construct. GUS activity was determined 48 h after bombardment. Figure 3 indicates that basal GUS activity for tobacco discs incubated on MS medium and bombarded with *35S*, *35S::GUS*, and *HB2p::GUS* was $1.5\pm0.3$, $11.4\pm1.1$, and $13.0\pm1.0$ nmol $mg^{-1}$ protein $min^{-1}$, respectively. The activity measured with *35S::GUS* and *HB2p::GUS* were significantly different from *35S* alone ($F$=25.5; df=5, 17; $P$<0.0001). Inclusion of BA in the incubation medium increased GUS activity in leaf discs bombarded with the *HB2p::GUS* construct to $37.5\pm10$ nmol $mg^{-1}$ protein $min^{-1}$, compared with $1.0\pm0.6$ and $11.4\pm3.6$ nmol $mg^{-1}$ protein $min^{-1}$ for discs bombarded with plasmids containing the *35S* and *35S::GUS* constructs, respectively. Therefore, BA application had a significant effect on the activity of the *HB2* promoter (3-fold) and no effect on the *35S* promoter (Fig. 3). These results are consistent with activation of the *OsNSHB2* promoter through a cytokinin-regulatory element, like that of the putative ARR1-binding element (Fig. 1).

To identify the specific element of the *OsNSHB2* promoter that was mediating the cytokinin activation, deletions or mutations of *HB2p* fused to the *GUS* reporter gene were analysed using transient bombardment assays. Figure 4A shows the regions of the promoter that were either deleted or mutated. The first deletion (Δ*960/652p*) removed the ACG box upstream at −689 bp. The second deletion (Δ*960/272p*) removed all of the identified canonical *cis*-acting elements including the W-box (−448 bp), GCC-box (−436 bp) and ARR1-site (−290 bp). To determine if the ARR1-binding element was primarily responsible for the observed cytokinin activation, the ARR1-binding element was mutated to a *Bam*HI restriction site (AGATT→GGATCC) to generate the mutant promoter *hb2p*. This mutation was confirmed by DNA sequencing and restriction enzyme analysis (data not shown).

GUS activity driven by the different promoter constructs in tobacco leaf discs incubated on MS agar supplemented with BA is shown in Fig. 4B. Tissue bombardment with the negative control construct (*35S)* showed minimal GUS activity ($1.0\pm0.6$ nmol $mg^{-1}$ protein $min^{-1}$). By contrast, bombardment with the positive control construct, *HB2p::GUS*, resulted in an approximately 30-fold increase in GUS activity ($33.5\pm3.2$ nmol $mg^{-1}$ protein $min^{-1}$), consistent with results obtained for this construct in earlier experiments (Fig. 3). Mutation of the ARR1-binding site (*hb2p*) significantly diminished GUS activity in the presence of BA ($7.3\pm3.2$ nmol $mg^{-1}$ protein $min^{-1}$), a 4.6-fold decrease in GUS activity compared with the activity observed in discs bombarded with the full length *HB2p*::*GUS* vector ($F$=20.16; df=4, 14; $P$<0.0001). Similarly, deletion of the putative ACG-box alone (Δ*960/652p::GUS)* caused a ∼2-fold decrease in GUS activity, which was significantly different from the GUS activity observed for all the other

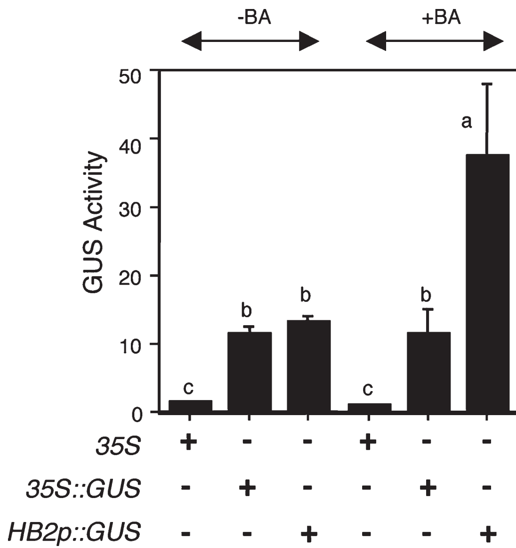


**Fig. 3.** The cytokinin, BA, activates the *OsNSHB2* promoter::*GUS* fusion in transient transformation assays. Tobacco discs incubated on either MS agar or MS agar+BA were bombarded with constructs containing *35S*; *35S::GUS*, or *HB2p::GUS*. GUS activity is expressed as nmol of 4-MU released $mg^{-1}$ protein $min^{-1}$. Data from three independent experiments were pooled and analysed. Error bars indicate standard deviation. Means indicated by the same letter are not significantly different ($P \geqslant 0.05$, LSD-test). Main effect: $F=25.5$; df=5, 17; $P < 0.0001$.

promoter elements (Fig. 4B). As expected, removal of all the identified putative *cis*-acting elements (*Δ960/272p::GUS)* resulted in GUS activity similar to the *35S* negative control, 2.8±2.2 versus 1.0±0.6 nmol $mg^{-1}$ protein $min^{-1}$ (Fig. 4B). Analysis of *hb2p::GUS*, *Δ960/652p::GUS*, and *Δ960/272p::GUS* in the absence of BA resulted in GUS activity with no significant difference with the negative control lacking the *GUS* gene (Fig. 4B). The *HB2p::GUS* shows some activity in the absence of BA, similar to the results shown in Fig. 3, whereas the mutated and deleted versions have less. The significance levels for the negative control in the treatment lacking and containing BA were equivalent, as seen in Fig. 3. These data demonstrated a striking activation of the *HB2p::GUS* construct in the presence of cytokinins that was abolished by mutation or removal of the ARR1-binding site.

ARR1-responsive genes are induced fairly rapidly upon perception of the cytokinin signal (Hutchinson and Keiber, 2002; Oka *et al.*, 2002; Sakai *et al.*, 2001). To verify that a similar response was occurring in the *HB2p::GUS* transformed plants, plants were sprayed with water or with 3 mg $l^{-1}$ BA in the presence of a wetting agent, Tween-20. All above-ground plant parts were harvested immediately following spraying, and 1 h and 2 h post-treatment. Cytokinin application enhanced *GUS* transcript levels within 1 h and were substantially elevated after 2 h, compared with the water controls (Fig. 4C). These data confirmed the early response of the *HB2p* promoter to exogenous cytokinins *in planta*. Accumulation of *HB2* transcripts was also detected in the native rice species using the same procedure (Fig. 4D).

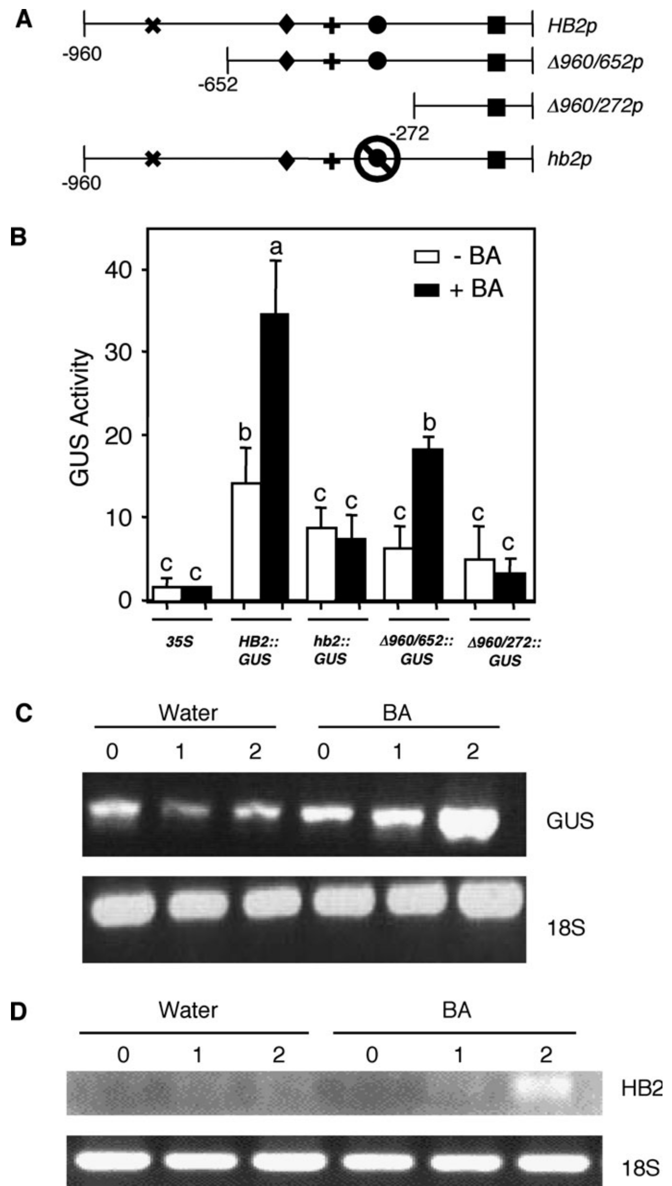


**Fig. 4.** Deletion or mutation of the *OsNSHB2* promoter affects activation by cytokinin. (A) Deletion and mutational strategy for the *OsNSHB2* promoter region. Promoters are designated as wild-type (*HB2p*); deleted 308 bp (*Δ960/652p*) and 688 bp *Δ960/272p*)*;* and full-length promoter with mutated ARR1-binding site (*hb2p*). Black spot, ARR1 binding element; black square, TATA box; black cross, ACG-box; plus sign, GCC-box; black diamond, W-box. (B) Cytokinin (BA) activation of derivations of the *OsNSHB2* promoter, analysed by transient bombardment assays. Tobacco discs were incubated on MS agar+BA and bombarded with either *35S*, *Δ960/272p::GUS*, *Δ960/652p::GUS*, *hb2p::GUS*, or *HB2p::GUS* constructs. GUS activity is expressed as nmol of 4-MU released $mg^{-1}$ protein $min^{-1}$. Data from three independent experiments were pooled and analysed. Error bars indicate standard deviation. Means indicated by the same letter are not significantly different ($P \geqslant 0.05$, LSD-test). Main effect: $F=20.16$*;* df=4, 14*;* $P<0.0001$. (C) Activation of GUS in of *HB2p*::GUS carrying *A. thaliana* plants. Plants were sprayed with distilled water containing 0.05% Tween-20 (Water) or 3 mg $l^{-1}$ BA containing 0.05% Tween-20 (BA). Total RNA was extracted at 0, 1, and 2 h after spraying and an RT reaction performed using GUS specific primers that would yield a 546 bp product. Loading was standardized using an 18S primer pair. (D) Activation of *HB2* by cytokinin in native *O. sativa*. Plants were treated in a similar manner as in

### *Activation of* O. sativa HB2p *promoter by co-bombardment with* 35S::ARR1

To investigate the role of the ARR1-binding element in the cytokinin-induced activation of the *OsNSHB2* promoter further, co-bombardment with the *AtARR1* gene was performed (Fig. 5). Tobacco leaf discs were co-bombarded with either *HB2p::GUS* or *hb2p::GUS* and *35S* or *35S::ARR1* on MS agar and MS agar supplemented with BA. Levels of GUS activity in discs maintained on MS medium alone and co-bombarded with *HB2p::GUS+35S*, *hb2p::GUS+35S*, or *hb2p::GUS+35S::ARR1* constructs were 7.8±4.9, 6.2±3.8, and 6.4±3.8 nmol $mg^{-1}$ protein $min^{-1}$, respectively (Fig. 5). These values were statistically similar to those obtained from tobacco leaf discs maintained on MS medium supplemented with BA and co-bombarded with the *hb2p::GUS+35S* constructs ($F$=53.89: df =7, 47; $P$<0.0001). Co-expression of *ARR1* in leaf discs maintained on MS medium alone enhanced GUS activity driven only by the wild-type *HB2* promoter. A 3-fold increase was observed in leaf discs maintained on BA-supplemented MS medium and co-bombarded with the *HB2p::GUS+35S* constructs (15.2±2.9 versus 21.9±6.8 nmol $mg^{-1}$ protein $min^{-1}$, respectively), indicating that there was a positive relationship between expression of the *ARR1* gene and activity of *HB2p*. There was no effect of co-bombardment of *ARR1* and the mutated *hb2p::GUS* constructs in the absence of BA (Fig. 5), suggesting that only the wild-type *HB2* promoter was able to respond to excess ARR1 in tobacco tissues.

In the presence of BA, co-bombardment of leaf discs with *35S::ARR1* with either the *HB2p::GUS* or *hb2p::GUS* constructs significantly enhanced GUS activity compared with the other treatments (Fig. 5). The highest level of GUS activity, 60.1±10.8 nmol $mg^{-1}$ protein $min^{-1}$, was observed in leaf discs incubated on MS agar supplemented with BA and co-bombarded with the wild-type, full-length promoter and *35S::ARR1*. Unexpectedly, in the presence of BA, coexpression with *ARR1* had a positive impact on GUS activity (34.0±7.9 nmol $mg^{-1}$ protein $min^{-1}$) driven by *hb2p*, with a mutated putative ARR1-binding element. In both instances there was an approximate 3-fold activation compared with the respective controls.

## Discussion

Plant non-symbiotic haemoglobins are encoded by multigene families, in species with significant genomic and/or EST sequences available. This observation raises the question of whether these proteins are essential (i.e. redundant) or have multiple physiological functions. Regardless, a better understanding of the expression patterns of different isoforms is essential to elucidating their individual physiological functions *in planta*.

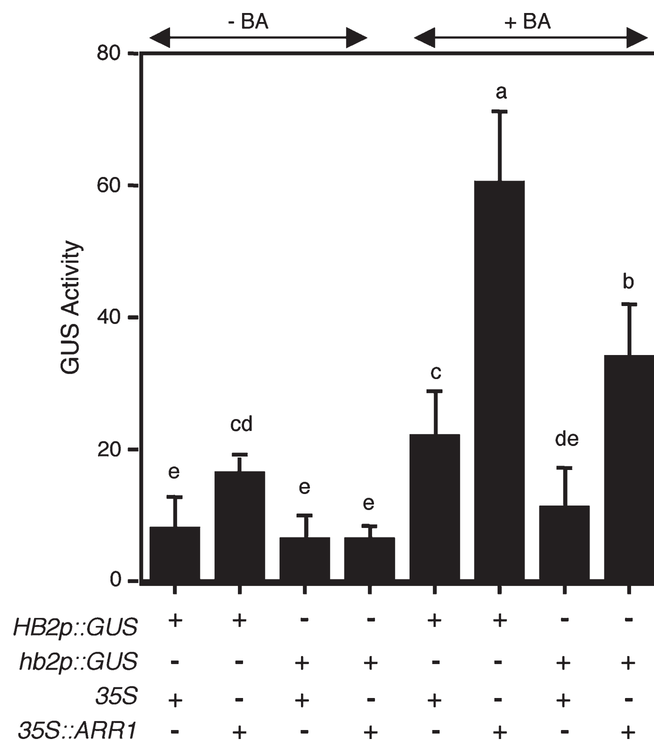


**Fig. 5.** Overexpression of the *A. thaliana* cytokinin response regulator, ARR1, activates the *NSHB2* promoter in the absence of exogenous cytokinin. Co-bombardment analysis of the wild-type and mutated *OsNSHB2* promoters in the absence and presence of *A. thaliana ARR1* under the control of the cauliflower mosaic virus *35S* promoter. Assays were conducted with *HB2p::GUS* or *hb2p::GUS* and *35S* or *35S::ARR1*. GUS activity is expressed as nmol of 4-MU released $mg^{-1}$ protein $min^{-1}$. Data from six independent experiments were pooled and analysed. Error bars indicate standard deviation. Means indicated by the same letter are not significantly different ($P$⩾0.05, LSD-test). Main effect: $F$=53.89; df=7, 47; $P$<0.0001.

*In silico* analyses of *O. sativa* and *A. thaliana NSHB* promoters (1 kbp upstream of the translation start sites) has revealed a number of putative *cis*-elements, implicating that *NSHB* expression may be regulated in part through hormones and abiotic stresses (Fig. 1). These elements include putative binding sites for the Arabidopsis Response Regulators (ARRs), specifically the type-B ARR1. ARR1 has been shown to be involved in early responses to cytokinins (Oka *et al.*, 2002; Sakai *et al.*, 2001). There are three major classes of ARRs: (i) the pseudo-response regulators that lack the critical Asp acceptor site; (ii) the type-A ARRs which lack a DNA binding domain and function as repressors of cytokinin signalling; and (iii) the type-B ARRs, which contain Myb-like DNA binding domains and function as transcriptional activators of cytokinin-induced gene expression (Haberer and Kieber, 2002; Hwang *et al.*, 2002; Imamura *et al.*, 1999).

The presence of these elements is consistent with previous reports regarding the activity of the *NSHB* promoters

Fig. 4C. RT reaction performed using *HB2*-specific primers that would yield a 477 bp product. Loading was standardized using an 18S primer pair.

*in planta*. For instance, cytokinin was shown to activate some *NSHB*s (Hendriks *et al.*, 1998; Hunt *et al.*, 2001), and hypoxia induces expression from the *AtNSHB1* promoter (Hunt *et al.*, 2001; Sowa *et al.*, 1999). It is likely that a combination of these *cis*-elements and their cognate *trans*-acting factors are required for the activation/deactivation of these promoters *in planta*.

The organ and tissue patterns of GUS activity driven by the *OsNSHB2* promoter in *A. thaliana* (Fig. 2) were largely similar to those observed for plants transformed with *AtNSHB1* and *AtNSHB2* promoter::*GUS* constructs (Hunt *et al.*, 2001), suggesting conservation of signal transduction pathways in monocots and dicots. The *AtNSHB1* promoter is activated during germination, in the roots and root tips of mature plants and under hypoxic conditions. By contrast, the *AtNSHB2* promoter drives GUS activity in older plants, in floral tissues, and in response to cytokinins (Hunt *et al.*, 2001). In *A. thaliana*, the *OsNSHB2* promoter-driven *GUS* expression occurs in cell types undergoing cellular differentiation or sensing some aspect of the environment (Fig. 2). The promoter activity correlates well with the localization of OsNSHB proteins in several differentiating cell types (Ross *et al.*, 2001). *OsNSHB2* activity was also abundant at the sites of tissue excision where a wound response has been initiated (Bouquin *et al.*, 1997; Peck and Kende, 1998; Rushton, 2002), indicating that endogenous hormonal status regulates expression of the *OsNSHB2* gene.

Analysis of transient bombardment assays indicates that cytokinin significantly (3-fold) activates the *OsNSHB2* promoter (Fig. 3). These data are within a similar activation range of ARR1 and ARR2 to other target sequences (Sakai *et al.*, 2000). Partial deletion (Δ*960/652p*) confers significantly less activation in the presence of cytokinin, deletion of the promoter to a minimial promoter (Δ*960/272p*), and mutation of the putative ARR1 binding site (*hb2p)* abolish cytokinin activation of the *OsNSHB2* promoter (Fig. 4). These data suggest that the *OsNSHB2* promoter is, in part, activated by a cytokinin-induced transcription factor or transcriptome, and that the putative ARR1-binding site is involved in this cytokinin-related transcriptional activation. Co-transformation of *ARR1* and the wild-type promoter resulted in enhanced activation in the absence of cytokinin, while a similar experiment with the mutant promoter resulted in the abolition of promoter activation. In the presence of cytokinin, activation of these promoters with ARR1 was 3-fold higher (Fig. 5) in both cases. These data indicate that other cytokinin-induced *trans*-acting factors are involved in the activation of both promoters (Rashotte *et al.*, 2003).

Mutation of the reported ARR1 binding site found in the promoters of *OsNSHB2* (this work) and *A. thaliana ARR6*, AGATT (Sakai *et al.*, 2001), to a *Bam*HI site, GGATCC, might not be expected to abolish DNA binding by ARR1, based on previous electrophoretic mobility shift assay (EMSA) analyses (Sakai *et al.*, 2000), in which the authors concluded that the central 'GAT' was critical for binding. However, this mutation clearly abolished activation of the *hb2p* promoter by exogenous cytokinin application (Figs 4B, 5).

The EMSA results, based on mutations of the sequence 5′-GGATT-3′, suggested that position one was irrelevant and specific mutations in positions two, three and four were critical for binding, leading to the conclusion that the central 'GAT' was critical for binding (Sakai *et al.*, 2000). However, the mutations analysed were not conservative, purine to pyrimidine and vice versa. Interestingly, non-conservative mutations in position 5 (from T to G and T to A; pyrimidine to purine) also abolished binding, while a conservative mutation in this position (T to C; pyrimidine to pyrimidine) actually enhanced binding. Therefore, the nature of the nucleotide at various positions seems critical. These observations raise doubts as to the importance of the central 'GAT' sequence. It is possible that conservative mutations of nucleotides two, three, or four would also have no effect on DNA binding. Therefore, the effect of the mutation cannot be accurately predicted from these experiments. However, it is clear that the mutation made in the *hb2* promoter resulted in the abolition of cytokinin responsiveness (Fig. 5).

A recent paper describing expression profiling of cytokinin action in *A. thaliana* identified a 5′-GATCTT-3′ consensus sequence prevalent in cytokinin-up-regulated gene promoters (Rashotte *et al.*, 2003). Because the Affymetrix GeneChip used (8300 genes) did not contain *AtNSHB1* and *2*, the potential up-regulation of these genes for cytokinin relative to other identified genes could not be ascertained.

A model for the activation of the *OsNSHB2* promoter by ARR1 and cytokinin is shown in Fig. 6. It has previously been suggested that native ARR1 is repressed on its receiver domain and that liberation of ARR1 from the endogenous repressor could be the result of a cytokinin-dependent phosphotransfer (Hwang *et al.*, 2002, 2001; Oka *et al.*, 2002). Therefore, in the absence of exogenous cytokinin, overexpression of ARR1 is sufficient to mimic cytokinin responses, thereby resulting in a moderate *trans*-activation of the wild-type promoter. The application of exogenous cytokinin induces other factors that amplify the cytokinin signal transduction pathway through a feed-forward mechanism in which cytokinin-dependent phosphorylation of ARR1 results in deactivation of the repressor. Activation of the cytokinin-responsive signalling mechanisms, coupled to the overexpression of ARR1, results in significantly higher activation of the *OsNSHB2* promoter. This model correlates well to the model of ARR1 activation proposed by others (Hwang *et al.*, 2002, 2001; Oka *et al.*, 2002). In addition, the data suggest that competent ARR1 binding to the ARR1 *cis*-element is required for maximal activation of the *OsNSHB2* promoter as previously

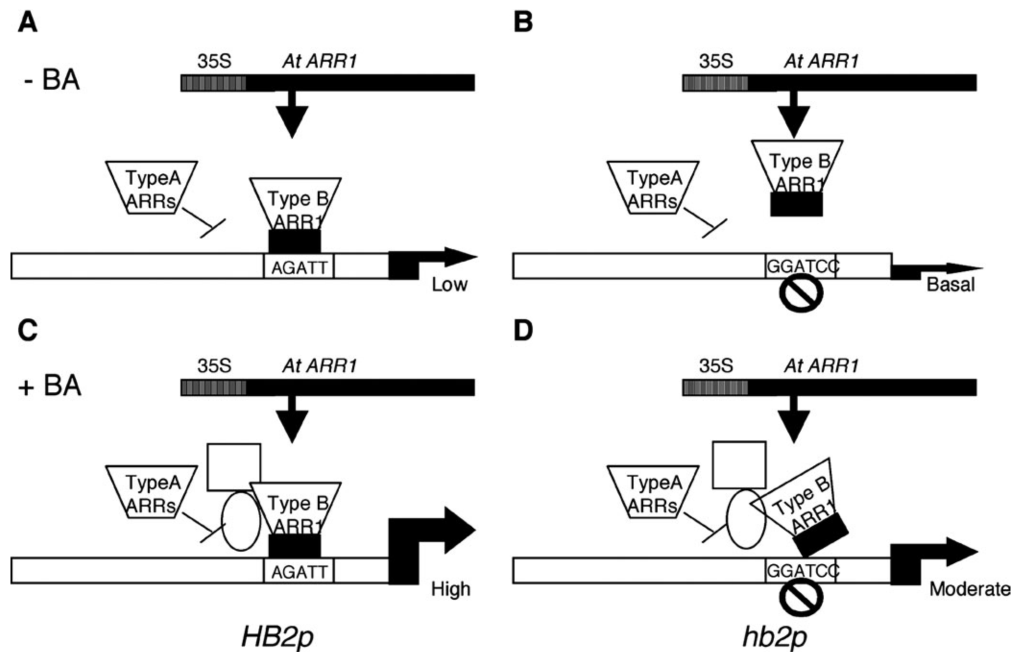


**Fig. 6.** Model for the activation of the *OsNSHB2* promoter by *A. thaliana* ARR1. (A) In the absence of cytokinin (BA), overexpression of the *A. thaliana* ARR1 protein activates the *OsNSHB2* promoter; and (B) mutation of the putative ARR1 binding site abolishes this activation. (C) Overexpression of ARR1 has a dramatic effect on the activity of the wild-type promoter in the presence of cytokinin; and (D) a moderate (but significant) effect on the activity of the mutated *hb2p* promoter in the presence of cytokinin.

suggested (Sakai *et al.*, 2000). Further dissection of the mechanism of ARR1 gene activation will give a better understanding of the regulation of *NSHB* gene expression by cytokinins.

Figure 1 supports the cytokinin-regulation of *AtNSHB2*, which has already been reported by Hunt *et al.* (2001). However, an equivalent number of ARR1 binding sites were also detected in the promoter of *AtNSHB1*, which are not in close proximity (further than 300 bp) from the start site. This promoter, however, was reported not to be activated by cytokinins in the same study (Hunt *et al.*, 2001). Perhaps the close proximity of the ARR1 binding sites to the translation start sites, as noted for the *ARR6* promoter by Sakai *et al.* (2001), are responsible for the observed cytokinin-mediated activation of both the *OsNSHB2* (this work) and the *A. thaliana NSHB2* (Hunt *et al.*, 2001). If so, it might be hypothesized that the *OsNSHB1*, but not *OsNSHB3* or *OsNSHB4*, is also activated by cytokinin (Fig. 1). A test of this hypothesis remains to be performed.

*OsNSHB2* is considered a class 1 NSHB based on phylogenetic analysis and oxygen-binding kinetics. Class 1 NSHBs are reported as being active in germination and roots and root tips under hypoxia (Hunt *et al.*, 2001). Class 2 NSHBs are reported as proteins that bind oxygen in a more similar fashion to the symbiotic HBs, are expressed during later development in floral tissues, and are induced by cytokinins. The *OsNSHB2* promoter is interestingly induced by cytokinins and is activated during all of development in tissues where *AtNSHB1* and *2* are activated. This suggests that, perhaps, the NSHB classification system requires more parameters and that functions of the two classes may overlap in some cases.

Cytokinins are involved in several differentiation pathways (Mok and Mok, 2001). Differentiation processes result in metabolic redirection and generally require high levels of energy and a high demand for oxygen for maintenance of cellular integrity during the differentiation process. Thus, NSHBs may function to maintain a specific cellular microenvironment that is part of a cellular protection mechanism during differentiation (Kundu *et al.*, 2003; Ross *et al.*, 2001). NSHBs could sense changes in the cellular energy status and may be involved in a signalling cascade that assists in the differentiation process. This understanding that *NSHBs* are activated in the presence of cytokinins by ARR1 through the ARR1-binding element can now be explored using the plethora of available *A. thaliana* mutants.

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Division, Lincoln, NE 68583. Journal Series No. 13844. We would like to dedicate this work to the late Dr Robert V Klucas.

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