



## Global expression analysis of *CESA* and *CSL* genes in *Arabidopsis*

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### Abstract

We have used Affymetrix gene chips to measure the expression of 10 *CESA* and 29 *CSL* genes of *Arabidopsis* in different developmental stages or organs. These measurements reveal that many of the genes exhibit different levels of expression in the various organs. While several *CESA* genes are highly expressed in all the tissues examined, very few *CSL* genes approach such high levels of expression. This suggests that the *CSL* genes either encode enzymes for the synthesis of minor components of cell walls or are expressed only in specific cell types. The expression data also highlights the potential importance of the *CESA* genes for primary and secondary cell wall formation during different developmental stages and in the different organs examined.

**Abbreviations:** FTIR – Fourier transform infrared spectroscopy; *CESA* – cellulose synthase; *cev 1* – constitutive expression of *VSP1*; *COL* – *Arabidopsis thaliana* ecotype *Columbia*; *CSL* – cellulose synthase like; *eli* – ectopic lignin; *kjk* – *kojak*; *prc* – *procuste 1*; *NaGsl1* – *Nicotiana glauca* glucan-synthase-like 1; *rat* – resistance to *Agrobacterium tumefaciens*; RPM – rounds per minute; *rsw1* – radially swollen 1; TAIR – the *Arabidopsis* information resource; *WS* – *Arabidopsis thaliana* ecotype *Wassilevskaja*.

### Introduction

The *Arabidopsis thaliana* genome contains 10 cellulose synthase a (*CESA*) genes that encode components of cellulose synthase and 30 cellulose synthase-like (*CSL*) genes that encode proteins of unknown function (Richmond and Somerville 2000, 2001). The sequence similarity of the *CSL* genes to the *CESA* genes originally suggested that they also encode processive glycosyl transferases (Cutler and Somerville 1997). Recently, Dhugga et al. (2004) showed that a *CSL* gene from guar encodes a  $\beta$ -1,4-mannan synthase. Thus, it seems likely that other *CSL* genes catalyze synthesis of various cell wall polysaccharides.

One of the strategies for investigating the function of the *CSL* genes has been to examine the effects of mutations in these genes (Favery et al. 2001; Goubet et al. 2003; Zhu et al. 2003a, b). Unfortunately, the characterizations have not yet provided an understanding of the protein function at the biochemical level. Although the phenotypes of these mutants are suggestive of changes in cell wall composition, no analysis of the cell wall composition of the mutants has been reported. FTIR analysis of cell walls indicates that the walls of some of the *CSL* mutants have an altered composition, but it is not yet possible to identify the corresponding change in polysaccharide composition (T. Raab, unpublished results). For all

but two *CSL* genes, TDNA insertion mutations are available in *Arabidopsis* (<http://www.signal.salk.edu/cgi-bin/tdnaexpress>). A prerequisite for the analysis of these mutants is to know when and where the *CSL* genes are expressed. It is also useful to quantitate the steady-state level of mRNA as an indication of the relative abundance of the corresponding enzyme. Because of the large number and relatively high degree of sequence similarity of the *CESA* and *CSL* genes, Northern blot analysis and RT PCR are not the methods of choice for measuring the steady state amount of mRNA for the various genes. In contrast, the oligonucleotide-based hybridization method employed in the Affymetrix gene chips allows to simultaneously measure the amount of mRNA of the genes without concern for cross-hybridization. The wide dynamic range of the DNA chips facilitates quantitative comparisons of mRNA amounts between and within samples. This comparison is facilitated by normalizing the signals from different chips so that the average signal intensity for each gene is arbitrarily set at 500 units. Although variation in the amount of mRNA in different tissue types might create a small bias due to differences in global transcriptional activity, this normalization method results in relatively similar numbers from one tissue to another for ubiquitously expressed genes such as cytoplasmic glyceraldehyde-3-phosphate dehydrogenase (Astrand 2003). We have exploited the availability of the Affymetrix DNA chip technology to measure the expression of all *CESA* and *CSL* genes in a variety of *Arabidopsis* organs and developmental stages.

## Results and discussion

The gene identifiers, available mutants and the isolated ESTs for the *CESA/CSL* genes of *Arabidopsis* are presented in Table 1. The sequences and the most recent gene models are available online in the *Arabidopsis* database TAIR (<http://Arabidopsis.org>). The *CSLB3* gene is not represented on the Affymetrix ATH-1 DNA chips. Thus, it was not possible to analyze its expression pattern in this context.

A key parameter in any measurement of gene expression concerns the degree to which variation arises from growth conditions or temporal factors. In an effort to minimize diurnal effects in the

greenhouse plant material, tissues for our experiments were harvested in the middle of the photoperiod. To evaluate the effect of growth conditions, we compared expression (in arbitrary units) of the *CESA* and *CSL* genes in leaves of plants grown for 15 days under natural light (Figure 1a) in greenhouses versus plants grown for 15 days under fluorescent illumination (Figure 1b) in growth chambers. The results of this comparison suggested that for most *CESA/CSL* genes, the growth conditions had apparently little effect on the steady state level of mRNA. The effect of leaf age was also examined by comparing mRNA levels in leaves of plants grown for 15 or 50 days (Figures 1a–c). At 15 days the leaves were still expanding, whereas at 50 days the leaves were fully expanded and beginning to senesce. As might be expected, the older leaves had strongly reduced levels of mRNA for many genes, possibly reflecting the reduced amount of biosynthetic activity in older leaves. However, in some cases, the expression of the genes was substantially higher in older leaves. The two most striking examples are *CSLD2* and *CSLE1*, which apparently exhibit strong increases in expression in old leaves versus young leaves. Relatively few studies of cell wall changes during vegetative tissue development have been reported. The only recent study of such changes was done on the composition of bean pods from *Phaseolus vulgaris* during development (Stolle-Smits et al. 1999). The most significant change observed was a steady increase in soluble homogalacturonan throughout development. Similar experiments detailing developmentally regulated changes in *Arabidopsis* wall composition have not been reported. However, if *Arabidopsis* walls exhibit similar changes to those observed in beans, a possible implication is that the changes seen in *CSLD2* and *CSLE1* expression may reflect a role in homogalacturonan synthesis.

A summary of the steady-state mRNA levels of the *CESA* and *CSL* genes in four different organs/developmental stages is presented in Figure 2. The data for each sample presented derive from between 3 and 8 replicates. For convenience, we refer to the amount of mRNA as the level of expression (in arbitrary units). Levels of expression below about 150 units are considered unreliable and could indicate very low expression or the absence of expression.

Table 1. Summary of expression data<sup>a</sup> for the *CESA* and *CSL* genes of *Arabidopsis*.

Gene	Locus <sup>a</sup>	Mutants <sup>b</sup>	Response <sup>c</sup>	ESTs <sup>a</sup>
CESA1	At4g32410	<i>rsw1</i>	Ethylene(↑), salt (↓), auxin (↑), circadian (↓,↑)	90
CESA2	At4g39350	<i>Ath-A</i>	Ethylene(↓), cytokinin (↑)	9
CESA3	At5g05170	<i>eli1</i> , <i>cev1</i> , <i>Ath-B</i> , <i>ixr1</i>	Ethylene(↑)	49
CESA4	At5g44030	<i>ixr5</i>	Ethylene(↓), jasmonate (↑), cytokinin (↑), circadian (↓,↑)	10
CESA5	At5g09870		Light (↓)	10
CESA6	At5g64740	<i>prc1</i> , <i>ixr2</i>	Ethylene (↓), salt (↑)	36
CESA7	At5g17420	<i>ixr3</i>	Vascular development (↑), circadian (↓,↑)	14
CESA8	At4g18780	<i>ixr1</i>		12
CESA9	At2g21770			1
CESA10	At2g25540			8
CSLA1	At4g16590		Anthers (↑), circadian (↓,↑)	9
CSLA2	At5g22740			15
CSLA3	At1g23480		Light (↓)	7
CSLA7	At2g35650	SGT4425		4
CSLA9	At5g03760	<i>rat4</i>	Ethylene (↓)	9
CSLA10	At1g24070			2
CSLA11	At5g16190			3
CSLA14	At3g56000			2
CSLA15	At4g13410			0
CSLB1	At2g32530		Ethylene (↓)	5
CSLB2	At2g32540		Ethylene (↓)	0
CSLB3	At2g32610			0
CSLB4	At2g32620			0
CSLB5	At4g15290			2
CSLB6	At4g15320		Ethylene (↓)	0
CSLC4	At3g28180		Cordecypin (↓), sucrose (↑), salt (↓), ethylene (↑)	20
CSLC5	At4g31590		Cordecypin (↓)	14
CSLC6	At3g07330		Light (↓)	24
CSLC8	At2g24630			4
CSLC12	At4g07960			3
CSLD1	At2g33100			1
CSLD2	At5g16910		Salt (↓)	12
CSLD3	At3g03050	<i>kjk</i>	Iron deficiency (↓), light (↓), sucrose (↑), cytokinin (↓), salt (↓)	17
CSLD4	At4g38190			2
CSLD5	At1g02730			8
CSLD6	At1g32180			0
CSLE1	At1g55850			7
CSLG1	At4g23990			3
CSLG2	At4g24000			4
CSLG3	At4g24010		Salt (↓)	3

<sup>a</sup>Assigned by TAIR (<http://www.arabidopsis.org>) as of July 1, 2003.

<sup>b</sup>*rsw*, radially swollen (Arioli et al. 1998); *ixr*, isoxaben resistant (Scheible et al. 2001); *eli*, ectopic lignin (Cano-Delgado et al. 2000); *cev*, constitutive expression of VSP1 (Ellis et al. 2002); *ixr*, irregular xylem (Turner and Somerville 1997); *prc*, procuste (Fagard et al. 2000); STG4425 (Goubet et al. 2003); *rat*, resistance to *Agrobacterium tumefaciens*. (Zhu et al. 2003a, b); *kjk* refers to *kojak*, a root-hairless mutant (Favery et al. 2001).

<sup>c</sup>Compiled from DNA microarray information available in TAIR as of July 1, 2003, or as previously described (Richmond and Somerville 2001). 'Light' refers to a series of experiments in which 4-day-old dark grown seedlings were exposed to white light for various times up to 120 min. '↑' indicates an increase, '↓' indicates a decrease in expression relative to controls treatments.

A simple test for the significance of the data is to see if the expression data presented here can be correlated with genetic or phenotypic data. The *CESA4* (*ixr5*), *CESA7* (*ixr3*), and *CESA8* (*ixr1*) genes are strongly expressed in stems and rather weakly expressed in other tissues. This is compat-

ible with genetic evidence indicating that these genes encode components of cellulose synthase complexes that mediate secondary cell wall synthesis in xylem cells (Turner and Somerville 1997; Taylor et al. 2003; Zhong et al. 2003). The high level of expression of the *CESA1* (*rsw1*), *CESA3*

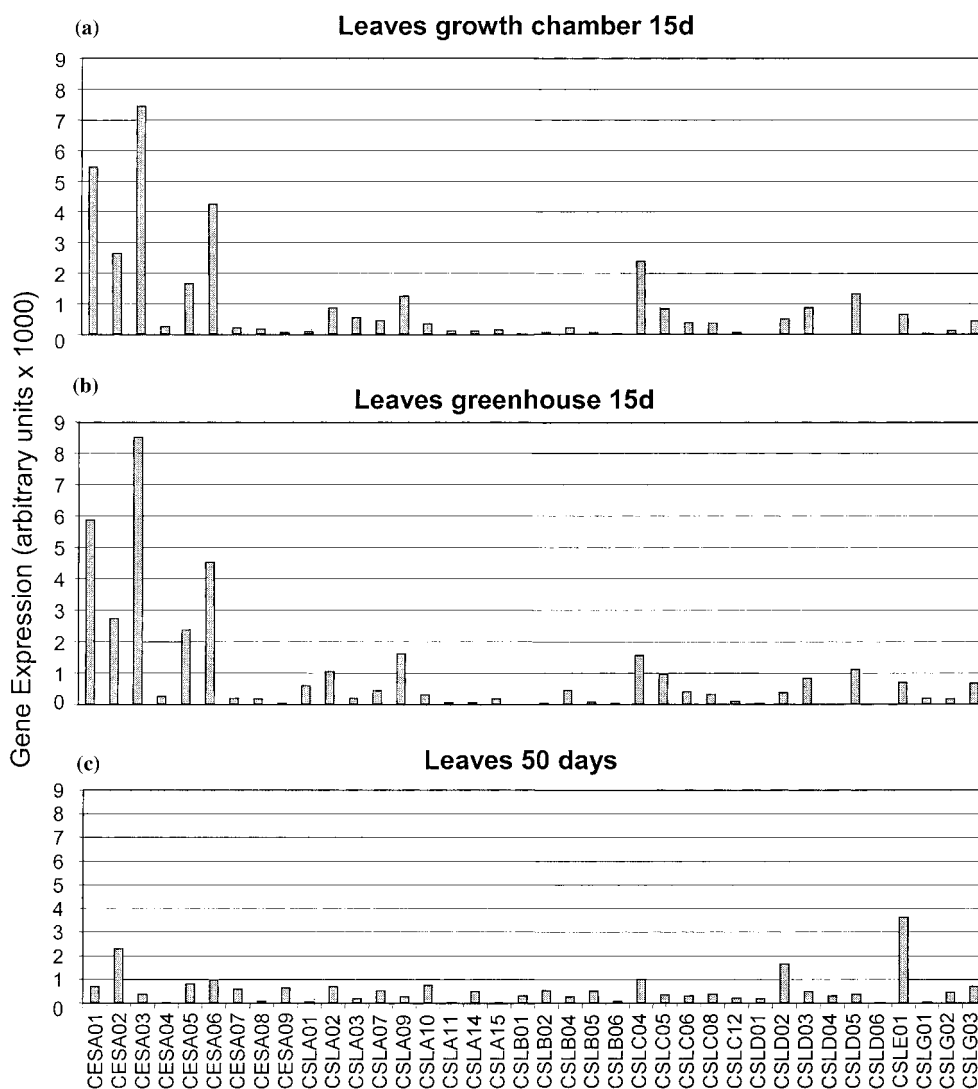


Figure 1. Steady-state mRNA levels in leaf tissues from plants grown for 15 or 50 days under various regimes. Each figure is based on a number of independent replicates: (a) leaves growth chamber 15 days (1); (b) leaves greenhouse 15 days (2); (c) leaves 50 days (1).

(*ixr1*) and *CESA6* (*prc*, *ixr2*) genes in most tissues is also compatible with the observations from genetic studies that these genes are involved in primary wall synthesis throughout the plants (also see below) (Arioli et al. 1998; Fagard et al. 2001; Scheible et al. 2001). A *CESA10::GUS* promoter fusion indicates that *CESA10* is expressed at the base of rosette leaves in *Arabidopsis*, which correlates with the very weak expression of the gene on the gene chips (Doblin et al. 2002). A mutation in *CSLD3* caused a defect in root hair cell wall formation (Favery et al. 2001). In accordance with the mutant phenotype, the expression analysis

showed the strongest expression in roots (Figure 2a). Knock out of the *CSLA9* gene with a TDNA insertion leads to resistance against *Agrobacterium tumefaciens* mediated transformation of roots and decreases the number of lateral roots formed in the mutant background (Zhu et al. 2003a, b). Our microarray results show that the *CSLA9* gene is expressed in roots. Two microarray based expression studies have shown *CSLD1* and *D4* to be expressed in hydrated pollen. In accordance with this, the highest expression for these genes in our expression studies is observed in flowers (Becker et al. 2003; Honys et al. 2003).

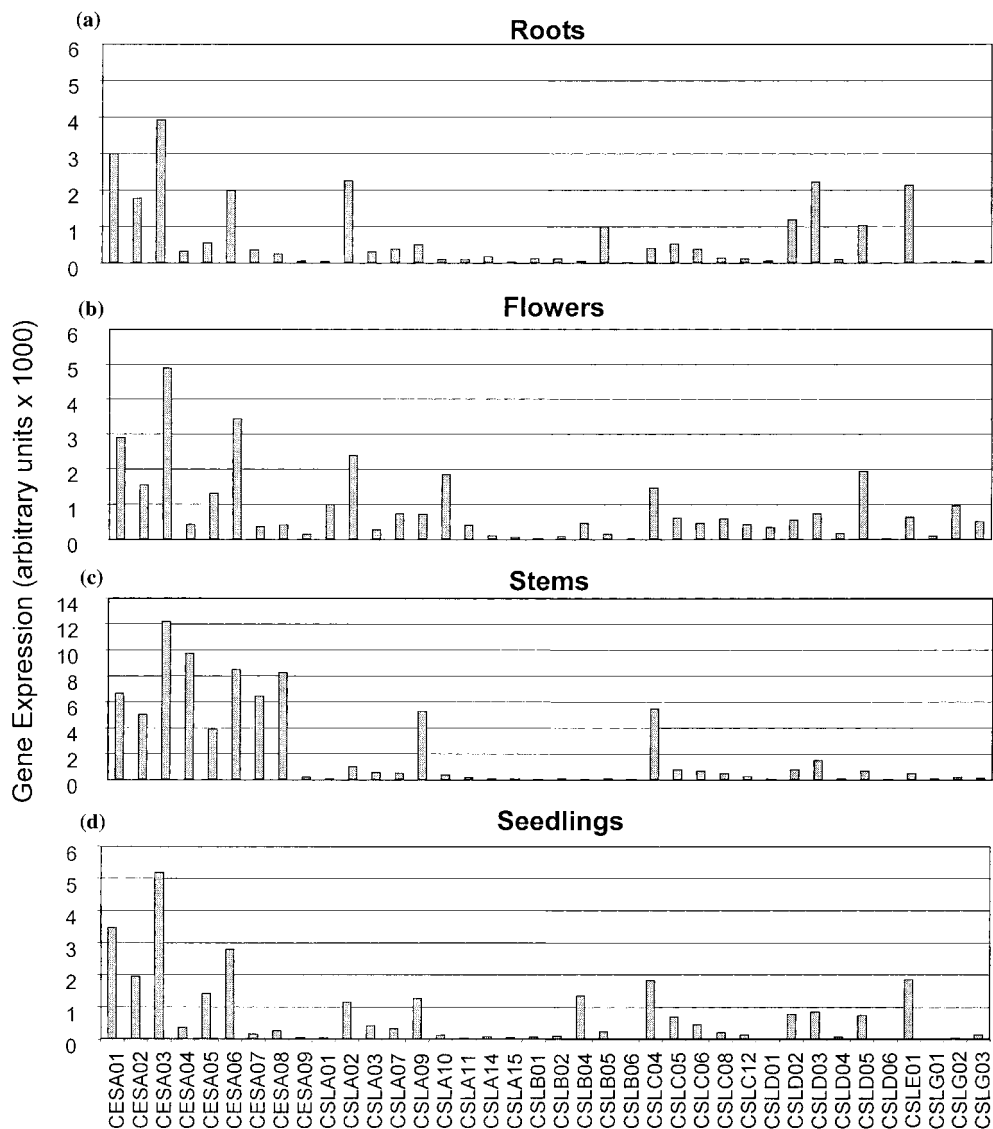


Figure 2. DNA chip measurements of RNA from various tissues of Arabidopsis. Each figure is based on a number of independent replicates: (a) roots (3); (b) flowers (4); (c) stems (4); (d) seedlings (8).

These observations confirm the *a priori* expectation that expression data can be correlated with mutant phenotypes and, thus, could provide starting points for analysis of mutants in *CSL* genes.

As can be seen in Figure 2a, several *CSL* genes (*CSLB5*, *CSLD2*, *CSLD3*) exhibited their strongest expression in roots. Intriguingly, *CSLA2* and *CSLD5* showed strong expression both in roots

and flowers, hinting at a potential role in dividing tissues (Figure 2a, b). *CSLE1*, on the other hand, exhibited a similar expression level in roots and seedlings, which could be explained by the fact that whole seedlings (including the primary root) were used to prepare the hybridization samples (Figure 2a-d). In this context it is of interest to note that *CSLB3* is apparently expressed in roots based on SAGE expression analysis (Fizames et al.

2004). Since *Arabidopsis* roots appear to have a similar level of compositional complexity to leaves (Peng et al. 2000), it is intriguing that *CSL* expression is disproportionately high in that tissue relative to leaf tissue (compare Figures 1a, b and 2a).

*CSLA10* and *CSLG2* exhibited elevated expression levels in flowers, hinting at the possibility that they might have a specific function during flowering similar to *NaCSLD1* and *NaGsl1* from tobacco, which are specifically expressed in pollen (Figure 2b) (Doblin et al. 2001). Interestingly, flowers also exhibit the highest absolute number of *CESA/CSL* genes expressed with a particular emphasis on the *CSLA* and *CSLC* gene families. A possible reason could be that the sample contained flowers of different developmental stages and the shoot meristematic region.

The results for the stem samples showed a strong bias in expression strength towards the *CESA* gene family with the only noteworthy exceptions being the *CSLA9* and *CSLC4* genes (Figure 2c). By analogy with the specific upregulation of the *CESA4*, 7, 8 genes in stems, this observation suggests that these *CSL* genes may also be involved in some aspect of secondary wall formation. Since *Arabidopsis* stems contain relatively large amounts of the xylose containing cell wall polymers xylan and xyloglucan, *CSLA9* and *CSLC4* genes are potential candidates for xylan or xyloglucan synthases (Richmond and Somerville 2001).

Looking at the expression data for seedlings, only *CSLB4* was specifically upregulated at this developmental stage compared to the other samples in this survey. Intriguingly, it was not expressed in the roots from 10 days old seedlings but in flowers, thus suggesting a developmentally regulated expression (see above) or expression in the shoot meristem.

In addition to the experiments reported here, some information concerning expression of the *CESA* and *CSL* genes is available from a large plant microarray project in which more than 10,000 cDNA clones were spotted on glass slides and used for 591 different gene expression profiling experiments (Wu et al. 2001; Finkelstein et al. 2002). Approximately half of the *CESA* and *CSL* genes were present on these arrays. The data obtained is available through the Stanford Microarray Database ([\[stanford.edu/MicroArray/SMD/\]\(http://genome-www5.stanford.edu/MicroArray/SMD/\)\). Some of the data is available in a more readily accessible but less powerful format in TAIR \(<http://arabidopsis.org>\). We examined the subset of the data that is available at TAIR and have annotated the genes for which relatively strong effects were observed in Table 1. A number of the genes appeared to be strongly regulated by circadian rhythm. Some of the genes were also regulated in response to the transition from dark to light, or by ethylene, cytokinin and salt stress. These preliminary observations suggest that detailed studies of the response of the genes to various treatments may be informative. Transcript abundance of several \*CSL\* genes was strongly decreased by cordecypin \(RNA synthesis inhibitor\) treatment, suggesting that the transcripts are rapidly turning over \(Gutiérrez et al. 2002\).](http://genome-www5.</a></p>
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In addition to simply providing an indication of which treatments alter the steady state level of mRNA for the various genes, the DNA microarray experiments have been analyzed by the clustering algorithm described by Eisen et al. (1998) to determine which genes have a pattern of expression that is most similar. These clusters are available at TAIR. An intriguing result from this analysis was that the *CESA3*, *CESA1* and *CESA6* genes cluster tightly. In addition, a putative endoglucanase (*At1g75680*) was co-regulated with this cluster. The endoglucanase may be a candidate for a similar function as the  $\beta$ -1,4-endoglucanase encoded by the *korrigan* gene (Mølthøj et al. 2002). The apparent clustering of three *CESA* genes is compatible with genetic evidence that they are co-expressed and required for formation of a functional cellulose synthase complex (Taylor et al. 2003). It is of interest to note that *CESA1*, *CESA2* and *CESA3* exhibited a conserved expression pattern in our experiments (Figures 1a, b and 2a–d). *CESA2* shows the weakest and *CESA1* an intermediate expression, while *CESA3* always shows the strongest expression of the three genes. This could be taken as an indication that certain ratios between the gene products have to be maintained.

The general pattern of expression of the *Arabidopsis CESA* genes does not appear to be highly correlated with a similar analysis of *CESA* gene expression in various tissues of maize (Dhugga 2001). However, because the maize genome sequence is not complete, it is currently unclear

which of the maize *CESA* genes is orthologous to each of the *Arabidopsis* genes.

In conclusion, the use of DNA microarrays and DNA chips provides useful insights into the relative level of expression of the *CESA* and *CSL* genes, and suggests various types of further experimental approaches that may provide insights into the biological function of the genes.

## Experimental protocol

### *Plant material*

The *Col-0* plants used as source for plant material were grown at a density of 4 plants per 5 in.<sup>2</sup> pot. One set of plants was grown in growth chambers at 25 °C during daytime (20 °C during night time) and a light period of 16 h with 125  $\mu\text{mol m}^{-2} \text{s}^{-1}$  fluorescent irradiation. A second set of plants was grown under natural light in greenhouses with a similar temperature regime. Expanding leaves were harvested 15 days post germination in the middle of the photoperiod. Stem and flower material was obtained from plants 29 days post germination when the inflorescence was 6 in. high. Siliques and pedicels were removed from the lower 5 in. of the stem and the remainder was used for RNA preparation. Flower samples consisted of developed flowers and unopened buds. Seedlings were grown in liquid culture for 6 days shaking (220 rpm) in a growth chamber at 23 °C under constant light. The culture medium contained 0.2% Murashige and Skoog salts and 1% sucrose (pH 5.8). Roots were harvested from *Ws* seedlings grown for 10 days under 14 h of light at 18/22 °C (125  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) on the surface of vertically oriented 1% agar medium with 0.04% Murashige and Skoog salts and 0.5% sucrose.

### *RNA isolation, preparation of hybridisation samples and data analysis*

RNA was isolated from leaves, stems and flowers using the Trizol method (Ramonell et al. 2002) for polysaccharide-rich samples. The hybridization samples were prepared according to instructions provided by Affymetrix using 20  $\mu\text{g}$  total RNA for cDNA synthesis and 20  $\mu\text{g}$  cRNA for hybridization (www.affymetrix.com). Seedling RNA was

isolated using the RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. Root RNA was isolated using the RNaid plus kit (Bio 101). cDNA synthesis was performed with an Invitrogen kit using 20  $\mu\text{g}$  of total RNA as starting material per sample. For the phenol/chloroform cleanup Phase lock tubes (Eppendorf) were used. The inverse transcription reaction was performed according to the Affymetrix Enzo protocol and cleanup was performed with the RNeasy mini kit (Qiagen). The hybridization samples contained 20  $\mu\text{g}$  of cRNA which had been fragmented for 20 min at 94 °C using the standard fragmentation buffer (Affymetrix). The samples were hybridized to Affymetrix ATH-1 chips for at least 16 h according to manufacturer's instructions. Washing, staining and scanning were performed as described by Affymetrix. The target value for analysis of the raw data with the Affymetrix microarray suite was set to 500. If data from more than three hybridizations per organ/developmental stage was available, medians were calculated for each gene using Microsoft Excel. Otherwise the average was determined and used to create the figures.

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