Transcription Analysis of Arabidopsis Membrane Transporters and Hormone Pathways during Developmental and Induced Leaf Senescence[1][W]

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A comparative transcriptome analysis for successive stages of Arabidopsis (*Arabidopsis thaliana*) developmental leaf senescence (NS), darkening-induced senescence of individual leaves attached to the plant (DIS), and senescence in dark-incubated detached leaves (DET) revealed many novel senescence-associated genes with distinct expression profiles. The three senescence processes share a high number of regulated genes, although the overall number of regulated genes during DIS and DET is about 2 times lower than during NS. Consequently, the number of NS-specific genes is much higher than the number of DIS- or DET-specific genes. The expression profiles of transporters (TPs), receptor-like kinases, autophagy genes, and hormone pathways were analyzed in detail. The Arabidopsis TPs and other integral membrane proteins were systematically reclassified based on the Transporter Classification system. Coordinate activation or inactivation of several genes is observed in some TP families in all three or only in individual senescence types, indicating differences in the genetic programs for remobilization of catabolites. Characteristic senescence type-specific differences were also apparent in the expression profiles of (putative) signaling kinases. For eight hormones, the expression of biosynthesis, metabolism, signaling, and (partially) response genes was investigated. In most pathways, novel senescence-associated genes were identified. The expression profiles of hormone homeostasis and signaling genes reveal additional players in the senescence regulatory network.

During its life span, a rosette leaf progresses through distinct developmental stages. Shortly after its initiation as a leaf primordium, the young leaf is a typical sink organ. Subsequently, the leaf undergoes a phase of rapid expansion, during which transition from sink to source organ takes place. After reaching its final (mature) size and a period as source organ, the rosette leaf enters the program of leaf senescence. During this process nutrients are recycled and transported to sink tissues, which in Arabidopsis (*Arabidopsis thaliana*) are developing flowers and seeds (Himelblau and Amasino, 2001).

Leaf senescence is a postmitotic senescence program (for review, see Gan, 2003) that occurs in aging, but also in stressed or detached leaves, and proceeds in a strictly ordered manner. Leaf senescence begins with the transition from anabolism of carbohydrates and other biomolecules to catabolism of proteins, nucleic acids, and lipids, and culminates in cell death. The onset and progression of leaf senescence is accompanied by changes in expression of a large number of genes, and activation of new genes in leaves is required for the onset of senescence (for review, see Noodén et al., 1997; Buchanan-Wollaston et al., 2003; Gan, 2003). Using various techniques, hundreds of senescence-associated genes (SAGs) were identified in Arabidopsis (e.g. He et al., 2001; Hinderhofer and Zentgraf, 2001; Chen et al., 2002; Buchanan-Wollaston et al., 2003, 2005).

Many, but not all, SAGs respond to induced senescence in a broadly similar fashion as upon natural leaf senescence or aging. However, different treatments induce different but overlapping sets of SAGs (Becker and Apel, 1993; Weaver et al., 1998). Incubation of detached leaves in darkness is highly effective in inducing SAGs, leaf yellowing, and chlorophyll loss (Weaver and Amasino, 2001), and it was suggested that the respective SAGs might be a superset of those induced by aging (Weaver et al., 1998). The evaluation of the overlap between artificially and aging-induced SAGs therefore gives an idea which SAGs are senescence specific or possibly more generally induced by stress conditions.

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In this study, the genome-wide changes in gene expression during leaf senescence were investigated employing the Agilent Arabidopsis version 2 chips with more than 80% genome coverage. To recognize changes in expression that are specific for developmental leaf senescence (NS) or aging, the respective expression profiles were compared to that of individually shaded leaves still attached to the plant (DIS) and detached dark-incubated leaves (DET). The emphasis of this study was to identify (putative) transporters (TPs), signaling kinases, and hormone pathway genes that are regulated during senescence. Many novel SAGs in various protein categories were identified. We discuss possible functions for several of these proteins during senescence and the involvement of hormone pathways in developmental and induced senescence.

RESULTS AND DISCUSSION

Experimental Setup

To study the progression of NS, leaves were harvested at six developmental stages from a sink leaf stage (4-week-old plants) to a late senescent stage (approximately 75% yellowing of the leaf surface; ≥80% chlorophyll loss; Table I). From each senescence stage, RNA was prepared, labeled, and hybridized to Agilent Arabidopsis 2 microarrays with 21,500 probes that represent more than 80% of Arabidopsis genes. In a recent study, it was reported that the sensitivity of these microarrays is comparable with Affymetrix ATH1 and CATMA arrays (Allemeersch et al., 2005). NS expression profiles were compared with profiles induced by DIS and by DET. These treatments were established to induce senescence (Becker and Apel, 1993; Oh et al., 1996; Park et al., 1998; Weaver and Amasino, 2001). As the expression profiles in senescing leaves are influenced both by the age (position) of the rosette leaf and plant age (Zentgraf et al., 2004), the use of whole plants as a source for RNA extraction could cause dilution, distortion, or blurring of senescence-associated expression profiles. Therefore, in this study, only the fifth and sixth rosette leaves were used.

In all samples, chlorophyll *a* and *b* contents were determined (Supplemental Fig. 1B), allowing correlation of the senescence stages of the NS, DET, and DIS experiments with each other and with experiments reported by others (Weaver et al., 1998; Weaver and Amasino, 2001). In DIS, similar chlorophyll degradation levels as during NS in the leaves were observed between 2 and 9 d after shading. After detaching of leaves, chlorophyll degradation occurs more rapidly, reaching comparably low levels already after approximately 6 d. The time scales in Figures 4 to 7 and Supplemental Figures 2 to 10 were superimposed based on approximately equal chlorophyll contents.

Expression Profiles during Leaf Senescence

In this study, structural, functional, and classification attributes were assigned to approximately 10,000 Arabidopsis genes (Supplemental Table I) and linked to the 21,500 genes represented on the microarrays. In Table II, the complete list of all figures and tables used for the discussion of individual protein families is given. The expression data for all 21,500 genes are summarized in Supplemental Table II. Supplemental Table III summarizes the data for all genes significantly regulated in both biological replicates (see "Materials and Methods"), including the classification tags.

The regulated genes were sorted into seven clusters by *k*-means clustering (Supplemental Fig. 1A; Supplemental Table III). Most of the 3,513 genes regulated during the NS experiment exhibit a gradual up-regulation (38%; cluster 1) or down-regulation (33%; cluster 7). Other genes are transiently up-regulated (17%; clusters 2–4) or down-regulated (11%; clusters 5 and 6). In the DIS and DET experiments, only 1,833 and 2,158 regulated genes were observed, and of these smaller fractions than during NS exhibit a gradual regulation (clusters 1 and 7). This difference presumably reflects that, during the 3 to 4 weeks of NS progression, developmentally programmed physiological processes are activated that remain inactive during the only 6- to 9-d DET and DIS senescence reactions. On the other hand, during DET and DIS, after only 2 d more genes show an intense response than at the 5-week-old NS stage, which is most obvious for the genes in DIS and DET clusters 3 to 5. The determination of expression levels at multiple time points allowed the recognition of SAGs with early and transient

Table I. *Developmental stages of fifth and sixth rosette leaves harvested for natural leaf senescence (NS)*

DAS, Days after sowing; w, week; y, percentage of yellow surface.

Stage	DAS	Description	Principle Growth[a]	Stage[a]
4w	28	Rapid expansion, sink leaf	Rosette growth	3.50–3.70
5w	35	Fully expanded, sink-to-source transition	Flower production	6.00–6.10
6w	42	Fully expanded, source leaf	Flower production	6.10–6.30
25y	48–50	Early senescence, approximately 25% yellow surface	Flower production	6.30–6.50
50y	50–53	Mid-senescence, approximately 50% yellow surface	Flower production	6.50–6.90
75y	52–56	Late senescence, approximately 75% yellow surface	Silique ripening	6.90–8.00

[a]Developmental stages according to Boyes et al. (2001).

Table II. *List of figures and tables*
Supp., Supplemental.

Name	Description
Table I	Description of natural senescence leaf material
Table II	This table
Table III	Expression profiles of SAGs
Table IV	Plant specificity of senescence-regulated genes
Figure 1	A, Sets of genes regulated during NS, DIS, and DET; B, MIPS functional categories
Figure 2	TP families with biased frequencies of up- or down-regulated genes
Figure 3	RLK families with biased frequencies of up- or down-regulated genes
Figure 4	Expression profiles of selected *ATG* genes
Figure 5	Expression profiles of selected CK-related genes
Figure 6	Expression profiles of selected JA-related genes
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Supp. Table I	10,127 genes with structural, functional, classification, and other tags
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Supp. Table III	4,109 genes regulated during NS, DIS, or DET with tags
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Supp. Table VII	Relative expression values of regulated kinases and RLKs
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Supp. Table XVI	BR-related genes
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Supp. Figure 1	*k*-means clustering of genes regulated during senescence
Supp. Figure 2	Expression profiles of senescence-associated *ATG* genes
Supp. Figure 3	Expression profiles of senescence-associated JA-related genes
Supp. Figure 4	Expression profiles of senescence-associated SA-related genes
Supp. Figure 5	Expression profiles of senescence-associated SA-responsive genes
Supp. Figure 6	Expression profiles of senescence-associated ET-related genes
Supp. Figure 7	Expression profiles of senescence-associated JA- and ET-responsive genes
Supp. Figure 8	Expression profiles of senescence-associated auxin-related genes
Supp. Figure 9	Expression profiles of senescence-associated auxin-signaling genes
Supp. Figure 10	Expression profiles of senescence-associated ABA-related genes

expression peaks, or slow and late up-regulation, that could be overlooked in single senescence stage experiments.

Developmental and Induced Senescence Share Many Commonly Regulated Genes

The expression profiles of 22 reported SAGs and of a chlorophyll *a*/*b* binding protein (CAB) were analyzed (Table III). The *CAB* gene is strongly and continuously down-regulated during NS, DIS, and DET. Applying stringent selection criteria (see "Materials and Methods"), all except two SAGs (*SAG101* and *YLS3*) are up-regulated. The 17 SAGs that show up-regulation during NS are also up-regulated in DET and/or DIS. These data indicate that our results are consistent and comparable with those of other studies. Three genes, *SEN1*, *DIN11*, and *SAG102*, are only in DET and/or DIS significantly up-regulated.

The Venn diagrams in Figure 1A illustrate the proportions of common and condition-specific regulated genes. During NS, the up-regulated genes outnumber the down-regulated genes, whereas in DIS and DET the fractions are similar or opposite. Of the 1,232 genes regulated in all three conditions, more genes are down-regulated (726 genes = 59%) than up-regulated (506 genes = 41%). This set of commonly regulated genes

Table III. *Temporal expression of SAGs*

Gene ID	Name	NS Cluster[a]	DIS Cluster[a]	DET Cluster[a]
At5g14930	SAG101	0	0	0
At5g66170	SEN1	0	2	3
At3g63210	SAG102	0	3	4
At1g62300	WRKY6	1	0	1
At2g29350	SAG13	1	1	0
At1g09500	SAG26	1	1	1
At5g11520	YLS4, AtASP3	1	1	1
At2g45570	YLS6	1	1	1
At5g13170	SAG29	1	1	1
At5g51640	YLS7	1	1	1
At1g66580	SAG24	1	1	2
At3g60130	YLS1	1	1	3
At5g45890	SAG12	1	1	3
At3g10980	SAG20	1	1	3
At5g20250	DIN10	1	1	3
At4g02380	SAG21	1	2	3
At5g51070	ERD1	1	2	3
At1g10140	SAG103	1	3	1
At4g30270	SEN4	1	3	3
At3g51430	YLS2	1	3	3
At2g44290	YLS3	3	0	0
At3g49620	DIN11	7	1	3
At1g29920	CAB	5	6	6

[a]Numbers refer to the clusters shown in Supplemental Figure 1. Clusters 1 to 4 in NS and 1 to 3 in DIS and DET represent upregulated genes, and clusters 5 to 7 in NS and 4 to 7 in DIS and DET represent down-regulated genes. "0" indicates no significant expression or regulation.

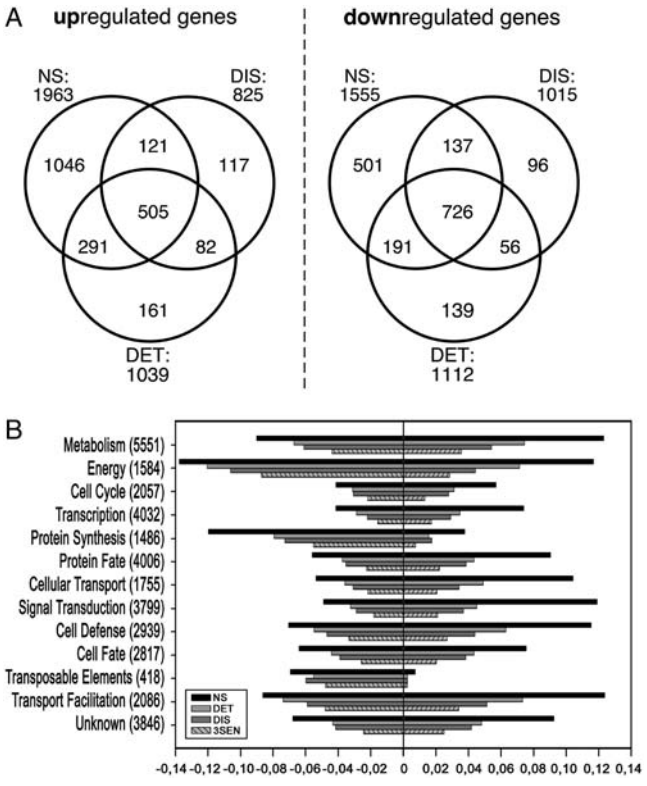

Figure 1. A, Venn diagrams showing the numbers of significantly up-regulated and down-regulated genes that are unique or commonly regulated during NS, DIS, and DET. B, Frequency of regulated genes that are assigned to a functional protein category according to the MAtDB. The number of genes on the array assigned to a category is shown in parentheses beside the category designation. Positive and negative values on the scale indicate the fraction of up- and down-regulated genes, respectively.

includes more than 50% of the genes regulated in DIS and DET, indicating that the developmental and induced senescence programs share many common pathways.

Categories of SAGs

For an overview, the frequency of regulated genes/proteins assigned to major protein functional categories in the Munich Information Center for Protein Sequences (MIPS) Arabidopsis Database (MAtDB; Schoof et al., 2004) was determined. In most categories, similar proportions of genes are regulated in DIS and DET, whereas in NS slightly more genes are up-regulated. However, in all three conditions, a bias toward down-regulation is observed for the ''transposable element,'' ''protein synthesis,'' and ''energy'' categories (Fig. 1B; Supplemental Table II). In the following sections, selected protein classes are discussed in more detail.

TPs

A key role of senescence in plant tissues is the ordered degradation of macromolecules and mobilization of the products, during which supposedly TPs are critically involved. However, until recently only few senescence-associated TPs were reported (Himelblau and Amasino, 2001; Quirino et al., 2001), and, only after the advent of large-scale expression profiling, more (putative) Arabidopsis TPs that are up-regulated during senescence were identified (Buchanan-Wollaston et al., 2003, 2005; Guo et al., 2004; Lin and Wu, 2004).

The Arabidopsis membrane proteins were systematically reclassified (see ''Materials and Methods''), resulting in 2,234 predicted Arabidopsis proteins with at least three transmembrane (TM) spans (Supplemental Table I, column F). A categorization by the Transporter Classification system (TC; Busch and Saier, 2004) returned 994 proteins assigned to 84 TP families, including 32 proteins with less than three TM spans. In addition, 69 putative TPs are listed that could not be assigned to a TC family (Supplemental Table IV).

Of the 963 putative TP genes on the array, during NS 153 are up-regulated (173 during NS or DIS or DET) and 60 are down-regulated (72 during NS or DIS or DET; Supplemental Table V). Remarkably, the proportion of up-regulated TPs is about 1.5 times higher than that of all genes in the three senescence experiments, whereas the overall proportion of down-regulated TPs is similar to that of all genes (Fig. 2).

In 27 of the 80 TC families, no significant regulation of genes is observed in any of the three senescence experiments, and 26 families are either too small to calculate a relative frequency or exhibit regulation of only a single gene in one experiment. In 21 of the remaining 27 families, a tendency toward up-regulation during senescence is apparent (Fig. 2). The only predominantly down-regulated families are the major intrinsic proteins, divalent anion:Na^+ symporters, and auxin efflux carriers. The preponderance of up-regulated TPs corresponds well with the substrates known to be transported from senescent leaves to sink organs (amino acids, inorganic phosphorus, sugars, purines, pyrimidines, and metal ions; Himelblau and Amasino, 2001; Soudry et al., 2005). The up-regulation for amino acid and oligopeptide TPs correlates with the high amount of protein degradation taking place during senescence and the subsequent need to export the breakdown products to the sink organs (Hörtensteiner and Feller, 2002). Many TPs are commonly regulated in the three types of senescence experiments, indicating that the nutrient fluxes might be largely similar (Fig. 2; Supplemental Table V). However, during NS a higher fraction of amino acid and oligopeptide TPs are up-regulated. This probably reflects the slower and developmentally controlled progression of the senescence process during aging compared to DET and DIS, presumably enabling a more efficient and thorough nutrient mobilization during NS.

Subcellular localization predictions for the TPs were retrieved from the ARAMEMNON database and are indicated in Supplemental Tables IV and V. The genes encoding TPs with predicted localization in the

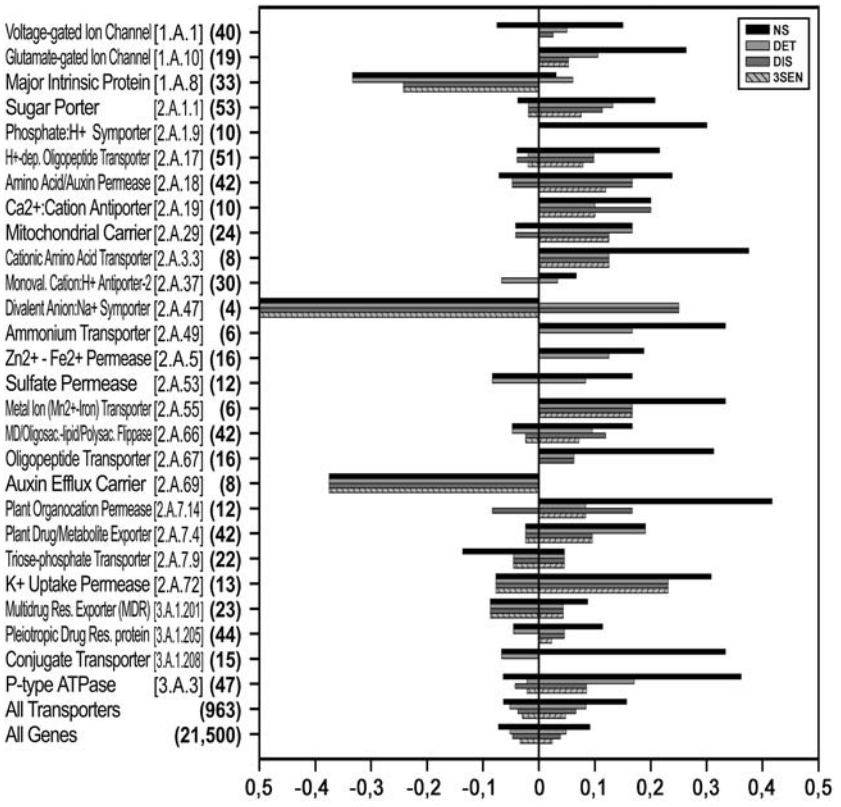

Figure 2. TP families with biased frequencies of significantly up- or down-regulated genes during senescence. Behind the TP family designation as defined in the TC system, the TC family code (in brackets) and the number of genes on the array assigned to this family (in parentheses) are indicated. Positive and negative values on the *x* axis indicate the fraction of up- and down-regulated genes, respectively.

plasma membrane (secretory pathway) exhibit a preferential up-regulation during NS and DET. Similar to the total set of regulated genes, the genes encoding (putative) plastidic localized TPs display a preferential down-regulation during all three senescence experiments. This might reflect the decline in photoassimilate export from plastids during the progression of senescence. Only 14 putative TPs with predicted plastidic localization are up-regulated during NS (Supplemental Table V). It is conceivable that these proteins are specifically involved in recycling of degradation products and energy allocation in the senescing plastids. The up-regulation of the putative aminophospholipid translocase *AtALA3* later in senescence might indicate a role in exporting thylakoid membrane degradation products.

In a recent study, 74 putative TPs up-regulated during developmental senescence were identified (Buchanan-Wollaston et al., 2005). Four of these are not represented on the microarrays used here, and five are not TM proteins according to the ARAMEMNON database. Forty-eight of the remaining genes also show up in the NS experiment (Supplemental Table V, column G). The remaining 17 genes were not rated as up-regulated in our experiment because they did not pass the significance criteria.

Kinases and Receptor-Like Kinases

In Arabidopsis, nearly 1,000 genes are estimated to encode proteins belonging to the eukaryotic protein kinase superfamily. More than 60% of these are receptor-like kinases (RLKs) whose kinase domains belong to the same family as the mammalian interleukin receptor-associated kinases and the *Drosophila melanogaster* Pelle proteins (Shiu and Bleecker, 2001).

The Agilent chip carries probes for 376 kinase and 610 *RLK* genes that fall into 62 (sub)families (Supplemental Table VI). More than 470 RLK/Pelle proteins are predicted by the ARAMEMNON database to contain a single α-helical TM domain.

Overall, 55 (9) kinase and 91 (42) *RLK* genes are up-regulated (down-regulated) during NS or DIS or DET (Supplemental Table VII). Thus, the same proportion (15%) of kinases and RLKs are up-regulated, whereas the proportion of down-regulated kinases is lower. Thirteen of the 61 kinase and RLK families are not regulated during senescence, and 32 families are either too small to calculate a relative frequency or exhibit regulation of only a single gene in one experiment. From 27 kinase and RLK families with more conspicuous patterns, 13 are shown in Figure 3. In contrast to the TP genes, the differences in expression patterns between NS, DIS, and DET are striking in some subfamilies. Some genes are up-regulated in all three senescence types; however, more frequently up-regulation is observed in NS, but not in DIS and DET. For example, 11 members of the DUF26 subfamily are rapidly up-regulated during the 5-week and 6-week NS stages, but not in DIS and DET. *AtCRK10* is even down-regulated during DIS and DET (Supplemental Table VII). *AtCRK7* and *AtCRK37* are salicylic acid (SA) responsive

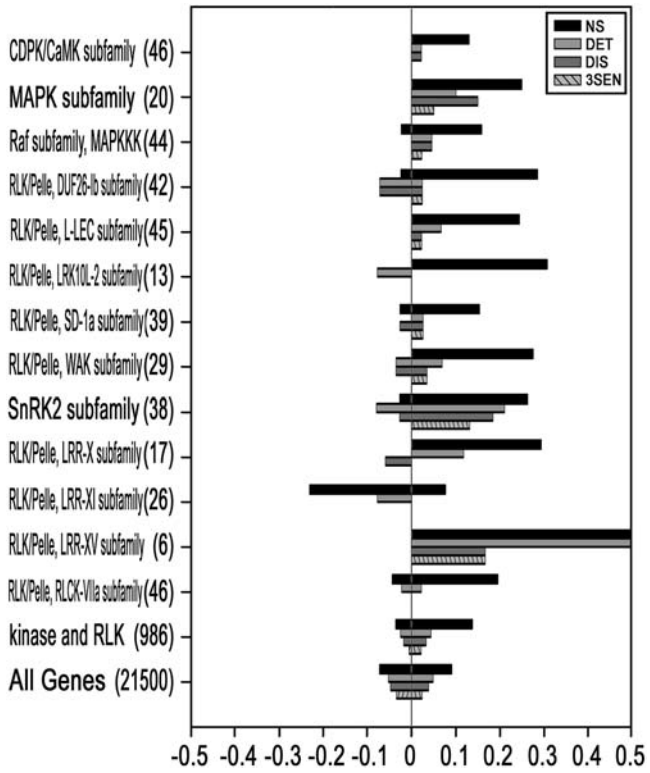

Figure 3. RLK families with biased frequencies of significantly up- or down-regulated genes during senescence. Behind the kinase family designations, the number of genes on the array assigned to this family is indicated in parentheses. Positive and negative values on the *x* axis indicate the fraction of up- and down-regulated genes, respectively.

(Supplemental Fig. 5, G and W; Buchanan-Wollaston et al., 2005), and several of these CRKs (for Ca^{2+}-dependent protein kinase-related kinases) were shown to be involved in pathogen defense responses and hypersensitive response-like cell death (Chen et al., 2003, 2004). Leu-rich repeat-Ia is another RLK subfamily with several NS up-regulated genes. Here, four of the five genes are also up-regulated during DET, but not during DIS. This group includes *AtSIRK*, which also is SA induced (Supplemental Fig. 5L; Buchanan-Wollaston et al., 2005). The senescence-associated expression of *AtSIRK* and a close relative in bean, *SARK*, was noted before (Hajouj et al., 2000; Robatzek and Somssich, 2002).

Plant-Specific Genes and Senescence

Leaf senescence is a unique plant-specific (PS) developmental program. We therefore examined a possible correlation between senescence-associated expression and PS genes. In Arabidopsis, 3,848 genes were identified that encode PS proteins and 2,436 genes with relatives in all other organisms (AO; Gutierrez et al., 2004a, 2004b). Of the 21,500 genes on the array, 2,996 are PS, whereas 2,391 have relatives in AO (Supplemental Table VIII). Relative to all genes on the array, the frequency of PS genes regulated during senescence is only slightly higher than the frequency of regulated AO genes (Table IV).

Of the 1,931 TM genes on the array, comprising TPs and other proteins with more than three TM spans (Supplemental Table I, column F), 10% are in the PS and 15% in the AO groups. The frequency of senescence-up-regulated TM genes in the AO group is approximately 3 times higher than that of the up- or down-regulated PS TM genes and the down-regulated TM genes common to all organisms, suggesting that the majority of TP functions involved in leaf senescence are common to all taxa and only few senescence-specific activities are required.

Differences become apparent in the two other protein categories. Of the 1,985 putative transcription factors (TFs) on the array, 32% are in the PS and only 1.6% in the AO groups. During developmental senescence, 133 (21%) of all PS TFs are either up- or down-regulated, whereas only four TFs in the AO group (12.5% of all AO TFs) are regulated. Even when taking into account that Arabidopsis has 2 to 3 times more TFs than *Caenorhabditis elegans* or *D. melanogaster* and only 8% to 23% of Arabidopsis TFs show similarity to TFs in non-plant eukaryotes (Arabidopsis Genome Initiative, 2000), there remains a disparity that suggests that PS TFs are key players in the regulatory pathways leading to and coordinating leaf senescence. An opposite bias is observed in the kinase and RLK categories. Apparently, there exist almost no strictly PS (receptor-like) kinases in Arabidopsis. All these proteins have relatives in other taxa, 65% of them even in AO (Table IV).

Autophagy Genes and Senescence

During senescence, different pathways contribute to degradation of proteins and other macromolecules, one of them being autophagy (ATG). In Arabidopsis, some *ATG* genes are encoded by small gene families, e.g. there are nine orthologs of the yeast ATG8 and five orthologs of the ATG18 protein. Knockout or RNAi mutants of *AtATG4a/b*, *AtATG5*, *AtATG7*, *AtATG9*, and *AtATG18a* display accelerated senescence and hypersensitivity to nutrient starvation (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Xiong et al., 2005), indicating that autophagy is not essential for the senescence program but plays an important role in nutrient remobilization. Consistently, in Arabidopsis suspension-cultured cells, several *ATG* genes are up-regulated at the onset of carbon starvation (Contento et al., 2004; Rose et al., 2006).

Remarkably, nineteen of the 21 Arabidopsis *ATG* genes (Supplemental Table IX) on the microarray are transcriptionally activated (Supplemental Fig. 2). It appears most *ATG* genes are coordinately up-regulated at a stage in developmental senescence when chlorophyll degradation starts to get visible, i.e. between the 6-week and 25%-bleaching stages. Figure 4 shows the expression profiles of the most dramatically up-regulated *ATG* genes, *7*, *8a*, *8e*, and *9*. The prolonged

Table IV. *Plant specificity of SAGs*

Up, Up-regulated; Down, down-regulated.

Category	PS[a] Total	PS SEN[b] Up	PS NS[c] Up	PS SEN Down	PS NS Down	AO[d] Total	AO SEN Up	AO NS Up	AO SEN Down	AO NS Down
All genes[e]	2,996/14%	392/1.8%	326/1.5%	316/1.5%	285/1.3%	2,391/11%	318/1.5%	273/1.3%	235/1.1%	188/0.9%
TM[f]	191/10%	22/1.1%	21/1.1%	18/0.9%	17/0.9%	297/15%	62/3.2%	54/2.8%	26/1.3%	22/1.1%
TF[g]	638/32%	104/5.2%	91/4.6%	48/2.4%	42/2.1%	32/1.6%	2/0.1%	2/0.1%	2/0.1%	2/0.1%
K[h]	0	0	0	0	0	252/67%	46/12%	42/11%	7/1.9%	4/1.1%
RLK[i]	1/0.1%	0	0	0	0	386/63%	65/11%	63/10%	31/5.1%	23/3.8%

[a]PS, Arabidopsis genes with similar protein sequences found only in other plants. [b]SEN, Genes that are regulated during NS, DIS, or DET. [c]NS, Genes that are regulated during NS. [d]AO, Arabidopsis genes with similar protein sequences in bacteria, archae, cyanobacteria, and eukaryotes. [e]All genes, All genes on the Agilent Arabidopsis 2 microarray. Given is the number of genes in the respective category. Percentages relate to the total 21,500 genes on the microarray. [f]TM, TPs and other membrane proteins with ≥3 TM spans. Given is the number of TM genes in the respective category. Percentages relate to the 1,931 TM genes on the microarray. [g]TF, Putative TFs. Given is the number of TF genes in the respective category. Percentages relate to the 1,985 TF genes on the microarray. [h]K, Putative kinases. Given is the number of K genes in the respective category. Percentages relate to the 376 K genes on the microarray. [i]RLK, Putative RLKs. Given is the number of RLK genes in the respective category. Percentages relate to the 610 RLK genes on the microarray.

observation time of induced and developmental senescence progression enabled the recognition of senescence-associated expression patterns of several *ATG* genes that had not been recognized in other studies (Buchanan-Wollaston et al., 2005; Xiong et al., 2005). It has been proposed that the Arabidopsis ATG system is ubiquitously present and not restricted to a certain developmental phase or nutritional state (for review, see Thompson et al., 2005). Consistent with this assumption is the significant background expression level of many *ATG* genes in 4-week-old plants (e.g. *AtATG4b*, *8c–f*, *8i*, and *18g*; Supplemental Table IX). On the other hand, the massive up-regulation of many *ATG* genes during all three senescence conditions suggests that autophagy is important for the efficient remobilization and recycling of nutrients.

Hormones

Cytokinin

Cytokinins (CKs) regulate cell division and various metabolic and developmental processes, including senescence. CK has a regulatory function early in senescence as CK levels are reduced in senescing leaves, whereas the exogenous application or endogenous overexpression of CKs delays senescence (Smart et al., 1991; Gan and Amasino, 1995).

For this study, 137 genes associated with various aspects of CK homeostasis were compiled (Supplemental Table X). The eight (putative) Arabidopsis adenylate isopentenyltransferase enzymes and two CK trans-hydroxylases that are possibly involved in CK biosynthesis are very weakly expressed in the three

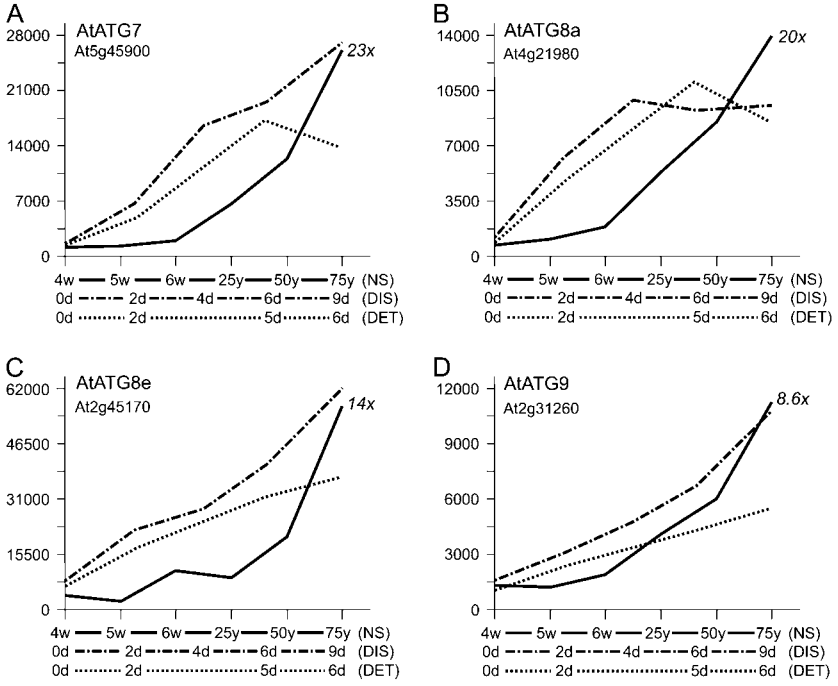

Figure 4. Autophagy. The expression profiles of four senescence-associated *ATG* genes are shown. Additional *ATG* gene expression profiles are shown in Supplemental Figure 2. The averages of the normalized signal values (see "Materials and Methods") were plotted against the sampling time points. The time scale of the *x* axis is not linear and different for the NS, DIS, and DET senescence experiments. For the developmental senescence (NS) curve, the signal ratio between the last sampling time point (75y for 75% yellow surface; see Table I) and the 4-week-old reference sample (4w/0 d) is indicated.

senescence experiments (''not well above background''), and none is up-regulated.

For the CK-degrading CK oxidase/dehydrogenases (CKX; Schmülling et al., 2003) and the CK-inactivating *N*- and *O*-glucosylases, the situation is different. Of the six *CKX* genes on the array, only *AtCKX5* is expressed at a high confidence level and increases weakly (2.1- to 2.9-fold) but continuously during NS, DIS, and DET. For *AtCKX2* and *AtCKX4*, similar patterns at lower levels are apparent. *AtCKX6* appears to be down-regulated. The role of CKX proteins in senescence is as yet unclear, which is illustrated by the observation that, unexpectedly, overexpression of *CKX* genes may result in retardation of leaf senescence (Werner et al., 2003). Four of the five active glycosyltransferases from Arabidopsis (Hou et al., 2004) are weakly expressed and barely regulated. However, the *O*-glycosyltransferase *AtUGT85A1* shows a remarkable activation in all three senescence conditions (Fig. 5A). It is thus conceivable that this protein is involved in senescence-specific inactivation of CKs by conjugation.

CK levels could also be reduced during senescence by translocation in the vascular system. Two members of the purine TP family, AtPUP1 and AtPUP2, can mediate CK uptake in yeast and thus are likely candidates for CK TPs (Bürkle et al., 2003). *AtPUP1* and three other family members are up-regulated (Fig. 5, B and C; Supplemental Table X) and none of the remaining *AtPUP*s is down-regulated (*AtPUP2* is not represented on the array), consistent with a role of purine permeases in exporting CKs during senescence.

The Arabidopsis CK-signaling pathway consists of CK perception by His protein kinases (AHKs) and activation of phosphorelay carriers, of B-type and eventually of A-type nuclear response regulators (ARRs), which control the primary CK-responsive genes by a negative feedback mechanism (for review, see Hwang et al., 2002). *AtAHK3* is the only CK receptor that is detectably expressed, but it is not (or only very weakly: 2.0- to 2.6-fold) up-regulated. Of two phosphorelay mediators and 11 B-type ARRs on the array, only *AtARR1* seems to be weakly (2.6- to 3.4-fold) up-regulated. Several A-type *ARRs* are rapidly induced by CK (Rashotte et al., 2003; Brenner et al., 2005; Kiba et al., 2005). Consistent with a depletion of CK during senescence progression, four of those are down-regulated in NS, DIS, and DET (Fig. 5, D–G). Down-regulation of these genes also was observed by Buchanan-Wollaston et al. (2005). A remarkable exception is *AtARR16*, which is strongly up-regulated upon CK treatment (D'Agostino et al., 2000; Rashotte et al., 2003; Kiba et al., 2005). This gene is also up-regulated during NS and DIS (but not DET; Fig. 5H), suggesting a special function in CK signaling that deviates from the other A-type ARRs.

Recently 71 up-regulated and 11 down-regulated immediate CK-response genes of Arabidopsis were identified (Brenner et al., 2005). From a simplistic viewpoint, one would expect that these genes are not regulated or down-regulated during senescence, if the CK level decreases. Indeed, 80% of the 69 CK-response genes on the array are not regulated (43 genes) or down-regulated (12 genes) during senescence, whereas the remaining 14 genes are up-regulated (Supplemental Table X). This suggests that one-fifth of the immediate CK-response genes are under positive control of other regulators during senescence.

Jasmonic Acid

Jasmonic acid (JA) is critically involved in senescence (for review, see He and Gan, 2003). JA application induces premature senescence and its level increases in senescing leaves (He et al., 2002). Eleven of 19 JA-biosynthesis and six of 11 JA-signaling or -response genes are up- or down-regulated, and some exhibit strikingly different regulation in NS, DIS, and DET (Supplemental Table XI; Supplemental Fig. 3, A–K). Eight JA-biosynthesis genes are specifically and transiently up-regulated in NS. These include the highly expressed lipoxygenase AtLOX2 (Supplemental Fig. 3C), allene oxide synthase AtAOS1 (Supplemental Fig. 3F), and 12-oxophytodienoate reductase AtOPR3 (Fig. 6A; Supplemental Fig. 3B) that catalyze sequential reactions in the JA-biosynthesis pathway. Their coordinate expression during NS, but not DIS or DET, suggests that this pathway is in leaves specifically activated by developmental stimuli. AtOPR3 is the isoenzyme also predominantly responsible for JA biosynthesis during pollen development, and, like AtAOS1, it is essential for male gametophyte development and anther elongation. *AtOPR1* and *AtOPR2* are also up-regulated during senescence (Supplemental Fig. 3, A and H), but the roles of these less efficient isoenzymes are not clear. Only one gene, the uncharacterized putative allene oxide cyclase family protein *AtAOC4* (Supplemental Fig. 3I), is weakly down-regulated. In striking contrast, two other genes, *AtαDOX1* encoding a fatty acid α-dioxygenase (Supplemental Fig. 3J) and *AtLOX1* (Fig. 6B; Supplemental Fig. 3K), are up-regulated to very high levels in detached leaves but not in NS and DIS, indicating that these genes are predominantly responding to wounding. Taken together, the expression profiles indicate that, during developmental senescence and upon wounding, different isozymes are active in the JA synthesis pathway and that in dark-induced senescence JA possibly does not play major role.

Several genes that are supposedly involved in JA signaling or response are up- or down-regulated during senescence (Supplemental Fig. 3, L–Q). The ribonuclease 1 gene *AtRNS1* is strongly up-regulated in later stages during NS, but not in DIS and DET (Fig. 6C; Supplemental Fig. 3L). *AtRNS1* also is very rapidly and transiently induced upon wounding but declines to almost noninduced level within 48 h (LeBrasseur et al., 2002), explaining that it is not detected in the DET experiment. The late expression peak of *RNS1* would be consistent with a participation in nucleic acid degradation late in senescence. The only JA-signaling

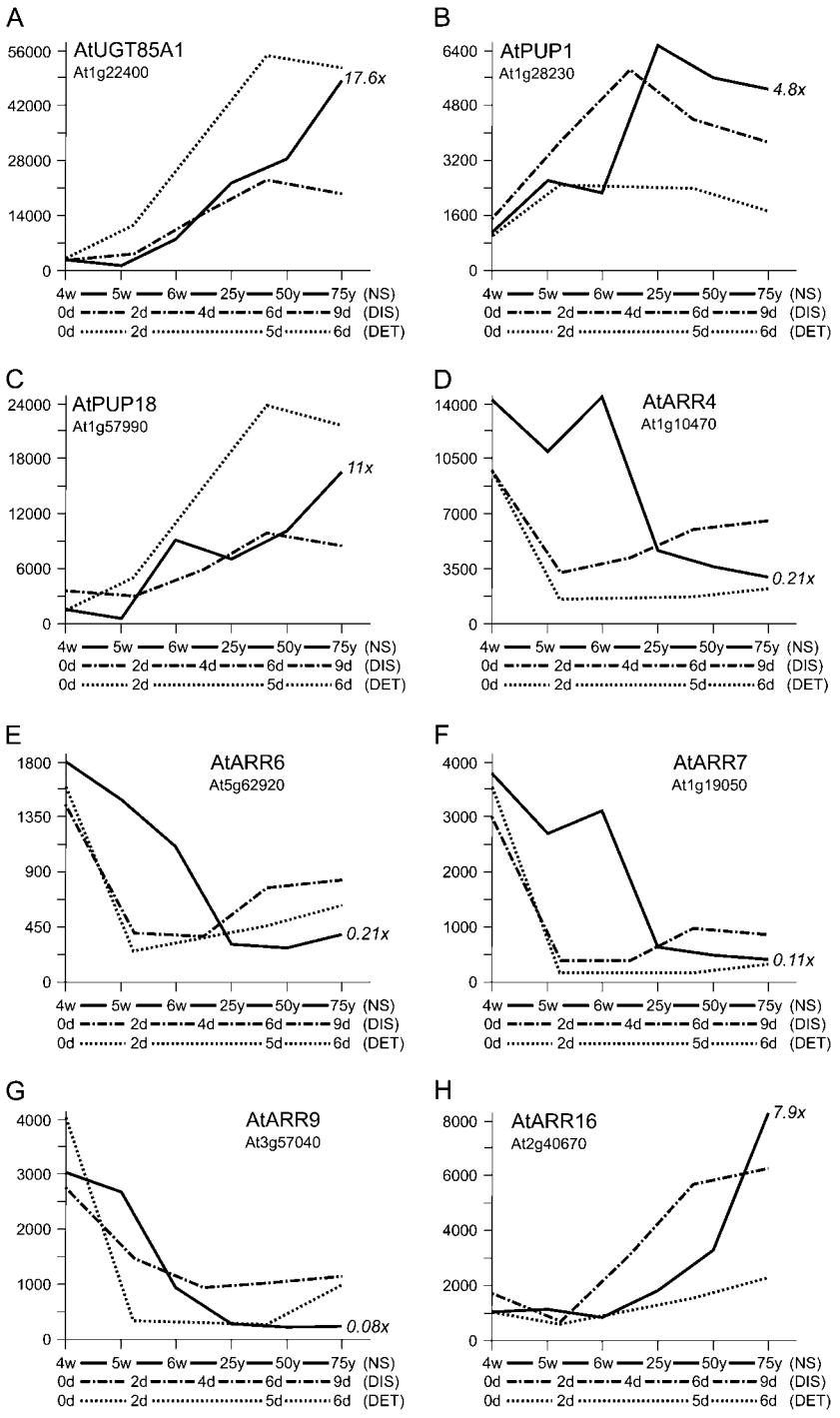

Figure 5. Cytokinin. The expression profiles of six senescence-associated CK genes are shown. The data were processed as described for Figure 4.

gene that is strictly down-regulated in all senescence conditions is *AtCOS1* (Fig. 6D; Supplemental Fig. 3Q). It encodes lumazine synthase, which catalyzes the penultimate step in riboflavin synthesis. It is suggested that *AtCOS1* acts in the riboflavin pathway as a suppressor of certain JA responses, including senescence (attenuated by the SCF/COI1 complex; Xiao et al., 2004). The strong down-regulation of *AtCOS1* during senescence corroborates this model.

Salicylic Acid

Salicylic acid (SA) plays a key role as mediator of plant stress responses, including disease and systemic acquired resistance. Recently, it was reported that the SA-signaling pathway is also active in the control of gene expression during developmental senescence (Morris et al., 2000). A transcriptome analysis in senescing Arabidopsis leaves from wild-type plants and

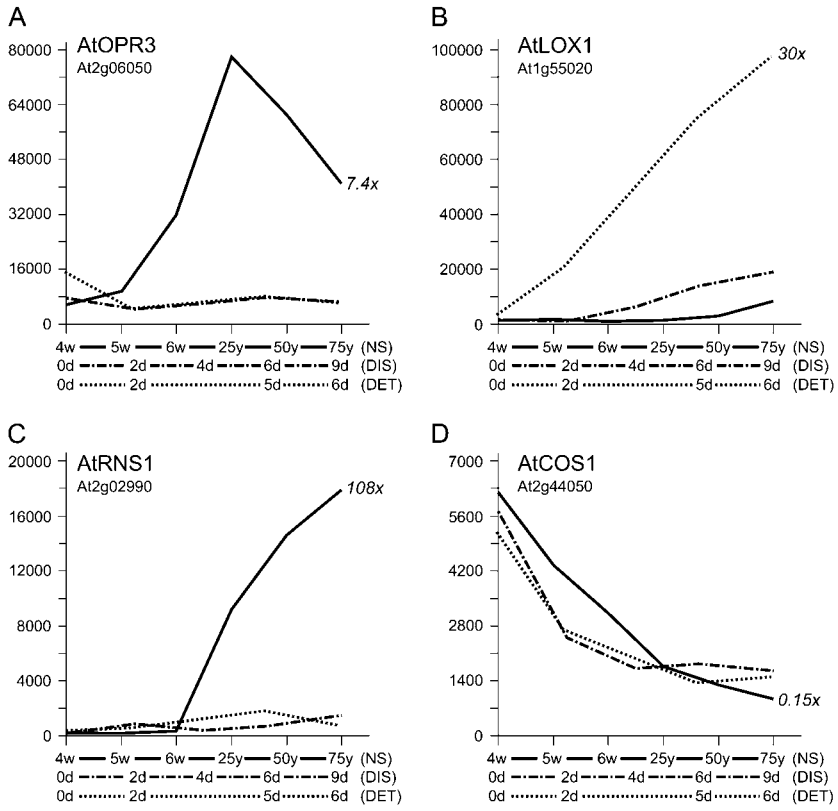

Figure 6. JA. The expression profiles of four senescence-associated JA genes are shown. Additional JA gene expression profiles are shown in Supplemental Figure 3. The data were processed as described for Figure 4.

SA-deficient *NahG* mutants revealed that many SAGs are dependent on the SA-signaling pathway (Buchanan-Wollaston et al., 2005).

Four of six genes that are (possibly) involved in SA biosynthesis are regulated during NS (Supplemental Fig. 4, A–D; Supplemental Table XII). Among them is the gene encoding isochorismate synthase (*AtICS1*), a central enzyme in the pathway. It is conspicuous that this gene is only induced during NS, but not after shading or detachment. In contrast, the *ICS1* homolog *At1g18870* is also induced by shading, but like *ICS1* not after detachment of leaves. The two Phe ammonia-lyase genes are only weakly regulated. Eight of 12 SA-signaling genes are regulated during NS, and also in this group of genes no or less regulation during DIS and DET is observed (Supplemental Fig. 4, E–L). Differential up-regulation is most prominent for *AtEDS4*, which encodes an orphan MATE-related efflux carrier. *AtEDS4* is induced by SA, UV light, and after pathogen infection, depending on the two other SA-signaling proteins (*AtEDS1* and *AtPAD4*; Nawrath et al., 2002). These also are up-regulated in NS, but not in DIS and DET (Supplemental Fig. 4, H and I). AtEDS1 and AtPAD4 interact with each other, and both genes are transducers of redox signals and important activators of SA signaling (for review, see Wiermer et al., 2005). Only one SA-signaling gene, *AtAGD2*, is down-regulated during senescence.

The expression profiles of 44 SA-responsive genes (Supplemental Table XII), including 30 of the 32 senescence-enhanced and SA-dependent genes identified by Buchanan-Wollaston et al. (2005), reveal an interesting trend. Twenty-six genes are up-regulated (Supplemental Figs. 5 and 10A), none is down-regulated, and all except one are genes identified in the *NahG* mutant. It is somewhat surprising that of the other 14 SA-responsive genes reported in the literature, only one (*AtAF2*; Supplemental Fig. 5P) is up-regulated during senescence. Remarkably, the majority of the senescence-associated SA-responsive genes are only up-regulated during NS, but not (or only weakly) during DIS or DET, resembling the expression profiles of the SA-biosynthesis and -signaling genes mentioned above. In summary, these data provide additional evidence for SA as a modulator of gene expression in naturally senescing leaves.

Ethylene

The plant hormone ethylene (ET) is well known as an accelerator of senescence. The importance of ET signaling during senescence is illuminated by a delayed senescence phenotype of ET-insensitive mutants like *ein2* in Arabidopsis (Oh et al., 1997). However, ET is not essential for the onset and progression of senescence (Grbic and Bleecker, 1995; Jing et al., 2002).

Of 69 genes (proposed to be) involved in ET biosynthesis or signaling (Supplemental Table XIII), 18 are up- or down-regulated (Supplemental Fig. 6). The 1-aminocyclopropane-1-carboxylic acid (ACC) synthases

(*AtACS*) catalyze the rate-limiting step in the ET-biosynthesis pathway. Arabidopsis encodes nine ACS proteins, of which eight form functional homodimers, that underly a complex spatial and temporal regulation (Tsuchisaka and Theologis, 2004). Only three of the functional ACC synthases (*AtACS2*, *AtACS5*, and *AtACS6*) are significantly expressed and up-regulated during NS (Supplemental Fig. 6, A–C). In the subsequent step, ACC is converted to ET by the ACC oxidase (*AtEAT1*), which is also up-regulated (Supplemental Fig. 6D). In addition, a nitrilase (*AtNIT4*) that is thought to play a role in cyanide detoxification during ET biosynthesis (Piotrowski et al., 2001) is up-regulated (Supplemental Fig. 6E). Possibly, this set of proteins is responsible for an increase in ET production in senescing leaves.

ET signal transduction supposedly follows a ''linear'' pathway, with membrane-bound receptors at the beginning, TFs at the end of the chain, and multiple positive and negative regulators in between. The five ET receptors or the next downstream protein, AtCTR1, are not regulated in NS, DIS, or DET, but several other components of ET signal transduction are. The TP-like AtRAN1 protein, which might be required to form functional ET receptors (Woeste and Kieber, 2000), and the downstream component *AtEIN3* are significantly up-regulated in all three senescence experiments, most rapidly in DIS and DET (Supplemental Fig. 6, H and I). *AtEIN2*, located between *AtCTR1* and *AtEIN3*, is not represented on the array. The *AtEIN3* and *AtEIL* TFs regulate gene expression by binding directly to primary ET-response elements in the promoters of various genes. One immediate target of *AtEIN3* is *AtERF1a* (Solano et al., 1998) that appears to be transiently up-regulated (Supplemental Fig. 6). AtERF1a is a TF whose transcriptional activation requires simultaneously the JA- and ET-signaling pathways (Lorenzo et al., 2003). Five other ERF proteins are also up-regulated but with different profiles (Supplemental Fig. 6, K–O). *AtERF1b*, *AtERF2*, and *AtEBP1* are ET-responsive activators of GCC-box genes (Büttner and Singh, 1997; Fujimoto et al., 2000).The weakly up-regulated *AtRCD1* modulates ET, abscisic acid (ABA), and methyl-JA responses (Ahlfors et al., 2004). Two uncharacterized putative TFs, *At5g07580* and *At5g25190*, annotated as possibly involved in ET signaling, are down-regulated (Supplemental Fig. 6, Q and R).

Buchanan-Wollaston et al. (2005) recently identified 21 SAGs that show reduced expression in ET- and JA-deficient plants. Of the 19 genes represented on the Agilent microarray (Supplemental Table XIII), one yields no significant signal. All others are strongly induced during NS (Supplemental Fig. 7) and four of them are known to be involved in JA signaling (*AtRNS1*; Supplemental Fig. 3L; *AtNAC3*; Supplemental Fig. 3O), JA biosynthesis (*AtαDOX1*; Supplemental Fig. 3J), and to be SA responsive (*At1g19250*; Supplemental Fig. 5M). However, as their expression patterns during DIS and DET differ, at least some of these genes are additionally regulated by other signaling pathways. A subgroup of six genes appears to be specific for developmental senescence as they do not respond at all to shading or leaf detachment, including the S-like RNAse *AtRNS1*, the exopolygalacturonases and endopolygalacturonases *AtPGA3* and *AtADPG1*, and the ABC-TP *AtPDR12*. Another subgroup of genes is most intensely and apparently transiently up-regulated in detached leaves and thus possibly responding to wounding (Supplemental Fig. 7, J–N).

In summary, these data indicate a coordinated up-regulation of ET-biosynthesis genes during leaf senescence in Arabidopsis that is accompanied by changes in expression of several ET-signaling components. It appears that some downstream response factors are NS-specifically and -transiently transcribed at later stages in senescence development.

Auxin

Although auxin effects on abscission and senescence were first reported more than 50 years ago (for review, see Noodén, 1988), the involvement of this hormone in the senescence development is much less understood than that of ET, JA, or CK. In Arabidopsis senescing leaves, the indole-3-acetic acid (IAA) concentration is 2-fold higher than in nonsenescing leaves (Quirino et al., 1999). However, auxin treatment leads to a transient decrease in *SAG12* expression (Noh and Amasino, 1999). Recently, it was described that the AUXIN RESPONSE FACTORs 1 and 2 (*AtARF1* and *AtARF2*) are involved in regulation of abscission and senescence (Ellis et al., 2005; Okushima et al., 2005). More than 110 proteins implicated in auxin biosynthesis, metabolism, transport, and signaling were compiled from Woodward and Bartel (2005), Remington et al. (2004), The Arabidopsis Information Resource Gene Ontologies, and gene descriptions (Supplemental Table XIV). The expression profiles of the regulated genes are shown in Supplemental Figure 8.

The observation that eight of the 15 (putative) Arabidopsis IAA-biosynthesis genes are significantly regulated suggests that leaf senescence is associated with changes in IAA synthesis. A moderate transcriptional increase of Trp synthase a (*AtTSA1*; Supplemental Fig. 8A) is observed in NS and DET, but not DIS. Downstream of Trp, the Arabidopsis amidohydrolase *AtAMI1* converts in vitro indole-3-acetamide to IAA (Pollmann et al., 2003). As *AtAMI1* transcription decreases during NS, DIS, and DET (Supplemental Fig. 8B), the indole-3-acetamide pathway is presumably not active in senescence. However, six genes encoding proteins that are supposedly acting in the IPA and/or IAOx pathway are heavily up-regulated (Supplemental Fig. 8, C–H). Most intriguingly, the IAA1d oxidase (*AtAO1*) and nitrilases *AtNIT1* to *3* that directly convert indole-3-acetaldehyde and indole-3-acetonitrile, respectively, into IAA are transcriptionally up-regulated. Quirino et al. (1999) have also reported the senescence-associated induction of *AtNIT1* to *3* and found that it is accompanied by an increase in IAA and decrease

in indole-3-acetonitrile. Suspiciously, transcription of *AtAO1*, *AtTSA1*, *AtSUR2*, and *AtCYP92B2* remains almost constant in dark-induced senescence.

IAA homeostasis also is affected by formation and degradation of IAA conjugates and indole-3-butyric acid. About one-third of the genes implicated in these processes are transcriptionally induced during leaf senescence. Three IAA-amino acid synthetases are massively up-regulated in NS, DIS, and DET (Supplemental Fig. 8, I–K). These enzymes presumably help to maintain auxin homeostasis by inactivating excess IAA (Staswick et al., 2005). On the other hand, the two IAA amidohydrolases *AtILR1* and *AtIAR3* also are induced (Supplemental Fig. 8, L and M) that release free IAA from IAA-Leu, -Phe, and -Ala and thus counteract the IAA-amino acid synthetases. Moreover, the three genes *AtACX1*, *AtACX4*, and *AtPED1* encoding acyl-CoA oxidases involved in conversion of indole-3-butyric acid into bioactive IAA (Zolman et al., 2000; Adham et al., 2005) are also up-regulated (Supplemental Fig. 8, N, P, and R).

Experiments with bean leaves suggested that changes in auxin gradients rather than the auxin concentration itself may modulate abscission and senescence development (Addicott et al., 1955). Of 13 TPs (suspected to be) involved in auxin transport, six are massively down-regulated in NS, DIS, and DET (Supplemental Fig. 8, S–X). The putative IAA permease AtAUX1 is localized asymmetrically in the plasma membrane of certain cells and facilitates IAA loading into the leaf vascular transport system and IAA unloading in the primary root apex. AtPIN3 is one of three down-regulated proteins from the PIN family involved in auxin efflux and expressed in gravity-sensing tissues like the columella, where it quickly relocates in the cell plasma membrane to the side oriented toward gravity when the gravity vector changes. ABC TPs of the PGP subfamily were recently identified as auxin TPs (for review, see Geisler and Murphy, 2006). AtPGP1 and AtPGP19 function in conjunction with TWISTED DWARF 1, supposedly as auxin exporters, and are probably involved in the proper polar distribution of auxin during plant development. *AtPGP19* is strongly down-regulated during senescence (Supplemental Fig. 8W), and *AtPGP1* also appears to be weakly down-regulated, but its expression level remains subsignificant (Supplemental Table XIV). Interestingly, *AtPGP4*, which presumably functions as auxin importer in root caps (Terasaka et al., 2005), is up-regulated in senescing leaves (Supplemental Fig. 8Y). These changes in expression of auxin TPs may contribute to the increased auxin concentration in senescing leaves that was observed by Quirino et al. (1999).

Auxin causes changes in the expression of many genes, and crucial factors in the response pathway are the ARF and Aux/IAA proteins. The Arabidopsis *AtARF* and *AtIAA* gene families consist of 23 and 29 members, respectively (Remington et al., 2004). Expression of most *AtARF* genes is below the confidence level in our experiments and does not change in NS, DIS, or DET. The only exception is *MONOPTEROS* (*AtARF5*/*AtMP*), which is induced late in natural senescence (Supplemental Fig. 9A). *AtARF5* has not been assigned a role in senescence yet. Recently, regulation of leaf senescence by *AtARF1* and *2* and a weak transcriptional repression/induction of *AtARF1*/*AtARF2* during senescence have been observed (Lin and Wu, 2004; Ellis et al., 2005). In our experiments also, a 2- to 3-fold decrease/increase in *AtARF1*/*AtARF2* expression can be seen, albeit below the significance threshold. In contrast to the *ARF*s, almost one-half of the 28 *Aux/IAA* genes are down-regulated during senescence (Supplemental Fig. 9, B–N). A function of Aux/IAA proteins in senescence has not yet been described, but the overall decline in *AtIAA* gene transcription suggests that the quality of auxin responses in leaves alters as a result of changes in signaling proteins.

A Role of GA and Brassinosteroids in Senescence?

Reports that demonstrate a link between GA activity and senescence are rare. In pea (*Pisum sativum*), exogenous GA3 can delay apical senescence, and endogenous GA concentration is lower in senescing than in flowering shoots (Zhu and Davies, 1997). In detached Arabidopsis leaves, CK and to a lower extent GA delay chlorophyll degradation. However, upon combining the two hormones, GA partially inhibits the CK effect, and it is suggested that the SPINDLY protein regulates this cross talk between the two signaling pathways (Greenboim-Wainberg et al., 2005).

Fifteen of the 18 genes on the array that are (putatively) involved in GA biosynthesis are expressed at subsignificant levels, and none is up-regulated (Supplemental Table XV). On the other hand, the functional and GA-inducible GA 2-oxidase 2 (*AtGA2OX2*) that deactivates GA (Thomas et al., 1999) is 18-fold up-regulated (Fig. 7A). These results suggest that during senescence at least some gibberellins are deactivated.

None of the 16 genes that encode (putative) GA-signaling proteins are up-regulated, and *AtGASA4* and *AtGASA5*, two members of the GASA family of short proteins of unknown function, are down-regulated (Fig. 7, B and C). Some GA-responsive genes display senescence-associated regulation. A well-known example is the GA-induced aquaporine *AtTIP1.1* (Phillips and Huttly, 1994) that is down-regulated, as many other intrinsic proteins too (Fig. 2). Intriguingly, the GA-responsive xyloglucan endotransglycosylase *AtMERI5* is massively up-regulated during senescence (Fig. 7D). *AtMERI5* and other xyloglucan endotransglycosylases are suggested to be involved in cell wall weakening that is necessary for cell expansion (Xu et al., 1996). Studies on *AtTIP1.1* and *AtMERI5* revealed that there is cross talk between the GA and brassinosteroid (BR) pathways. *AtTIP1.1* is antagonistically regulated by GA and BR, whereas transcription of *AtMERI5* was shown to be positively and additively regulated by either GA or BR treatment (Kauschmann

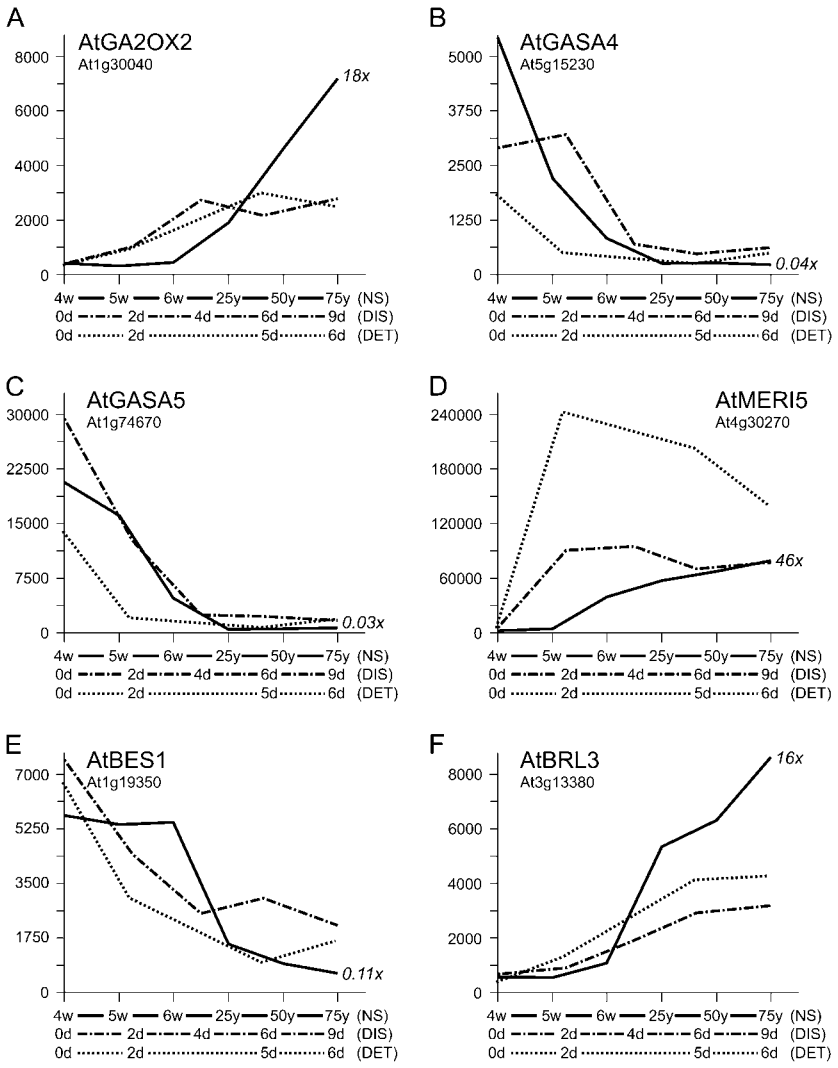

Figure 7. GA and BRs. The expression profiles of four senescence-associated GA (A–D) and two BR genes (E and F) are shown. The data were processed as described for Figure 4.

et al., 1996). As during leaf senescence GA activity appears to be reduced, the senescence-associated induction of *AtMERI5* must be effected by a different regulator, possibly BR.

BRs appear to promote developmental senescence, as mutants deficient in BR biosynthesis (e.g. *det2*) or the BR receptor BRI1 have a retarded senescence progression (for review, see Clouse and Sasse, 1998). However, from a set of 99 BR-related genes on the array (14 biosynthesis and inactivation genes, 11 receptor and signaling genes, 52 BR-induced genes, and 29 BR-repressed genes; compiled from Müssig and Altmann [2003] and Lisso et al. [2005]), no clear picture emerges (Supplemental Table XVI). Of the BR-induced genes, 15 and 14 were significantly expressed and up- or down-regulated during NS, respectively. Of the BR-repressed genes, 13 were up- and 5 were down-regulated. Two of the 14 (putative) biosynthesis genes are down-regulated (*AtCPD*/*At5g05690* and *At3g55360*; data not shown) and none is up-regulated, suggesting that BR biosynthesis does not significantly increase during senescence. Central transmitters of the BR signal from the receptor(s) to the target genes are the *AtBIN2* kinase and the two nearly identical transcriptional modulators and regulators of BR signaling, *AtBES1* and *AtBZR1. AtBIN2* and *AtBZR1* show no significant change in expression, whereas *AtBES1* is down-regulated (Fig. 7E).

The BR receptors AtBRL1 and AtBRL3 supposedly function specifically in provascular differentiation (Cano-Delgado et al., 2004). As *AtBRL3* is strongly up-regulated in later stages of NS, DIS, and DET (Fig. 7F), one could speculate that BR modulates the properties of the vascular tissue in senescing leaves toward efficient export of metabolites.

ABA

As treatment with ABA can induce leaf senescence, ABA is considered a promoter of senescence (Noodén, 1988). In Arabidopsis, ABA induces expression of several SAGs. Vice versa, during senescence several

genes are up-regulated that are involved in ABA synthesis, metabolism, or signaling (Oh et al., 1996; Weaver et al., 1998; He et al., 2001; Buchanan-Wollaston et al., 2005). We investigated the expression profiles of 58 ABA-biosynthesis, -metabolism, and -signaling genes (Supplemental Table XVII). Four of 11 ABA-biosynthesis genes are up-regulated, including the 9-cis-epoxycarotenoid dioxygenases *AtNCED2* and *3* that catalyze the cleavage of neoxanthin and the abscisic aldehyde oxidases *AtAAO3* and 4 (Supplemental Fig. 10, A–D). On the other hand, the ABA-inactivating glucosylase *UGT71B6* (Priest et al., 2005) is also up-regulated during NS (Supplemental Fig. 10E).

Of 30 ABA-signaling genes, eight are up-regulated and only two are down-regulated (Supplemental Fig. 10, F–O). Of 12 negative regulators of ABA responses, five protein phosphatases 2C are up-regulated (Supplemental Fig. 10, P–T). The ABA-responsive *AtSEN1* gene is up-regulated during senescence (Supplemental Fig. 10U) as described by Oh et al. (1996). The ABA-repressible *AtPIP1.5* and *AtPIP2.6* genes (Jang et al., 2004), but also the ABA-inducible *AtPIP2.2*, are down-regulated during senescence (Supplemental Fig. 10, V–X). These complex and, for several ABA-related genes, opposite regulation patterns during natural and induced senescence support the notion that ABA is a modulator of senescence-associated physiological changes.

CONCLUSION

Leaf senescence is a slow-going, PS cellular reorganization process that ensures the mobilization and export of nutrients from the cells before they die. In young and old rosette leaves, different nutrient and assimilate fluxes take place, raising the question whether the same TPs are active during these developmental stages. During NS, the fraction of up-regulated TPs is approximately 1.5 times higher than that of all genes and 2.5 times higher than that of down-regulated TPs. This supports the notion that during senescence there is an increased demand for transport across membranes and that specific TPs fulfill this need.

In this study, different gene categories were analyzed for senescence-associated transcriptional regulation of PS genes versus genes found also in AO. Senescence-associated transport functions appear not to be predominantly provided by PS TPs, as similar fractions of the regulated TPs are PS or AO. Also, signal recognition and transmission processes active during senescence and involving RLKs are not executed by PS proteins, although during NS (but not DIS and DET) almost 3 times more *RLK* genes are up- than down-regulated. The plant lineage has a dramatically expanded number of RLKs compared to animals. It was hypothesized that this expansion in plants represents a PS adaptation for extracellular signal sensing and involves mostly defense/resistance-related genes (Shiu and Bleecker, 2003; Shiu et al., 2004). However, the RLK clade has not diverged enough from the RLKs in non-plants to be classified as PS (Gutierrez et al., 2004a), possibly indicating that plants have no distinct, PS, RLK-dependent signal transduction mechanisms. An opposite bias of PS versus AO genes regulated during senescence is observed for the TFs. This suggests that PS TFs are key players in the regulatory pathways leading to and coordinating leaf senescence.

Gan (2003) and Buchanan-Wollaston et al. (2005) presented models illustrating the pathways that operate in senescence. The analysis in this study of hormone homeostasis and signaling genes adds more details to this model and reveals additional SAGs in most pathways of the regulatory network. The expression profiles of genes involved in CK homeostasis are mostly consistent with a depletion of this hormone during senescence. However, the expression of CK-signaling genes indicates a more complex picture, as about 20% of the immediate CK-response genes (Brenner et al., 2005) are up-regulated during NS, among them the A-type response regulator *AtARR16*. In the JA pathway, the senescence-type specificity of some genes is striking. The expression profiles are consistent with the hypothesis that, during NS and upon wounding, different JA synthesis isozymes are activated, whereas during DIS JA biosynthesis is not induced. Differential activation depending on the senescence condition is also apparent for the SA pathway. The expression profiles of SA-biosynthesis and -response genes support the notion that SA is an important and specific regulator in developmental senescence. In addition, they suggest that SA is not involved in artificially induced senescence. In the ET pathway, a coordinated up-regulation of ET-biosynthesis genes during senescence is observed. Differences between the senescence types are not as obvious as in the JA and SA pathways, except a tendency of ET-response factors for transient up-regulation during NS. Previous evidence for a role of auxin in the senescence program is rather circumstantial; however, the complex expression patterns of auxin homeostasis genes suggest a participation of this hormone in Arabidopsis leaf senescence regulation. Auxin-biosynthesis genes are preferentially up-regulated, but also auxin conjugate formation and metabolism genes, whereas IAA TPs and AUX/IAA genes are mostly down-regulated. Also, GAs and BRs have only rarely been reported as regulators of senescence. The data shown here are compatible with a decrease in GAs during senescence. For the BR-related genes, no clear picture emerges from the expression studies. ABA can promote senescence and induce expression of several SAGs. The expression profiles of ABA-related genes are consistent with increasing ABA levels and senescence-dependent alterations in ABA signaling. However, as some ABA-response genes are up- and others down-regulated, an estimate of the impact of ABA as a regulator during senescence is not straightforward.

MATERIALS AND METHODS

Plant Material

Wild-type Arabidopsis (*Arabidopsis thaliana*; Columbia-0) fifth and sixth rosette leaves grown under greenhouse conditions supplemented with artificial light (16 h light, 21°C; 8 h dark, 19°C) were used throughout all experiments. In each senescence experiment, the fifth and sixth rosette leaves from 4-week-old leaf material served as reference for the other time points. Each senescence experiment was repeated once with independently grown plants as a biological replicate. The two batches of plants were grown May to June 2003 and December 2003 to January 2004.

NS

Leaves were harvested at six developmental stages from the start of the rapid expansion phase at the 4-week-old (4w) stage (28 d after germination) until the late senescent stage, with up to 75% of the leaf surface exhibiting chlorophyll degradation (yellowing) during the silique ripening stage (53 d after germination), corresponding to principal growth stages 3.50 to 8.00 (Boyes et al., 2001; Table I).

DIS

Leaves were wrapped in aluminum foil at the 4-week-old stage and harvested 2, 4, 6, and 9 d later.

DET

Four-week-old leaves were detached and harvested after incubation for 2, 5, and 6 d in petri dishes on water in the dark.

Chlorophyll Content

The chlorophyll content in the different leaf samples was determined as described by Graan and Ort (1984).

Hybridization of Arabidopsis Microarrays and Data Analysis

Total RNA was purified using the RNeasy kit (Qiagen), and quality was assayed using an Agilent 2100 Bioanalyzer. Total RNA (500 ng) was labeled with either Cyanine 3-CTP or Cyanine 5-CTP (Perkin-Elmer) using the Agilent low-input linear amplification kit, and dye incorporation was determined using a NanoDrop spectrophotometer. Labeled RNA was hybridized to Agilent Arabidopsis 2 microarrays according to manufacturer's instructions.

The experiments were designed following a common reference model (König et al., 2004). Each slide within one experiment (NS, DIS, or DET) was hybridized with Cy3-labeled 4-week reference cDNA derived from one single labeling reaction and Cy5-labeled stage-specific cDNA. Each microarray hybridization was repeated with Cy5-labeled reference cDNA and Cy3-labeled stage-specific cDNA (dye-swap). Thus, for each gene and stage, four data points were generated (two biological replicates times two technical replicates).

Microarrays were scanned on an Agilent G2565 scanner, and images were extracted and quality assessed with the Agilent Feature Extraction version 7.5 software using the default settings. The expression values of all individual hybridizations were normalized by setting the median expression value of the Arabidopsis genes (21,500 genes) in the control channel (4w for NS or 4w/0 d for DET and DIS) for each microarray slide to 1,000. The significantly regulated genes in both biological replicates were clustered by the *k*-means algorithm in seven groups based on their averaged $\log_2$-based expression ratios with the Cluster 3.0 program (de Hoon et al., 2004; for details, see supplemental file "Data Analysis").

Sources for Protein Classification

Arabidopsis protein designations were from The Institute for Genomic Research Arabidopsis database release 5. The general functional categories were adopted from MIPS (http://mips.gsf.de/projects/funcat; Ruepp et al., 2004). The classification of Arabidopsis TPs was performed on the basis of the TC system (http://www.tcdb.org; Saier, 2000; Busch and Saier, 2004) and incorporated into the ARAMEMNON plant membrane protein database (http://aramemnon.botanik.uni-koeln.de; Schwacke et al., 2003). The classification of (receptor-like) kinases was adopted from Shiu and Bleecker (2003) and Shiu et al. (2004). The PS genes were obtained from the Plant Specific Database (PSDB; http://genomics.msu.edu/plant_specific/; Gutierrez et al., 2004a, 2004b). TFs were compiled from DTAF (http://datf.cbi.pku.edu.cn/), AtTFDB (http://arabidopsis.med.ohio-state.edu/AtTFDB/), and RARTF (http://rarge.gsc.riken.jp/rartf/). The complete list of (re)classified and partially renamed proteins is provided in Supplemental Table I.

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