Transcription Analysis of Arabidopsis Membrane Transporters and Hormone Pathways during Developmental and Induced Leaf Senescence¹[W]

Eric van der Graaff², Rainer Schwacke, Anja Schneider³, Marcelo Desimone, Ulf-Ingo Flügge, and Reinhard Kunze⁴*

Institute of Botany II, University of Cologne, 50931 Cologne, Germany (E.v.d.G., R.S., A.S., U.-I.F., R.K.); and Center of Plant Molecular Biology, University of Tübingen, 72076 Tuebingen, Germany (M.D.)

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.

¹ This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung und Forschung, and the Fonds der Chemischen Industrie.
² Present address: Institute of Biology III, University of Freiburg, Schanzlestrasse 1, 79104 Freiburg, Germany.
³ Present address: Botanical Institute, Ludwig-Maximilians-University Munich, Menzinger Str. 67, 80638 Munich, Germany.
⁴ Present address: Institute of Biology/Applied Genetics, Free University Berlin, Albrecht-Thaer-Weg 6, 14195 Berlin, Germany.
* Corresponding author; e-mail rkunze@zedat.fu-berlin.de; fax 49–30–838–54345.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Reinhard Kunze (rkunze@zedat.fu-berlin.de).

[W] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.079293.
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 expression.

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

<table>
<thead>
<tr>
<th>Stage</th>
<th>DAS</th>
<th>Description</th>
<th>Principle Growtha</th>
<th>Stageb</th>
</tr>
</thead>
<tbody>
<tr>
<td>4w</td>
<td>28</td>
<td>Rapid expansion, sink leaf</td>
<td>Rosette growth</td>
<td>5.00–7.00</td>
</tr>
<tr>
<td>5w</td>
<td>35</td>
<td>Fully expanded, sink-to-source transition</td>
<td>Flower production</td>
<td>6.00–6.10</td>
</tr>
<tr>
<td>6w</td>
<td>42</td>
<td>Fully expanded, source leaf</td>
<td>Flower production</td>
<td>6.10–6.30</td>
</tr>
<tr>
<td>25y</td>
<td>48–50</td>
<td>Early senescence, approximately 25% yellow surface</td>
<td>Flower production</td>
<td>6.30–6.50</td>
</tr>
<tr>
<td>50y</td>
<td>50–53</td>
<td>Mid-senescence, approximately 50% yellow surface</td>
<td>Flower production</td>
<td>6.50–6.90</td>
</tr>
<tr>
<td>75y</td>
<td>52–56</td>
<td>Late senescence, approximately 75% yellow surface</td>
<td>Silique ripening</td>
<td>6.90–8.00</td>
</tr>
</tbody>
</table>

aDevelopmental stages according to Boyes et al. (2001).
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”), 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 during NS, DIS, and DET. Applying stringent selection criteria (see “Materials and Methods”), 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 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”), 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

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Name</th>
<th>NS Cluster</th>
<th>DIS Cluster</th>
<th>DET Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5g14930</td>
<td>SAG101</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A5g66170</td>
<td>SEN1</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>A3g63210</td>
<td>SAG102</td>
<td>0</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>A1g63300</td>
<td>WRKY6</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>A2g29350</td>
<td>SAG13</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>A1g09500</td>
<td>SAG26</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A5g11520</td>
<td>YLS4, AiasP3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A2g45570</td>
<td>YLS6</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A5g13170</td>
<td>SAG29</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A5g51640</td>
<td>YLS7</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A1g66580</td>
<td>SAG24</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A3g60130</td>
<td>YLS1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>A5g45890</td>
<td>SAG12</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>A3g10980</td>
<td>SAG20</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>A5g20250</td>
<td>DIN10</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>A4g02380</td>
<td>SAG21</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>A5g51070</td>
<td>ERD1</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>A1g0140</td>
<td>SAG103</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>A4g0270</td>
<td>SEN4</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>A3g51430</td>
<td>YLS2</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>A2g4290</td>
<td>YLS3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A3g49620</td>
<td>DIN11</td>
<td>7</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>A1g29920</td>
<td>CAB</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

*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.*
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 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.
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) plastid 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. *AtCRK10* is even down-regulated during DIS and DET (Supplemental Table VII). *AtCRK7* and *AtCRK37* are salicylic acid (SA) responsive.
supplemental fig. 5, g and w; buchanan-wollaston et al., 2005), and several of these crks (for ca\textsuperscript{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 1, 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 rnaai 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, 8c, and 9. the prolonged

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{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.}
\end{figure}
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 8g; 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 Table IV. Plant specificity of SAGs

<table>
<thead>
<tr>
<th>Category</th>
<th>PS Total</th>
<th>PS SENb Up</th>
<th>PS NSb Up</th>
<th>PS SEN</th>
<th>PS NS</th>
<th>AO Total</th>
<th>AO SEN</th>
<th>AO NS</th>
<th>AO SEN</th>
<th>AO NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>All genesa</td>
<td>2,996/14%</td>
<td>392/1.8%</td>
<td>326/1.5%</td>
<td>316/1.5%</td>
<td>285/1.3%</td>
<td>2,391/11%</td>
<td>318/1.5%</td>
<td>273/1.3%</td>
<td>235/1.1%</td>
<td>188/0.9%</td>
</tr>
<tr>
<td>TMc</td>
<td>191/10%</td>
<td>22/1.1%</td>
<td>21/1.1%</td>
<td>18/0.9%</td>
<td>17/0.9%</td>
<td>297/15%</td>
<td>62/3.2%</td>
<td>54/2.8%</td>
<td>26/1.3%</td>
<td>22/1.1%</td>
</tr>
<tr>
<td>TFd</td>
<td>638/32%</td>
<td>104/5.2%</td>
<td>91/4.6%</td>
<td>482/2.4%</td>
<td>42/2.1%</td>
<td>32/1.6%</td>
<td>2/0.1%</td>
<td>2/0.1%</td>
<td>2/0.1%</td>
<td>2/0.1%</td>
</tr>
<tr>
<td>Kc</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>252/67%</td>
<td>46/12%</td>
<td>42/11%</td>
<td>7/1.9%</td>
<td>4/1.1%</td>
</tr>
<tr>
<td>RLKf</td>
<td>1/0.1%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>386/63%</td>
<td>65/11%</td>
<td>63/10%</td>
<td>31/5.1%</td>
<td>23/3.8%</td>
</tr>
</tbody>
</table>

aPS, Arabidopsis genes with similar protein sequences found only in other plants. bSEN, Genes that are regulated during NS, DIS, or DET. cNS, Genes that are regulated during NS. dAO, Arabidopsis genes with similar protein sequences in bacteria, archae, cyanobacteria, and eukaryotes. eAll 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. TM, TPs and other membrane proteins with $\geq 3$ TM spans. Given is the number of TM genes in the respective category. Percentages relate to the 1,931 TM genes on the microarray. fTF, Putative TFs. Given is the number of TF genes in the respective category. Percentages relate to the 1,985 TF genes on the microarray. 

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 (75% 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 AtPUPs 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 phosphoryl 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; Rashotte 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.

The Arabidopsis CK-signaling pathway consists of CK perception by His protein kinases (AHKs) and activation of phosphoryl 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 phosphoryl 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 was also 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, AtaDOX1 encoding a fatty acid α-dioxgenase (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 (Le Bras 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
A gene that is strictly down-regulated in all senescence-associated CK genes is \textit{AtCOS1} (Fig. 6D, Supplemental Fig. 3Q). It encodes lumazine synthase, which catalyzes the penultimate step in riboflavin synthesis. It is suggested that \textit{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 \textit{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
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 AtEIBP1 are ET-responsive activators of GCC-box genes (Büttner and Singh, 1997; Fujimoto et al., 2000). The weakly up-regulated AtRCD1 modulates ET, ascorbic 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 (AtADOX1; 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 40 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 SAGI2 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 AtAMII1 converts in vitro indole-3-acetamide to IAA (Pollmann et al., 2003). As AtAMII1 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, AtTSAs1, AtTSUR2, 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 AtILR3 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 AtPDE1 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 exporters, and are probably involved in the proper polar distribution of auxin during plant development. AtPGP1 and AtPGP19 function in conjunction with TWISTED DWARF 1, supposedly as auxin exporters, and are putatively involved in auxin-induced aquaporine AtTIP1.1 localization 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. AtTIP1.1 is involved in auxin export (Xu et al., 1996). Studies on 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 Hutty, 1994) that is down-regulated, as many other intrinsic proteins too (Fig. 2). Intriguingly, the GA-responsive xylan glucan endotransglycosylase AtMER15 is massively up-regulated during senescence (Fig. 7D). AtMER15 and other xylan glucan 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 AtMER15 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 AtMER15 was shown to be positively and additively regulated by either GA or BR treatment (Kauschmann et al., 2005).
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 BRII1 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 acid 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 italiana; 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 1).

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 RNasey 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 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 Cy5-labeled 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 Cy5-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 hybridizations) to 1.00. The significantly regulated genes in both biological replicates were clustered by the k-means algorithm in seven groups based on their averaged log2-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/tuncat; 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) kinasen 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). TPs were compiled from DTAF (http://data.ctb.pku.edu.cn/), AITFDB (http://arabidopsis.med.ohio-state.edu/AITFDB/), and RARFT (http://rarge.gsc.riken.jp/rarft/). The complete list of (re)classified and partially renamed proteins is provided in Supplemental Table I.

ACKNOWLEDGMENTS

We thank Sonja Hetfeld and Carmen Zilmleimer for excellent technical work on the array hybridizations; Dr. Andreas Oberthür for granting us access to the Agilent scanner, nanodrop, and bioanalyser; Dr. Andreas Polten for technical support; and Dr. Christine Rausch for critical reading of the manuscript.

Received February 15, 2006; revised April 4, 2006; accepted April 5, 2006; published April 7, 2006.

LITERATURE CITED


van der Graaff et al.
different senescence-inducing factors in Arabidopsis. Plant Mol Biol 37: 445–454


Pollmann S, Neuf D, Weiler EW (2003) Molecular cloning and characterization of an amidase from Arabidopsis thaliana capable of converting indole-3-acetamide into the plant growth hormone, indole-3-acetic acid. Phytochemistry 62: 293–300


