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Transcription factors relevant to auxin signalling coordinate broad-spectrum metabolic shifts including sulphur metabolism

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Abstract

A systems approach has previously been used to follow the response behaviour of *Arabidopsis thaliana* plants upon sulphur limitation. A response network was reconstructed from a time series of transcript and metabolite profiles, integrating complex metabolic and transcript data in order to investigate a potential causal relationship. The resulting scale-free network allowed potential transcriptional regulators of sulphur metabolism to be identified. Here, three sulphur-starvation responsive transcription factors, IAA13, IAA28, and ARF-2 (ARF1-Binding Protein), all of which are related to auxin signalling, were selected for further investigation. *IAA28* overexpressing and knock-down lines showed no major morphological changes, whereas *IAA13-* and *ARF1-BP*-overexpressing plants grew more slowly than the wild type. Steady-state metabolite levels and expression of pathway-relevant genes were monitored under normal and sulphate-depleted conditions. For all lines, changes in transcript and metabolite levels were observed, yet none of these changes could exclusively be linked to sulphur stress. Instead, up- or down-regulation of the transcription factors caused metabolic changes which in turn affected sulphur metabolism. Auxin-relevant transcription factors are thus part of a complex response pattern to nutrient starvation that serve as coordinators of the metabolic shifts driving sulphur homeostasis rather then as direct effectors of the sulphate assimilation pathway. This study provides the first evidence ever presented that correlates auxin-related transcriptional regulators with primary plant metabolism.

Key words: Auxin, sulphur metabolism, systems biology, transcription factors.

Introduction

Plants cannot physically relocate to avoid nutrient stress. Instead, they rely on complex biochemical and physiological adaptive responses to ensure survival and reproduction. Several authors have reported signal perception and transduction cascades that trigger alterations in gene expression involved in abiotic stress responses like drought and cold (Bohnert *et al*., 2006; Fujita *et al*., 2006; Valliyodan and Nguyen, 2006; Van Buskirk and Thomashow, 2006; Yamaguchi-Shinozaki *et al*., 2006). However, no cascades involved in sulphur availability have been elucidated in higher plants.

As a first step to achieve a systems understanding of plant adaptive responses to sulphur limitation and transcript and metabolite profiling of plants grown under normal and sulphur-depleted conditions was performed previously (Hirai *et al*., 2003; Maruyama-Nakashita *et al*., 2003; Nikiforova *et al*., 2003). The resulting transcriptome data were overlaid with >100 non-redundant compounds of known chemical structure (Nikiforova *et al*., 2005*a*). Integration of these data sets allowed the first multifactorial correlation network to be created, revealing potential relationships among genes and metabolites under sulphur limitation (Nikiforova *et al*., 2005*b*). The deduced

network indicated, among other potential links, a relationship between sulphur–serine metabolism and tryptophan–glucosinolate–auxin metabolism. Based on these data, it was hypothesized that auxin might be involved in communicating the nutrient status of the shoot to the root, inducing root formation when sulphur is limiting (Nikiforova *et al.*, 2005*b*).

Auxins are phytohormones that play an essential role in coordinating many growth-related processes throughout the plant life cycle, including the induction of lateral root formation (Woodward and Bartel, 2005; Kepinski, 2006; Paciorek and Friml, 2006; Prusinkiewicz and Rolland-Lagan, 2006). The biologically most active auxin is indole-3-acetic acid (IAA). The predominant biosynthetic pathway for IAA is still uncertain, but both tryptophan (trp)-dependent and trp-independent pathways are known (Woodward and Bartel, 2005). Under sulphur limitation, trp accumulation occurs simultaneously with the activation of IAA biosynthetic genes such as tryptophan synthase β-subunit (AT5G38530), myrosinases (AT1G51470, AT2G33070, AT5G25980, AT3G14210), and nitrilase 3 (AT3G44320) as outlined in Nikiforova *et al.* (2003). The degradation of glucosinolates—another potential source of auxin and of sulphate—also occurs concomitant with trp accumulation (Kutz *et al.*, 2002; Nikiforova *et al.*, 2003, 2005*a*, *b*; 2006; Hirai *et al.*, 2004, 2005).

Physiological responses to auxin involve changes in gene expression. Auxin regulates the cell-specific transcription of target genes via two types of transcription factors, auxin response factors (ARFs) and Aux/IAA proteins. Aux/IAA proteins prevent ARFs from activating auxin response genes. When cellular levels of auxin are low, Aux/IAA repressors form dimers with ARF activators in the promoters of the response genes. The repression domain in Aux/IAA proteins is dominant over the activation domain in ARF proteins, preventing gene expression. When auxin levels rise, the Aux/IAA repressors are targeted for degradation. The loss of repressors allows the subsequent activation of auxin response genes by ARFs by increasing their interaction with SCF[Tir1] ubiquitin ligase, leading to the ubiquitination of substrates and their subsequent degradation (Woodward and Bartel, 2005; Kepinski, 2006; Paciorek and Friml, 2006; Prusinkiewicz and Rolland-Lagan, 2006).

The *Aux*/*IAA* and *ARF* gene families are represented by 29 and 23 loci, respectively, in *Arabidopsis thaliana*. Among them, gene duplications have likely resulted in some functional redundancy (Remington *et al.*, 2004). Several transcription factors of both families, including IAA13, IAA28, and ARF2 (formerly denoted ARF1-binding protein; Ulmasov *et al.*, 1999*a*, *b*), are moderately up-regulated during sulphur depletion (Nikiforova *et al.*, 2005*b*). IAA28 exhibits a high degree of connectivity and so was identified as a hub of the transcript/metabolite co-response network responding to sulphur starvation

(Nikiforova *et al.*, 2005*b*). A gain-of-function *Arabidopsis* mutant of *IAA28* has been reported to exhibit suppressed lateral root formation (Rogg *et al.*, 2001). Further studies revealed that IAA28 represses transcription, perhaps of genes that promote lateral root initiation in response to auxin signals. This finding might explain the phenotypic changes in root morphology seen under sulphur limitation (Nikiforova *et al.*, 2005*b*).

A recent publication by Weijers *et al.* (2005) considered *IAA13* as a functional paralogue of its sister gene *BDL*/*IAA12*, which was shown to be involved in embryonic root initiation when a mutated version of *IAA13* (Ser$_{80}$→Pro) was expressed (Hamann *et al.*, 1999, 2002).

ARF2 was identified as a transcription factor binding to AuxRE in promoters of auxin response genes, thus activating flowering, senescence, and abscission. It has also functions as a light-independent repressor of cell growth and of differential hypocotyl growth during seedling hook formation (Ulmasov *et al.*, 1999*a*; Li *et al.*, 2004; Ellis *et al.*, 2005; Okushima *et al.*, 2005*a*, *b*). ARF2 is a pleiotropic developmental regulator. *ARF2* knockout mutants develop a phenotype with increased leaf size, enhanced flower formation, and increased seed size (Ellis *et al.*, 2005). Overexpression of ARF2 is likely to be lethal as it was impossible to retrieve *ARF2* overexpressing lines, while co-suppression lines could be isolated.

Studies concerning the function of members of the *Aux*/*IAA* and *ARF* families usually focus on the impact these transcription factors have on plant development and morphogenesis. In this study, novel insights into the roles of IAA13, IAA28, and ARF2 were gained in the context of sulphur limitation and amino acid metabolism based on a previously executed systems approach (Nikiforova *et al.*, 2003, 2005*a*, 2006). The aim was to evaluate these transcription factors with respect to their putative roles in plant sulphur metabolism. To this end, transcription factor knock-down or overexpression lines were analysed with a particular emphasis on sulphate assimilation pathways and amino acid homeostasis that were studied at transcript and metabolite levels. Both, transcript and metabolic changes were observed. Results were dependent not only on the respective genotype (mutant) but also on the culture conditions. Soil-grown plants were affected more severely than hydroponically grown plants.

Although alterations were observed in the contents of sulphur-containing metabolites in response to the manipulation of Aux/IAA and ARF transcription factor expression, especially of the key metabolites cysteine and glutathione (GSH), many other metabolic changes were also identified. These changes were interpreted as broad-spectrum shifts in amino acid metabolism and carbon/nitrogen balance caused by changes in the expression of AUX/IAA and ARF target genes, rather than a direct effect of auxin on sulphate metabolism. Based on these results, a complex, multilevel regulatory scheme for

sulphate metabolism that operates in plants challenged by altered sulphate supply is proposed.

Materials and methods

Overexpression of IAA13, IAA28, and ARF1-BP transcription factors

Polymerase chain reaction (PCR) was used to amplify the *IAA13*, *IAA28*, and *ARF1-BP* coding regions (locus numbers At2g33310, At5g25890, At5g62010, respectively) employing the Advantage–HF2 PCR Kit (Clontech, Heidelberg, Germany) according to the manufacturer's protocol. *Arabidopsis thaliana* (L.) Heynh. Col-0 cDNA, established from leaves, was used as template. Primer sequences were as follows: *IAA28*fwd (5′-primer), 5′-GTTTAAACATGGAAGAAGAAAAGAGATTGG-3′; *IAA28*rev (3′-primer), 5′-TTAATTAATTACTCCCAAGGAACATCCCCG-3′, *IAA13*fwd (5′-primer), 5′-GTTTAAACATGGATTACTGAACTTGAGATGG-3′; *IAA13*rev (3′-primer), 5′-TTAATTAACTAAACCGGCTGCTTTCGCTGT-3′, *ARF-1BP*fwd (5′-primer), 5′-GTTTAAACATGCCTAGGGCCTAAGAGGCCCA-3′; *ARF-1BP*rev (3′-primer), 5′-TTAATTAATTAAGAGTTCCCAGCGCTGGAC-3′ (added restriction sites underlined: *Pme*I in forward and *Pac*I in reverse primers). PCR products were analysed by agarose gel electrophoresis. Individual fragments were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and subcloned into pUni/V5-His-TOPO® (Invitrogen, Karlsruhe, Germany). After sequence confirmation, the *IAA13*, *IAA28*, and *ARF-BP1* fragments were cloned via *Pme*I/*Pac*I sites into a modified pGreen 0229 plant transformation vector (Roger *et al.*, 2000) containing a cauliflower mosaic virus (CaMV) 35S promoter (Skirycz *et al.*, 2006). The final construct was transferred into *Arabidopsis thaliana* using *Agrobacterium tumefaciens*-mediated gene transfer. Transgenic plants were selected in the greenhouse by spraying with the herbicide BASTA.

Plant cultivation for physiological experiments on sulphur depletion

Arabidopsis thaliana ecotype Col-0 and transgenic plants were grown in a growth chamber (16 h light/8 h dark cycle) on sterile agarose medium in 12.5 cm square Petri dishes on a solidified sulphur-sufficient medium (0.5 N Murashige–Skoog salts). After 3 weeks on this medium, plants were transferred to blue 1 ml tip boxes filled with the same liquid medium for 2 weeks to adapt them to hydroponic culture conditions. Per line, two conditions were tested: a control with sufficient sulphur content and a depleted with a sulphate content of about 0.1 mM (from Fe-EDTA). The macro-element $MgSO_4$ was replaced with an equimolar amount of $Mg(NO_3)_2$, and the micro-salts $CuCl_2$, $MnCl_2$, and $ZnCl_2$ were used instead of $CuSO_4$, $MnSO_4$, and $ZnSO_4$, correspondingly. These conditions were maintained for 10 d. Several boxes per treatment were pooled, resulting in around 100 plants per line per treatment.

RNA extraction, northern blot analysis, cDNA synthesis, and RT-PCR

Total RNA was isolated from leaves using TRIzol® reagent (Invitrogen/BRL, Karlsruhe, Germany) according to the manufacturer's instructions. Ten micrograms of total RNA was loaded per lane on 1.5% agarose gels and separated by electrophoresis under denaturing conditions. RNA was blotted onto Hybond-N nylon membrane (Amersham Biosciences, Freiburg, Germany) and cross-linked by UV illumination. Hybridization at 60 °C was performed using sodium phosphate buffer (0.25 M, pH 7.2) containing 7% SDS, 1% bovine serum albumin, and 1 mM EDTA. [^{32}P]dCTP-labelled *IAA13*, *IAA28*, or *ARF1-BP* cDNAs were used as a probe. The membranes were washed twice for 15 min at 65 °C (first wash: 10× SSC, 0.5% SDS; second wash: 5× SSC, 0.5% SDS; third wash: 2× SSC, 0.5% SDS). Membranes were exposed to X-ray films (Kodak X-OMAT AR) at –70 °C for 4–10 d.

To screen for the T-DNA insertion in the *IAA28* knock-down line (SALK_129988; Alonso *et al.*, 2003; Li *et al.*, 2004), polymerase chain reactions were performed on a Biometra PCR machine (Germany) using genomic DNA, 0.4 µM specific primers, 1 mM $MgCl_2$, 2 mM dNTPs, 1× PCR buffer, and 5 U Taq Polymerase (Gibco BRL, Germany) in a 100 µl volume. After 35 cycles, 10 µl samples were taken for electrophoresis. The PCR samples were electrophoresed on 1% agarose gels containing ethidium bromide for staining and photographed on top of a 280 nm UV light box. The gel images were digitally captured with a CCD camera. PCR products amplified using T-DNA border primers and gene-specific primers were sequenced to verify the sites of the insertions. DNA fragments of about 100 bases were generated by specific oligonucleotides for the T-DNA and gene, respectively (oligonucleotides used: *LBa* (left border) 5′ TGGTTCACGTAGTGGGCCATCG; *LBb* (left border) 5′ GCGTGGACCGCTTGCTGCAACT; *IAA28-LP* (LP) 5′ GAGACCCCACACGGCTATGAA; *IAA28-RP* (RP) 5′ CGGCATGTGAAAGCTGTTGGT; *IAA28*-cDNA (ATG; 28 fw) 5′ ATGGAAGAAGAAAAGAGATTGG, (stop; 28 rw 5′ CTATTCCTTGCCATGTTTTCTA; oligonucleotides were purchased from TIB MOLBIOL, Berlin, Germany).

Quantitative RT-PCR

In order to analyse the expression of sulphur assimilation pathway-related genes in the overexpressing and knock-down plants and wild-type control plants, RNA was extracted for quantitative RT-PCR. Total RNA was isolated from shoots using TRIZOL reagent (Invitrogen), according to Czechowski *et al.* (2004). RNA concentrations were measured in an Eppendorf biophotometer, and 150 µg of total RNA digested with RNase-free DNaseI (Sigma-Aldrich, Taufkirchen, Germany), according to the manufacturer's instructions. Absence of genomic DNA contamination was subsequently confirmed by PCR, using primers designed based on the intron sequence of a control gene (At5g65080). RNA integrity was checked on a 1.5% (w/v) agarose gel prior to and after DNaseI digestion. Poly(A)$^+$ RNA was purified with an Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany), using the supplier's batch protocol. RT reactions were performed with SuperScript™ III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The efficiency of cDNA synthesis was assessed by real-time PCR amplification of control genes encoding:

At3g18780F (*actin*2) 5′-TCCCTCAGCACATTCCAGCAGAT-3′, At3g18780R 5′-AACGATTCCTGGACCTGCCTCATC-3′;

At4g05320F (*UBQ10*) 5′-CACACTCCACTTGGTCTTGCGT-3′, At4g05320R 5′-TGGTCTTTCCGGTGAGAGTCTTCA-3′;

At5g60390F (*elongation factor 1α*) 5′-TGAGCACGCTCTTCTTGCTTTCA-3′, At5G60390R 5′-GGTGGTGGCATCCATCTTGTTACA-3′.

Only cDNA preparations that yielded similar C_T values (e.g. 20±1) for the control genes were used for comparing sulphur assimilation pathway transcript levels. The following oligonucleotides were used for gene-specific amplification:

At5g25890 (*IAA28*) fw: 5′GCTCCTCCTTGTCACCAATTCACT, rw: 5′ACTGGAGCTACCTCAACCCTGTTA;

At2g33310 (*IAA13*) fw: 5′GCTAATGGACTCGCTGCACGAAA, rw: 5′TAAACCGGCTGCTTTCGCTGTCTC;

At5g62010 (*ARF1-BP*) fw: 5′GGAGTTGCACAAAGGTTCACAAGC, rw: 5′-TTGAAAGATCCACTGAACGGCCAA;

At5g10180 (sulphate transporter [*Sultr*] *2.1*) fw: 5′-CATCTTGTTACGCTGCTACTGG, rw: 5′-GTTGGACATTGCTGTCTCACAT;

At1g77990 (*Sultr 2.2*) fw: 5′-ATCCAATGGCAAATAA-GACTGC, rw: 5′-AAAATTGGCATTAGCAAAGCAT;

At3g51895 (*Sultr 3.1*) fw: 5′-GGCGATATCTATAGC-GAGGTTG, rw: 5′-TCATGCTGTTTGGTATGTTTCC;

At1g23090 (*Sultr 3.3*) fw: 5′-AGAGATCTAGCAGTGGGAC-CAG, rw: 5′-ACGGGAGATACTTGTTGCCTTA;

At3g15990 (*Sultr 3.4*) fw: 5′-AGTCCAAGATCATTGAGG-CATT, rw: 5′-GTTGATGAGAGATCAGCCACAG;

At5g13550 (*Sultr 4.1*) fw: 5′-AACCCGAACAAAGATGTT-CACT, rw: 5′-AAGAACCATTCCTTGCCAACTA;

At1g80310 (*Sultr 5.1*) fw: 5′-CGATTCCTCAGATTC-CACTCTC, rw: 5′-TCCTTGTCAAACAAGTCATTGG;

At2g25680 (*Sultr 5.2*) fw: 5′-GCAATGGGTGATCTTGG-TACTT, rw: 5′-CAGTGTTGTGCCTAAATCCAAA;

At1g19920 (ATP sulphurylase [*APS*]2) fw: 5′-CACCAAC-CAAATCTCCTTCC, rw: 5′-CAGATTGCATTGTTAGGTTTCG;

At2G14750 (APS kinase [*APK*]1) fw: 5′-GGGAGATTTTGTT-GAGGTGTTC, rw: 5′-CAAGCTTGTAAAGACCCTTTGG;

At3G03900 (*APK(1)*) fw: 5′-GTGTCCTTCCCCTGTAGC-TATG, rw: 5′-TCGTTTTGAAGGAAACCTTTGT;

At4g39940 (*APK2*) fw CCTTAACCGTGACCTCACTTTC, rw ACAACTTAGCCACCTCACCAAT;

At5G67520 (*APK(2)*) fw: 5′-AGAACATTGTCTGGCATGAT-TG, rw: 5′-AAATCACACATCCCTTTTGCTT;

At1g55920 (serine acetyltransferase [*SAT*]1) fw: 5′-AGAT-GCTTGAAGAAGCCAAATC, rw: 5′-TGAGATGTGATCGAAG-CGTAGT;

At3g13110 (*SAT A1*) fw: 5′-TCCTGATCGATCTTCCTTCAAT, rw: 5′-AGATCTTCAAGCAAAGGACGAG;

At5g56760 (*SAT52*) fw: 5′-ACAAGCTATGGACACAAT-CACG, rw: 5′-AACAGCGAATACATCGGAGATT;

At3g61440 (*O*-acetylserine(thiol)lyase [*OASTL*]-*C1*) fw: 5′-CCACACCGTGAGGAAACTCT, rw: 5′-GCGTTGGTAGAGGGGAAGTC;

At5g28030 (*OASTL*) fw: 5′-CGGTACGACGTTCTTTTTGG, rw: 5′-CCAAAATCAGAAAATGGACGA;

At3g57050 (cystathionine beta-lyase [*Cbl*]) fw: 5′-CTTGC-TGTAAAGGCGAAAAAT, rw: 5′-TCGAAAGGAGCTAATC-CAGAAC (TIB MOLBIOL, Berlin, Germany).

Real-time PCR conditions and analysis

Polymerase chain reactions were performed in an optical 384 well plate with an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany), using SYBR® Green to monitor dsDNA synthesis. Reactions contained 5 µl 2× SYBR® Green Master Mix reagent (Applied Biosystems), 1.0 ng cDNA, and 500 nM of each gene-specific primer in a final volume of 10 µl. A master mix of sufficient cDNA and 2× SYBR® Green reagent was prepared prior to dispensing into individual wells to reduce pipetting errors and ensure that each reaction contained an equal amount of cDNA. The following standard thermal profile was used for all PCRs: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analysed using the SDS 2.0 software (Applied Biosystems).

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) versus cycle number, baseline data were collected between cycles 3 and 15. All amplification plots were analysed with an R_n threshold of 0.3 to obtain C_T (threshold cycle) values. In order to compare data from different PCR runs or cDNA samples, C_T values for all genes were normalized to the C_T value of *UBQ10*, which was the most constant of the four housekeeping genes included in each PCR run. The average C_T value for *UBQ10* was 20.04 (±0.89) for all plates/templates measured in this series of experiments. PCR efficiency (E) was estimated in two ways. The first method of calculating efficiency utilized template

dilutions and the equation $(1+E)=10^{(-1/\text{slope})}$, as described previously by Pfaffl (2001). The second method made use of data obtained from the exponential phase of each individual amplification plot and the equation $(1+E)=10^{\text{slope}}$ (Ramakers *et al.*, 2003). Gene expression was normalized to that of *UBQ10* by subtracting the C_T value of *UBQ10* from the C_T value of the gene of interest. S/R expression ratios were then obtained from the equation $(1+E)^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ represents ΔC_{TS} minus ΔC_{TR}, and E is the PCR efficiency. For every data point, the threshold cycle (C_T) value was the average of the C_T values obtained from the triplicate PCR analyses. For every mutant, two biological replicates were analysed.

Extraction and analysis of soluble thiol compounds

Individual soluble thiols were determined as the sum of their reduced and oxidized forms. One hundred milligrams of fresh ground leaf material were added to 100 mg of polyvinylpolypyrrolidone (previously washed with 0.1 M HCl) and 1 ml of 0.1 M HCl. The samples were shaken for 60 min at room temperature. After centrifugation (15 min at 13 000 *g*; 4 °C), the supernatants were frozen at –20°C until reduction/derivatization. Thiols were reduced by incubation with 10 mM dithiothreitol for 40 min at room temperature and derivatized for 15 min in the dark according to Kreft *et al.* (2003). Column eluent was monitored by fluorescence detection (λ_{ex} 380/λ_{em} 480). Mixed standards treated exactly as the sample supernatants were used as a reference for the quantification of cysteine and GSH content.

Extraction and analysis of soluble amino acids

Soluble amino acids were determined according to Kreft *et al.* (2003). Leaf tissues (about 100 mg per plant) were ground to a fine powder in liquid nitrogen in a bead mill and extracted three times for 20 min at 80 °C: once with 400 µl of 80% (v/v) aqueous ethanol (buffered with 2.5 mM HEPES-KOH, pH 7.5), once with 400 ml of 50% (v/v) aqueous ethanol (buffered as before), and once with 200 µl of 80% (v/v) aqueous ethanol. Between the extraction steps, the samples were centrifuged for 10 min at 13 000 *g*, and the supernatants were collected. The combined ethanol/water extracts were stored at –20 °C or directly subjected to RP-HPLC using an ODS column (Hypersil C18; 150×4.6 mm i.d.; 3 µm; Knauer GmbH, Berlin) connected to an HPLC system (Dionex, Idstein, Germany). Amino acids were measured by precolumn derivatization with OPA in combination with fluorescence detection (Lindroth and Mopper, 1979). Peak areas were integrated using Chromeleon 6.30 software (Dionex) and subjected to quantification by means of calibration curves made from standard mixtures.

Statistical analysis

Statistical test procedures, such as Student's *t*-test (Sokal and Rohlf, 1995) were executed with the Microsoft Excel software. All descriptive statistical analyses were implemented in and executed with R (R Development Core Team, 2007). The heat-map graphics drawn with cluster trees were generated with the gplot package (Warnes, 2007) implemented in R. Data presented in Table S1 and Fig. S2 in Supplementary data available at *JXB* online were used for the generation of the heat maps. To aid interpretation and avoid division by zero, missing and zero values were replaced by an arbitrary detection limit for each metabolite. These arbitrary values represent 1/100 of the minimum non-zero column (metabolite) value obtained. All values were expressed as ratios to the respective wild-type value. The heat-map graphs were generated by using log base 2-transformed values. The hierarchical cluster analyses were performed as unweighted average linkage clustering algorithm

(UPGMA) on the basis of Euclidean distance (Mirkin, 1996) between the column (metabolites) and rows (genotypes).

Results

Generation of CaMV 35S overexpression lines

The transcription factors IAA13, IAA28, and ARF2 are up-regulated during sulphur starvation in *Arabidopsis thaliana* seedlings (Nikiforova *et al.*, 2003). To study their functions, their respective cDNAs were overexpressed in *Arabidopsis* under the control of the CaMV 35S promoter harboured in the binary vector pGreen 0229 (http://www.pgreen.ac.uk). Since the overexpression of the complete *ARF2* is probably lethal (Okushima *et al.*, 2005b), it was decided to use the 3' part of the gene encoding domains II and III responsible for protein stability and homo/heterodimerization with other Aux/IAA gene family members, respectively (Kim *et al.*, 1997; Worley *et al.*, 2000; Ouellet *et al.*, 2001). The product of this region has been previously denoted as ARF1-binding protein, so the construct was named *ARF1-BP* (Paciorek and Friml, 2006). For each construct, several lines were identified that displayed high expression of the respective gene, whilst expression remained below the detection limit for wild-type and empty-vector control plants.

Three lines per construct were selected for detailed analyses (Fig. 1). The highest transcript levels were observed for plants expressing *IAA28* (lines #16, #14, #6), while *IAA13* levels (lines #31, #15, #14) were generally lower, and *ARF1-BP* RNA levels (lines #16-1, #16-2, #16-5) low but clearly detectable (Fig. 1A).

Quantitative RT-PCR (q-RT-PCR) was applied to verify the RNA blot data: transcript levels of *IAA28* increased up to 14-fold, *IAA13* up to 215-fold, and *ARF1-BP* up to 112-fold. The apparent difference between quantification data from blotting and q-RT-PCR analyses is probably due to the low (nonexistent) expression level of the genes in control plants, which boosts the apparent ratio of expression when q-RT-PCR is applied (Fig. 1B). Since cross-effects between various IAA and ARF transcription factors are to be expected, it was tested how these three transcription factors were transcriptionally regulated in the transgenic lines (Fig. 1B). For *IAA28* overexpressing lines, the *IAA13* transcription level was not altered while the *ARF1-BP* transcript was down-regulated by up to 88%. In *IAA13* overexpressing lines, only line 15-4 exhibited reduced *IAA28* transcript content (a reduction of 60%). However, *ARF1-BP* transcript was reduced by 86%. In *ARF1-BP* overexpressing lines, both *IAA28* and *IAA13* transcript accumulated 3-fold and 2-fold, respectively. All comparisons are to wild-type.
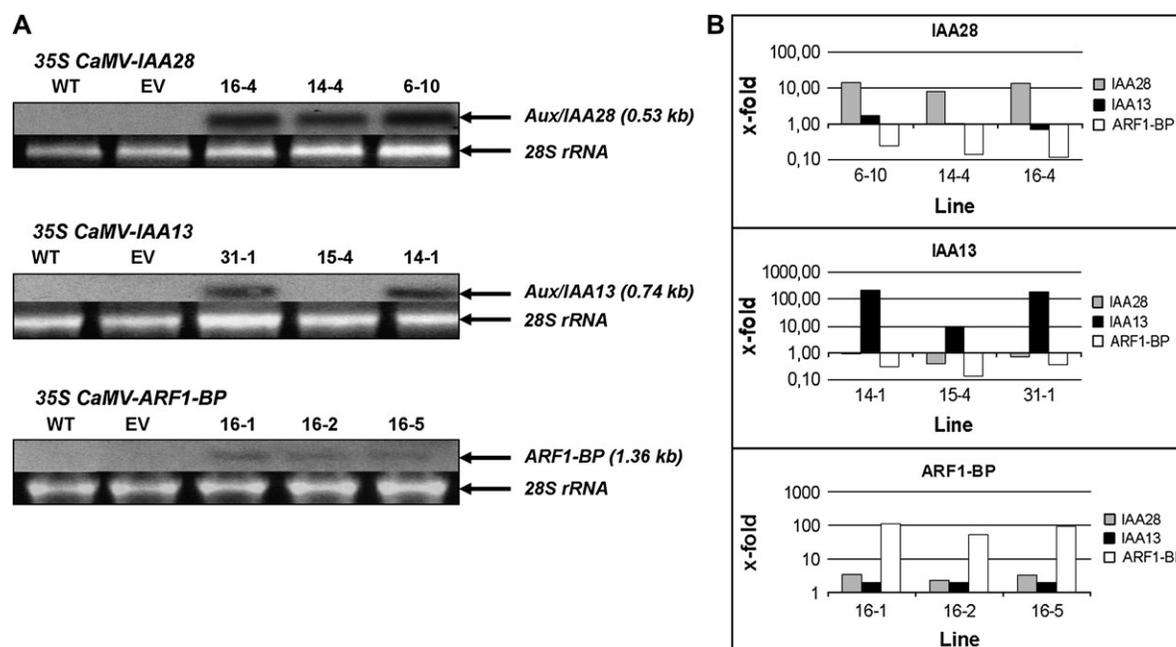
Fig. 1. Expression analysis of *IAA28*, *IAA13*, and *ARF1-BP*, respectively, in overexpressing lines from 28-d-old soil-grown plants with northern blot hybridization and q-RT-PCR, respectively. (A) Wild-type and empty-vector plants served as controls. RNA from 10 plants per treatment was extracted and 10 μg total RNA was subjected to northern blot analysis. Gene-specific probes were radioactively labelled and hybridized as described in Materials and methods. Equal loading in each track is demonstrated by comparing the amount of the 28S rRNA. Transcript sizes are given in brackets. (B) Quantification of gene expression in transgenic plants overexpressing *IAA28*, *IAA13*, and *ARF1-BP*, respectively. Gene expression was assessed by q-RT-PCR as described in Materials and methods. The data represent the average relative to the gene expression in control plants.

Overexpression of *IAA28* did not result in severe growth retardation (Fig. 2A–C). This was unexpected since it was hypothesized that *IAA28* is a potential hub in the sulphate-starvation response network (Nikiforova *et al.*, 2005*b*). Further, overexpression of *Aux/IAA* genes typically resulted in growth-retardation phenotypes in *Arabidopsis*, especially when overexpressing mutated domain II regions of Aux/IAA proteins (Reed *et al.*, 2001). When compared with wild-type and empty-vector control plants, flowering time was not delayed and the height of the transgenic lines did not change. However, as shown later, the overexpression of *IAA28* does affect gene expression and metabolite composition in the mutant plants.

Phenotypic changes were observed for transgenic plants overexpressing *IAA13* or *ARF1-BP*, although the encoded proteins were not mutated in domain II to generate stable proteins. Expression of both constructs resulted in severe growth retardation (Fig. 2D–F, G–J, respectively). Interestingly, this result for ARF1-BP is quite opposite to results seen in ARF2 knockout lines (Okushima *et al.*, 2005*a*, *b*). When compared with empty-vector control plants, flowering time of the transgenic plants was not

delayed. However, the height of the transgenic lines was reduced by two-thirds, and rosette leaves of the transgenic lines were smaller (Table 1). While *IAA13* lines exhibited a bushy phenotype with a significantly increased number of side branches, *ARF1-BP* lines exhibited extended shoots with only a slightly increased number of side branches with more flowers. Furthermore, rosette leaves were dramatically reduced in length (again by two-thirds), and the total number of leaves decreased by three-quarters. Rosette leaves of *ARF1-BP*-expressing plants developed a curly phenotype (Fig. 2D). The result for *IAA13* is also of special interest since non-stabilized domain II IAA overexpressions usually do not yield phenotypic changes (Weijers *et al.*, 2005).

Isolation of an Aux/IAA28 T-DNA insertion mutant

In addition to overexpressing *Aux/IAA28*, a PCR-based approach was used to identify a T-DNA insertion mutant in this gene to test the hypothesis that it is a network hub (Nikiforova *et al.*, 2005*b*). A search of the Salk T-DNA express line collection (http://signal.salk.edu) revealed a line containing a T-DNA insertion line in the *IAA28*
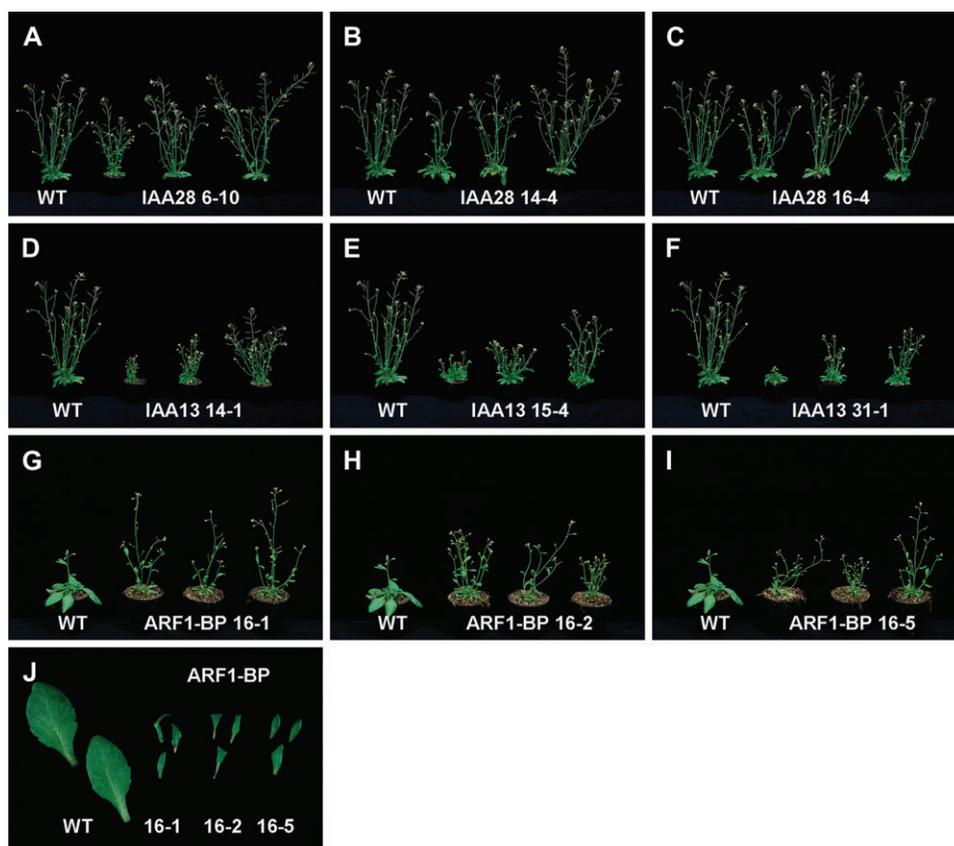
Fig. 2. Growth phenotypes of overexpressing lines of *IAA28*, *IAA13*, and *ARF1-BP*, respectively. Wild-type plants (left in each picture) and three independent transgenic lines per construct and three plants per line were grown simultaneously in soil for 10 weeks. (A–C) Lines expressing *IAA28*, (D–F) lines expressing *IAA13*, and (G–J) line expressing ARF1-BP, respectively. The wild-type plants were grown for 6 weeks in soil and are shown for comparison of regular and mutant shoot phenotypes. (J) Close-up of leaf morphology of lines overexpressing *ARF1-BP* in comparison with leaves of wild-type plants (10 weeks old). Two wild-type leaves are shown in the left part of the picture (10 weeks old).

Table 1. *Measurements of wild-type (WT)/empty-vector (EV) control and mutant plants*

*Values given are mean $\pm$SD (n).

IAA28-OE		IAA13-OE		ARF1-BP-OE	
Leaf length					
6–10	5.63±0.70	14–1	3.37±0.94*	16–1	2.08±0.21*
14–4	6.69±0.61[a]	15–4	4.31±0.93[a]	16–2	2.68±0.46[a]
16–4	6.85±0.55[a]	31–1	4.04±1.13[a]	16–5	2.28±0.57[a]
WT/EV	5.44±0.79	WT/EV	5.44±0.79	WT/EV[b]	4.96±0.54
Leaf width					
6–10	2.26±0.25	14–1	1.14±0.54[a]	16–1	0.50±0.09[a]
14–4	2.42±0.32	15–4	1.11±0.53[a]	16–2	0.63±0.17[a]
16–4	2.53±0.42	31–1	1.02±0.42[a]	16–5	0.50±0.16[a]
WT/EV	2.01±0.54	WT/EV	2.01±0.54	WT/EV[b]	1.82±0.33
Stem					
6–10	29.55±3.35	14–1	10.70±4.10[a]	16–1	9.89±2.77[a]
14–4	31.84±3.61	15–4	11.07±4.93[a]	16–2	7.24±3.21
16–4	29.62±1.94	31–1	9.22±4.35[a]	16–5	8.32±4.09
WT/EV	28.89±2.49	WT/EV	28.89±2.49	WT/EV[b]	5.14±3.14
Branch					
6–10	6.30±0.82[a]	14–1	7.20±2.66	16–1	4.70±0.67[a]
14–4	6.90±1.29[a]	15–4	8.80±2.86[a]	16–2	4.22±0.67
16–4	7.20±0.79[a]	31–1	8.00±2.40[a]	16–5	3.90±0.87
WT/EV	5.50±0.85	WT/EV	5.50±0.85	WT/EV[b]	3.77±1.09
				Leaf number	
				16–1	4.70±1.06[a]
				16–2	7.33±1.12[a]
				16–5	5.00±1.25[a]
				WT/EV[b]	17.44±3.13

[a] Single mutant values are distinguishable from corresponding wild-type/empty-vector control values of 10-week-old plants by t-test ($P < 0.05$).
[b] Single mutant values are distinguishable from corresponding wild-type/empty-vector control values of 6-week-old plants by t-test ($P < 0.05$).

gene (At5g25890). The line was backcrossed three times with wild-type Columbia (Col-0), and characterized phenotypically and genetically. Three selected plants of the knock-down mutant (#6, #11, and #13) were analysed further.

The down-regulation of *IAA28* did not result in gross phenotypic changes (Fig. 3A). The T-DNA insertion was located in the 5' UTR of the gene. Two T-DNA insertions separate the promoter from the translation start point (Fig. 3B). Though *IAA28* transcript could not be detected by RNA gel blot hybridization in *IAA28* T-DNA insertion lines (data not shown), transcription was not completely down-regulated as revealed by q-RT-PCR analysis. At best, a reduction to 65% in the *IAA28* knock-down plants was detected (Fig. 3C). Despite the partial inhibition of *IAA28* expression, *IAA13* expression was down-regulated to between 34% and 40% of the control plant level in these lines. Additionally, in knock-down line 6, *ARF1-BP* expression was reduced to 84%.

Does the expression or mutation of AUX/IAA and ARF genes influence metabolite levels?

To investigate the potential involvement of the transcription factors IAA13, IAA28, and ARF1-BP in signalling sulphur status, the levels of free amino acids and of the thiols cysteine, γ-glutamylcysteine (GEC), and GSH were determined in 8-week-old soil-grown plants. In plants, thiol and amino acid levels are strictly regulated and amounts scarcely fluctuate under nutrient-sufficient growth condition (Hesse and Hoefgen, 2003). Amino acid homeostasis, in general, is difficult to perturb beyond fluctuations of the stress amino acid proline or diurnal shifts of the N-storing amides glutamine and asparagine (Lam *et al.*, 1996; Buchanan *et al.*, 2000).

In the plant lines investigated, however, thiol levels were altered, some significantly, in comparison with wild-type and empty-vector control plants (Fig. 4). In the *IAA28*-overexpressing plants, thiol levels generally increased (Fig. 4A–C), with cysteine levels rising 3-fold, GEC 2-fold, and GSH 1.25-fold. No major changes in these metabolites were detected in *IAA28* knock-down mutants, where cysteine levels decreased only weakly and GEC and GSH remained constant. The latter is consistent with the fact that these plants still displayed 65% of wild-type *IAA28* transcript level.

Lines overexpressing *IAA13* exhibited an increase of up to 3.5-fold in cysteine levels, of ~1.5-fold for GEC, and of ~1.3-fold for GSH (Fig. 4D–F). The levels of cysteine and GSH were markedly increased in plants expressing *ARF1-BP* (Fig. 4G, H). While cysteine increased up to 6-fold, GEC was not detectable, probably because of its efficient conversion to GSH, which again increased up to 7-fold.

At a first glance, the above data hint at the immediate control of sulphate metabolism by these transcription
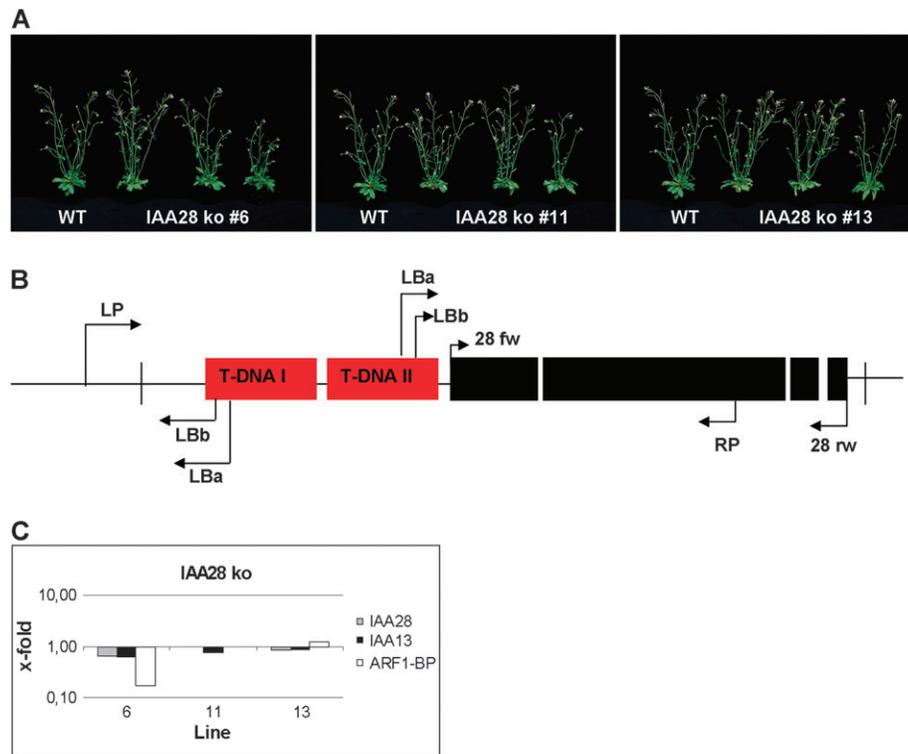
Fig. 3. Phenotype of mature *IAA28* knock-down plants, a sketch depicting the location of the T-DNA insertions, and quantification of gene expression in mutant plants. (A) Wild-type plants (left in each picture) and three mutant plants (right) at 10 weeks. Each of three individually selected homozygote mutant plants is presented. The plants were grown at the same time. (B) Identification of *IAA28* T-DNA insertion. Boxes in black represent exons, lines represent non-coding regions, and boxes in grey indicate T-DNA insertions. (C) q-RT-PCR analysis of plants derived from the *IAA28* knock-down screen. RNA was extracted from 28-d-old soil-grown plants. Ratios to controls are shown.

factors. But since Aux/IAA proteins are general regulators of plant development, it was wondered whether they exert additional effects on plant metabolism, especially amino acid biosynthesis. Even though *IAA28* was initially identified as a hub in the network generated from data sampled during sulphate starvation (Nikiforova *et al.*, 2005*b*), mutant plant lines affected in this gene exhibited no adverse effects on growth or phenotype. When scoring overall plant metabolism, especially as regarding amino acid contents (Fig. 5, and Table S1 in Supplementary data available at *JXB* online), the ratios for a few amino acid levels changed in the *IAA28* knock-down mutant, although plants still express about 65% of the wild-type transcript level. The knock-out mutant lines cluster together with wild-type and empty-vector control plants (Fig. 5). Moreover, all overexpression lines cluster together and thus are distinguishable from wild-type plants. The empty-vector control plants displayed an intermediate metabolic phenotype, however, only due to the lost resolution by converting the data to a heat-map presentation. Minor reductions were seen in histidine (–1.4-fold), isoleucine (2-fold), and serine (–1.8-fold) levels, marginal reductions were observed for alanine, valine, and tryptophan, the rest of the amino acids remaining at constant levels (Table S1 in Supplementary data available

at *JXB* online). In all overexpressing lines, a general effect, i.e. the total free amino acid content increased up to 3-fold compared with control plants, was observed and, for some of the constructs, the levels of some individual amino acids increased significantly, indicated by the dark blue colour code (Fig. 5, and Table S1 in Supplementary data available at *JXB* online).

Three patterns of changes in amino acid levels were seen (Table S1 in Supplementary data available at *JXB* online): The first group includes the amino acids glutamate, arginine, methionine, lysine, tryptophan, tyrosine, and isoleucine, which did not change significantly in content compared with the respective control plants. The second group consists of the amino acids glutamine, aspartate, asparagine, alanine, leucine, serine, threonine, and glycine, which, depending on the line, significantly increased, in some cases up to 7-fold (e.g. leucine in *IAA13*). The third group comprises amino acids with inconsistent responses such as histidine, which decreased only in the *ARF1-BP* lines, valine, down in *IAA28*, up in *IAA13*, and phenylalanine, up in *IAA13*, down in *ARF1-BP*.

It is noteworthy that approximately half of the 19 proteinogenic amino acids measured here were altered in content. Mass amino acids such as glutamine, aspartate, and asparagine increased drastically suggesting that the
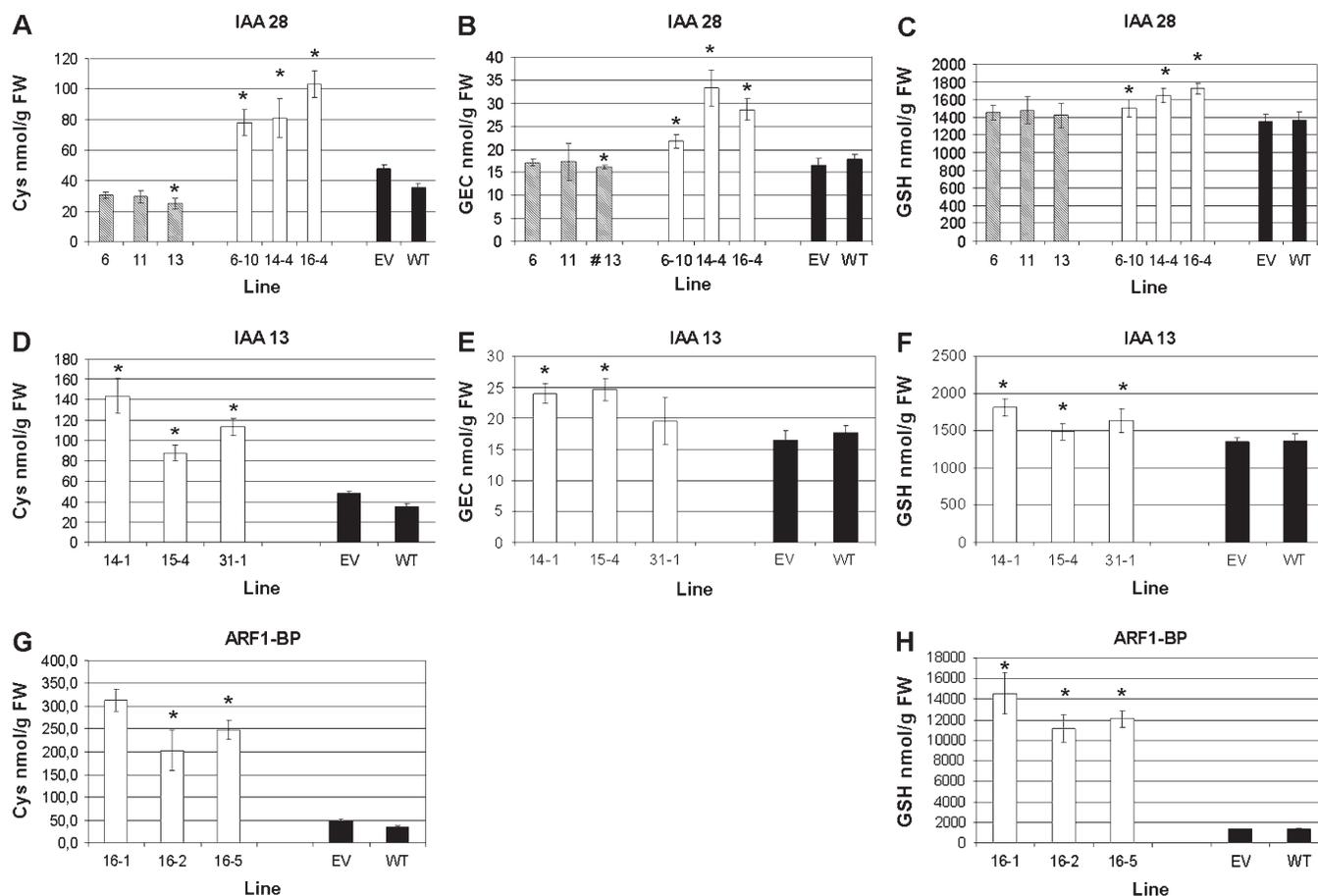
Fig. 4. Contents of cysteine (upper row), γ-glutamylcysteine (GEC; middle row), and glutathione (GSH; lower row) are shown for *Arabidopsis* plants overexpressing *IAA28*, *IAA13*, and *ARF1-BP*, respectively, or down-regulated with respect to *IAA28*. Plants were grown for 10 weeks on soil before thiol extraction. *IAA28* knock-downs are represented by cross-hatched columns, overexpressing lines by white columns, and wild-type (WT) and empty-vector control lines (EV) by black columns. Values are the mean ±SD of three independent experiments. Asterisks indicate that the difference between the wild-type plants and the manipulated transgenic plants was significant using *t*-tests ($P \leqslant 0.05$).

carbon/nitrogen ratio has been altered. If this is the case, it is likely to be the result of changes in photosynthesis and/ or photorespiration, which is further on supported by increases in serine and glycine contents (derived from phosphoglycolate/3-phosphoglycerate). Since serine itself is the carbon backbone precursor for cysteine synthesis and since cysteine accumulates in all overexpressing lines investigated, the increase might be driven by increased carbon supply rather than by the demands of sulphate metabolism. Usually cysteine and GSH levels display a fixed ratio in plant cells, but in *IAA13* and *IAA28* overexpressors, the bulk of cysteine is not converted to GSH. These consistent metabolite-responses are remarkable and speak for a general regulatory pattern, especially since the *IAA13* and *ARF1-BP* transgenic lines show developmentally impaired phenotypes, while *IAA28* knock-down or overexpressing lines are morphologically like wild type. Thus, auxin response genes affect sulphur metabolism in the wider context of amino acid metabolism and carbon/nitrogen balance.

Effect of transcription factor expression on pathway gene expression in transgenic plants

To see if downstream genes were affected in transgenic lines, and to investigate the molecular basis for the observed changes in metabolite levels, the expression of selected genes of each mutant line was compared with that of the wild-type and/or empty-vector control using quantitative RT-PCR (qRT-PCR) (Fig. S1 in Supplementary data available at *JXB* online). All 21 selected genes responded to the transcription factor expression (data not shown in full; Nikiforova *et al.*, 2003). Genes encoding sulphate transporter isoforms, ATP-sulphurylase, APS kinase, serine acetyltransferase, and *O*-acetylserine(thiol)-lyase were coordinately down-regulated in *IAA28* and *IAA13* overexpressing lines, while in *IAA28* knock-down and *ARF1-BP* overexpressing plants these genes were jointly activated. Even though *IAA28* was not completely knocked down, genes of the sulphate pathway were affected. It may appear at first glance that the respective transcription factors regulate the expression of these target

genes, but these effects are interpreted to be consequences of the pleiotropic metabolic alterations described above, since the changes in amino acid metabolism and carbon/nitrogen balance may themselves affect the expression of sulphate metabolism pathway genes (Hesse *et al.*, 2004*a*).

Does knock-down and overexpression of IAA13, IAA28, or ARF1-BP influence plant adaptations to nutrient stress?

Since overexpression of the transcription factors resulted in pleiotropic effects including those to sulphate metabolism, it was decided to limit sulphate using more easily controlled hydroponic culture conditions. A start was made with the hypothesis that, if *IAA13*, *IAA28*, and *ARF2* do indeed play direct roles in the plant's responses to sulphate status, it should be possible to detect an impact in the mutant lines grown under sulphate-limiting conditions, perhaps even a marked predisposition for withstanding sulphate stress. If the transcription factors were to improve or regulate part of the response behaviour to sulphur stress, then their respective metabolite levels would reflect this shift. Therefore, the fact that the metabolic changes previously observed in soil-grown plants were not observed in *IAA28* overexpressing or knock-down lines or in *IAA13* overexpressing lines when grown hydroponically was quite surprising. This fact is visualized in Fig. 6 showing the ratio of amino acid
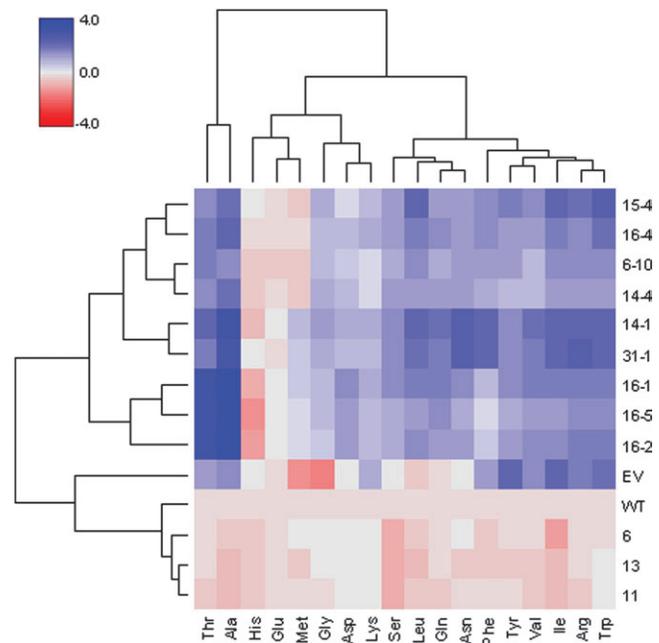
contents under control and sulphur starvation condition (see also Figs S2, S3 in Supplementary data available at *JXB* online). The cluster analysis shows a rather scattered distribution of the different plant lines. Neither thiol levels nor amino acid levels were altered to the degree seen in plants grown in soil (Fig. 6, and Figs S2, S3). Moreover, hydroponically grown plants tested for their response to sulphur limitation behaved in a manner quite similar to that of control plants with regard to the levels of all but a few metabolites.

This indicates that overexpression of *IAA28* and *IAA13* in hydroponically grown plants provided no beneficial effects such as fostering or decelerating the breakdown of thiol compounds (Fig. 6, Fig. S2 in Supplementary data available at *JXB* online). *IAA13* overexpressors displayed a slight increase in the amino acids threonine, isoleucine, asparagine, arginine, and tyrosine under sulphate-deprived growth conditions, indicating an effect on the methionine–threonine control branch point (Hesse and Hoefgen, 2003), and on nitrogen-containing amino acids as a result of a disturbed sulphate/nitrogen balance (Hesse *et al.*, 2004*a*; Nikiforova 2005*a*, 2006). Again, though, the

Fig. 5. Heat map generated from amino acid measurements reflecting log base 2-transformed and normalized amino acid levels and its similarity among themselves and the genotypes. The top colour bar indicates the relative log base 2-fold changes ranging between reduced relative (red) and increased relative (blue) contents of amino acids with respect to the wild-type.
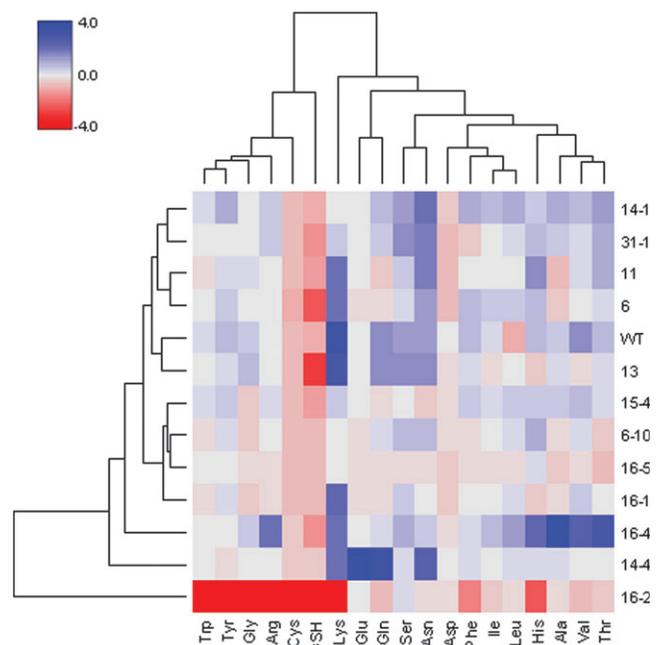
Fig. 6. Heat-map visualization and cluster tree representations of amino acid contents and genotypes. Data were obtained from experiments where plants were starved of sulphate for 10 d. The heat-map was generated by using log base 2-transformed fold changes. The given data represent the ratio of the determined amino acids for control and starved plants. Each amino acid is represented by a single column and each genotype by a single row. Red indicates decreased relative metabolite content whereas blue indicates increased relative contents of amino acids compared with the wild-type. Separated heat-map visualization of amino acid contents in control and mutant plants are presented in Fig. S3 in Supplementary data available at *JXB* online and the respective diagrams in Fig. S2.

effects are more pleiotropic than specific and mutant plants do not survive starvation better than controls.

Under sulphur limitation, *ARF1-BP* overexpression resulted in enhanced thiol synthesis in these lines (cys 50–60%; GSH 50%) such that plants retained levels of thiols similar to those found under normal growth conditions. This metabolic alteration, although probably triggered indirectly, has interesting implications for sulphur metabolism homeostasis. In the same plants, other amino acids such as alanine, threonine, isoleucine, valine, asparagine, and phenylalanine are elevated even under sulphate-sufficient nutrient conditions. Leucine levels, however, rise when sulphur is scarce. In comparison with the changes observed in wild-type plants during sulphate starvation (Nikiforova *et al.*, 2005*a*, 2006), these changes are rather inconsistent. Therefore, again, a direct link between ARF1-BP, IAA13, and IAA28 and sulphur metabolism was not observed. The increases in metabolite levels in soil-grown plants overexpressing *IAA28*, *IAA13*, and *ARF1-BP* may simply be a pleiotropic effect due to artificially initiating a part of the auxin response network mimicking auxin alterations. In this context it is important to note that tryptophan increased only marginally in overexpressing lines and thus cannot be considered to be a determining factor in the line of evidence, suggesting that auxin might be involved in signalling sulphur depletion (Kutz *et al.*, 2002; Nikiforova *et al.*, 2003).

The underlying mechanisms of the auxin response appear to be quite diverse in the mutant plant lines. Though *IAA13* and *ARF1-BP* overexpressing lines do show comparable phenotypic alterations, the differences in their respective metabolite compositions indicate other modes of action. The increase in metabolites observed for soil-grown plants overexpressing *IAA28*, *IAA13*, and *ARF1-BP* reflects the general control effects of auxin as exerted through these transcription factors instead of their immediate involvement in regulating the sulphur assimilation pathway. The effects observed in plants grown in soil were drastically reduced in plants grown hydroponically and other amino acids were changed with respect to their levels (Figs S2 and S3 in Supplementary data available at *JXB* online). The levels of the amino acids threonine, tyrosine, valine, isoleucine, and (marginally) arginine increased in all overexpressing lines. Among the overexpressing lines, only *ARF1-BP* exhibited higher contents of aspartate, alanine, serine, asparagine, and (marginally) glutamine. *IAA28* and *IAA13* lines showed no increase of these amino acids.

Discussion

Sulphur, nitrogen, phosphorus, calcium, magnesium, and potassium are the key macro-elements needed for plant nutrition. While data concerning the biochemistry of sulphur metabolism has accumulated steadily, little information is available concerning its regulation at the level of expression control. Recently, however, some evidence of *cis*-elements (enhancers) and DNA-binding proteins has emerged (Howarth *et al.*, 2005; Ide *et al.*, 2005; Lewandowska *et al.*, 2005; Maruyama-Nakashita and Takahashi, 2005; Maruyama-Nakashita *et al.*, 2005; Takahashi, 2005). Among them the transcription factor SULFUR LIMITATION RESPONSES LESS MUTANT 1 (SLIM1) (Maruyama-Nakashita *et al.*, 2006). The respective *Arabidopsis* mutant exhibits reduced sulphate uptake and plant growth under sulphur starvation conditions compared with the wild type. However, *SLIM1* expression is constitutive and not altered upon sulphur starvation (Maruyama-Nakashita *et al.*, 2006), potentially indicating it being rather a signal cascade element then a regulator of the pathway *per se*. Very recently, *Arabidopsis* MYB transcription factors 28 and 29, also named 'production of methionine-derived glucosinolate' (PMG) 1 and 2, were identified to be involved in the regulation of aliphatic glucosinolate biosynthesis using omics-based co-expression analysis (Hirai *et al.*, 2007).

A systems-oriented approach has been used to identify genes involved in sulphur homeostasis in plants. In this and previous studies *Arabidopsis* plants at different developmental stages have been subjected to sulphate starvation and their transcriptomes and metabolomes analysed, developing a network that integrates both data sets (Nikiforova *et al.*, 2003, 2005*a*, *b*). Auxin regulates the cell-specific transcription of target genes via two types of transcription factors, ARFs and Aux/IAA proteins. From these groups of transcription factors, *IAA28*, *IAA13*, and *ARF2* were observed to be up-regulated during sulphur starvation in *Arabidopsis* (Nikiforova *et al.*, 2003). It is thus hypothesized that auxin might have signalling or signal-integrating properties pertinent to the regulation of sulphur homeostasis (Nikiforova *et al.*, 2005*b*).

To test this hypothesis, transgenic plants overexpressing the three auxin-related transcriptional regulators IAA28, IAA13, and ARF1-BP (the C-terminal part of ARF2) were studied in detail. Also included in the present analysis is a knock-down mutant line exhibiting reduced *IAA28* transcript abundance due to a T-DNA insertion. IAA28 was of particular interest since it exhibits high connectivity to other network elements in the metabolite/transcript network of sulphate-starved *Arabidopsis* plants (Nikiforova *et al.*, 2005*b*). The hub position of IAA28 in the network led to the hypothesis that it might act as a cellular element controlling the integration of sulphur- and auxin-related pathways.

IAA13- and *ARF1-BP*-overexpressing plants displayed growth retardation resulting in dwarfed plants, the effect being more pronounced in soil-grown than in hydroponically grown plants. By contrast, neither knock-down nor

overexpression of *IAA28* affected growth. The present results indicate that *IAA28*, *IAA13*, and *ARF1-BP* over-expression influences multiple phenotypic traits, specifically with respect to metabolite composition and sulphate pathway-relevant gene expression. Elevated thiol levels may indicate a direct effect of these transcription factors on sulphate assimilation (cys, and GSH, and in some cases GEC), as corroborated by RNA expression data, but the general, broad spectrum changes observed in the levels of other amino acids argue against direct or exclusive links between sulphur assimilation and auxin signalling.

Even though the *IAA28* gene was tagged by two T-DNA insertions separating the promoter from the coding sequence, the total reduction of the transcript abundance in knock-downs was only 35%. Still, this partially reduced expression resulted in a metabolic phenotype displaying altered levels of a number of metabolites. However, the observed phenotypic changes in the knock-downs were not directly attributable to the effects of *IAA28* on sulphate metabolism, several pathway genes being up-regulated. Depending on the gene and plant, inductions in expression of up to 20-fold were observed. Genes influenced ranged from the uptake system (sulphate transporter) to cysteine formation (SAT/OASTL complex). Interestingly, a gene encoding cystathionine beta-lyase (CbL), catalysing the formation of homocysteine (the precursor of methionine) was up-regulated. However, it is known from previous studies in potato that CbL does not influence net biosynthesis when overexpressed (Maimann *et al.*, 2001), and further, that CbL is not induced during sulphate starvation in either potato or *Arabidopsis* (Maimann *et al.*, 2001; Nikiforova *et al.*, 2003).

They did, however, exhibit metabolic phenotypes with altered thiol and amino acid levels. A gain-of-function mutant of *IAA28* identified by Rogg *et al.* (2001) exhibited severe defects in lateral root formation and subsequently reduced adult plant size and apical dominance. The mutation (in protein domain II) led to super-stabilization of IAA28, resulting in more pronounced changes than did the expression of the wild-type allele described here.

Thiol and amino acid composition were used as the basis of comparison between control and sulphate-starved plants (Nikiforova *et al.*, 2003, 2005*a*). In soil-grown plants, *IAA28* overexpressors clearly showed increased thiol (but not GSH) levels and increases in some amino acids. These results are largely consistent with those gathered from *IAA13*- and *ARF1-BP* overexpressing lines. However, one has to assume that a gene exhibiting an extraordinary position in a calculated network would largely affect the response to sulphate starvation if over-expressed or down-regulated. The observed minor changes seen in *IAA28* knock-down plants might be attributable to gene redundancy adjusting the reduced

gene expression level of *IAA28*, but may not be suited to explain the results seen in overexpressors with its metabolic consequences described above. Taking this into consideration one has to rethink the term 'hub' and its biological consequences. From a mathematical/statistical point of view IAA28 serves as a hub in the sulphate starvation co-response network but might have no measurable biological relevance for the investigated stress. Furthermore, the experimental procedures which resulted in this prediction have to be re-evaluated in this light. One explanation might well lie in the dimensionality problem (Bellman, 1961; Scholz *et al.*, 2004, 2005); i.e. a relatively small number of experimental data were used when calculating a network with a high degree of variance. The network might be better determined using more time points per data set and by analysing 'transition' phases between various response states. Further, significance thresholds might need to be re-assessed to align with data concerning the inherent variability of gene expression in all biological systems (data not shown).

In contrast to the results obtained with *IAA28* lines, *IAA13* and *ARF1-BP* overexpressing lines developed severe developmental defects. *IAA13* was identified as a sister gene of *BDL*/*IAA12*, leading to embryonic and post-embryonic phenotypes if expressed with a mutated domain II (Weijers *et al.*, 2005). Notably, in this study, CaMV 35S-driven overexpression of the wild-type *IAA13* allele resulted in short, bushy plants representing morphological changes similar to those described by Weijers *et al.* (2005).

ARF2 has been previously described as HSS1, a suppressor of the seedling hook formation mutant, HLS1 (Lehmann *et al.*, 1996; Li *et al.*, 2004). *ARF2* knock-out mutants (Okushima *et al.*, 2005*b*) exhibit pleiotropic developmental effects with generally improved plant growth, which is opposite to the effect seen with *ARF2* 3' end (*ARF1-BP*) overexpression in this study. Okushima *et al.* (2005*b*) also reported that overexpression of *ARF2* merely yielded co-suppression lines indicating a lethal effect of *ARF2* overexpression. Here, overexpression of the 3' *ARF1-BP* part of *ARF2* yielded transgenic plants that are dwarfed. A gain-of-function mutant or *ARF1-BP* overexpressing line has not been reported so far (Ellis *et al.*, 2005). ARF2 has been shown to act in a redundant manner with ARF1, enhancing many ARF2 phenotypes such as processes related to plant ageing, including flower initiation, rosette leaf senescence, floral organ abscission, and silique ripening (Li *et al.*, 2004; Okushima *et al.*, 2005*a, b*). In this study the overexpression of *ARF1-BP* resulted in the loss of apical dominance, enhanced shooting, and reduced leaf size.

A novel aspect of this study is the linking of the expression of transcription factors to metabolic changes, specifically of thiol and amino acid levels, rather than to developmental features. Surprisingly, all three

transcription factors show quite related responses in soil-grown plants. While *ARF1-BP* overexpressors show similar responses in both soil-grown and hydroponically grown plants, the other lines do not. In these plants, thiol levels increased up to 6-fold (*ARF1-BP*) with respect to cys levels and 7-fold for GSH. Such high accumulation is well beyond everything achieved so far through over-expression of pathway genes (Blaszczyk *et al*., 1999; Harms *et al*., 2000).

A change in the levels of reduced sulphur compounds would be expected from altering the expression of transcription factors assumed to be involved in the regulation of sulphate metabolism and the response to sulphate starvation. This expectation was further born out by the fact that some genes, although not all, of the sulphate assimilation pathway were affected, i.e. they were induced in *ARF1-BP* overexpression and *IAA28* knock-down lines, and reduced in *IAA28* and *IAA13* overexpressing lines. Simultaneously, however, several amino acids displayed changes, mainly increases in concentration, resulting in a total increase in amino acid content by a factor of three. These affected amino acids—serine, glycine, alanine, valine, leucine and mass amino acids such as aspartate, asparagine, and glutamine (though not glutamate)—are directly linked to carbon skeleton production via photosynthesis and photorespiration.

The increases in asparagine and glutamine might also be attributable to alterations in C/N balance and higher amounts of nitrogenous compounds (Hesse *et al*., 2004*a*; Nikiforova *et al*., 2005*a*, 2006). A similar result was obtained when the transcription factor DOF1 was overexpressed in *Arabidopsis* (Yanagisawa *et al*., 2004). In addition, the total increase of amino acids can be interpreted as an overall elevation of nitrogenous compounds. Imbalances in the C to N ratio have been reported to affect development (Rademacher *et al*., 2002). Although changes were not seen in glutamate levels, its derivative glutamine increased 2.5-fold in all overexpression lines. Similar accumulations at approximately the same rates were observed for aspartate, asparagine, alanine, leucine, serine, glycine, and cysteine. On the other hand, some amino acid levels were reduced.

A hallmark of sulphate starvation is the accumulation of shikimate and tryptophan and, eventually, anthocyanins (Nikiforova *et al*., 2003, 2005). This typical stress response was not observed in the overexpressing lines; only tyrosine and phenylalanine accumulate, albeit slightly. This indicates that the transcription factors analysed here are not involved in this part of the sulphur stress response. Rather they are triggered through other processes such as, for example, the depletion of GSH under sulphate deprivation, leading to misbalances in ROS detoxification and the activation of alternative ROS-scavenging systems. It appears that *IAA28*, *IAA13* and,

especially ARF1-BP, act on C/N ratio homeostasis thus affecting various amino acid biosynthetic pathways, maybe even through carbon metabolism. The effects on sulphate metabolism, though evident, are thus part of a wider response scheme.

In the context of sulphur metabolism, it is intriguing that genes involved in sulphur uptake and assimilation were down-regulated in *IAA28* and *IAA13* overexpressing lines, but up-regulated in *IAA28*-knock-down and *ARF1-BP*-expressing plants. This result is contradictory to earlier transcript profiling studies in which genes related to sulphur uptake and assimilation (except CbL) were up-regulated in sulphur-depleted plants, resulting in higher cysteine levels (Maruyama-Nakashita *et al*., 2003; Nikiforova *et al*., 2003). The decreased expression levels of sulphur pathway-relevant genes do not coincide with the elevated cysteine levels of *IAA28* and *IAA13* overexpression lines. Moreover, these results are difficult to reconcile with the observation that all three transcription factors were identified as being up-regulated during sulphate starvation.

The assumption that overexpressing or down-regulating these transcription factors would alter plant growth under sulphur-limited conditions was not confirmed. Responses of transgenic plants were similar to those of wild-type plants, leading to reduced growth rates and reduction of thiol levels under sulphur starvation. Though repeated several times, the differences between soil-grown and hydroponically grown plants remained. It is possible that hydroponic culture *per se* is stressful to plants, thus masking the nutrient stress response. However, stress indicators have not been identified in other experiments with hydroponically grown plants (Nikiforova *et al*., 2003, 2005, and references therein). None of the genetically altered plant lines fared markedly better during sulphur starvation than wild-type plants as determined by thiol measurements. However, prior to sulphur deprivation, *ARF1-BP* overexpressing plants accumulate high amounts of thiols. During deprivation, these thiol pools remained longer than in wild-type plants. However, since the plant phenotype is drastically affected, the advantage of thiol accumulation might not manifest itself through improved growth parameters. The interesting question here is whether the disadvantageous growth phenotype and the advantageous metabolic phenotype might be separable, for example, through identification of suppressor mutants.

Based on the comprehensive transcript and metabolite profiling of plants grown under normal and sulphur-depleted conditions (Nikiforova *et al*., 2005*a*), several potential regulators of sulphate metabolism were targeted for this study. Looking at plant metabolism during sulphur stress adds layers of complexity beyond those associated with the sulphate-assimilation pathway alone. The layers are, of course, interrelated, and part of a larger network

that has yet to be resolved. Based on the results reported here, it is suggested that IAA28, IAA13, and ARF1-BP transcription factors are involved in mediating plant responses to sulphate starvation. However, it appears that the transcription factors do not act on sulphate metabolism *per se* but rather work to establish a new balance of metabolites under these growth conditions. It is speculated that this response is triggered through the depletion of the downstream sulphur metabolite, *S*-adenosylmethionine (SAM), an important intermediate in metabolism and a regulatory molecule itself (Hesse and Hoefgen, 2003, Hesse *et al.*, 2004*b*; Onouchi *et al.*, 2005).

Under SAM depletion, ethylene supply would drop, triggering a cascade via HLS1 and ARF2 affecting further downstream regulators such as IAA13 and IAA28 and, hence, shown here for the first time, metabolic changes induced by the Aux/IAA regulators. Although IAA28 would be involved in such a response, it is unlikely to be a key regulator of sulphate starvation as had previously been hypothesized since its effects were weak and inconsistent. With respect to the architecture of putative sulphate starvation response networks it is noteworthy that general stress responses such as flavonoid biosynthesis are likely not to be part of this regulatory network.

The Aux/IAA and ARF transcriptional regulators identified via the sulphate starvation screen are likely to trigger a general response mechanism, rather than to elicit a direct response via the regulation of sulphate metabolism. However, redundant effects of transcription factors on multiple targets cannot be excluded entirely. It is speculated that the effects on sulphur metabolism result from alterations in the plant's carbon/nitrogen balance leading to changes in the supply of the necessary carbon backbones for sulphur assimilation. This increase in supply would in turn trigger changes in sulphur-pathway gene expression. Additionally, depletion of central metabolites such as SAM, and hence ethylene, might be involved in triggering the Aux/IAA response, coordinating sulphate metabolism in the context of overall plant metabolism.

Supplementary data

Figure S1 Effect of *IAA28*, *IAA13*, and *ARF1-BP* expression, respectively, or down-regulation of *IAA28* on pathway-relevant gene expression in untreated soil grown control samples

Figure S2 Effects of *IAA28* over-expression and reduced expression, *IAA13* over-expression, and *ARF1-BP* over-expression on metabolite contents in Arabidopsis plants grown under plus/minus sulfur conditions

Figure S3 Heat map visualisation and cluster tree representations of amino acid contents of control and sulfate starved plants and genotypes.

Table S1 Amino acid concentration of *Arabidopsis* plants grown in soil.

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