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Emily Ross
*University of Nebraska-Lincoln*

Julie M. Stone
*University of Nebraska-Lincoln*, jstone2@unl.edu

Christian Elowsky
*University of Nebraska-Lincoln*, celowsky@unl.edu

Raul Arredondo-Peter
*Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Colonia Chamilpa, 62210 Cuernavaca, Morelos, México*, ra@uaem.mx

Robert V. Klucas
*University of Nebraska-Lincoln*

*See next page for additional authors*

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Emily Ross, Julie M. Stone, Christian Elowsky, Raul Arredondo-Peter, Robert V. Klucas, and Gautam Sarath
RESEARCH PAPER

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

Emily J. H. Ross¹,*, Julie M. Stone¹, Christian G. Elowsky², Raul Arredondo-Peter³, Robert V. Klucas¹,† and Gautam Sarath⁴

¹ Department of Biochemistry and Plant Science Initiative, University of Nebraska-Lincoln, Lincoln, Nebraska 68588, USA
² Johns Hopkins University, School of Medicine, Division of Gastroenterology, 720 Rutland Ave., Baltimore, MD 21205, USA
³ Laboratorio de Biofísica y Biología Molecular, Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Colonia Chamilpa, 62210 Cuernavaca, Morelos, México
⁴ USDA-ARS, Wheat, Sorghum and Forage Unit, University of Nebraska-Lincoln, Lincoln, Nebraska, USA

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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* *NSHB*1-2 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
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 iso-genomes 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 NSHBs (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.afrc.go.jp/htdocs/PLACE.html). 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 HindIII restriction site (underlined): 5’-AAGCTTTAGGCTGGTGCTGGAAGCT-3’, and a reverse primer (HB2p.RVS) engineered with an EcoRI restriction site (underlined): 5’-GAATTCTAAAGAGGTTGCTTCCT-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 MgCl₂, 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 HindIII site (5’-AAGCTTTCCAGGTTAGGAAAAGG-3’) and the HB2p.RVS primer. The 272 bp promoter (Δ960/272p; Δ960 to 273) was amplified using a HindIII-engineered forward primer (5’-AAGCTTGCATATGGCCGCAGTCTCCA-3’) and the HB2p.RVS primer. The predicted ARR1-binding element (AGATT; (Sakai et al., 2001), of the wild-type HB2p was mutated to a BamHI site (GGATC) using the megaprimr method (Sarker and Sommer, 1990), resulting in the hb2p mutant promoter. The primer used to mutate this site was 5’-GTCGATCCGGCTGCTGCGAATGATG-3’ and a reverse primer (960/272p, 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’- HindIII and 3’- EcoRI 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 XLI-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 T₂ and T₃ populations of A. thaliana were harvested and analysed for GUS activity by incubating plant organs in the GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-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 dH₂O+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’-CCATGGCTCTCGTGGAGGAGAACACCGGTGTCGGGGGA-3’ and R: 5’-CATTCTACCTAGAGCGGCTTCACA-3’ to generate a 477 bp product. GUS transcripts in A. thaliana were analysed using the oligonucleotide primers F: 5’-CCACGGACACACCTGCGTGGACG-3’ and R: 5’-GAGAGGTTAAGCCGACAGCTGC-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 Ncol restriction site (underlined): 5’-CCATGGGTATTCTCTGGTGATTCAATGGACG-3’ and a reverse primer engineered with a BamHI site (underlined): 5’-GGATCCTCAACGGATGGGAATGATG-3’, and a reverse primer engineered with a BamHI site (underlined): 5’-GGATCCTCAACGGATGGGAATGATG-3’, and a reverse primer engineered with a BamHI site (underlined): 5’-GGATCCTCAACGGATGGGAATGATG-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 MgCl₂, 1× PCR buffer, and 1 unit of Intragen 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 NcoI and BamHI 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 transformed tobacco (Nicotiana tabacum cv. Xanthi) leaf discs using microparticle 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-methylumbellifereone (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<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 (AGCGGCC) was only detected in OsNSHB2. 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.

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 T2 and T3 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. 2H–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 wounded sites (Fig. 2M). Expression of the OSNSHB2 promoter overlaps with the activities of the promoters of both AtNSHB1 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

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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.
Regulation of non-symbiotic haemoglobin gene expression by cytokinins

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±0.3, 11.4±1.1, and 13.0±1.0 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±10 nmol mg⁻¹ protein min⁻¹, compared with 1.0±0.6 and 11.4±3.6 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 (∆960/652p) removed the ACG box upstream at –689 bp. The second deletion (∆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 BamHI 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±0.6 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±3.2 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±3.2 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 (∆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. 2. The *OsNSHB2* promoter is active in *A. thaliana*. Expression of the *OsNSHB2* promoter::GUS construct in *A. thaliana* (Col-0) T₂ and T₃ 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.](image-url)
promoter elements (Fig. 4B). As expected, removal of all the identified putative cis-acting elements \((\Delta 960/272p::GUS)\) 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 \(hb2p::GUS\), \(\Delta 960/652p::GUS\), and \(\Delta 960/272p::GUS\) 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).
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. Regard-

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 NSHBs (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 (A960/652p) confers significantly less activation in the presence of cytokinin, deletion of the promoter to a minimal promoter (A960/272p), 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 BamHI 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
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 \textit{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 \textit{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 \textit{ARR6} promoter by Sakai et al. (2001), are responsible for the observed cytokinin-mediated activation of both the \textit{OsNSHB2} (this work) and the \textit{A. thaliana NSHB2} (Hunt et al., 2001). If so, it might be hypothesized that the \textit{OsNSHB1}, but not \textit{OsNSHB3} or \textit{OsNSHB4}, is also activated by cytokinin (Fig. 1). A test of this hypothesis remains to be performed.

\textit{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 \textit{OsNSHB2} promoter is interestingly induced by cytokinins and is activated during all of development in tissues where \textit{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 \textit{NSHBs} are activated in the presence of cytokinins by ARR1 through the ARR1-binding element can now be explored using the plethora of available \textit{A. thaliana} mutants.

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