Nicotiana tabacum EIL2 directly regulates expression of at least one tobacco gene induced by sulphur starvation

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Abstract

Sulphur deficiency severely affects plant growth and their agricultural productivity leading to diverse changes in development and metabolisms. Molecular mechanisms regulating gene expression under low sulphur conditions remain largely unknown. AtSLIM1, a member of the EIN3-like (EIL) family was reported to be a central transcriptional regulator of the plant sulphur response, however, no direct interaction of this protein with any sulphur-responsive promoters was demonstrated. The focus of this study was on the analysis of a promoter region of UP9C, a tobacco gene strongly induced by sulphur limitation. Cloning and subsequent examination of this promoter resulted in the identification of a 20-nt sequence (UPE-box), also present in the promoters of several Arabidopsis genes, including three out of four homologues of UP9C. The UPE-box, consisting of two parallel tefs sequences (TEIL binding site), proved to be necessary to bind the transcription factors belonging to the EIL family and of a 5-nt conserved sequence at the 3'-end. The yeast one-hybrid analysis resulted in the identification of one transcription factor (NtEIL2) capable of binding to the UPE-box. The interactions of NtEIL2, and its homologue from Arabidopsis, AtSLIM1, with DNA were affected by mutations within the UPE-box. Transient expression assays in Nicotiana benthamiana have further shown that both factors, NtEIL2 and AtSLIM1, activate the UP9C promoter. Interestingly, activation by NtEIL2, but not by AtSLIM1, was dependent on the sulphur-deficiency of the plants.

Key words: EIL family, promoter, reporter gene, sulphur deficit, sulphur metabolism, tobacco, transcription factor, yeast one-hybrid.

Introduction

Sulphur is an important macro-element for all organisms. Plants are able to assimilate inorganic sulphur and incorporate it into organic compounds, while animals rely entirely on organic sources of this element. In recent decades sulphur availability in soils has become the major limiting factor for plant production in many geographical regions due to a significant reduction in anthropogenic sulphur emission. A sufficient supply of sulphur is needed for the appropriate development and performance of crops and for their resistance to environmental stresses (Haneklaus et al., 2005). To resist sulphur deficiency, plants must demonstrate physiological flexibility. In response to sulphur deficiency, as in other environmental challenges they must tune the expression of an extensive set of genes and gene regulators. Micro- and macro-array experiments have revealed hundreds of up- and down-regulated genes in Arabidopsis thaliana under sulphur-deficient conditions (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003). Not only gene expression but also translation efficiency and activity of enzymes needed for sulphate assimilation and the synthesis of sulphur-containing metabolites are regulated. Although most of these regulatory steps are not yet fully characterized, a general outcome of such multilevel regulation is the adjustment of the entire sulphur flux of the plant to meet the new conditions (for a recent review and references, see Lewandowska and Sirko, 2008)).

Regulation of gene expression at the level of transcription is a major control point in many biological processes, and plant genomes devote approximately 7% of their coding sequence to transcription factors that bind to DNA cis-elements located in gene promoters and introns (Udvardi
Despite the identification of sulphur-responsive genes, little is known about the mechanisms leading to transcriptional gene activation in response to sulphur deficiency. Only recently, the first cis-element SURE (sulphur response element) was identified in the promoter of the *Arabidopsis* sulphur transporter gene *SULTR1;1* (Maruyama-Nakashita *et al.*, 2005). It was demonstrated that a 5-nt core of SURE (GAGAC or GTCTC) is sufficient to drive the sulphur-deprivation dependent transcriptional response. Although the SURE element occurs in the promoter regions of many sulphur-responsive genes, it can also be found in the promoters of genes not regulated by sulphur supply; therefore, additional regulatory factors must determine the specificity of the response.

SLIM1 (sulphur limitation 1), found during screening for mutations influencing the activity of the *Arabidopsis* sulphur transporter *SULTR1;2* promoter (Maruyama-Nakashita *et al.*, 2006), is the only so far identified trans-acting factor involved in the regulation of the genes during sulphur starvation. Growth of *Arabidopsis* slim1 mutants is affected by severe sulphur deficiency conditions, with a 30% reduction in their root lengths and a 60% decrease in sulphate uptake rates. AtSLIM1 appears to be identical to the transcription factor AtEIL3 (EIN3-like 3), belonging to a small plant-specific multigenic family of which several members have been cloned and characterized in different species (Kosugi and Ohashi, 2000; Tieman *et al.*, 2001; Lee and Kim, 2003; Rieu *et al.*, 2003; Iordachescu and Verlinden, 2005; Mao *et al.*, 2006). In the *Arabidopsis* genome, six genes are annotated to encode the EIL family proteins (AtEIN3 and AtEIL1 to AtEIL5) (Guo and Ecker, 2004). AtEIN3 is a transcription factor controlling the expression of ethylene-responsive genes, and AtEIL1 and AtEIL2 are the closest functional homologues of AtEIN3 (Chao *et al.*, 1997; Solano *et al.*, 1998; Guo and Ecker, 2004). The functions of AtEIL4 and AtEIL5 have not been identified so far (Guo and Ecker, 2004). Since only AtSLIM1/AtEIL3 was able to complement the *Arabidopsis* slim1 mutant, its function seems to be specific for the sulphur response. AtSLIM1 is expressed constitutively with predominant localization in vascular tissues (Maruyama-Nakashita *et al.*, 2006). It was also demonstrated that AtSLIM1 mRNA was not modulated by the changes in sulphur conditions, suggesting regulation at the post-transcriptional level, similar to AtEIN3 whose protein level is strictly regulated by ethylene and carbon status (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Yanagisawa *et al.*, 2003). Only after the perception of ethylene does AtEIN3 become stabilized and bind as a dimer to primary ethylene response DNA elements, which are 28-nt imperfect palindromes found in the promoters of various ethylene-responsive genes (Solano *et al.*, 1998).

Five different EIL cDNAs from tobacco that encode deduced proteins with high similarity to the *Arabidopsis* EIN3 protein have been isolated. The highest homology was found between the N-termini (60–89% identity), which contain the DNA-binding domain and the dimerization domain (Solano *et al.*, 1998; Kosugi and Ohashi, 2000). All *NtEILs* are expressed in all organs constitutively (Rieu *et al.*, 2003). So far, mainly the *NtEII/TEIL* (tobacco EIN3-like) gene was studied. TEIL-expressing *Arabidopsis* seedlings showed a triple response phenotype indicating that TEIL is functionally similar to AtEIN3 or AtEIL1 (Kosugi and Ohashi, 2000). It was shown that TEIL binds to the promoter of the tobacco *PR1a* gene as a putative negative transacting factor (Kosugi and Ohashi, 2000). However, it was also demonstrated that, in tobacco leaves, TEIL positively regulates basic PR gene expression (wound-, jasmonate- and ethylene-inducible), but not acidic PR gene expression (salicylic acid-inducible) (Hibi *et al.*, 2007). TEIL transcript was found at various levels in all organs examined; it was lower in flowers and higher in stems and roots compared with the mature leaves (Hibi *et al.*, 2007). Also, the cis-element responsible for TEIL binding was determined by Kosugi and Ohashi (2000) by random binding selection analysis revealing that the TEIL binding site (tebs) was A[T/C][G/A][T/A][C/T]CT. The tebs sequence is present in the 5′-upstream region of various ethylene-inducible genes, but also in a large number of other promoter regions (Kosugi and Ohashi, 2000). The relatively redundant nature of the DNA-binding specificity indicates a wide prevalence of potential TEIL-binding sites in plant genomes. Although the similarity between the region responsible for DNA binding of TEIL and AtEIN3 was identical or highly similar to that of TEIL, the sequence essential for AtEIN3 binding was bound by TEIL with considerably less affinity than the TEIL consensus sequence showing differences in the binding preference between TEIL and AtEIN3 (Kosugi and Ohashi, 2000). It was proved during *in vitro* studies that the DNA binding domain of AtSLIM1 is able to bind to tebs (Yamasaki *et al.*, 2005). Also, the presence of tebs was reported in the upstream regions of several sulphur-deficiency induced genes of *Arabidopsis*, the regulation of which was also dependent on AtSLIM1 (Maruyama-Nakashita *et al.*, 2006).

The direct identification of sulphur-starvation responsive cis-regulatory elements and trans-acting factors should contribute to elucidating the molecular mechanisms regulating sulphur starvation elicited gene expression. To this end, we have made use of the *UP9C* gene of *Nicotiana tabacum* as a model to determine the molecular mechanisms of sulphur-dependent gene regulation. A 20-nt sequence was identified in the promoter of *UP9C* that was also conserved in the promoters of several *Arabidopsis* genes that were reported to be regulated by sulphur deficit. Search for transcription factors binding this sequence resulted in the identification of *NiEIL2*. This regulator appeared to induce *in planta* expression of a reporter gene controlled by the *UP9C* promoter in a sulphur deficiency-dependent manner.

**Materials and methods**

**DNA manipulations and plasmid construction**

To construct the bait plasmid for a yeast one-hybrid screen, the UPE-box of the *UP9C* promoter was PCR amplified with

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the UPEF and HISR primers (Table 1) flanking 1437 bp of the pHS2.1 vector (Clontech, http://www.clontech.com). The UPEF-box was added to the 5′-end of the forward primer UPEF. The PCR product was inserted into the MluI/BamHI sites of the pHS2.1 vector, and the corresponding construct was named pUPE-HIS. UPE-box deletions and mutations were generated in the similar way by PCR using respective forward primers (Table 1) in combination with the reverse primer HISR. The products were ligated into MluI/BamHI sites of the pHIS2.1 vector and verified by sequencing using the forward primer complementary to the vector sequence.

For trans-activation studies in Nicotiana benthamiana, NtEIL2 and AtSLIM1 coding sequences were amplified by PCR using cDNA from N. tabacum and A. thaliana as the respective templates. EILIVF and EILIVR primers were used for NtEIL2 amplification, while SLIMIVF and SLIMIVR primers were used for AtSLIM1 amplification (Table 1). All primers contained restriction sites at the 5′-end enabling, after digestion, the direct cloning of the PCR product into the NeoI/BglII site of the ImpactVector1.1-tag (ImpactVector™, Plant Research International, http://www.pri.wur.nl/UK/). The inserts were subcloned into the Asel/PacI sites of the binary plasmid pBINPLUS (ImpactVector™, Plant Research International, http://www.pri.wur.nl/UK/). The DNA sequence of the UP9C promoter was PCR amplified using the N. tabacum genomic DNA and two primer pairs. The reverse primer was always prUP9R (Table 1) and, depending on the desired size of a product, either prUP9shF or prUP9lgF was used as the forward primer. Both PCR products were digested with NotI and XbaI and cloned into the respective sites of the modified pGreenII0029 binary vector (Hellens et al., 2000). Accuracy of the cloned DNA sequence was confirmed with sequencing using forward or reverse primers homologous to the vector sequence.

To determine whether the UPE-box can, by itself, direct sulphur-deficiency induced expression in plants, a gain-of-function construct comprising a one copy of this sequence fused to the –46 bp cauliflower mosaic virus (CaMV) 35S minimal promoter was constructed. First, the –46/0 region of the CaMV 35S promoter was created by annealing the two complementary synthetic oligonucleotides 35Sm1F and 35Sm1R (Table 1) and cloned into the XbaI site of pGG (Zientara et al., 2009). This plasmid was designated as pMIN-GG. The 20-nt UPE-box was created by adding this sequence to the 5′-end of the forward primer of the subsequent PCR with primers UPEMINF and GUSR using pMIN-GG as template. The 1.8 kb fragment obtained was cloned between the EcoRI and NotI sites of pMIN-GG resulting in the pUPEMIN-GG plasmid and sequenced.

Table 1. Oligonucleotides used as primers (underlined are sequences added for restriction enzymes facilitating further cloning steps)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′-3′)</th>
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<tbody>
<tr>
<td>UPEF</td>
<td>GCACGGCGTAGATACATTTGAACCTGGACAGATCCACTAGTAATTCCTGGCAT</td>
</tr>
<tr>
<td>HISR</td>
<td>GCACGGCGTAGATACATTGAACCTGGACAGATCCACTAGTAATTCCTGGCAT</td>
</tr>
<tr>
<td>UPE-1stEIL</td>
<td>GCACGGCGTAGATACATTGAACCTGGACAGATCCACTAGTAATTCCTGGCAT</td>
</tr>
<tr>
<td>UPE-2ndEIL</td>
<td>GCACGGCGTAGATACATTGAACCTGGACAGATCCACTAGTAATTCCTGGCAT</td>
</tr>
<tr>
<td>UPEmut</td>
<td>GCACGGCGTAGATACATTGAACCTGGACAGATCCACTAGTAATTCCTGGCAT</td>
</tr>
<tr>
<td>UPE1stmut</td>
<td>GCACGGCGTAGATACATTGAACCTGGACAGATCCACTAGTAATTCCTGGCAT</td>
</tr>
<tr>
<td>UPE1stmut+3</td>
<td>GCACGGCGTAGATACATTGAACCTGGACAGATCCACTAGTAATTCCTGGCAT</td>
</tr>
<tr>
<td>UPE2nd+3</td>
<td>GCACGGCGTAGATACATTGAACCTGGACAGATCCACTAGTAATTCCTGGCAT</td>
</tr>
<tr>
<td>UPE2nd+3mut</td>
<td>GCACGGCGTAGATACATTGAACCTGGACAGATCCACTAGTAATTCCTGGCAT</td>
</tr>
<tr>
<td>EILIVF</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>EILIVR</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>SLIMIVF</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>SLIMIVR</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>prUP9shF</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>prUP9lgF</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>prUP9R</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>35SminiF</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>35SminiR</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>UPEMINF</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>GUSR</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>UP9CF</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>UP9CR</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>ACT1</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>ACT2</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>U9Gsp1</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
</tr>
<tr>
<td>U9Gsp2</td>
<td>GCGGGCCCATCTACCTACCCCAACCCGGGAAACATCTTTG</td>
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Primary PCR was performed using adaptor primer 1 (API) and a UP9C cDNA specific primer U9Gsp1. The second PCR was performed using adaptor primer 2 (AP2) and UP9C cDNA specific primer U9Gsp2 (Table 1). The amplified PCR products were cloned into the T/A cloning vector pGEM-T-Easy (Promega, http://www.promega.com), sequenced and assembled. S' RACE library was constructed using the SMART™ RACE cDNA Amplification Kit (BD Biosciences, http://www.bdbiobreurope.com) according to the procedure recommended by the manufacturer. Two gene-specific primers U9Gsp1 and U9Gsp2 (Table 1) were used to amplify the transcribed sequence upstream of ATG.

Yeast one-hybrid screen

Yeast one-hybrid screening was performed to isolate genes encoding proteins associated with the UPE-box. Total RNA was isolated from N. tabacum leaves from plants sulphur-starved for 2 d using the TRI REAGENT (Molecular Research Centre, Inc., http://www.mrcgene.com) and used for the preparation of MATCHMAKER One-Hybrid System cDNA libraries according to the manufacturer’s protocol (Clontech, http://www.clontech.com). Yeast strain Y187 was cotransformed with a cDNA library from sulphur-starved N. tabacum, linearized pGADT7-Rec2 and UPExpHis2.1 plasmid, and plated on medium lacking leucine, tryptophan, and histidine, but in the presence of 75 mM 3-amino nitrosoazole plates. The yeast screening procedure was performed essentially as described in the Yeast MATCHMAKER System 3 manual (Clontech, http://www.clontech.com). Putative positive clones were verified by cotransformation with the bait plasmid into yeast, and their sequences were analysed by HindIII digestion, to identify redundant clones, and direct sequencing.

Plant growth conditions

After bleach sterilization, seeds of N. tabacum cv. LA Burley 21 (Legg et al., 1970) and N. benthamiana were germinated on agar-solidified (0.8%) half-strength MS medium (Murashige and Skoog, 1962). Seedlings were grown in a climate chamber at 23 °C with a 16/8 h light/dark photoperiod for 3 weeks. The plates were maintained vertically to allow gravitational elongation of the root apex. After 3 weeks, plantlets were transferred to the AB liquid agar-solidified (0.8%) half-strength MS medium (Murashige and Skoog, 1962) and cultivated hydroponically in a growth chamber (16/8 h 22/18 °C, light/darkness), with weekly changes of the medium.

For UP9C expression studies 2-month-old N. tabacum plants were divided into separate groups and treated depending on the specific experiment. The control groups were transferred to the optimal medium (AB) while the others were transferred either to the sulphur-free medium (AB-S) containing 1 mM MgCl2 instead of 1 mM MgSO4; sulphur-surplus medium (AB+S), containing 10 mM MgSO4; AB-GSH medium, containing 2 mM GSH; phosphate-free medium (AB-P), containing 1 mM KCl instead of 1 mM KH2PO4; nitrate-free medium (AB-N), containing 13.5 mM KCl instead of 3.5 mM KNO3 and 10 mM NH4NO3, and 1.7 mM MgCl2 instead of 1 mM Mg(NO3)2; AB+NaCl medium, containing 100 mM NaCl; AB+Ca medium, containing 15 μM CdCl2. Unless specified otherwise, after 2 d, five plants from each group were harvested and pooled.

For transient expression experiments, after 2 weeks of hydroponic growth in AB medium N. benthamiana plants were divided into two groups. Plants from the control group were transferred to the optimal medium and plants from the sulphur-starved group were transferred to the AB-S medium, containing 1 mM MgCl2 instead of 1 mM MgSO4. After 48 h the leaves of the plants were used for A. tumefaciens infiltration.

Transient expression assays in N. benthamiana plants

The plasmids with the uidA reporter gene (pUPE-GG, pPUP9Csh, and pPUP9Clg) were introduced into the LBA4404 strain of A. tumefaciens while the plasmids for transcription factors expression (pBIN-SLIM and pBIN-EIL) were introduced into the GV3101 strain of A. tumefaciens. Five millilitres of A. tumefaciens cultures were grown overnight at 28 °C in the presence of 100 μM acetosyringone (3,5-dimethoxy-4'-hydroxy-acetophenone). Next, the cells were harvested by centrifugation and resuspended in water with 100 μM acetosyringone at an OD600=0.8 for experiments involving coinjection of two strains and an OD600=0.4 when injected individually. Prior to infiltration, a suspension of A. tumefaciens harbouring a binary plasmid containing the uidA gene was mixed in a 1:1 ratio with a bacterial suspension carrying one of the transcription factors in the binary plasmid. Three well-expanded leaves of 5-week-old hydroponically grown N. benthamiana plants were infiltrated with a 2 ml syringe without a needle. Samples were collected for protein extractions 70 h after inoculation and either used directly for histochemical GUS assays or frozen in liquid nitrogen and stored at −80 °C until processing.

β-glucuronidase (GUS) activity assays

For the qualitative assay, the activity was determined histochemically in the fresh plant tissues using X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) as a substrate (Jefferson et al., 1987; Jefferson, 1989). Three to four discs of 8 mm diameter were cut from a leaf infiltrated with A. tumefaciens and used for staining. Plant tissue was submerged in the reagent mixture (0.075% X-Gluc, 0.1% Triton X-100, 50 mM sodium phosphate, pH 8.0), treated with vacuum for 5 min and left overnight in darkness at 37 °C with gentle shaking. Next, the tissues were bleached with three changes of 70% ethanol at 65 °C for 2 h. The stained discs were photographed and stored in 70% ethanol.

For the quantitative assay, the frozen plant material, combined from three discs (8 mm diameter) collected from three different leaves of transiently transformed N. benthamiana, was ground with a pestle in the extraction buffer (100 mM sodium phosphate pH 8.0, 10 mM EDTA, 14 mM β-mercaptoethanol, 0.1% Triton X-100) and centrifuged at 11 000 g for 5 min at 4 °C. Soluble crude protein in the supernatant was quantified using the Bradford method (Bradford, 1976). For GUS activity assay 10 μl of a crude extract containing 10–20 μg of total protein was transferred to 140 μl of the extraction buffer containing 1 mM PNPG (p-nitrophenyl-β-D-glucuronide) and the mixture was incubated at 37 °C for 30 min. After colour development the absorbance was measured at 415 nm in microtitre plates and corrected by the absorbance of the sample without PNPG. The activity was calculated as μmol processed substrate mg−1 total soluble protein min−1.

Results

The UP9C gene is specifically induced in tobacco by sulphur starvation stress

The UP9C (accession number AY547446) and UP9A (accession number DQ444223) genes, both encoding proteins of unknown function have been identified as being strongly activated by sulphur deficit (Lewandowska et al., 2005; Wawrzynska et al., 2005). Both genes (UP9A and UP9C) seem to be regulated by sulphur deficit in the same manner and contribute to the total UP9 mRNA pool more or less equally (data not published). The work described in this study was performed only with UP9C gene and its promoter. To determine whether the observed induction of the UP9C transcription is specific to sulphur-deficiency stress, the level of UP9C expression was monitored under...
various plant growth conditions (Fig. 1). An elevated level of \( \text{UP9C} \) mRNA was observed after 2 d of sulphur-deficit, but not after 2 d of nitrogen- or phosphorus-starvation. In addition, neither the presence of 10-fold sulphate excess nor exogenous glutathione nor the 2 d of salt stress affected \( \text{UP9C} \) expression. Prolonged sulphur starvation (6 d) caused further accumulation of \( \text{UP9C} \) transcript. Interestingly, a 3 week exposure to 15 lM CdCl\(_2\) also resulted in an elevated level of \( \text{UP9C} \) which is in contrast to a previous observation, when 10 mM CdCl\(_2\) apparently repressed \( \text{UP9} \) expression (Wawrzynska et al., 2005). This discrepancy probably results from the differences in the experimental conditions, such as plant age and cadmium concentration.

**Homologues of \( \text{UP9C} \) exist in other plants**

A family of four \( \text{UP9C} \)-like genes, denoted \( \text{LSU1-4} \) (low sulphur) and located in pairs as direct neighbours on two chromosomes, exists in the \( \text{Arabidopsis} \) genome. It was possible to follow the expression of three of them, namely At3g49580 (\( \text{LSU1} \)), At5g24660 (\( \text{LSU2} \)), and At5g24655 (\( \text{LSU4} \)) by GENEVESTIGATOR (Zimmermann et al., 2004). The fourth gene, At3g49570 (\( \text{LSU3} \)), was not included on Affymetrix ATH1 genome arrays. Two genes, \( \text{LSU1} \) and \( \text{LSU2} \), were strongly induced by sulphur-deficit, hydrogen peroxide, salt stress, and AgNO\(_3\), while the expression of the third one, \( \text{LSU4} \), was apparently more inert (Fig. 2). Apart from the conspicuous response to the four stresses mentioned above, the expression of these genes remained at the low level during other treatments.

Database searches revealed that at least seven homologues of the \( \text{UP9C} \) gene exist in the tobacco genome (data not shown). Families of \( \text{UP9C} \)-like proteins are also present in other plant species, such as tomato, potato, soybean, rice, corn, grape wine, and poplar; however, no data on their function (nor gene expression) are available.

**Cloning and analysis of the \( \text{UP9C} \) promoter**

The 5′-RACE analysis showed that the \( \text{UP9C} \) gene has only one transcription start site (data not shown), and the transcription begins at an adenine residue located 109 bp upstream of the translational start site, which conforms to the principle of the transcription start site in plant genes (Joshi, 1987). Subsequently, an approximately 1.1 kb DNA fragment, upstream of the translation start site of the \( \text{UP9C} \) gene, has been cloned by Genome Walking (accession number GQ396657). An analysis using the PLACE database (http://www.dna.affrc.go.jp/PLACE) indicated that the \( \text{UP9C} \) promoter contains the basal elements (TATA box and CAAT box) near the ATG, responsive cis-elements for light, salt, and several phytohormones such as abscisic acid, ethylene, and cytokinins. A core of the sulphur-responsive element (SURE) (Maruyama-Nakashita et al., 2005) was also found 351 bp upstream of the ATG.

A question was raised whether the \( \text{UP9C} \) promoter and the promoters of its four \( \text{Arabidopsis} \) homologues share sequence motifs that might have a role in sulphur-deprivation-induced expression. Computational analysis using MEME software (http://meme.sdsc.edu; (Bailey and Elkan, 1994)) revealed the presence of a 20-nt DNA motif (AGATACATTGAACCTG-GACA) in all but one (\( \text{LSU4} \)) promoters analysed. This sequence was designated the UPE-box. The localization of UPE-boxes and SURE sequences in the searched promoters is shown in Fig. 3.
Further examination of the UPE-box revealed that it consists of two parallel tebs sequences (TEIL binding site). tebs is a 8 nt consensus $AG(G/A)T(T/A)C(A/G)T$ with the underlined nucleotides appearing to be required for strong binding to TEIL (Kosugi and Ohashi, 2000). The UPE-box contains two partially overlapping tebs in opposite orientation to each other. It was of interest to evaluate the number of existing UPE-boxes in the plant genomes. Due to the incomplete genomic sequence of tobacco, there are limited data on promoters of sulphur-starvation regulated genes. Thus, the Arabidopsis genome was searched for the presence of UPE-box, allowing for degenerate nucleotides in the two tebs and five nucleotides at the 3′ end of the motif. It was only found in the promoters of eight genes (Table 2), all of which being strongly inducible during sulphur deprivation (Maruyama-Nakashita et al., 2006). Surprisingly, the full UPE-box is also present 48 nt downstream of the 3′ UTR of At5g10180 encoding sulphur transporter Sultr2;1. Taking into consideration the presence of degenerated nucleotides found in the UPE-boxes of the genes in Table 2, the final consensus of the UPE box is $AG(G/A)T(T/A)CATTGAA(T/C)CT(A/G)GAC(A/G)$.

In order to identify the transcription factors mediating gene activation during sulphur starvation, a yeast one-hybrid screen was performed using the UPE-box from the tobacco UP9C gene as a bait. A Y187 yeast reporter strain containing a HISTIDINE3 (HIS3) gene under the control of a chimeric promoter comprising a single UPE-box sequence fused to the HIS3 minimal promoter was used to screen GAL4-fused cDNA libraries derived from the leaves of tobacco grown without a sulphur source for 2 d (see Experimental procedures). This UPE-box reporter yeast strain is unable to grow on selective medium (without histidine and supplemented with 3-aminotriazole), thus allowing efficient screening. After the screening of $5 \times 10^6$ yeast transformants, 35 candidates were selected for subsequent testing on more stringent selective medium, followed by retransformation of yeast with isolated plasmids. Finally, only three clones were positively verified. Sequence analysis revealed that all three clones corresponded to the in-frame GAL4-AD fusions with the full-length coding region of NtEIL2 cDNA, encoding a member of the EIL family of transcription factors.

Mutations in the UPE-box affect the binding strength of NtEIL2 and AtSLIM1

To examine the specificity of the interactions of NtEIL2 with the UPE-box, the plasmid with the NtEIL2-GAL4-AD fusion was used to transform yeast strains harbouring the HIS3 reporter fused to several mutated or truncated versions of the UPE-box (Fig. 4). Since AtSLIM1, proved to have a role in transcription activation during sulphur deficiency (Maruyama-Nakashita et al., 2006), also belongs to the EIL family of transcription factors, it was decided to include AtSLIM1 as an additional control (Fig. 4). The respective positive and negative controls were supplied by the manufacturer (Matchmaker; Clontech, http://www.clontech.com). Significant yeast growth was only detected when the NtEIL2 or AtSLIM1 clones were
expressed in the presence of the unmodified UPE-box (Fig. 4, variant 1). Interestingly, deletion of the first tebs (variant 2) had a lesser effect on yeast growth than the deletion of the second tebs (variant 3). The latter resulted in severe growth retardation (NtEIL2) or no growth at all (AtSLIM1). To maintain the same distance from the promoter, five nucleotides from the second tebs were removed in the 3’ end of the UPE-box (variant 7) or added to the first tebs (variant 8). These changes had no impact on the ability of the transformed yeast to grow on the selective media. This result suggests that differences in the sequences of the two tebs, rather than differences in their distance from the promoter, are responsible for the observed effects on yeast growth.

The effects of the mutations of the crucial nucleotides in two tebs of the UPE-box on their binding ability (variants 4–6) were also examined. Again, it appeared that the second tebs in the motif enabled stronger binding of the NtEIL2 or AtSLIM1, since mutations in its sequence had a greater impact on yeast growth than mutations in the first tebs. It is of note that whenever AtSLIM1 was used to interact with the UPE-box and its mutated versions, the yeast growth was always weaker. That might indicate that binding of AtSLIM1 to the UPE-box is weaker than that of NtEIL2.

Discussion

Plants are able to sense sulphur deficiency and process this information to bring about changes in gene expression that allow effective adaptation to such conditions. Identification of cis-acting elements and trans-acting factors that work upstream of genes that comprise the first wave of sulphur-deficiency-responsive genes might help to understand the molecular bases of these processes. Here, the focus was on factors regulating the expression of a tobacco gene UP9C, which is specifically induced during sulphur deficit and cadmium exposure (Fig. 1). Up-regulation of UP9C by cadmium also supports the role of the UP9C protein in sulphur metabolism because this toxic metal induces genes encoding proteins involved in the sulphate assimilation.
pathway by generating a strong sink for reduced sulphur metabolites (Mendoza-Cozatl et al., 2005). In the conditions of our experiment, no effects of salt stress on the expression of UP9C in the leaves was detected, while in Arabidopsis two out of three tested UP9C-like genes (LSU1 and LSU2) are induced by salt stress (Fig. 2). However, the salt stress response in Arabidopsis was monitored after 24 h post-induction, while the UP9C expression in tobacco was tested after 2 d. If the salt stress response of UP9C is transient it would explain why no induction was observed in the conditions of our experiment. Interestingly, in Arabidopsis the two LSU genes responsive to sulphur starvation were also induced by hydrogen peroxide and AgNO3 (Fig. 2).

While the hydrogen peroxide response could easily be explained by oxidative stress being the strong sink for protective thiol compounds (a condition resembling sulphur starvation) the AgNO3 induction indicates connections with the ethylene signalling pathway. AgNO3 is an inhibitor of ethylene action that interferes with ethylene binding to the receptor (Beyer, 1976). On the contrary, there was no impact on the expression of LSU genes when Arabidopsis was treated with ethylene itself or another ethylene inhibitor, aminoethoxyvinylglycine (AVG, Fig. 2). AVG stops ethylene biosynthesis by inhibiting ACC synthase (Mattoo et al., 1979). Therefore, it is reasonable to speculate that the observed induction by AgNO3 is connected with ethylene action rather than with its biosynthesis. It is also possible that this induction may result from the cytotoxic effect of the heavy metal, Ag. No data on ethylene involvement in the regulation of UP9C in tobacco are available.

A palindromic UPE-box, AGATACATTGAACCTGGACA, was found in the 5’ upstream region of the tobacco UP9C gene. Database searches of the Arabidopsis genome sequence revealed that this consensus could only be found in the promoters of several genes, all up-regulated by sulphur deficiency. It was presumed that the UPE-box is a target for a transcription factor controlling gene expression. From the sequence composition, a member of the EIL family could be expected to bind to the UPE-box since it consists of double tebs elements, specifically targeted by NtEIL1/TEIL (Kosugi and Ohashi, 2000) and then proven to bind other members of the EIL family (Lee and Kim, 2003; Yamasaki et al., 2005). However, no conclusive evidence indicates that any of these proteins (apart from AtSLIM1) function as an actual transcription factor in sulphur deficiency-responsive gene expression. The presence of palindromic repeats in the UPE-box with double tebs, suggests that NtEIL2 interacts with its target as a dimer, a situation already observed for AtEIN3 (Solano et al., 1998). Therefore, it was checked whether the deletion of one of the tebs from the UPE-box or changes of crucial nucleotides would have any impact on the binding efficiency. The same studies were also performed using AtSLIM1, a transcription factor from Arabidopsis regulating the sulphur response (Maruyama-Nakashita et al., 2006). The major conclusion from our yeast experiments is that the second tebs is more critical for DNA-protein
interaction than the first one. Since the second tebs is followed by five additional nucleotides of the UPE-box, it could not be excluded that it was a distance effect and different positioning of the particular tebs to the minimal promoter. However, yeast growth was still similar when these five nucleotides were added to the first tebs or omitted from the second one (Fig. 4, variants 8 and 7, respectively), indicating that the last five nucleotides in the 3'-end of the UPE-box had no effect on binding of NtEIL2 or AtSLIM1. It was also noticed that AtSLIM1 interactions with the UPE-box and its mutated versions were, in every case, somewhat weaker than those of NtEIL2. Binding of AtSLIM1 to the tebs sequence can be unstable, as it was only detectable with surface plasmon resonance (Yamasaki et al., 2005) but not by the electro-mobility shift assay (Solano et al., 1998). Therefore, it was suggested that these differences in binding kinetics may imply a distinct function of AtSLIM1 in transcriptional regulation, separated from the ethylene signalling pathways and tebs to be more specific for EIL proteins governing the ethylene response (Maruyama-Nakashita et al., 2006).

To check how NtEIL2 and AtSLIM1 trans-activate the UP9C promoter, the constructs with reporter cassettes were transiently introduced into N. benthamiana leaves together with the constructs containing the expression cassettes for any of the two proteins. The transformed plants were grown either in sulphur-sufficient or in sulphur-deficient conditions. In addition, it was tested whether the presence of any other cis-acting sites in the UP9C promoter might have an influence on the activation of the reporter gene and the UP9C promoter fragment of two different lengths being used. In the conditions of sufficient sulphur supply, the NtEIL2 failed to activate reporter gene expression driven by the UP9C promoter. It suggests that some kind of repression of the activating or binding domain is taking place to protect the responsive genes from unnecessary induction. Whether this repression is due to intramolecular interactions or is mediated by other factors that bind and indirectly repress the transcriptional activation domain is unknown. There are several examples of intramolecular repression in the βHLH family of transcription factors (Dai and Cserjesi, 2002) and a similar mechanism might occur in the EIL family. Notably, AtSLIM1 did not show any sulphur-deprivation-dependent activation of the reporter and was able strongly to induce the expression even during the sulphur-sufficient conditions. It is therefore more plausible that a cofactor rather than an intramolecular mechanism is responsible for the observed transcriptional repression in sulphur-sufficient conditions. In the heterologous N. benthamiana cells, AtSLIM1 could not be properly recognized by such a potential interacting partner and failed specifically to turn off transcription in sulphur-sufficient conditions. Differences in regulation might also be associated with differences in growth rate or size between species, a situation already observed for E2F from rice and Arabidopsis (Kosugi and Ohashi, 2002). Finally, it is also possible that NtEIL2 is lacking the autonomous nuclear localization signal and needs a specific partner triggering effective nuclear import during sulphur deficiency. In the case of AtSLIM1 protein, it was demonstrated that its nuclear localization was not affected by sulphur conditions (Maruyama-Nakashita et al., 2006). To identify potential cofactors of NtEIL2 and AtSLIM1, it will be useful to screen for the interacting proteins using a yeast two-hybrid assay. Solano et al. (1998) showed that AtEIN3, AtEIL1, and AtEIL2 are capable of forming homodimers and, as such, bind to DNA. By contrast, the isolated DNA binding domain of AtSLIM1 existed in a monomeric form (Yamasaki et al., 2005), suggesting the importance of dimerization for the stable binding to a pseudo-palindromic sequence recognized by AtEIN3, but also a possibility of a monomeric domain binding to a single tebs. Alternatively, it is possible that the EIL family members from different species might possess slightly different DNA-binding specificity (e.g. distinct target sequences and different binding activities) or differential signal thresholds and sensitivities, resulting in the activation of different sets of gene expression.

Another interesting observation from our experiments is the higher expression of the reporter in the case of the shorter fragment of the UP9C promoter (192 bp upstream of ATG) than the longer fragment (419 bp upstream of ATG). It suggests the presence of a negative cis-element located between –419 bp and –192 bp of the UP9C promoter. It might indicate that the SURE element, the first cis-acting sequence responsive to sulphur-deficiency conditions to be discovered (Maruyama-Nakashita et al., 2005), which locates there, is actually binding a transcriptional repressor preventing transcription during sulphur sufficiency. Such protein might be degraded or modified in a way enabling efficient transcription during sulphur deficiency. However, we were unsuccessful in isolation of any factors able to bind to SURE element via yeast one-hybrid screening (not shown). It may be due to the fact that such a factor binds as a heterodimer or it might require plant-specific modifications for binding. The SURE element resembles an auxin response factor (ARF) binding sequence (GAGACA). However, SURE is not responsive to auxin, indicating its specific function in sulphur response (Maruyama-Nakashita et al., 2005). Nevertheless, it is possible that a not-yet-identified member of the ARF family of transcription factors (Guilfoyle and Hagen, 2007) is capable of binding to the SURE sequence. Recently, several transcription factors relevant to auxin signalling (IAA13, IAA28, and ARF-2) were proved to respond to sulphur starvation (Falkenberg et al., 2008). However, they probably serve as co-ordinators of the metabolic shift driving sulphur homeostasis rather than as direct effectors of the sulphate assimilation pathway.

Although the involvement of NtEIL2 was demonstrated in the regulation of the UP9C promoter, we failed to prove that the UPE-box alone is able to drive the sulphur-deficiency-dependent transcription in plant cells (Fig. 5). Using more than one copy of the UPE-box might give positive results, since multimers have been shown to confer expression to minimal promoters more efficiently than
monomers, probably by compensating for the absence of general positive regulatory elements (Pontier et al., 2001; Rushton et al., 2002). Other explanations would be that either the spacing between the UPE-box and the minimal 35S promoter is incorrect in this artificial construct or that the UPE-box requires additional essential regulatory sequences. cis-elements, called coupling elements that are active in combination with an ABRE element but not active alone have been identified in ABA responsive genes (Shen and Ho, 1995). Alternatively, the last five nucleotides conserved in the UPE-box, GGACA, might bind a transcription factor that negatively regulated the expression of the reporter gene. However, computational searches indicated that such sequence does not correspond to any known regulatory motif.

Although direct involvement of NtEIL2 in sulphur-deficiency-dependent regulation of UP9C expression seems to be certain, NtEIL2 is more similar to AtEIN3 and AtEIL1 than to AtSLIM1, which, in turn, shares 97% amino acid identity with NtEIL1 protein proven to function in ethylene signalling (Solano et al., 1998; Hibi et al., 2007). On the other hand, some results suggest that the phylogeny tree is not the best predictor of the specific function of members of the EIL family. Firstly, attempts to demonstrate that AtEIN3 is a functional orthologue of NtEIL1 failed, despite the fact that they were the most closely related from the EIL families of these two species (Solano et al., 1998). Secondly, no correlation between the amino acid structure of banana EIL proteins and their ability to complement the Arabidopsis ein3 mutant was observed (Mbegue et al., 2008). Therefore, it is plausible that NtEIL2 is a functional orthologue of AtSLIM1, despite not being its closest homologue. The straightforward verification of this hypothesis will be the ability of NtEIL2 to complement the Arabidopsis slim1 mutant. Further studies in planta will also be required to assess if other related members of the tobacco EIL family are able to act as regulatory factors of UP9C gene expression. Therefore, in future experiments, other tobacco EIL proteins will be tested for their ability to activate UP9C promoter, also during other stresses.

The NtEIL2 binding site (the UPE-box, present in the UP9C promoter) greatly contributes to the up-regulation of transcription. However, our results suggest that there might also be other elements responsible for the specific induction of UP9C during sulphur starvation. In addition, the UPE-box is not universally present in all genes dependent on AtSLIM1 and could only be found in the promoters of several genes with expression influenced by sulphur starvation (Table 2). Three of these genes, encoding APR1, APR3, and Sultr2;1, have the UPE-box consensus in their UTRs and all three of them show up-regulation during sulphur deficiency and this is not influenced by the slim1 mutations (Maruyama-Nakashita et al., 2006). All in all it suggests that NtEIL2 and AtSLIM1 are not the single trans-acting factors of plant sulphur metabolism. Since a proper metabolic response is critical for plant survival during sulphur starvation, it is not surprising that plants have evolved multiple regulatory mechanisms to control gene expression depending on sulphur status in the environment. NtEIL2 and AtSLIM1 are probably just part of a complex signalling cascade, with multiple elements needed for an adjustment of plant metabolism to the conditions of sulphur deficiency. It is tempting to speculate that UP9C would be part of this network; however, our preliminary results from the yeast two-hybrid screen show that NtEIL2 and UP9C are not interacting directly (data not shown). Whether the situation is true for AtSLIM1 and any of the four Arabidopsis UP9C homologues or whether UP9C is a part of such sulphur-nutrition status signalling or sulphur-deficiency response network, just not directly connected with NtEIL2, awaits further investigation.

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