RESEARCH PAPER

Using a suppression subtractive library-based approach to identify tobacco genes regulated in response to short-term sulphur deficit

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Abstract

Monitoring expression at the transcriptional level is an essential first step for the functional analysis of plant genes. Genes encoding proteins directly involved in sulphur metabolism constitute only a small fraction of all the genes affected by sulphur deficiency stress. Transcriptional responses to various periods of sulphur deprivation have been extensively studied in Arabidopsis thaliana; however, no corresponding data are available for Solanaceae sp. To address this problem, a subtractive library-based approach to search for tobacco genes regulated by a short-term sulphur starvation has been adopted. In this work, 38 genes were identified, of which 22 were regulated positively and 16 were regulated negatively. The transcript levels of the representative genes were monitored in four parts of the plants (mature and immature leaves, stems, and roots), which exhibited differential sulphur deficiency. Interestingly, some genes exhibit different regulation of expression in different parts of the plants. Database analysis allowed assignment of the potential function for many of the identified genes; however, the functions of a small number of genes strongly regulated by sulphur starvation remain unknown. The genes were grouped into nine functional categories, each including both up- and down-regulated genes. The possible links between the identified regulated genes and sulphur metabolism are considered, and compared where possible with expression patterns in Arabidopsis thaliana. Although no obvious regulatory genes were identified, the genes encoding proteins of unknown function remain as potential components of the regulatory processes.

Key words: Starvation, stress signaling, sulphur metabolism, suppression subtractive hybridization, tobacco.

Introduction

Recent studies involving high-throughput technologies have shown that almost all environmental and physiological transitions in bacteria, yeast, and plants induce massive and genome-wide changes in gene expression (Mahalingam et al., 2003; Orlandi et al., 2004; Palma et al., 2004; Schuller et al., 2004). Responses to environmental stresses such as drought and cold stress (Seki et al., 2001) and oxidative stress (Desikan et al., 2001) were studied in plants using microarrays in order to obtain the global patterns of gene expression in such conditions. In Arabidopsis, over 1000 genes were found to respond within 20 min to the change of the nitrogen source from ammonium to nitrate (Wang et al., 2003). Among the genes induced by nitrate were nitrate transport- and nitrate assimilation-related genes, glycolysis-related genes, and genes involved in sulphur metabolism including sulphate transporters and APS-reductase. Moreover, many potential regulatory genes were also found to respond to nitrate. The
complexity of signalling events associated with the sensing of stress and the activation of defence and acclimation pathways is believed to involve ROS, calcium, calcium-regulated proteins, mitogen-activated protein cascades, and crosstalk between different transcription factors (Sirko et al., 2003).

Sulphur availability plays an important role in the growth and development of higher plants (Hell and Hillebrand, 2001). It is not known how S-availability is perceived and transmitted within the plant or within individual plant cells. Untargeted analyses of gene expression in response to sulphur starvation have been performed primarily in two plant species, maize and Arabidopsis thaliana. In maize, mRNA differential display analysis resulted in the identification of a novel gene encoding a polypeptide with a strong homology to isoflavone-reductase. The gene is up-regulated in response to the lowering of glutathione levels due to growth under sulphate-deprived conditions (Petrucco et al., 1996). In Arabidopsis, the results obtained by three independent groups using microarray approaches give complementary views of the transcriptional responses upon various periods of S-deprivation (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003). For Solanaceae, information about transcriptional changes in response to sulphur starvation is rather scarce and has been limited to monitoring of the expression of a small number of nitrogen metabolism-related genes in tobacco after prolonged S-starvation (Migge et al., 2000). Despite the fact that overall organization of sulphate assimilation and S-metabolism seems to be very similar in most plants, specific differences in the mechanisms controlling methionine biosynthesis have been reported between Arabidopsis thaliana and potato (Nikiforova et al., 2002). Thus, identification and comparing genes regulated by sulphur deficiency in various plants, not only in Arabidopsis, may be advisable. In addition, the subtractive approach is preferable to microarrays for the identification of genes with a low level of expression.

Here, tobacco (Nicotiana tabacum) has been chosen as a model system for Solanaceae sp. Even though the analysis of global transcription responses in tobacco might be complicated by the lack of the complete genomic sequence of this organism, many research groups use this plant for ‘global’ approaches. For example, expression of tobacco genes can be studied by using cDNA microarrays from tomato (Rizhsky et al., 2002), because of the significant homology between these two closely related plant species, although usefulness of this approach is limited to the more abundantly expressed genes. Recently, a cDNA-AFLP method has been developed and demonstrated to be useful for genome-wide expression analysis in tobacco (Breene et al., 2003; Vandenabeele et al., 2003). Identification of the genes encoding proteins with regulatory functions for adaptation to sulphur-limiting conditions is the major challenge of the research on plant sulphur metabolism. The genes encoding regulatory proteins are usually expressed at low-levels and, therefore, a suppression subtractive library-based approach optimized for fast and easy identification of weakly expressed genes with strongly (several-fold) regulated expression, was used (Luo et al., 1999).

Materials and methods

Plant materials and growth conditions

Surface-sterilized seeds of Nicotiana tabacum LA Burley 21 (Legg et al., 1970) were germinated on MS (Murashige and Skoog, 1962) plates containing 0.8% agar. Three-week-old plantlets were transferred to the liquid AB medium (3.5 mM KNO₃, 5 mM Ca(NO₃)₂, 1.7 mM Mg(NO₃)₂, 10 mM KH₂PO₄, 1 mM MgSO₄, 0.9 mM MgCl₂, 2 mM CaCl₂, 0.1 mM NaCl, 50 μM FeNaEDTA, 0.64 μM Cu(NO₃)₂, 10 μM Mn(NO₃)₂, 0.82 μM (NH₄)₂MoO₄), 0.096 μM (CH₃COO)₂Zn, 0.11 μM CoCl₂, 50 μM H₂BO₄) and cultivated hydroponically in the growth chamber (16 h light, 3000 lx, 20 °C/8 °C darkness, 18 °C) for about 2 months. Every 2 weeks the plants were transferred to fresh AB medium. At this point the plants were divided into two groups: the plants from the control group were transferred to optimal medium and the 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 plants were harvested, fractioned into four parts (young developing leaves, mature leaves, stems, and roots) and pooled from ten plants per condition.

Seedlings used for the experiment to test the effects of the stress conditions were germinated for 10 d on two layers of Whatmann 3MM filter paper placed on the surface of the MS plates. They were transferred with the filter paper for 2 d onto fresh plates in the stress conditions indicated.

RNA isolation

Total RNA was isolated from frozen leaf tissue using the cold phenol method (Linthurst et al., 1993). Briefly, tissue was ground to a fine powder in liquid nitrogen and about 500 mg was homogenized in mixture of 400 μl of phenol and 400 μl of buffer consisting of 350 mM glycine, 48 mM NaOH, 340 mM NaCl, 40 mM EDTA, 4% SDS; then allowed to stand at room temperature for 5–10 min. Samples were mixed by vortexing and then centrifuged for 10 min at 10 000 g. The upper aqueous layer was transferred to a clean tube and 400 μl of phenol was added. Again, samples were vortexed and centrifuged. Subsequently, two more extractions were performed with 400 μl of phenol/chloroform (1:1, v/v) and 400 μl of chloroform. RNA was precipitated overnight with 1:3, v/v of 8 M LiCl₂ at 4 °C. Samples were centrifuged for 15 min at 10 000 g and the pellet was resuspended in 400 μl of water and again precipitated with ethanol. After centrifugation for 15 min at 10 000 g the RNA pellet was washed with 75% ethanol and resuspended in RNase-free water. The typical yield of total RNA was 30–70 μg 100 mg⁻¹ leaf tissue. Poly(A)⁺ RNA was purified from 600 μg of total RNA using Oligotex mRNA Midi Kit (Qiagen).

Suppression subtractive hybridization (SSH)

SSH was carried out using the PCR-Select Subtractive Hybridization Kit (Clontech). Experimental and control samples for each treatment were processed simultaneously to reduce false positives. The amount of mRNA was increased to 3–4 μg instead of the 2 μg recommended by the manufacturer to compensate for the loss of mRNA during the phenol–chloroform extractions. cDNA prepared from the treated...
samples (sulphur deficiency) was spotted after heat denaturation (5 min, 95°C) with 100 mg l⁻¹. The 500 bp fragment from exon3 of the Tac9 gene encoding Nicotiana tabacum actin (GenBank accession no. X69885) was amplified with the specific primers (supplementary material is available at JXB online) using the adaptor-ligated cDNA as the template in order to test the ligation efficiency, as recommended by the manufacturer. Two rounds of PCR amplification were performed under low stringency conditions according to the manufacturer’s protocol in order to enrich differentially regulated genes.

Cloning and differential screening
Secondary PCR products of SSH were cloned into the T/A cloning vector pGEM-T Easy (Promega) according to the manufacturer’s instructions and introduced into DH5α E.coli cells. About 1000 colonies from each subtraction experiment were obtained. Randomly picked single colonies were grown O/N in 5 ml of liquid LB medium with 100 mg l⁻¹ ampicillin. The plasmid DNA isolated from these cultures was spotted after heat denaturation (5 min, 95°C) in duplicate onto nylon membranes. The membranes were hybridized under stringent conditions with equivalent amounts of the probes labelled with digoxigenin (DIG) generated from either unsubtracted or subtracted cDNAs. DNA probes were non-radioactively labelled using the PCR DIG Probe Synthesis Kit (Roche) following the manufacturer’s protocol. Primers from the SSH Kit were used in PCR reactions in the conditions exactly as secondary PCR in the SSH experiment with primary PCR products as template. Reaction products were applied directly for hybridization without purification. Blots were hybridized and washed according to standard procedures (Sambrook et al., 1989). An immunological detection was carried out using anti-DIG antibodies, conjugated with alkaline phosphatase (DIG Detection Kit from Boehringer Mannheim) following the manufacturer’s protocol. A chemiluminescent substrate (CDPStar Ready-to-use, Roche) was used for signal development. Membranes were exposed to an X-ray film (X-Omat AR, Kodak) for 1–10 min depending on the strength of the chemiluminescent signals.

Sequencing, sequence analysis and accession numbers
Sequencing was carried out by the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, IBB PAS. DNA fragments were automatically sequenced in an ABI377 DNA Sequencer (Applied Biosystems) using the universal forward or reverse primers homologous to vector sequence. Each sequence was edited to correct sequencing ambiguities and to remove the primer sequence. The edited sequences were used to query the GenBank database at NCBI (http://www.ncbi.nlm.nih.gov) using the BLAST sequence comparison algorithms.

The sequences of the tobacco cDNA clones identified in this work were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) and their accession numbers are listed in Table 1.

Northern blot analysis
Total RNA was fractionated on a 1% agarose RNA gel and transferred to Hybond N nylon membrane (Amersham). RNA immobilized on nylon membranes was visualized by staining in 0.02% methylene blue (Herrin and Schmidt, 1988) and photographed prior to hybridization.

DNA probes specific to the identified cDNA clones were non-radioactively labelled with digoxigenin using universal M13 forward and reverse primers and in PCR reactions. The probes for the detection of the transcript specific to APR (AY648056), parB (D10524), SR (D83583), SOD (X55974), rbcS (X02353), and actin/Tac9 (X69885) were generated by PCR using the total N. tabacum DNA as a template and the pairs of respective primes (supplementary material is available at JXB on-line). Blots were hybridized and washed according to standard procedures. Detection was carried out following the manufacturer’s protocol for DIG-labelled probes. A chemiluminescent substrate (CDPStar Ready-to-use, Roche) was used for signal development. Membranes were exposed to an X-ray film (X-Omat AR, Kodak) for at least 5 min (up to 1 h) depending on the strength of the chemiluminescent signals.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis
For the cDNAs whose transcription level was undetectable on northern blots, semi-quantitative RT-PCR was conducted. Total RNA purified separately from all pooled parts of the tobacco plants served as templates. The 20 μl RT reaction contained 5 μg RNA, 2 pmol of specific antisense primer, 1 mM dNTPs mix, 10 mM DTT, and 1 μl of PowerScript™ Reverse Transcriptionase (Clontech) in buffer supplied by the manufacturer. The RNA and primers were preheated to 70 °C for 10 min and snap-cooled in ice-water before adding the remaining components; the RT reactions (1 h, 42 °C) were terminated (15 min, 70 °C). 1 μl of the reaction mixture was then used for the PCR with the specific primers designed for selected cDNAs. The variable number (25, 30, or 35) of the cycles (30 s denaturation at 94 °C, 30 s annealing at 50 °C, and a 1 min extension at 72 °C) were then performed. Actin cDNA amplification using the same temperature profile served as the internal control. DNA samples were separated by electrophoresis on 1% agarose gels, and the images of the ethidium-bromide-stained bands were obtained with BioRad Imaging System.

Determination of soluble (non-protein) thiols
Soluble thiols present in leaf samples were determined after forming conjugates with DTNB (5,5-dithio-2-nitrobenzoic acid). About 250–450 mg of frozen, ground material was put into microcentrifuge tubes, weighed, and subsequently 500 μl of ice-cold 0.1 N HCl was added. After vortexing, all the samples were spun down (14 000 g, 10 min, 4 °C) and supernatants collected for analysis. Aliquots of 200 μl were added to 800 μl of neutralization buffer (0.5 M K2HPO4 containing 25 μl of 10 mM DTNB). The absorbance of the samples was immediately measured at 412 nm and corrected by the absorbance of the samples without DTNB. The amount of the soluble thiols was expressed as nmol g⁻¹ FW. Using this method the sum of all reduced forms of non-protein thiols was detected.

Determination of ascorbate and dehydroascorbate
Ascorbic (AA) and dehydroascorbic (DHA) acid content was determined by the formation of a ferrous-dipyridyl complex according to Okamura (1980) with the modification of Knörrer et al. (1996). 200–400 mg of frozen, ground material was extracted with 1 ml of 0.1 N HCl and centrifuged (14 000 g, 10 min, 4 °C). Then, to 500 μl of the reaction mixture was then used for the PCR with the specific primers designed for selected cDNAs. The variable number (25, 30, or 35) of the cycles (30 s denaturation at 94 °C, 30 s annealing at 50 °C, and a 1 min extension at 72 °C) were then performed. Actin cDNA amplification using the same temperature profile served as the internal control. DNA samples were separated by electrophoresis on 1% agarose gels, and the images of the ethidium-bromide-stained bands were obtained with BioRad Imaging System.

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<table>
<thead>
<tr>
<th>Clone</th>
<th>Length (bp)</th>
<th>GenBank accession no.</th>
<th>Homology/putative ID</th>
<th>BLAST program&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Matching sequence&lt;sup&gt;b&lt;/sup&gt; (Probability)/plant</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>UP-1</td>
<td>175</td>
<td>AY547439</td>
<td>Catalase</td>
<td>N</td>
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<td>711</td>
<td>AY547440</td>
<td>1-Aminocyclo-propane-1-carboxylic acid oxidase (ACC oxidase)</td>
<td>N</td>
<td>X83229 (e-110)/N. tabacum</td>
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<td>UP-3</td>
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<td>Thioredoxin peroxidase</td>
<td>N, X</td>
<td>NP_176072 (3e-46)/A. thaliana</td>
</tr>
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<td>UP-5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>350</td>
<td>AY547442</td>
<td>Similar to pectin acetyl esterase, putative, (C-terminus)</td>
<td>N</td>
<td>X</td>
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<td>UP-6</td>
<td>162</td>
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<td>Elicitor responsive protein (Gly-, His-rich; unknown function)</td>
<td>N</td>
<td>AB040409 (8e-48)/N. tabacum</td>
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<tr>
<td>UP-7</td>
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<td>Acidic endochitinase (PR-P; PR-Q)</td>
<td>N</td>
<td>X51426 (0.0)/</td>
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<td>40S ribosomal protein S23 (S12)</td>
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<td>X</td>
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<td>N, X</td>
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<tr>
<td>UP-11</td>
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<td>Cysteine proteinase</td>
<td>N</td>
<td>AB032168 (e-162)/N. tabacum</td>
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<tr>
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<td>Ubiquitin activating enzyme (E1)</td>
<td>N</td>
<td>AB124879 (0.0)/N. tabacum</td>
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<td>UP-15&lt;sup&gt;e&lt;/sup&gt;</td>
<td>479</td>
<td>AY547449</td>
<td>Unknown function</td>
<td>N, X, E</td>
<td>E: CK282032 (e-134)/N. benthamiana</td>
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<td>484</td>
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<td>Calmodulin–NtCaM4</td>
<td>N</td>
<td>AB050840 (0.0)/N. tabacum</td>
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<td>CO046507</td>
<td>LHC-I chlorophyll a/b binding protein</td>
<td>N</td>
<td>X64198.1 (e-100)/</td>
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<td>L13441.1 (5e-47)/N. tabacum</td>
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<td>X</td>
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<td>Plastidic cysteine synthase</td>
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<td>UP-23</td>
<td>273</td>
<td>CO046511</td>
<td>NADP-dependent malic enzyme</td>
<td>N</td>
<td>AB029513 (4e-14)/S. tuberosum</td>
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<td>UP-24</td>
<td>216</td>
<td>CO046512</td>
<td>GTP-binding, TypA, involved in stress response</td>
<td>N</td>
<td>AB029514 (1e-10)/S. tuberosum</td>
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<td>UP-27</td>
<td>258</td>
<td>CO046513</td>
<td>Ribosomal protein S15 (40S)</td>
<td>N</td>
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<tr>
<td>UP-28</td>
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<td>CO046514</td>
<td>Histone H3</td>
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<td>NM_101535.1 (e-17)/A. thaliana</td>
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<tr>
<td>UP-30&lt;sup&gt;f&lt;/sup&gt;</td>
<td>256</td>
<td>CO046515</td>
<td>Elicitor-induced, belongs to the family of Gly-rich proteins (GRP) of unknown function, possibly cell-wall located</td>
<td>N</td>
<td>AB041515 (e-72)/N. tabacum</td>
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<td>UP-31</td>
<td>307</td>
<td>CO046516</td>
<td>Chlorophyll ab binding protein, similar to cab9 encoding CP29</td>
<td>N</td>
<td>BT014450 (e-118)/L. esculentum</td>
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<tr>
<td><strong>Down-regulated</strong></td>
<td></td>
<td></td>
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<tr>
<td>D-1</td>
<td>395</td>
<td>AY547451</td>
<td>Similar to pectin acetyl esterase (C-terminus)</td>
<td>N, X, T</td>
<td>BT014450 (e-118)/L. esculentum</td>
</tr>
<tr>
<td>D-3</td>
<td>328</td>
<td>AY547452</td>
<td>Cystatin (cysteine proteinase inhibitor)</td>
<td>N</td>
<td>X</td>
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<tr>
<td>D-4</td>
<td>174</td>
<td>AY547453</td>
<td>Similar to glycolate oxidase</td>
<td>N</td>
<td>T</td>
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<td>D-6&lt;sup&gt;g&lt;/sup&gt;</td>
<td>374</td>
<td>AY547454</td>
<td>Tumour-related protein (Cys-rich; unknown function)</td>
<td>N, X</td>
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<tr>
<td>D-7</td>
<td>473</td>
<td>AY547455</td>
<td>Aquaporin 1</td>
<td>N</td>
<td>T</td>
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<tr>
<td>D-8&lt;sup&gt;h&lt;/sup&gt;</td>
<td>391</td>
<td>AY547456</td>
<td>Subunit X(PsaK) of PSI</td>
<td>N</td>
<td>X</td>
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<tr>
<td>D-10&lt;sup&gt;i&lt;/sup&gt;</td>
<td>465</td>
<td>AY547457</td>
<td>H subunit of glycine decarboxylase multi-enzyme complex</td>
<td>N</td>
<td>T</td>
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<tr>
<td>D-12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>308</td>
<td>CO046498</td>
<td>Ribosomal protein L12 (chloroplastic)</td>
<td>N</td>
<td>X</td>
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<tr>
<td>D-13</td>
<td>352</td>
<td>CO046499</td>
<td>NiptL, 10kDa peptide of PSI (PsbR)</td>
<td>N</td>
<td>X</td>
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<td>D-15</td>
<td>340</td>
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<td>Similar to ribosomal protein L41</td>
<td>N</td>
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<td>D-17</td>
<td>340</td>
<td>CO046501</td>
<td>Universal stress protein family</td>
<td>N, X</td>
<td>X</td>
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</tbody>
</table>

<sup>a</sup>BLAST program searches were conducted against national institutes of a number of plant species, including Arabidopsis thaliana, Nicotiana tabacum, Medicago truncatula, Oryza sativa, and A. benthamiana.

<sup>b</sup>Matching sequences are given with their E-values.

<sup>c</sup>Clone D-15 has unknown function.

<sup>d</sup>Clone D-6 has unknown function.

<sup>e</sup>Clone D-2 has unknown function.

<sup>f</sup>Clone D-3 has unknown function.

<sup>g</sup>Clone D-4 has unknown function.

<sup>h</sup>Clone D-6 has unknown function.

<sup>i</sup>Clone D-10 has unknown function.
between total ascorbate and AA levels. The assays were performed in three independently prepared extracts.

### Determination of chemical elements and sulphate content

For determination of the total amounts of the selected chemical elements 50–150 mg of lyophilized plant material was digested overnight in 85% (v/v) HNO₃ and 15% (v/v) HClO₄. Then 10 ml of 4% (v/v) HCl was added and the sulphur content measured by inductively coupled plasma-atomic emission spectroscopy (Applied Research Laboratories, Accuris) at 182 nm. The assay was performed in duplicate.

Sulphate was extracted from 10–25 mg of lyophilized plant material in 1 ml of deionized water at 80 °C for 30 min. After centrifugation (14 000 g, 15 min) the supernatant was filtered through a 0.2 μm filter. Sulphate was determined by autosuppressed ion chromatography (Dionex 2000i/sp, Camberley, Surrey) using an AS9SC separation column. The eluent solution consisted of 1.8 mM Na₂CO₃ and 1.7 mM NaHCO₃. Sulphate measurements were performed four times from each plant material.

### Results

#### Preparation and initial characterization of the plant material

Tobacco plants grown hydroponically in the sulphur-sufficient medium AB (see Plant materials and growth conditions) were transferred for 2 d into either the sulphur-deficient medium AB-S (S-starvation) or into the same growth medium AB (control conditions, sulphur-sufficient). Tissue from ten plants grown in each condition was pooled together after dividing each plant into four parts: young (=immature) leaves, mature leaves, stems (stalks), and roots. No visible physiological stress symptoms were apparent at the time of collecting the plant material. In order to determine whether the short-term S-deficiency stress had any physiological consequences, the levels of selected compounds in the tissues were evaluated (Fig. 1). Two days of S-starvation resulted in the reduction of total non-protein thiols in all tissues, with young leaves and roots being most affected. The level of dehydroascorbate (DAA) was significantly elevated in the young leaves and in the stems of the starved plants compared with the plants maintained in the sulphur-sufficient medium. Although in the mature leaves, the total level of the sum of AA and DAA remained almost unchanged, the stress resulted in the elevation of the AA/DAA ratio. No significant changes of AA and DAA levels were observed in the roots. Finally, as expected, diminished levels of sulphate, as well as of total sulphur, were found in all tissues of the starved plants compared with the control.

Expression of several known genes was examined: plasma membrane high affinity sulphate transporter (ST, AJ745720), APS reductase (APR, AY648056), sulphite reductase (SR, D83583), Cu/Zn superoxide dismutase (SOD, X55974), small subunit of Rubisco (rbcS, X02353), actin (X69885), and a glutathione S-transferase (parB, D10524). The results are shown in Fig. 2. The rbcS and Tac9 (actin) transcripts served as a control of the level of total RNA used for the northern blots and the RT-PCRs. The mRNA abundances of ST, APR, and parB were increased by the sulphur deficiency stress imposed on the plants. As expected, the parB transcript was detected mainly in the roots. The SR transcript showed a slight increase of abundance in the leaves and a slight decrease in the roots of the sulphur-starved plants compared

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**Table 1. Continued**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Length (bp)</th>
<th>GenBank accession no.</th>
<th>Homology/putative ID</th>
<th>BLAST program⁵</th>
<th>Matching sequenceb (Probability/plant)</th>
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<tr>
<td>D-18</td>
<td>276</td>
<td>CO046502</td>
<td>Ribosomal protein S7</td>
<td>N</td>
<td>NM_121618 (e-26)/A. thaliana</td>
</tr>
<tr>
<td>D-21</td>
<td>617</td>
<td>CO046503</td>
<td>Chlorophyll αβ binding protein, CAB36 (type I)</td>
<td>N</td>
<td>X82300 (0.0)/N. tabacum</td>
</tr>
<tr>
<td>D-22</td>
<td>564</td>
<td>CO046504</td>
<td>Rca, Rubisco activase</td>
<td>N</td>
<td>Z14980 (0.0)/N. tabacum</td>
</tr>
<tr>
<td>D-23</td>
<td>330</td>
<td>CO046505</td>
<td>Subunit H (psaH) of PSI</td>
<td>N</td>
<td>X14981 (0.0)/N. tabacum</td>
</tr>
<tr>
<td>D-25</td>
<td>351</td>
<td>CO046506</td>
<td>Subunit A of chloroplast glyceraldehyde-3-phosphate dehydrogenase</td>
<td>N</td>
<td>M14417 (e-165)/N. tabacum</td>
</tr>
</tbody>
</table>

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⁵ BLAST program used for homology search: N, blastN and X, blastX using nr databases; E, blastN using EST database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), T, blastn at TIGR using databases for the selected plants (http://tigrblast.tigr.org/tgi/).

b GenBank/EMBL/DDJ/TIGR accession number for known sequences according to the BLAST search.
with the control plants. However, these changes were within the range of variation observed for the control genes, \textit{rbcS}, and \textit{Tac9}. \textit{SOD} transcript levels were slightly lower in the sulphur-starved plants compared with the controls in leaves as well as in roots.

\textit{Identification of differentially expressed genes}

Young (immature) leaves, which had the largest decrease in content of the non-protein thiols and sulphate levels upon sulphur starvation, were used for preparing the subtractive libraries. Two sets of the subtracted cDNAs were cloned into the pGEM-T vectors. The ‘forward’ set provided identification of the clones up-regulated by sulphur starvation, while the ‘reverse’ set enabled identification of the down-regulated clones. Approximately 1000 plasmids from each set were used for initial differential screening (hybridization) with DIG-labelled probes, prepared either from the control or from the S-starved plants. Only about 5% of the clones were confirmed to be differentially expressed in this screen (Fig. 3). The candidate clones were subsequently retested in the second and third rounds of hybridization. Finally, 32 up-regulated and 25 down-regulated clones were sequenced. In summary, the entire procedure resulted in the identification of cDNA fragments corresponding to 22 up-regulated and 16 down-regulated independent genes (Table 1). Several representative clones were selected as probes for northern blots or semi-quantitative RT-PCR (Fig. 4). The predicted pattern of expression
was observed most strongly in the young (immature) leaves. In some cases (for instance, UP-8, UP-11), almost no regulation of expression was observed in other tissues. Moreover, the RT-PCR results did not indicate any transcriptional regulation for clone UP-18. Surprisingly, in the case of clone D-4 that was identified as down-regulated, the results of RT-PCR showed an opposite regulation in the leaves, namely the increased amount of the transcript after the stress. In the case of controversial results (as the results for UP-18 and D-4), analyses were repeated at least 3-fold using two sets of separately grown plants in order to confirm reproducibility. The results of RT-PCR for UP-18

Fig. 3. Example of the macroarray analysis of cDNAs expression. Randomly chosen cloned subtracted products were spotted in duplicate onto two nylon membrane sheets and probed with subtracted probes (labelled cDNA pools). The spots differing in their intensity between membranes were recognized as either up- (circled by continuous line) or down-regulated (circled by dotted line).

Fig. 4. Verification of differential expression of the selected cDNA in the analysed plant material using either a northern blot technique or RT-PCR. The plant organs are specified at the top; C and S− denotes the control and minus S conditions, respectively. Expression of the genes represented by the cDNA clones, indicated on the left-hand side of each row, was tested either by northern blot (UP-2, UP-7, UP-15, D-1, D-6, D-7, D-8, D-10, D-16, D-22; each lane contained 20 µg of total RNA) or by RT-PCR using 30 cycles except for UP-11 and UP-12 (20 cycles), UP-22 and UP-23 (25 cycles), and D-3 (35 cycles).
performed with a smaller number of amplification cycles (20 and 25, respectively) were essentially the same as for 30 cycles and did not reveal differences in expression. Since UP-18 encodes a member of a large family of conserved proteins (calmodulins), the PCR product may represent a mixture of products from several genes. Consequently, the regulation of the gene represented by UP-18 may be masked by the expression of other genes from this family. However, the discrepancy between the expected and the observed regulation of clone D-4 encoding glycolate oxidase cannot be explained. Expression of UP-22, encoding plastid cysteine synthase, was tested only in leaf tissues, both from mature and immature leaves. The up-regulation of expression was confirmed mostly in the young leaves and could not be validated for the mature leaves (Fig. 4). In the case of two clones (UP-23 and D-13), the expression was tested only in the young leaves and in the case of two clones (D-16 and D-22), only in the mature leaves. The analyses confirmed the expected up-regulation of the expression of the clone UP-23 and the down-regulation of the expression of D-13, D-16 and D-22.

Expression of the selected cDNAs in the seedlings in response to stress

The abundance of the transcripts of several selected clones, UP-2 (ACC oxidase), UP-7 (acidic endochitinase), UP-9 (unknown function), D-7 (aquaporin), and D-8 (PsaK), identified as regulated by short-term sulphur starvation, were analysed in response to other stress conditions for 48 h (Fig. 5) using 10-d-old tobacco seedlings germinated and grown in vitro. Transcription of the acidic endochitinase appeared to be strongly induced, while the transcription of UP-9 was repressed by copper. Cadmium almost entirely repressed the expression of all clones with the exception of a slight induction of UP-7. The effects of hydrogen peroxide were more variable, with a slight inhibition of UP-2 and D-8, strong inhibition of UP-9, and a slight induction of UP-7 and D-7 expression. In the dark, expression of UP-2, UP-9, and D-8 was much lower than in the control conditions, while UP-7 and D-7 transcripts were induced.

Discussion

Nutritional status of the tobacco plants

This is the first description of an attempt of global and untargeted analysis of the transcriptional responses of tobacco plants to short-term sulphur deprivation. Tissue analyses validated the starting material to quantify the effect of the 48 h sulphate-deprivation on tissue sulphur status and plant metabolism. As expected, lower contents of total sulphur, sulphate, and non-protein thiols were observed in the plants grown for 2 d without a S-source. The differences in thiols and sulphate levels were most pronounced in immature leaves, which were selected as a starting material for making the libraries. Accordingly, many analysed genes were most significantly regulated in this part of the plant (Fig. 4).

In plants, only a limited number of the genes encoding proteins involved in sulphur metabolism (such as ST encoding sulphate transporter and APR encoding APS reductase) are induced in response to sulphur-deficiency stress (Hirai et al., 2003; Nikiforova et al., 2003; Takahashi et al., 1997). The SR gene encoding sulphite reductase showed an expected pattern of expression, with almost no effect of S-starvation and slightly higher expression in the shoots than in the roots (Bork et al., 1998; Takahashi et al., 1997; Yonekura-Sakakibara et al., 1998). In addition, the genes encoding Cu/Zn SOD and ParB were expected to be regulated in tobacco; the transcription of the gene encoding cytosolic Cu/Zn SOD was activated in the protoplasts of Nicotiana plumbaginifolia by antioxidant sulphhydryl reagents such as GSH, cysteine, or DTT (Herouart et al., 1993) and a similar pattern of transcriptional activity of the Cu/Zn SOD gene was confirmed in N. tabacum plants (Youssefian et al., 2001). The product of the parB gene is localized in the cytoplasm and has glutathione S-transferase activity (Takahashi and Nagata, 1992). In the regulatory region of the tobacco parB gene, an auxin-responsive element has been identified (Takahashi et al., 1995). Since the genes involved in auxin biosynthesis pathway were induced upon S-starvation (Nikiforova et al., 2003), the increased abundance of parB transcript in the roots of S-starved plants was expected (Fig. 2).
Functional categories of the regulated cDNAs

A number of new tobacco cDNAs were identified in this study. Many have characterized homologues in other plants while in a few cases it was impossible to assign any homologues with known functions (Table 1). The genes may be divided into several functional categories (Table 2). The categories include both up- and down-regulated cDNAs. This divergent regulation may aid the maintenance of the optimal levels of the proteins required for the fine-tuning of complex processes including photosynthesis, protein translation and carbon metabolism, to the specific conditions imposed by S-deprivation.

Stress- and pathogen-related genes

A significant group of up-regulated clones with homology to known sequences represents pathogen-related and stress-responsive genes. They encode proteins such as catalase (UP-1), ACC oxidase (UP-2), thioredoxin peroxidase (UP-3), acidic chitinase (UP-7), calmodulin (UP-18), and proteins of unknown function (UP-6 and UP-24). The UP-6 clone has strong similarity to a cDNA (AB040409), which was induced by fungal elicitors and encodes a Gly- and His-rich protein. Clone UP-24 has some similarity to bacterial TypA proteins which are GTPases required for stress adaptation as well as for symbiosis (Kiss et al., 2004). In the same functional category of stress-related genes, several down-regulated clones encoding such proteins as aquaporin (D-7), Cu/Zn SOD (Fig. 2), and a protein of unknown function (D-17) possessing the similarity to the bacterial universal stress protein family may be included (Sousa and McKay, 2001). The level of non-protein thiols (consisting in tobacco mostly from the reduced glutathione, GSH) drops down after S-starvation stress. Therefore, not only the sulphur status but also the redox status of the tissues of S-starved plants is affected, as not only the GSH/GSSG ratio, but also total concentration of glutathione is a determinant of the redox state in the cell (Schafer and Buettner, 2001). Diminished levels of GSH can mimic the conditions of environmental (biotic and abiotic) stresses since less antioxidant is available for scavenging of the reactive oxygen species produced during normal metabolism.

Table 2. Functional categories of the tobacco cDNAs regulated by short-term sulphur starvation identified in the subtraction approach or as assayed by RT-PCR or northern blots

<table>
<thead>
<tr>
<th>Category</th>
<th>Up-regulated clones</th>
<th>Down-regulated clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress-related, pathogen-related</td>
<td>Catalase (UP-1)</td>
<td>Aquaporin 1 (D-7)</td>
</tr>
<tr>
<td></td>
<td>ACC oxidase (UP-2)</td>
<td>Universal stress protein family (D-17)</td>
</tr>
<tr>
<td></td>
<td>Thioredoxin peroxidase (UP-3)</td>
<td>Superoxide dismutase (SOD)</td>
</tr>
<tr>
<td></td>
<td>Acidic endochitinase (UP-7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calmodulin (UP-18)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TypA-related (UP-24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Elicitor-responsive, Gly-, His-rich (UP-6)</td>
<td></td>
</tr>
<tr>
<td>Cell wall</td>
<td>Pectin acetyltransferase (UP-5)</td>
<td>Pectin acetyltransferase (D-1)</td>
</tr>
<tr>
<td></td>
<td>Cys-rich, extensin-like (UP-20)</td>
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</tr>
<tr>
<td></td>
<td>Gly-rich family (GRP) (UP-30)</td>
<td>Cystatin (D-3)</td>
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<tr>
<td>Protein degradation</td>
<td>Cysteine protease (UP-11)</td>
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</tr>
<tr>
<td></td>
<td>Ubiquitin activating enzyme, E-1 (UP-12)</td>
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</tr>
<tr>
<td>Photosynthesis</td>
<td>LHC-I chlorophyll a/b binding protein (UP-19)</td>
<td>Subunit X (psaK) of PSI (D-8)</td>
</tr>
<tr>
<td></td>
<td>Chlorophyll a/b binding protein CP29 (UP-31)</td>
<td>Subunit H (psaH) of PSI (D-23)</td>
</tr>
<tr>
<td>Carbon metabolism</td>
<td>NADP-dependent malic enzyme (UP-23)</td>
<td>NtpII from PSII (PsbR) (D-13)</td>
</tr>
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<td></td>
<td>Plastidic cysteine synthase (UP-22)</td>
<td>Chlorophyll a/b binding CAB36, PSI (D-21)</td>
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<tr>
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<td>APS reductase (APR)</td>
<td>Rubisco activase (D-22)</td>
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<td></td>
<td>Sulphate transporter (ST)</td>
<td>Glycolate oxidase (D-4)</td>
</tr>
<tr>
<td></td>
<td>Glutathione S-transferase (parB)</td>
<td>H subunit of glycine decarboxylase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>multienzymatic complex (D-10)</td>
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<td></td>
<td></td>
<td>Chloroplast glyceraldehyde-3-phosphate dehydrogenase, subunit A (D-25)</td>
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<td>Sulphur metabolism</td>
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<td>Ribosomal proteins</td>
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(Mittler, 2002). In addition, it has been reported that various nutrient deficiencies, including S-deficiency, trigger oxidative stress (Kandlbinder et al., 2004), and a set of genes related to the oxidative stress response was identified in A. thaliana as up-regulated by the short-term S-deprivation (Hirai et al., 2003; Maruyama-Nakashita et al., 2003). Finally, in non-stressing conditions, the expression of several defence genes was induced in A. thaliana containing a reduced level of glutathione due to mutations in the gene encoding γ-glutamylcysteine synthase (Ball et al., 2004). Thus, it is not surprising that many stress- and pathogen-related genes were identified in this study.

Catalase is an indispensable enzyme for stress defence in plants and is involved in hydrogen peroxide degradation (Scandalios, 2002). Interestingly, it was recently suggested that plant calmodulins may play a critical role in controlling hydrogen peroxide homeostasis in plants by binding to catalase and stimulating its activity (Yang and Poovaiah, 2002). A potential involvement of the calmodulin represented by clone UP-18 in this process is possible. In Arabidopsis, expression of genes encoding three isoforms of catalase, Cat1, Cat2, and Cat3 was monitored in response to S-starvation and only the expression of Cat2 was significantly induced in the conditions of the experiment (Kandlbinder et al., 2004). In fact, the up-regulated clone UP-1 is more significantly similar (probability: 2e-25) to Cat2 (At4g35090) than to other Cat genes of A. thaliana.

The production of ethylene is regulated by internal signals during development and in response to biotic and abiotic stresses. Multiple gene families in plants encode both of the enzymes involved in ethylene production from S-adenosyl-methionine: ACC synthase and ACC oxidase. These genes are subject to transcriptional as well as post-transcriptional regulation (Wang et al., 2002), however, the components involved in this regulation have not yet been identified. Clone UP-2, identified in this study, encodes ACC oxidase and is up-regulated significantly in the young leaves after S-starvation.

Most plants possess a large number of peroxidase isoenzymes encoded by multigene families. In the presence of hydrogen peroxide the enzymes oxidize a vast array of compounds and possess several physiological functions including removal of hydrogen peroxide, oxidation of toxic reductants, biosynthesis and degradation of lignin in cell walls, auxin catabolism, and defence responses to wounding and biotic stresses (Kawano, 2003; Yoshida et al., 2003). The clone homologous to thioredoxin peroxidase identified as up-regulated upon S-starvation (UP-3) was previously identified in a two-hybrid assay as interacting with the catalytic subunit of NADPH oxidase required for the accumulation of reactive oxygen after pathogen infection (Simon-Plas et al., 2002). The interaction of these two proteins suggests that the product of the gene represented by UP-3 is localized near to the cell membrane-located NADPH oxidase and may be involved in stress defence-related mechanisms. No link to S-metabolism except potential thioredoxin dependence is obvious in this case. There was no significant increase of the transcripts encoding peroxidases as previously reported for S-starved A. thaliana (Hirai et al., 2003; Kandlbinder et al., 2004).

Down-regulation by S-starvation of a gene encoding a plasma membrane intrinsic aquaporin (clone D-7), which is a member of the major intrinsic protein (MIP) superfamily of channel proteins (Otto and Kaldenhoff, 2000; Siefritz et al., 2002) was observed mainly in the roots and to a lesser extent in the green parts of tobacco plants (Fig. 4). This protein functions in cellular and whole-plant water transport (Siefritz et al., 2002). The significance of the reduction of expression of this gene during S-deficiency stress is not obvious, however, it would be in agreement with the hypothesis that plants tolerate abiotic stresses better if the total integrated amount of symplastic water transport via plasma membrane aquaporins is reduced during the stress (Aharon et al., 2003). On the other hand, the induction of D-7 transcript was observed in seedlings exposed to stresses (Fig. 5), which may make the regulation of this gene even more puzzling. In agreement with these data, down-regulation of two Arabidopsis genes encoding aquaporins by short-term S-deficiency has previously been reported (Hirai et al., 2003). Although an aquaporin is not strictly a stress-related protein, D-7 is included in this category.

In summary, the up-regulated clones included in this category may be considered as important elements of the network of stress signalling pathways. Those with assigned functions are associated with calcium and MAPK signalling (calmodulin), ROS production (catalase, peroxidase), and ethylene production (ACC oxidase). Therefore, the term ‘S-deficiency stress’ seems to be justified as an appropriate description of the conditions met by the plants deprived of any S-source for at least 2 d.

Cell-wall structure

Pectin esterases are involved in de-esterification of pectin, a major constituent of the cell wall. This modification changes the structure/deposition of pectin components and influences such properties of the cell wall as porosity or water-holding capacity (Vincken et al., 2003). In addition, it has been proposed that interaction with pectin esterases is necessary for the cell-to-cell movement of the tobacco mosaic virus (Chen et al., 2000). Plant pectin esterases are encoded by multiple gene families whose members have specific patterns of expression (Castillejo et al., 2004). In the present study, one gene encoding pectin esterase-like protein (UP-5) was identified as up-regulated, while another (D-1) was down-regulated upon sulphur starvation. The observed differences in pectin acetyl-esterase transcript levels may be a part of as yet unidentified regulatory mechanisms resulting in the differential plant cell growth leading to the increased root/shoot ratio that is observed upon long-term S-starvation (Hell and Hillebrand, 2001).
In *Arabidopsis thaliana*, an increased transcription of a gene encoding a pectin acetyltransferase (At3g05910) was observed after infection with nematodes (Vercauteren *et al*., 2002) and induction of a gene encoding putative pectin esterase (At4g02330) was observed in the *A. thaliana* mutant with reduced glutathione levels (Ball *et al*., 2004).

Clone UP-20 was identified as the 3′-uncoding region of the tobacco gene encoding a protein of unknown function, but which is known as a small glycoprotein containing Cys-and Pro-rich domains and a highly charged C-terminus, located to the cell wall and found mainly in the floral tissues of tobacco (Wu *et al*., 1993). However, its homology to the putative lipid transfer proteins alternatively suggests that it might be involved in systemic signalling of stress, as has been suggested for the *A. thaliana* putative apoplastic lipid transfer protein (Maldonado *et al*., 2002).

Clone UP-30 is homologous to a previously reported cDNA induced by a fungal elicitor (Takemoto *et al*., 2001) and encodes an acidic and Tyr-rich protein with the characteristic Cys-domain responsible for cross-linking to cell walls (Domingo *et al*., 1999).

**Protein degradation**

Ubiquitin-mediated proteolysis is a fundamental process regulating diverse cellular events, including plant responses to environmental stresses controlled by jasmonate, ethylene, and abscisic acid (Sullivan *et al*., 2003; Vierstra, 2003). Target proteins are ‘labelled’ for degradation in the 26S proteasome by the addition of a polyubiquitin chain. The first step in this process is the activation of ubiquitin by the ATP-dependent formation of a thioester bond with a ubiquitin-activating enzyme. Clone UP-12, identified in this study as up-regulated in the young leaves, encodes a ubiquitin activating enzyme, E1.

In this functional category, are included two clones, UP-11 and D-3. The up-regulated clone UP-11 encodes a cysteine protease. The mRNA of the gene *NTCP-23*, to which UP-11 is highly homologous, accumulates during natural senescence and is regulated by wounding and the circadian rhythm (Ueda *et al*., 2000). The product of the gene is most probably located in the vacuole and in addition to protease activity, it may also possess aminopeptidase activity and can take part in releasing amino acids from degraded proteins. The down-regulated clone, D-3, encodes an inhibitor of cysteine protease, cystatin. This gene is strongly down-regulated in the young leaves and stems and rather unaffected or even up-regulated in the other tissues. In plants, cysteine protease inhibitors are involved in the regulation of protein turnover and play an important role in resistance against insects and pathogens (Belenghi *et al*., 2003). The down-regulation of the gene encoding cystatin coincides with the up-regulation of the clone encoding the cysteine protease. The observed patterns of gene regulation would indicate a need for increased protein turnover in S-starved plants. Changes of the protein composition between plants grown in S-sufficient and S-deficient conditions were previously reported: for example, S-deficiency results in a redistribution of proteins in a wheat grain with the S-poor proteins increasing at the expense of S-rich proteins (Naeem and MacRitchie, 2003). It is notable that the up-regulation of the *A. thaliana* gene (At4g16190) encoding cysteine proteinase was previously reported after both, short-term (Hirai *et al*., 2003) and long-term (Nikiforova *et al*., 2003) S-limitation.

**Photosynthesis**

Optimum energy distribution in photosynthesis is dependent on the balanced expression of genes encoding photosynthesis-related proteins and adjustments to plant nutritional status. Changes in the reduction/oxidation (redox) state of components of the photosynthetic machinery act as signals, which regulate the expression of genes encoding chloroplast proteins in both chloroplasts and nucleus and help to co-ordinate the expression both in compartments (Pfannschmidt, 2003; Pfannschmidt *et al*., 2003). Therefore, regulation of photosynthesis genes is closely linked to the redox status of the cell and decreases of the glutathione level observed in the S-starved plants might either directly or indirectly affect the expression of these genes.

Two clones of this category were identified as up-regulated, UP-19 encoding chlorophyll a/b binding protein of LHC-I and UP-31 encoding the CP29 protein from photosystem II (PSII). Five photosynthesis-related clones were identified as down-regulated. One of the down-regulated clones (D-21) encodes another chlorophyll a/b binding protein CAB36 (type I). The clone D-8 encodes PsAK, one of the subunits of photosystem I (PSI). PsAK is involved in the organization of the light-harvesting complexes on the core of photosystem I (Jensen *et al*., 2000) and in state transitions in photosynthesis (Varotto *et al*., 2002). Clone D-23 encodes Psah, another subunit of PSI with a proposed role as a docking site for LHCII (Ben-Shem *et al*., 2003). One of the down-regulated clones (D-13) encodes the 10 kDa component of PSII, the PsbR protein. Recently, it has been shown that the gene encoding PsbR is down-regulated by dehydration stress and up-regulated by rehydration in *Xerophyta humilis* (Collet *et al*., 2003).

Finally, a down-regulated clone, D-22, was identified as *rca* cDNA. The product of this gene, the enzyme Rubisco activase, is specifically involved in the activation of Rubisco through carbamylation. It induces conformational changes and the reactivation of the catalytic site of Rubisco enzyme. The activity of Rubisco is regulated by the activase in response to light intensity and energy needs (Houtz and Portis, 2003). Notably, the level of *rbcS* transcript, encoding the small subunit of Rubisco, remained unchanged after S-starvation (Fig. 2).

In *Arabidopsis* plants, a short-term S-deficiency stress resulted in down-regulation of several genes encoding
chlorophyll $a/b$ binding proteins and components of PSII as well as Rubisco activase (Hirai et al., 2003).

**Carbon metabolism**

The down-regulated clones of this functional category represent mainly the genes whose products are required for maintaining regular metabolism. They include D-4 encoding glycine decarboxylase activase (Hirai et al., 2003), other three subunits of GDC (Douce et al., 2003), a small (15 kDa) lipoamide-containing enzyme which acts as a mobile substrate that commutes successively between the glyoxisomes and using glycolate as a substrate) is used subsequently for glycine production, which is one of the constituents of glutathione. Apparently, this gene is up-regulated and was erroneously identified in the category of down-regulated genes.

GDC is localized in the mitochondrial matrix and catalyzes the conversion of two glycine molecules into serine, carbon dioxide, and ammonia. The H subunit of GDC is a small (15 kDa) lipoamide-containing enzyme which acts as a mobile substrate that commutes successively between the other three subunits of GDC (Douce et al., 2001). The level of mRNA of the H subunit is high in the light and was reported as unaffected by environmental stresses such as high salinity, flooding, and low temperature (Ho and Saito, 2001). The slight reduction of the transcript abundance for this enzyme upon S-starvation detected in this study might reflect the lower demand for serine under such conditions, since serine is a donor of the carbon–nitrogen skeleton for cysteine, the first sulphur-containing organic compound.

Chloroplast GAPDH contributes to glycolysis using NAD(H) and plays a role in CO2 assimilation through the Benson–Calvin cycle using NADPH (Graciet et al., 2004), and forms a complex with phosphoribulosokinase (Graciet et al., 2004). Lower levels of the transcript encoding subunit A of GAPDH in the conditions of S-starvation might reflect the reduced requirements for CO2 assimilation. Additionally, since it is a chloroplast enzyme this gene might be also under redox control.

Clone UP-23 encodes NADP-dependent malic enzyme, which converts malate to pyruvate and synthesizes NADPH in the cytoplasm, playing an important role in accumulation and increased supply of these metabolites. The gene encoding NADP-dependent malic enzyme was identified as one of the elicitor-activated genes in potato (Nakane et al., 2003). Then, from this point of view, it can also be considered a stress-related gene.

**Ribosomal proteins and translation**

Six cDNAs representing ribosomal proteins from either small or large subunits were identified. The genes encoding S12, S15, and L20 proteins represented by the clones UP-8, UP-27, and UP-21, respectively, were up-regulated, while the genes encoding S7, L41-like, and L12 proteins represented by the clones D-18, D-15, and D-12a, respectively, were down-regulated. The exact number of ribosomal proteins in higher plants is not known. They form a complex molecule in which a co-ordinated regulation of subunit expression is essential, however, in Arabidopsis some ribosomal proteins are encoded by multigene families which complicates the control of their synthesis (Barakat et al., 2001). The up-regulated cDNAs encode: the S12 protein of 40S subunit located in a ribosomal accuracy centre and possessing RNA chaperone activity, the S15 protein of 40S which is involved in early steps of ribosome assembly, and L20 protein of subunit 60S which is necessary for in vitro assembly of the large subunit. The down-regulated cDNAs encode: the S7 protein of the 40S subunit that may play a regulatory role in translation initiation, the L12 protein of the chloroplast 50S subunit that is responsible for interaction with elongation factors and GTPase activity (Bocharov et al., 2004; Chandra Sanyal and Liljas, 2000). Down-regulation of the gene encoding this protein might slow down the translation process.

One of the down-regulated clones (D-15) encodes protein similar to L41 protein, the smallest and the most basic eukaryotic protein. It is dispensable in yeast ribosomes, however, it optimizes peptidyltransferase activity and affects translocation (Dresios et al., 2003). The L41 must possess also some extra-ribosomal activity. It associates with protein kinase CKII and can modulate its activity toward a specific substrate (Ahn et al., 2003).

Although the significance of modulation of the ribosomal gene expression in response to S-starvation is not obvious it can be linked to the changes in protein composition between S-sufficient and S-deficient plants. Modification of transcripts of some ribosomal proteins was previously reported for S-starved Arabidopsis (Nikiforova et al., 2003). In agreement with the above data is the reduced amount of the transcript for L12 protein from chloroplastic ribosomes (D-12a) observed in this work.

**Sulphur metabolism**

Surprisingly, only one gene from this category was found amongst the up-regulated clones (UP-22). It has the closest homology to two mRNAs encoding plastidic isoforms of potato cysteine synthase (Maruyama et al., 2001). The homology on protein level is not apparent because the clone UP-22 covers only a few amino acids of the C-terminal part.
of the open reading frame, which is not strongly conserved, and the 3′-end of the non-coding region of the gene. The other sulphur metabolism-related genes, which are up-regulated after S-starvation (Fig. 2), but which were not identified in the subtraction screen, include APR encoding APS reductase (AY648056), sultr1;1 encoding a high affinity sulphate transporter (AJ745720), and an auxin-regulated gene, parB (Takahashi et al., 1995, 1997) encoding glutathione S-transferase. The parB gene is apparently expressed mainly in the roots, therefore, the absence of its cDNA in the libraries prepared from the leaves is not surprising. It is also likely that a masking of differential expression of some of these genes occurred in the subtraction approach resulting in a failure of their identification. This would occur because many of them are members of large gene families and that not all members are differentially regulated in specific tissues (Buchner et al., 2004). Cross-hybridization would therefore mask signals of specific genes in screening protocols. It is also the case that extended periods of deficiency are required for differential expression of some sulphate transporter isoforms (Buchner et al., 2004). An alternative explanation of identification of only the small number of genes from this category is that too few clones were analysed and the search was not ‘saturated’.

Histone H3
Clone UP-28 encodes histone H3. It is known that some variants of H1 histone are up-regulated during abiotic stresses in plants (Scippa et al., 2004) and a fluctuation of histone gene expression during the cell cycle was observed in plants (Moshi et al., 2000). The significance of up-regulation of the transcription of H3 histone during sulphur-starvation is not clear. Moreover, it is not known whether the induction is specific to a single gene or represents multiple genes. The gene for putative histone H3 (At1g09200) was down-regulated in the leaves and up-regulated in the roots after short-term S-starvation (Hirai et al., 2003).

Proteins of unknown function
Interestingly, some of the most frequently identified clones in both up-regulated (UP-9, UP-15, UP-30) and down-regulated (D-6) sets do not have significant homologies to cDNAs with assigned functions for the products. The identification of numerous clones for these genes strongly affected by the S-starvation treatment represents new leads for investigation.

Final remarks
The appropriateness of the subtractive library approach for identification of tobacco genes regulated by S-starvation is confirmed by studies indicating that their counterparts in the Arabidopsis genome are also regulated by S-starvation (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003). This work does not supply a complete profile on the transcriptional response to short-term S-deprivation in tobacco, however, this is the first untargeted approach for the analysis of such responses in Solanaceae species. The libraries constructed and described here are available for further analysis and results of analyses indicate that 5% of the clones present in each of the two libraries represent regulated clones. Perhaps, the percentage of the regulated clones would be higher if the criteria used for identification of differentially expressed genes were less stringent. Moreover, additional searches may result in increasing the number of genes in particular categories, especially in the category of sulphur-related genes.

Interestingly, a fraction of the clones identified in multiple copies (e.g. UP-9) displayed a small heterogeneity of their nucleotide sequence. This might be the indication of their various origins (they might be products of cognate but distinct genes), mRNA editing (heterogeneity), or errors that appeared in the process of library construction due to the action of Taq polymerase. Unfortunately, at this stage it is not possible to distinguish between these alternatives. Therefore, the most frequent sequence was arbitrarily selected as a representative for the group of such clones.

It is worth emphasizing that in some cases, in the various plant tissues, opposite changes of the mRNA level of the same gene were observed upon the treatment. Therefore, the regulation of expression of such genes could be overlooked (be unnoticed) if the similar experiment is performed with the non-fractionated plant material.

Regulatory aspects of sulphur deficiency stress are not well characterized in plants at the molecular level. Despite the identification of several S-regulated genes encoding transcription factors and proteins involved in cellular communication and signalling in A. thaliana (Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003), no proof for their function as S-starvation-specific regulators was provided. None of the genes identified in the present study with homology to the genes with established function is a good candidate for a gene encoding protein involved in the regulation of the sulphur-starvation response in plants. However, some genes were identified, both down- and up-regulated, for which the function could not be defined. It is possible that some of these encode proteins which may have a function in regulation/signalling of S-starvation stress. The products of some of these genes have homologues in A. thaliana (UP-9), while in other cases it is not possible to identify such homologues (D-6, UP-15, UP-30). Investigations of the functions of these genes will help in the understanding of S-starvation responses and will identify both general elements and species-specific factors.

Supplementary data
A table listing the primers used to generate the specific probes for northern blots and to perform the semi-quantitative RT-PCR reactions can be found at JXB online.
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