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FOCUS REVIEW

Nitric oxide-responsive genes and promoters in *Arabidopsis thaliana*: a bioinformatics approach

M. Cristina Palmieri[1], Simone Sell[1], Xi Huang[1,*], Matthias Scherf[2], Thomas Werner[2], Jörg Durner[1,†] and Christian Lindermayr[1]

[1] *Institute for Biochemical Plant Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany*

[2] *Genomatix Software GmbH, Munich, Germany*

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Abstract

Due to its high reactivity and its ability to diffuse and permeate the cell membrane, nitric oxide (NO) and its exchangeable redox-activated species are unique biological messengers in animals and in plants. Although an increasing number of reports indicate that NO is an essential molecule in several physiological processes, there is not a clear picture of its method of action. Studies on the transcriptional changes induced by NO permitted identification of genes involved in different functional processes such as signal transduction, defence and cell death, transport, basic metabolism, and reactive oxygen species (ROS) production and degradation. The co-expression of these genes can be explained by the co-operation of a set of transcription factors that bind a common region in the promoter of the regulated genes. The present report describes the search for a common transcription factor-binding site (TFBS) in promoter regions of NO-regulated genes, based on microarray analyses. Using Genomatix Gene2Promotor and MatInspector, eight families of TFBSs were found to occur at least 15% more often in the promoter regions of the responsive genes in comparison with the promoter regions of 28 447 *Arabidopsis* control genes. Most of these TFBSs, such as ocs element-like sequences and WRKY, have already been reported to be involved in particular stress responses. Furthermore, the promoter regions of genes involved in jasmonic acid (JA) biosynthesis were analysed for a common TFBS module, since some genes responsible for JA biosynthesis are induced by NO, and an interaction between NO and JA signalling has already been described.

Key words: Arabidopsis, gene expression, microarray, nitric oxide, signal transduction.

Introduction

Nitric oxide (NO) is a small, highly reactive, membrane-permeable molecule, which has turned out to be an important biological messenger in animals and plants (Stamler *et al*., 1992; Schmidt and Walter, 1994; Wendehenne *et al*., 2001). In the last few years, many studies have described NO as both a cytotoxic and a cytoprotecting regulator involved in different physiological processes in plants. It has been implicated in disease resistance, stomata closure, seed germination, iron homeostasis, different development processes, and the response of plants to abiotic stresses such as drought, UV-B, salinity, and high temperature (Delledonne *et al*., 1998; Durner *et al*., 1998; Garcia-Mata and Lamattina, 2002; Zhao *et al*., 2004).

Many of the biological functions of NO arise as a direct consequence of chemical reactions between proteins and NO or NO oxides generated as NO/O_2 or NO/superoxide reaction products. The reactions of NO with metal ions of haem groups or the formation of dinitrosyl complexes are demonstrated to play important roles in NO signalling. In mammalian cells, NO regulates the production of the important second messenger cGMP by interacting with the iron ion of the haem moiety of guanylate cyclase (Russwurm and Koesling, 2004). An increase in the

* Present address: Institute of Biological Science and Technology, Hainan University, Haikou, PR China.
† To whom correspondence should be addressed. E-mail: durner@gsf.de

endogenous cGMP in tobacco leaves and suspension cells after NO treatment was demonstrated by Durner *et al.* (1998), suggesting the existence of an NO-dependent cGMP pathway in plants, too.

However, NO is also an important redox-active signalling molecule and after the cGMP signalling mechanism, *S*-nitrosylation of cysteine residues of redox-sensitive proteins is the second most important principle of NO signalling. Recently, >100 proteins were identified in *Arabidopsis* representing candidates for protein *S*-nitrosylation (Lindermayr *et al.*, 2005). However, until now there is experimental evidence for only a few plant proteins being regulated by *S*-nitrosylation, including haemoglobin 1, glyceraldehyde 3-phosphate dehydrogenase, *S*-adenosylmethionine synthetase, and metacaspase, and the K^+ channels in guard cells of *Arabidopsis thaliana* are regulated by NO via *S*-nitrosylation (Perazzolli *et al.*, 2004; Sokolovski and Blatt, 2004; Lindermayr *et al.*, 2005, 2006).

Besides the regulation of signalling pathways, NO can control physiological processes directly by encroaching upon gene transcription. Transcriptional changes in *A. thaliana* in response to NO have been analysed using different techniques such as cDNA-amplified fragment length polymorphism (Polverari *et al.*, 2003), microarray, and real-time PCR (Huang *et al.*, 2002; Parani *et al.*, 2004). Identified NO-modulated genes are involved in different functional processes such as signal transduction, defence and cell death, transport, basic metabolism, and reactive oxygen species (ROS) production and degradation. An important number of these modulated genes correspond to proteins with a regulatory role as components of the signal transduction cascade or transcription factors.

How can NO regulate gene expression? The transcription of genes is regulated by transcription factors. These proteins bind to defined promoter sequences to enhance or repress gene expression by assisting or blocking RNA polymerase binding, respectively. As well as direct DNA binding, transcription factors often interact with other proteins and bind to promoter regions as multiprotein complexes. The DNA binding affinity of transcription factors can be altered, for example, by phosphorylation or redox-dependent modifications of transcription factors, such as protein *S*-nitrosylation. For example, the activity of the thiol-containing transcriptional activator OxyR, whose oxidation controls the expression of genes involved in H_2O_2 detoxification, is modulated by *S*-nitrosylation (Hausladen *et al.*, 1996).

The use of whole genome transcript analyses to identify co-regulated genes has been rapidly becoming a widespread approach to understanding the regulation of physiological processes. Similar expression profiles might be caused by the co-ordinated action of transcription factors. Thus, a systematic and logical approach to study genes with similar expression patterns is to analyse their promoter sequences in order to identify transcription factors that might be responsible for the co-expression.

Here the screening of promoter regions of NO-regulated genes for common transcription factor-binding site (TFBS) patterns is described. Using Agilent microarrays, the transcriptional changes in *Arabidopsis* plants in response to gaseous NO and in cell suspension cultures after treatment with an NO donor were investigated. A total of 28 genes which were up-regulated in both plant and cell culture experiments were identified. Furthermore, 121 and 79 genes were induced exclusively in plant and cell culture experiments, respectively. Twenty-six genes were found to be down-regulated. Using the Genomatix Gene2Promotor program, the promoter regions of the NO-regulated genes were identified, which were then screened for common TFBSs with Genomatix MatInspector.

Material and methods

Cell culture and plant treatment

Plants (*A. thaliana*, ecotype Columbia) were grown for 4–5 weeks in a growth chamber (at 69% relative humidity, 10 h dark) at 23 °C during the day and 18 °C at night. The experimental set-ups to study the effect of NO on plants consisted of controlled environment cabinets as well as complete instrumentation to adjust and control gaseous NO concentrations of 1250 ppm for 10 min. At this concentration, the plants did not show symptoms. After the treatment, plants were put back into the growth chambers until they were harvested.

Cell suspension cultures of *A. thaliana* (ecotype Columbia) were grown in liquid PS-medium as described in Huang *et al.* (2002). A 7-d-old cell suspension culture was treated with 0.5 mM of the NO donor NOR3 {(E)-ethyl-2-[(E)-hydrxyimino]-5-nitro-3-hexene-amide}. After the treatment, cells were harvested by filtration at different time points. Harvested plants and cell cultures were immediately frozen in liquid nitrogen and stored at –80 °C.

Microarray

Microarray analyses were performed as described in Huang *et al.* (2002) with some modifications. For microarray analyses, Agilent whole genome *Arabidopsis* arrays were used (Agilent, Santa Clara, CA, USA). Probes were made by using an indirect aminoallyl labelling method with Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia, Freiburg, Germany) and purified according to standard protocols. The arrays were scanned by using an Axon GenePix 4000 scanner (Axon Instruments, Union City, CA, USA) and the GENEPIX PRO 4.1 and ACUITY (Axon Instruments) software packages.

Fluorescent probes

Target RNA from NOR-3-treated *Arabidopsis* cells was extracted using the TRIzol reagent according to the supplier's instructions (Invitrogen, Karlsruhe, Germany). Poly(A) RNA was purified from total RNA with the Fast Track mRNA isolation kit (Invitrogen) according to the manufacturer's instructions. Probes were made using the indirect aminoallyl labelling method (see http://www.tigr.org/tdb/microarray/protocols.shtml). Each mRNA sample (one control and one treated sample) was reverse-transcribed in the

presence of Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech), and purified according to standard protocols.

Hybridization and scanning

Hybridization was done as previously described (Huang *et al*., 2002). GenePix Pro3.0 software was used to identify differentially expressed genes. Background fluorescence was calculated as the median fluorescence signal of non-target pixels around each gene spot. The induction or repression of a gene is defined as a minimum 2.5-fold change in its transcript level. Presented data show the mean of four experiments.

Screening for putative TFBSs (Genomatix analyses)

Identification of the potential promoter regions and TFBSs was conducted using the Genomatix suite of programs (http://www.genomatix.de, Genomatix Software GmbH, Munich, Germany) (Quandt *et al*., 1995). The Gene2promotor program from the Genomatix software package was used to locate the correlated genes within the genome and define 601 bp of the promoter regions (500 bp upstream of and 100 bp into the transcription start site) for each gene. The 601 bp sequences obtained from the Gene2promotor program were then used as the target sequences for putative transcription factor recognition site identification using the MatInspector Version 4.3 program (Cartharius *et al*., 2005). The parameters used were the standard (0.75) core similarity and the optimized matrix similarity. To identify common TFBS modules in the promoter regions of the jasmonic acid (JA) biosynthetic pathway genes, the Genomatix Frameworker program was used. The parameters used were: (i) minimum number of sequences with framework, 27%; (ii) maximum distance variance between two elements, 25 bp.

Results and discussion

NO is an important signalling molecule, which fulfils many different physiological functions in plants. Gene expression in response to NO was analysed in several laboratories, where an induction of several pathogenesis-related proteins and an array of antioxidant genes encoding peroxidases, glutathione *S*-transferases (GST), protein kinases, and transcription factors could be demonstrated.

Microarray analyses

Whole genome microarray (Agilent 1/2) was used to explore transcriptional changes in *Arabidopsis* cell suspension cultures and plants in response to the NO donor NOR-3 and gaseous NO. Array hybridizations were based on four replicates. Rigorous criteria were applied in the selection procedure so that those genes with <2.5-fold signal compared with the background were ignored (see Materials and methods). To obtain specific gene expression, the NO treatment was adjusted to yield only moderate changes in transcriptional activity. Under the conditions applied (0.5 mM NOR-3) the cells did not show any symptoms such as cell death. Modifying the treatment (i.e. lower NO concentrations for a prolonged time) resulted in a slightly different expression array (data not shown).

In summary, 28 genes which were up-regulated in almost all experiment were observed (Fig. 1; Supplementary data 1 available at *JXB* online). Furthermore, 79 and 121 genes were exclusively induced in cell culture and plant experiments, respectively. Additionally, 26 genes were found to be down-regulated in cell culture experiments. The difference in the set of regulated genes is probably due to the difference between the biological system used (plant/cell culture) but also to the different mechanism of action of the NO applications (NO donor, NOR-3/gaseous NO).

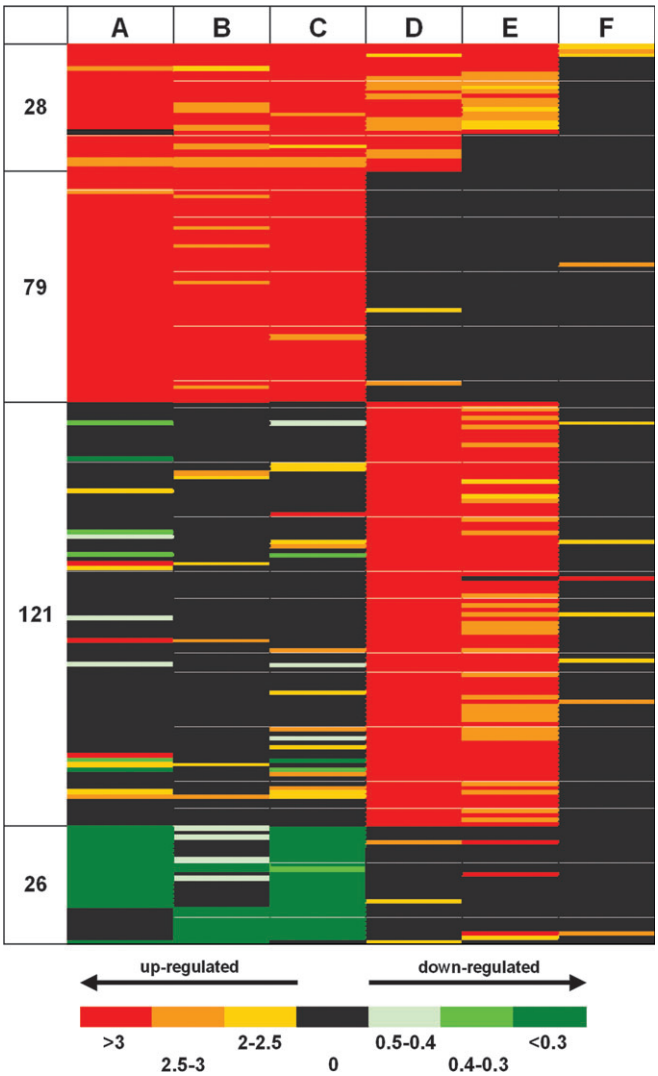


Fig. 1. Schematic display of the expression profile of NO-regulated *Arabidopsis* genes. Expression profile of NO-regulated genes in cell cultures (A–C) and plants (D–F) treated with 0.5 mM of NOR-3 or gaseous NO, respectively. The transcriptional profile of the cell cultures and plants was analysed using Agilent microarrays (generation 1 for A and D and generation 2 for B, C, E, and F). Cell cultures and plants were treated as described in Materials and methods for 1 h (A, B, D, E) or 3 h (C, F). NO-regulated genes were grouped intp genes which were up-regulated in almost all experiments (28), up-regulated in cell culture experiments only (79), up-regulated in plant experiments only (121), and down-regulated genes (26).

Common TFBSs in NO-regulated genes

Based on the results of the microarray analyses, the possibility of identifying common TFBSs in the promoter regions of the co-expressed genes was explored. The Gene2promotor program from the Genomatix software package was used to locate the correlated genes within the genome and define 601 bp of the promoter regions (500 bp upstream of and 100 bp into the transcription start site) for each gene (Genomatix Software GmbH, Munich, Germany). The 601 bp sequences obtained from the Gene2promotor program then were used as the target sequences for putative transcription factor recognition site identification using the Genomatix MatInspector Version 4.3 program. The parameters used were the standard (0.75) core similarity and the optimized matrix similarity (Quandt *et al*., 1995). As a control, the occurrence of the TFBSs within a set of 28 447 *Arabidopsis* genes as a percentage was used (Genomatix database, http://www.genomatix.de).

The MatInspector analyses showed a large number of putative TFBSs in the promoter of the NO-regulated genes. In Table 1 all TFBSs which are common to at least 25% of the analysed promoters are listed. The given values represent the percentage of promoters in which a match to the matrix family is found with optimized matrix similarity. The detailed results are given as Supplementary data at *JXB* online (Supplementary data 2–5). Most of the TFBSs were found to occur with the same frequency as the 28 447 *Arabidopsis* genes, e.g. MADS, AHBP, and IBOX elements (Table 1). However, eight families of TFBSs occurred at least 15% more often in the promoter regions of the analysed groups of genes in comparison with the promoter regions of the control genes (Table 1). GBOX, OCSE, and L1BX elements are enriched in genes up-regulated in both cell culture and plant experiments. Moreover, GBOX and OCSE elements, together with MYCL and OPAQ, are highly present in promoters of up-regulated cell culture genes. In genes induced in plants only, WRKY-binding sites are enriched in their promoters. In the down-regulated genes of cell cultures an increased occurrence of TBPF and MIIG elements was observed.

In plants, basic region/leucine zipper motif (bZIP) transcription factors are involved in the regulation of many different physiological processes such as biotic and abiotic stress signalling, seed maturation, flowering, and light signalling (Lebel *et al*., 1998; Hobo *et al*., 1999; Ratcliffe and Riechmann, 2002). The bZIP transcription factors contain a DNA-binding motif and a leucine zipper domain, which is responsible for dimerization (Ellenberger *et al*., 1992; Izawa *et al*., 1993; Metallo and Schepartz, 1997; Choi *et al*., 2000). The core motif for DNA binding is ACGT. However, using recombinant bZIP proteins, it has been demonstrated that neighbouring nucleotides affect binding affinity. Dependent on the nucleotide flanking the 3′ site of the motif, three different types of ACGT elements have been defined: a G-box (CACGTG), C-box (GACGTG), and A-box (TACGTA). bZIP transcription factors seem to play a pivotal role in regulation of plant physiology, since *Arabidopsis* has ~4 times as many bZIP-encoding genes as yeast, worm, and human (Riechmann *et al*., 2000). The memebers of the bZIP family of G-box-binding factors have been implicated in the expression of a number of genes during pathogen attack (Kim *et al*., 1992). Interestingly, in almost all promoters of the genes up-regulated in plants and cell cultures treated with NO, GBOX elements were located within the first 250 bp upstream of the putative transcription start (Fig. 2A).

Another class of bZIP-binding elements implicated in the plant defence response is formed by octopine synthase (ocs). In *Arabidopsis*, ocs element-like sequences (OCSEs)

Table 1. *Frequency of occurrence of different transcription factor-binding sites in the promoter region of NO-regulated genes*

The promoter regions of the different groups of NO-regulated genes were screened for common transcription factor-binding sites (TFBSs) using the Genomatix program tools. Promotor regions of 28 447 *Arabidopsis* genes were used to determine the unspecified distribution of the TFBSs (control). TFBSs occurring at least 15% more often in the promoter regions of the NO-regulated genes compared with the control are highlighted in bold.

Transcription factor	Up-regulated genes			Down-regulated genes	Control
	Cell cultures and plants	Plants	Cell cultures	Cell cultures	Genomatix
WRKY	50%	**71%**	49%	42%	54%
GBOX	**85%**	70%	**90%**	65%	62%
OCSE	**82%**	76%	**82%**	61%	67%
TBPF	75%	82%	82%	**88%**	70%
L1BX	**75%**	61%	58%	65%	58%
MYCL	53%	47%	**66%**	38%	46%
MIIG	35%	35%	33%	**57%**	40%
OPAQ	75%	69%	**82%**	73%	67%
GAGA	21%	37%	31%	42%	45%
GAPB	32%	44%	33%	65%	53%
HMGF	57%	51%	39%	50%	43%
AHBP	92%	91%	92%	100%	92%
CCAF	57%	65%	45%	53%	67%
DOFF	71%	89%	86%	84%	93%
GTBX	92%	98%	98%	96%	97%
HEAT	46%	35%	41%	34%	49%
IBOX	60%	77%	76%	73%	80%
IDDF	42%	40%	45%	26%	47%
LREM	57%	65%	74%	73%	67%
MADS	85%	83%	84%	92%	88%
MSAE	42%	46%	45%	38%	48%
MYBL	100%	92%	88%	88%	96%
MYBS	60%	68%	56%	57%	69%
NCS1	57%	70%	62%	46%	70%
NCS2	46%	41%	49%	46%	47%
PSRE	32%	50%	47%	23%	50%
SPF1	35%	55%	56%	65%	54%
STKM	32%	47%	45%	30%	44%
CAAT	39%	53%	54%	44%	49%

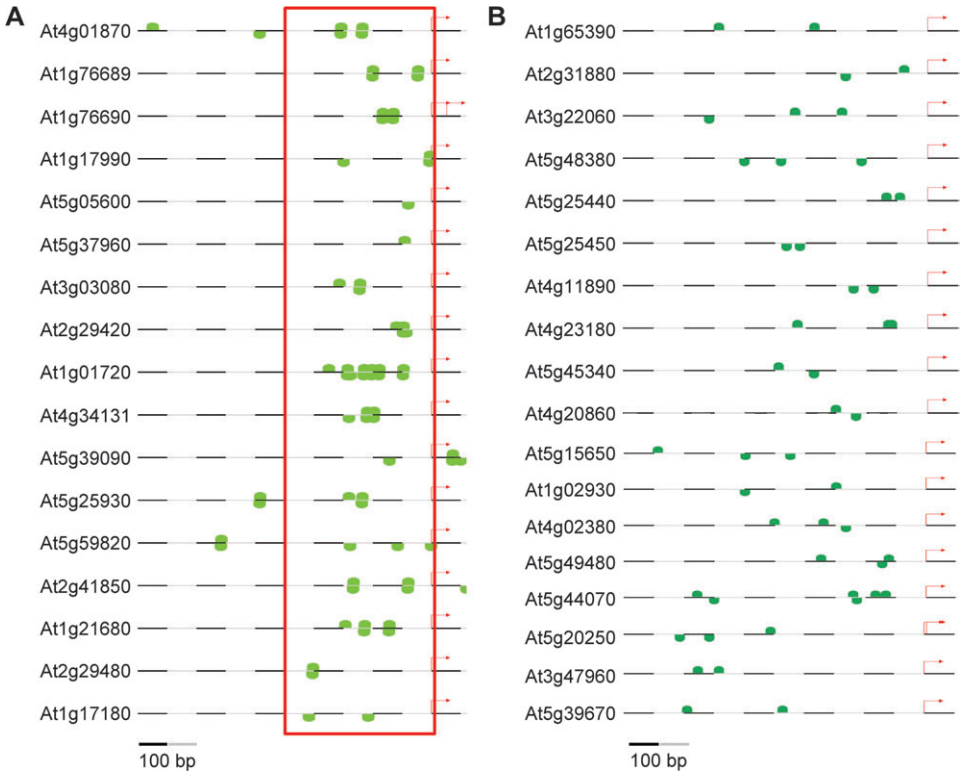


Fig. 2. Illustration of putative transcription factor-binding sites in the promoter regions of NO-induced *Arabidopsis* genes. For each gene, the promoter region 500 bp upstream and 100 bp downstream from the putative transcription start site (arrow) is shown. The positions of significant binding sites for GBOX transcription factors (A) and WRKY proteins (B) are indicated. Genes which were up-regulated in almost all experiments are enriched in GBOX elements (A), whereas genes induced in plant experiments only were charged with multiple WRKY elements (B). GBox elements are concentrated within the first 250 bp upstream of the putative transcription start (A, red box).

are important for the expression of specific GST- and pathogenesis-related genes such as the GST6 and the PR1 genes (Lebel *et al*., 1998; Chen and Singh, 1999). This relationship between ocs elements and plant defence responses was supported by the discovery that *Arabidopsis* TGA/ocs element-binding factors (OBFs) interact with NPR1, a key component in the salicylic acid defence signalling pathway (Zhou *et al*., 2000). Moreover, there are several members of the TGA/OBF family which plays a role in xenobiotic stress responses and development (Johnson *et al*., 2001).

Opaque-2-like transcriptional activators (OPAQs) are also a well-characterized family of plant bZIPs with an extended leucine zipper with up to nine heptad repeats. One of the main processes modulating OPAQ activity is the heterodimerization with other bZIP transcription factors. Opaque-2 regulates the expression of α and β prolamines, the main storage proteins in seeds of cereals such as maize and *Coix* (Takatsuji, 1998). Other members of the OPAQ family, such as CPFR2 and G/HBF-1, might be involved in responses to environmental or pathogen challenge (Droge-Laser *et al*., 1997; Lara *et al*., 2003). The stimulation of G/HBF-1 kinase activity and G/HBF-1 phosphorylation after treatment with glutathione or avirulent *Pseudomonas syringae* pv. *glycinea* are terminal events in a signal pathway for activation of early transcription-dependent plant defence responses (Droge-Laser *et al*., 1997).

Similar to bZIP proteins, WRKY family members are involved in the regulation of various physiological processes, including pathogen defence, senescence, and trichome development (Robatzek and Somssich, 2001; Johnson *et al*., 2002; Dong *et al*., 2003). Their DNA-binding domain (WRKY domain) comprises ~60 amino acids, but the overall structures of WRKY proteins are highly divergent and can be categorized into distinct groups, which might reflect their different functions (Eulgem *et al*., 2000). Additional domains of WRKYs are restricted to subgroups of this family and include, for example, conserved regions of nuclear localization signals, calmodulin-binding sites, or putative leucine zippers (Cormack *et al*., 2002; Sun *et al*., 2003; Park *et al*., 2005). Multiple studies have demonstrated the ability of WRKYs to bind the W box element (TTGACC/T) (Rushton *et al*., 2002; Yamasaki *et al*., 2005), which is found in the promoters of many plant defence genes (Maleck *et al*., 2000; Chen *et al*., 2002). W box or W box-like sequences often occur in clusters within promoters, suggesting a possible synergistic action with other WRKY proteins and/or other classes of transcription factors (Maleck *et al*., 2000). The

transcription of WRKY genes is strongly and rapidly up-regulated in numerous plant species in response to wounding, pathogen infection, or abiotic stresses, such as drought, cold adaptation, or heat-induced chilling tolerance (Eulgem *et al*., 2000; Robatzek and Somssich, 2001; Rizhsky *et al*., 2002). A total of 74 *Arabidopsis* WRKY genes respond to bacterial infection or salicylic acid (SA) treatment (Dong *et al*., 2003), and *Arabidopsis* WRKY70 was identified as a common regulatory component of SA- and JA-dependent defence signalling, mediating cross-talk between these antagonistic pathways. Overexpression and antisense lines indicated that WRKY70 plays a positive role in SA signalling and functions as a negative regulator of JA-inducible genes (Li *et al*., 2004). In contrast, WRKY18, WRKY40, and WRKY60 may function redundantly as negative regulators in SA-dependent pathways but play a positive role in JA-mediated pathways (Xu *et al*., 2006). Moreover, NPR1 is functionally linked to WRKYs during plant immune responses. Intriguingly, WRKYs control NPR1 expression on the one hand, while on the other hand they seem to operate downstream from NPR1 (Yu *et al*., 2001; Wang *et al*., 2006). Additionally, WRKY transcription factors are involved in co-ordination of plant development and/or ageing, since the expression of several *Arabidopsis* WRKY genes is strongly up-regulated during plant senescence (Robatzek and Somssich, 2001).

Many of the genes induced in *Arabidopsis* plants after NO treatment have more than one WRKY-binding site in their promoter region (Fig. 2B). Typically, *WRKY* promoters are enriched for W boxes, and multiple studies have revealed interactions of WRKYs with either their own promoters or those of other family members, suggesting that these transcription factors engage extensively in auto- and cross-regulation (Eulgem *et al*., 1999; Turck *et al*., 2004).

The L1 box (L1BX) was identified as an essential promoter sequence for the expression of the *A. thaliana* PROTODERMAL FACTOR1 (PDF1). The PDF1 gene encodes a putative extracellular proline-rich protein that is exclusively expressed in the L1 layer of shoot apices and the protoderm of organ primordia. Electrophoretic mobility shift assays demonstrated that the L1-specific homeodomain protein ATML1 can bind to the L1 box sequence *in vitro* (Abe *et al*., 2001). This protein belongs to the HD-ZIP IV class homeodomain (HD) proteins that are expressed exclusively in the L1 layer of the shoot apical meristem (SAM) (Lu *et al*., 1996). The homeodomain consists of ~60 amino acids starting with the N-terminal arm followed by three helical regions. Helix 3 of the HD binds in the major groove of DNA, with helices 1 and 2 lying outside the double helix. The N-terminal arm is located in the major groove and makes additional contacts. Different genes coding for the HD-ZIP IV class have been shown in the *Arabidopsis* genome. Intriguingly, a similar target sequences of the L1 box has been identified in the upstream region of a parsley PR2 gene (Korfhage *et al*., 1994). Interaction of this element with PRHP, an HD protein isolated as a binding factor to it, has been suggested to play a role in the elicitor-inducible expression of PR2 (Korfhage *et al*., 1994).

The TATA box is a *cis*-regulatory element found in the promoter region of many eukaryotic genes. This binding site can interact with transcription factors or histones (Godde *et al*., 1995; Smale and Kadonaga, 2003). It is normally bound by the TATA-binding protein (TBP) in the process of transcription. The segment coding for the evolutionarily conserved C-terminal DNA-binding domain is unique. Binding of TBP is the first step in the assembly of a transcription complex and is essentially the only step where the particular base sequence of the DNA is read and recognized during this process (Smale and Kadonaga, 2003).

The characteristic feature of the MYCL transcription factors is their basic helix–loop–helix (bHLH) structure, a stretch of 40–50 amino acids containing two amphipathic α-helices separated by a linker region (the loop) of varying length. bHLH proteins have a region with conserved positive charges immediately adjacent to the first helix (Murre *et al*., 1994). Proteins in this group form both homodimers and heterodimers by means of interactions between the hydrophobic residues on the corresponding faces of the two helices. A dimer in which both subunits have the basic region can bind to DNA. While the bHLH domain is evolutionarily conserved, there is little sequence similarity between clades beyond the domain (Morgenstern and Atchley, 1999). The bHLHs that have been characterized function in the transcriptional regulation of genes associated with anthocyanin biosynthesis, phytochrome signalling, globulin expression, fruit dehiscence, carpel and epidermal development, and the circadian clock (Heisler *et al*., 2001; Rajani and Sundaresan, 2001; Makino *et al*., 2002). AtMYC2, a bHLH transcription factor, has been shown to function in abscisic acid (ABA)-inducible gene expression under drought stress in plants. Moreover, as a positive regulator of ABA signalling, it plays a role in negative regulation of JA/ethylene-responsive defence genes in *Arabidopsis* (Abe *et al*., 2003; Anderson *et al*., 2004; Boter *et al*., 2004; Lorenzo *et al*., 2004).

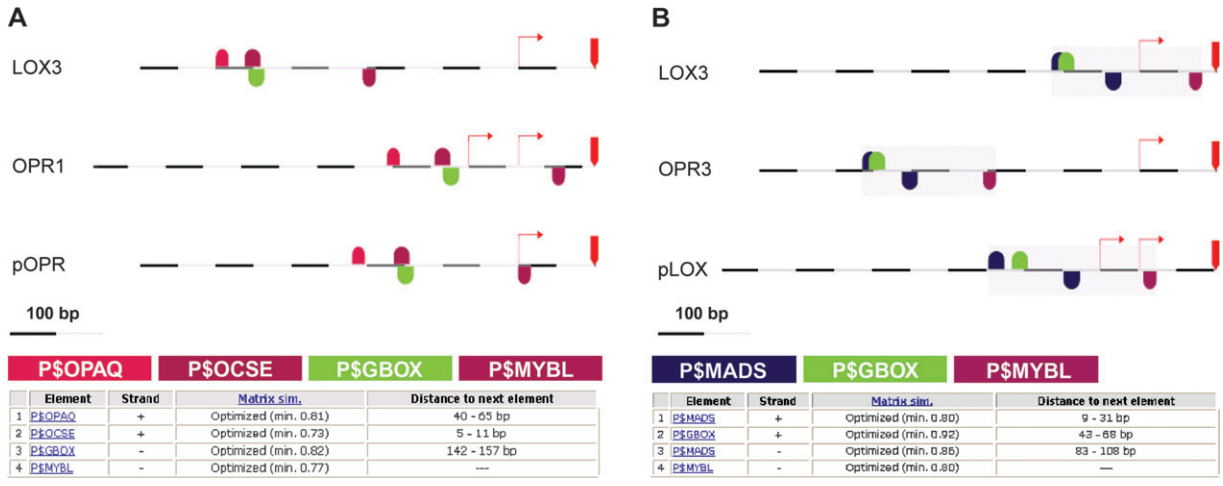
The myb domain of vertebrates consists of three imperfect tandem repeats (R1, R2, and R3), each forming a helix–turn–helix (HTH) structure of ~53 amino acids. Three regularly spaced tryptophan residues, which form a tryptophan cluster in the three-dimensional HTH structure, are characteristic of a MYB repeat. Myb proteins usually have only one (R1) or two imperfect tandem repeats (R2, R3). MYB genes containing two repeats constitute the largest MYB gene family in plants and is subdivided into three groups (MI, MII, MIIG) depending on the sequence of the binding site. An important function for R2R3-type MYB factors is the control of development and determination of cell fate and identity (Oppenheimer *et al*.,

1991; Lee and Schiefelbein, 1999); some are activated in plants in response to environmental factors and hormones (Iturriaga *et al*., 1996; Hoeren *et al*., 1998). Most members of the family seem to be involved in control of secondary metabolism or response to secondary metabolites such as flavonoid and phenylpropanoid metabolism and the anthocyanin pathway (Logemann *et al*., 1995; Moyano *et al*., 1996; Grotewold *et al*., 1998). Moreover, MYB factors play a role in the plant defence reactions. NtMYB2 is involved in the stress response of retrotransposon- and defence-related genes. Overexpression of NtMYB2 cDNA in transgenic tobacco plants induced expression of Tto1 and a phenylalanine ammonia lyase (PAL), a gene involved in the defence response to plant development and in response to light, pathogen ingress, mechanical damage, and other stresses (Sugimoto *et al*., 2000).

Common TFBS modules

Interestingly, several genes of the JA biosynthetic pathway are up-regulated after NO treatment: all three 12-oxophytodienoate reductases (OPR1, OPR2, and OPR3) and two lipoxygenases (LOX3 and a putative lipoxygenase protein). The Genomatix program Frameworker was used to search for common TFBS modules in the promoter regions of the JA biosynthetic pathway genes. Two modules common to three genes each and consisting of four TFBS elements were identified (Fig. 3). One is present in the promoter region of LOX3, OPR1, and a putative OPR, and is formed by OPAQ, OCSE, GBOX, and MYBL elements (Fig. 3A). The second module is present in LOX3, OPR3, and a putative LOX gene, and is composed of MADS, GBOX, and MYBL (Fig. 3B). These modules inside the promoters represent a possible explanation of the co-expression of some genes of the JA biosynthetic pathway after NO treatment. As already shown, a particular physiological process can induce a distinct set of genes responsible for the elevation of JA levels in plants (He *et al*., 2002). For example, LOX2 is required for the wound-induced synthesis of JA in leaves (Bell *et al*., 1995). Furthermore, OPR1, OPR3, and LOX3 are reported to be overexpressed during leaf senescence, while OPR2 appears to be constitutively expressed throughout the different stages of leaf development, and LOX2 is down-regulated in senescent leaves (He *et al*., 2002).

In several reports, the correlation between NO and JA is discussed controversially. Huang *et al*. (2004) showed JA- and wounding-dependent NO production by diaminofluoresceins (DAFs). Using a cDNA microarray made up of ~330 defence-related genes, they observed the induction of three genes involved in wounding-induced JA biosynthesis (allene oxide synthase, LOX2, and OPR3). Although NO activates genes involved in JA biosynthesis, it did not affect JA levels in *Arabidopsis* and showed an extremely weak or almost no accumulation of JA-dependent late defence genes (Huang *et al*., 2004). In *Taxus* cell cultures, exogenously supplied methyl jasmonate (MeJA) induced rapid production of NO, and the MeJA-induced intracellular malondialdehyde (MDA), LOX, and PAL were all enhanced by an NO donor, but suppressed by NO inhibitors, underlining the connection between NO and JA signalling (Wang and Wu, 2005). In leaf senescence, the role of NO and JA is still controversial. Although it was demonstrated that exogenous application of JA or MeJA induces leaf senescence (Ueda and Kato, 1980) and that the endogenous JA level in senescing leaves increased to nearly 500% of that in non-senescent leaves (He *et al*., 2002), the role of JA in this process remains unclear. NO also seems to affect this phase of the plant life cycle. It is



	Element	Strand	Matrix sim.	Distance to next element
1	P$OPAQ	+	Optimized (min. 0.81)	40 - 65 bp
2	P$OCSE	+	Optimized (min. 0.73)	5 - 11 bp
3	P$GBOX	-	Optimized (min. 0.82)	142 - 157 bp
4	P$MYBL	-	Optimized (min. 0.77)	---

	Element	Strand	Matrix sim.	Distance to next element
1	P$MADS	+	Optimized (min. 0.80)	9 - 31 bp
2	P$GBOX	+	Optimized (min. 0.92)	43 - 68 bp
3	P$MADS	-	Optimized (min. 0.86)	83 - 108 bp
4	P$MYBL	-	Optimized (min. 0.80)	---

Fig. 3. Illustration of the common framework of elements from the promoter region of several genes of the JA biosynthetic pathway. The Genomatix program Frameworker was used to search for common frameworks between the promoter regions of the JA biosynthetic pathway genes. (A) Four-element module present in the promoter region of LOX3 (At1g17420), OPR1 (At1g76680), and a putative OPR (At1g17990). It is composed of OPAQ, OCSE, GBOX, and MYBL elements. (B) Three-element module present in LOX3 (At1g17420), OPR3 (At2g06050), and a putative LOX gene (At1g72520), composed of MADS, GBOX, and MYBL.

hypothesized that a stoichiometric relationship between the two gases NO and ethylene probably determined whether senescence took place or not (Leshem and Haramaty, 1996; Leshem and Pinchasov, 2000).

Conclusion

Taken together, such a bioinformatics approach is a useful tool to identify common TFBSs and promoter modules in co-regulated genes, which might be involved in establishing specific expression profiles. Since co-expression can be due to a variety of co-regulatory mechanisms, such analyses may be a first step to provide the basis to understanding the regulatory networks involved in gene expression profiles. Next, the involvement of the identified motifs in the NO response should be experimentally validated.

Supplementary data

Supplementary data containing the microarray and the MatInspector analyses results are available at *JXB* online.

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