Comprehensive Dissection of Spatiotemporal Metabolic Shifts in Primary, Secondary, and Lipid Metabolism during Developmental Senescence in Arabidopsis1[W]

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Developmental senescence is a coordinated physiological process in plants and is critical for nutrient redistribution from senescing leaves to newly formed sink organs, including young leaves and developing seeds. Progress has been made concerning the genes involved and the regulatory networks controlling senescence. The resulting complex metabolome changes during senescence have not been investigated in detail yet. Therefore, we conducted a comprehensive profiling of metabolites, including pigments, lipids, sugars, amino acids, organic acids, nutrient ions, and secondary metabolites, and determined approximately 260 metabolites at distinct stages in leaves and siliques during senescence in Arabidopsis (Arabidopsis thaliana). This provided an extensive catalog of metabolites and their spatiotemporal cobebehavior with progressing senescence. Comparison with silique data provides clues to source-sink relations. Furthermore, we analyzed the metabolite distribution within single leaves along the basipetal sink-source transition trajectory during senescence. Ceramides, lysolipids, aromatic amino acids, branched chain amino acids, and stress-induced amino acids accumulated, and an imbalance of asparagine/aspartate, glutamate/glutamine, and nutrient ions in the tip region of leaves was detected. Furthermore, the spatiotemporal distribution of tricarboxylic acid cycle intermediates was already changed in the presenescent leaves, and glucosinolates, raffinose, and galactinol accumulated in the base region of leaves with preceding senescence. These results are discussed in the context of current models of the metabolic shifts occurring during developmental and environmentally induced senescence. As senescence processes are correlated to crop yield, the metabolome data and the approach provided here can serve as a blueprint for the analysis of traits and conditions linking crop yield and senescence.

Senescence is a developmental process that ultimately leads to cell death (Gregersen et al., 2013). During developmental senescence, catabolic processes such as the degradation of chlorophylls, proteins, carbohydrates, lipids, and nucleic acids increase, while anabolic processes decrease. This metabolic shift is key for the mobilization and recycling of nutrients from senescing leaves to sinks, such as young leaves or developing seeds (Li et al., 2007). Nutrient mobilization during senescence has been reported in several plant species, including soybean (Glycine max), wheat (Triticum aestivum), and trees (Hill, 1980; Mauk and Nooden, 1992; Hocking, 1994; Nieminen and Helmisari, 1996). In Arabidopsis (Arabidopsis thaliana), the content of various elements, namely nitrogen, potassium, molybdenum, phosphorus, chromium, sulfur, iron, copper, zinc, and carbon, was reduced by more than 25% compared with the presenescent stage of development, indicating that they are mobilized at the onset of and during leaf senescence (Himelblau and Amasino, 2001). A large number of genes generally called senescence-associated genes (SAGs) and senescence down-regulated genes (SDGs) have been reported to be transcriptionally regulated during senescence in various plant species. This indicates that developmental senescence is a highly ordered and controlled process. SAGs encoding hydrolytic enzymes as well as transporters have been identified to be responsible for either the degradation or mobilization of the various macromolecules and breakdown products. In addition, many transcription factors are expressed that coordinate this complex developmental process (Nam, 1997; Lers et al., 1998; Thompson et al., 1998; Rubinstein, 2000; Bhalarao et al., 2003; Buchanan-Wollaston et al., 2003, 2005; Gepstein et al., 2003; Yoshida, 2003; Guo et al., 2004; Balazadeh et al., 2008). As well as developmental leaf senescence, adverse biotic or abiotic conditions can interfere with or induce senescence (Gregersen et al., 2013). Thus, complex physiological and molecular processes have to be properly integrated with developmental programs and respond to many environmental cues, such as nonoptimal

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temperature, drought, limited nutrient supply, pathogen attack, and unfavorable light conditions (Smart, 1994; Ono et al., 1996; Thomas and deVilliers, 1996; Crafts-Brandner et al., 1998; Saulescu et al., 2001).

The transport of metabolites from mature and senescing source leaves to sinks, such as young leaves or developing seeds, is one important aspect of developmentally or environmentally controlled senescence and has a massive bearing on crop yield and crop quality (for review, see Gregersen et al., 2013). In terms of the regulation of senescence, not only transcription factors and hormones but also altered plant source-sink relationships and the accumulation of some specific metabolites such as sugars and amino acids have been suggested to regulate the timing and developmental progression of leaf senescence (Noodén, 1980; Yoshida, 2003; van Doorn, 2004; Wingler et al., 2006). However, there is still little known about the involvement of metabolites in controlling senescence. For example, the induction of senescence by sugar accumulation, or conversely by sugar starvation, has long been a topic of debate (van Doorn, 2008; Wingler et al., 2009). A metabolic effect triggering leaf senescence has recently been shown through the detailed characterization of the onset of leaf death5 mutant, where a mutation in the quinolate synthase gene caused changes in pyrimidine nucleotide levels (Schippers et al., 2008). Such studies suggest that there is a mechanistic interaction between metabolism and the senescence process, but this is likely to be complex and remains to be fully resolved. Therefore, in order to consider the evidence of specific roles for metabolites in senescence, a more precise analysis of metabolite levels in different tissues and cell types during the process of senescence is required. Although, to date, a number of targeted metabolite profiles, particularly those focused on lipids as seed storage compounds (Yang and Ohlrogge, 2009; Seltmann et al., 2010) and sugars, amino acids, and nutrient ions (Masclaux et al., 2000; Quirino et al., 2001; Stessman et al., 2002; Diaz et al., 2005; Masclaux-Daubresse et al., 2005, 2007; Pourtau et al., 2006; Wingler et al., 2006, 2012), have been generated from maturing and senescing tissues, a broad overview of metabolic changes during senescence, including the interactions between various metabolic pathways, is still lacking. Therefore, we performed a metabolomics study to obtain comprehensive metabolite profiles, including primary and secondary metabolites and lipids, over the course of developmental senescence in rosette leaves before and after bolting and siliques of Arabidopsis in order to investigate the metabolic response and spatiotemporal distribution of metabolites during senescence at the whole-plant level and within single leaves. Furthermore, as senescence does not progress synchronously in all leaves of an Arabidopsis rosette but rather is correlated with the respective leaf age, we investigated detailed metabolic shifts during senescence within single leaves. In Arabidopsis, developmental senescence, visibly indicated by leaf yellowing, generally begins at the tip of the leaf and proceeds toward the leaf base and petiole (Lohman et al., 1994; Weaver et al., 1998). Similarly, nutrient mobilization has been observed to occur in a basipetal manner from tip to base (Jones and Eagles, 1962; Fellows and Geiger, 1974; Pitcher and Daie, 1991). In dicotyledonous plants, this sink-source transition begins at the tip of leaves that are 30% to 60% fully expanded. The transition has been imaged by using autoradiography (Jones and Eagles, 1962; Fellows and Geiger, 1974; Pitcher and Daie, 1991) or fluorescent tracers (Roberts et al., 1997; Imlau et al., 1999), which showed the unloading patterns of pho-toassimilates. Furthermore, comprehensive transcript profiling during the development of a distinct Arabidopsis leaf over a 3-week period to senescence has been reported (Breeze et al., 2011). As a result of transcriptional changes of many regulatory and catabolic genes, metabolites eventually need to be mobilized and transported to sink tissues. However, currently, detailed reports of metabolite profiles during developmental transitions within a single leaf are limited (Pick et al., 2011). Therefore, in addition to the analysis of whole leaves of different ages and various positions on the plants and of siliques as the major sink organ, we performed metabolite profiling of single defined leaves spatially dissected into three zones (tip, middle, and base) using leaves of three consecutive developmental stages. This allowed us to capture metabolic changes during the sink-source transition within a single leaf and to identify differences in metabolic patterns between presenescent and senescent tissues. This provides a spatiotemporal standard catalog of metabolites with which experiments affecting developmental processes, such as nutrient deficiency-induced senescence (Diaz et al., 2005; Watanabe et al., 2010; Obata and Fernie, 2012) or others, can be compared as well as marker metabolites for distinct senescence stages. The results are discussed in the context of current models of the metabolic shifts underlying developmental senescence.

RESULTS

Developmental Leaf Senescence in Arabidopsis

For the analysis of developmental leaf senescence, Arabidopsis plants were grown on soil under long-day conditions. Rosette leaves (leaf nos. 1–12), which appeared before bolting, upper leaves (leaf nos. 13–41), which developed as rosette leaves after bolting, and siliques with developing seeds were harvested at various developmental stages (Fig. 1A). As leaf yellowing is a typical visible symptom of leaf senescence, we determined the extent of chlorophyll loss to define the stages of senescence. In a first approach, the numbers of green and yellowish rosette leaves were counted to ascertain the percentage of leaves that were senescent. Following this diagnostic survey, plants were harvested at four different stages: 0%, 20%, 50%,
and 100% senescent. Rosette leaves (RL), upper leaves (UL), and siliques (SI) were harvested at four, three, and two developmental time points (RL1–RL4, UL2–UL4, and SI3 and SI4), respectively (Fig. 1A). Total chlorophyll levels in rosette leaves decreased with the progression of senescence and correlated with the above-mentioned color score (Fig. 1B). Upper leaves also displayed decreased chlorophyll contents over time in a manner similar to rosette leaves, although the timing was delayed. In addition to the loss of chlorophyll, the transcript abundance of SDGs and SAGs was analyzed to aid in the classification of leaf senescence stages. Two SDGs encoding chlorophyll a/b-binding protein1 (CAB1) and ribulose bisphosphate carboxylase small subunit1A (RBCS1A), as well as three SAGs encoding SAG12 (Cys-type peptidase), SAG21 (AtLEA5; for late embryogenesis abundant-like protein), and senescence-associated protein1 (SEN1; for dark inducible1, DIN1), which are commonly used as leaf senescence marker genes in Arabidopsis, and the three transcription factors WRKY53 (Miao et al., 2004), ANAC029 (Guo and Gan, 2006), and ANAC092 (Kim et al. 2009; Balazadeh et al., 2010), which are known to regulate senescence, were analyzed (Fig. 1, C and D). Consistent with the loss in chlorophyll, the transcript abundance of SDGs, CAB1 and RBCS1A, continuously decreased throughout leaf senescence, while the expression levels of SAGs increased. The highest transcript abundance of SAGs was observed at stages RL2 (WRKY53) or RL3 (SAG12, SAG21, SEN1, ANAC029, and ANAC092) in rosette leaves. In upper leaves, almost all gene transcripts reached maximal abundance at UL4, with the exception of the constant high-level expression of WRKY53 between UL2 and UL4. Thus, the transcriptional regulation of these SAGs follows the expected pattern and correlates to the chlorophyll data and corroborates the visual scoring of senescence stages.

Alteration in Lipophilic, Primary, and Secondary Metabolites during Leaf Senescence

In order to investigate metabolic changes, including the onset of metabolite remobilization, during developmental senescence in rosette leaves, upper leaves, and siliques, profiling of lipophilic metabolites by ultra-performance liquid chromatography (UPLC)-mass spectrometry (MS; Giavalisco et al., 2011), primary

Figure 1. Developmental leaf senescence in Arabidopsis. A, Senescence stages of Arabidopsis plants during the experiment. Rosette leaves (leaf nos. 1–12) at four developmental stages (stages 1–4), upper leaves (leaf nos. 13–41) at three stages (stages 2–4), and siliques containing developing seeds at two stages (stages 3 and 4) were harvested. B, Chlorophyll changes though leaf senescence. Data represent mean values of five biological replicates for each time point. F.W., Fresh weight. C and D, Transcript abundance of SDGs and SAGs. Expression levels of SDGs (C), CAB and RBCS1A, and SAGs (D), SAG12, SAG21, SEN1, ANAC029, ANAC092, and WRKY53, were measured by qRT-PCR. Transcript abundance of the genes in rosette leaves and upper leaves is presented as mean fold changes from RL1. Expression values were normalized to relative levels of PDF2. Data represent mean values of two biological replicates for each time point, each measured in two technical replicates. Error bars in B to D represent so. Different letters represent statistically significant differences (P < 0.05) using Tukey’s test.
metabolites by HPLC and gas chromatography (GC)-MS (Lisee et al., 2006; Erban et al., 2007), and secondary metabolites by liquid chromatography (LC)-MS (Tohge and Fernie, 2010) was performed (Fig. 2). Senescence-dependent metabolic changes are shown in false color by log2 fold changes of metabolite levels, with each sample being normalized to RL1; we referenced metabolite concentrations of the various developmental stages and tissues (rosette leaves, upper leaves, and siliques) to one stage (RL1) to allow for a better comparison and visualization of the measured differences. In addition, to identify metabolic changes between different developmental stages of a tissue, we referenced metabolite contents in rosette leaves, upper leaves, and siliques to the values of RL1, UL2, and SL3, respectively (Supplemental Table S1). In both rosette and upper leaves, a reduction of the chlorophyll a/b ratio was apparent during senescence as a consequence of large reductions in chlorophyll a. Other pigments, such as the magnesium-free chlorophyll pigment pheophytin and the xanthophyll cycle pigment violaxanthin, were also decreased. Loss of leaf chlorophyll content was associated with a decline in most chloroplast-localized lipid species, such as the monogalactosyldiacylglycerols (MGDGs) and digalactosyldiacylglycerols (DGDGs), as well as the phosphatidylglycerols (PGs) and minor chloroplast lipids such as sulfoquinovosyldiacylglycerols. There was also a decline in 12-oxo-phytodienoic acid (OPDA)-containing galactolipids (OPDA-MGDGs, -DGDGs, and -PGs). Some phospholipids, phosphatidylethanolamines, and phosphatidylcholines displayed a mixed pattern of changes (i.e. some increased while others decreased). Most other lipid species, for example diacylglycerols, triacylglycerides (TAGs), ceramides, glucosylceramides, and lysolipids such as lysophosphatidylethanolamines, lysophosphatidylcholines, and the quinone molecules plastoquinone and ubiquinone continuously increased during leaf senescence. Similarly, almost all of the metabolites associated with sugar metabolism, such as the monosaccharides Fru, Glc, and Xyl and the disaccharides Suc, trehalose, and maltose, continuously increased. In contrast, a slight reduction of the starch content was observed during the progression of senescence. Alongside the increase of sugars during leaf senescence, sugar derivatives such as sugar alcohols (galactitol, sorbitol, erythritol, and arabitol) and aldonic acids (gluconic acid and galactonic acid) were increased.

Despite the constant increase in sugars, tricarboxylic acid (TCA) cycle intermediates displayed a rather mixed pattern. Regarding amino acid metabolism, the most remarkable increases were observed in the branched chain amino acids (BCAAs) Ile, Val, and Leu and in the aromatic amino acids (AAAs) Trp, Tyr, and Phe, most likely reflecting their well-defined role as alternative respiratory substrates under stress conditions, including dark-induced senescence (Araujo et al., 2010, 2011). Significant increases were observed for other amino acids and their derivatives. For example, Thr, Lys, His, Cys, O-acetyl-serine (OAS), Asn, β-Ala, γ-aminobutyric acid (GABA), and 4-Hyp all increased with the progression of senescence, whereas Gln, Ala, and pyro-Glu displayed a biphasic pattern in response to senescence in rosette leaves, decreasing at the second or third stage but increasing at the fourth stage. Glu, Asp, and Arg contents also showed a biphasic change in rosette leaves, but increases at the fourth stage were not significant. In spite of the increase of the sulfur-containing metabolite Cys and its immediate precursor OAS, their precursors Ser and in particular Gly were highly decreased at the second developmental stage in both rosette leaves and upper leaves. Furthermore, glutathione (GSH), glyceralic acid, Met, Met-derived-glucosinolates (GLSs; 4-methylsulfinylbutyl-glucosinolate [4MSOB], 5-methylsulfinylpentyl-GLS, 6-methylsulfinylhexyl-GLS, 7-methylsulfinylheptyl-glucosinolate, 8-methylsulfinyloctyl-glucosinolate, and 4-methylthiobutyl-glucosinolate [4MTB]), and ascorbic acid also displayed biphasic patterns, with a maximum reduction at RL2 or RL3 in rosette leaves and at UL4 in the upper leaves. Further secondary metabolites, including anthocyanins and indole-derived GLSs (1-methoxyindol-3-ylmethyl-glucosinolate [1MI3M] and 4-methoxyindol-3-ylmethyl-glucosinolate [4MI3M]), largely increased; however, indolyl-3-methyl indole-glucosinolate (IM), flavonol glycosides, and sinapate conjugates decreased continuously throughout leaf senescence.

The nutrient ion sulfate (SO4²⁻) was increased, but nitrate (NO3⁻) and phosphate (PO4³⁻) were decreased, during the senescence process. These changes of nutrient ions are consistent with a previous report by Diaz et al. (2005). The total soluble protein content generally decreased during senescence, exhibiting a maximal reduction at stage RL3.

**Metabolites in Siliques Containing Developing Seeds**

Source-sink balances in plants have long been suggested to regulate leaf senescence (Noodén, 1980; Yoshida, 2003; van Doorn, 2004; Wingler et al., 2006), with the developing siliques being proposed as a key sink organ that “draws” nutrients from vegetative (source) organs in order to ensure the reproductive success of the plant (Robinson and Hill, 1999). Figure 2 displays the changes in metabolite levels on a fresh weight basis in siliques during plant developmental senescence. Some metabolite concentrations in siliques containing developing seeds were significantly different from those in leaves. For example, high accumulation of Met-GLSs and flavonols, whose production is regulated in a tissue-specific manner (Matsuda et al., 2010), was observed in siliques compared with leaves (Fig. 2B). The main storage lipids in seeds, TAGs, also showed much higher accumulation in siliques than in senescent leaves, whereas TAGs also accumulated (Fig. 2A). In order to investigate metabolic changes during siliques development, the ratio of...
Figure 2. Heat map of metabolite changes through plant senescence. Lipids, pigments, and quinones (A) and primary and secondary metabolites (B) are displayed on a metabolic pathway representation. Log_2 ratios of fold changes from RL1 are given by shades of red or blue colors according to the scale bar. Data represent mean values of three to five biological replicates for each tissue and time point. Statistical analysis was performed using Tukey’s test (Supplemental Table S3). Abbreviations not already defined are as follows: Cer, ceramide; Chl, chlorophyll; DAG, diacylglycerol; GlcCer, glucosylceramide; flavonols [K-3RG-7R, K-3G-7R, K-3R-7R, and Q-3RG-7R, for kaempferol 3-O-rhamnosyl(1→2)glucoside-7-O-rhamnoside, kaempferol 3-O-glucoside-7-O-rhamnoside, kaempferol 3-O-rhamnoside-7-O-rhamnoside, and quercetin 3-O-glucoside-7-O-rhamnoside, respectively]; Met-GLSs (5MSOP, 6MSOH, 7MSOH, and 8MSOO, for 5-methylsulfinylpentyl-GLS, 6-methylsulflylhexyl-GLS,
metabolite levels between SI3 and SI4 (SI4/SI3) was calculated (Table 1; Supplemental Table S1). Only 12 metabolites increased by more than 2-fold between these silique stages: maltose, sorbitol, galactinol, TAGs (two out of a total of 38 species), 4MTB, 1MI3M, Ile, and OPDA-containing galactolipids (four out of a total of nine species; Table I).

### Metabolic Changes across a Single Leaf during Development and Early Senescence

In addition to the mobilization of nutrients from senescent leaves to young leaves or developing seeds, mobilization also occurs within a single leaf across the sink-source transition. Therefore, in order to obtain a clear picture of metabolic changes within a single leaf during senescence, single leaves were investigated at different senescence stages after separation into three parts each (Fig. 3A). Here, we used plants cultivated under short-day conditions to eliminate effects that might otherwise be caused by the transition to flowering. Individual leaves (13th or 14th leaf emerging after germination) of Arabidopsis were harvested at three stages from leaf expansion to early senescence: stage 1 leaves were 50% expanded, stage 2 leaves were fully expanded, and stage 3 leaves showed 10% to 20% senescence on a leaf area basis. Upon harvesting, the leaf was divided into three parts of equivalent lengths, termed tip (T), middle (M), and base (B; Fig. 3A). To evaluate senescence stages in the harvested samples, total chlorophyll content and transcript accumulation of the SAG12 gene were analyzed (Fig. 3B). A significant decrease of chlorophyll content was observed at T3 compared with M3 and B3, indicating a chlorophyll gradient from tip to base. Furthermore, a clear elevation of SAG12 expression according to the senescence stages of samples was observed at T3 compared with M3 and B3.

As metabolite changes on a fresh weight basis during the sink-source transition were very complex, several calculations were conducted for proper evaluation. The respective figures were focused on spatio-temporal metabolite changes during leaf development (Fig. 3, C and D), early senescence (Supplemental Fig. S1), and metabolite distributions within a single leaf (Fig. 4). Normalization was done specifically for the various data set comparisons. For comparison 1 (changes during leaf development; Fig. 3, C and D), log$_2$ ratios of fold changes from the average at stage 1 (the average value of T1, M1, and B1) were calculated. For comparison 2 (changes during early leaf senescence; Supplemental Fig. S1), log$_2$ ratios of fold changes from the average at stage 2 (the average value of T2, M2, and B2) were calculated. For comparison 3 (metabolite distributions; Fig. 4), log$_2$ ratios of fold changes from the average values of all parts at each stage were calculated (e.g. T1/the average of T1, M1, and B1, T2/the average of T2, M2, and B2). Due to the large number of possible comparisons within this data set, we chose to highlight some representative examples in the histograms of Figure 5.

Changes of lipophilic metabolites during leaf expansion (Fig. 3C) and early senescence (Supplemental Fig. S1A) in single leaves were similar to the changes reported above for leaf senescence in the whole-plant experiment (Fig. 2A). Chloroplast lipid species such as MGDGs, DGDGs, PGs, and sulfoquinovosyldiacylglycerols decreased during leaf expansion (Fig. 3C), although not as strongly as the developing chlorophyll, especially at T3, suggested (Supplemental Fig. S1A), while ceramides, glucosylceramides, lysolipids, and quinones increased during leaf expansion (Fig. 3C) and early senescence (Supplemental Fig. S1A). Some phosphatidylcholines and TAGs, which displayed reduced levels in the second or third stage senescent leaves in the whole-plant analyses (Fig. 2A), also decreased in single leaves during early senescence (Supplemental Fig. S1A). When the metabolite distributions within a single leaf at the first stage were assessed (Fig. 4A), MGDGs, DGDGs, and OPDA-containing galactolipids behaved like chlorophyll in showing high accumulation at T1. However, TAGs showed the opposite pattern, with low accumulation in T1.

When comparing changes in primary and secondary metabolite levels between the whole-plant experiment (Fig. 2B) and the single-leaf experiment (Fig. 3D), the majority of the metabolites showed similar changes. However, there was one prominent exception, since sugar metabolism behaved quite distinctly. Figure 3D

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Table I. Accumulation of metabolites in silique.

Metabolites in siliques that were 2-fold higher at SI4 compared with SI3 are shown. For abbreviations of metabolite names, see Figure 2.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>SI4/SI3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>5.5</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>4.7</td>
</tr>
<tr>
<td>Galactinol</td>
<td>4.1</td>
</tr>
<tr>
<td>1MI3M_Indole-GLS</td>
<td>3.1</td>
</tr>
<tr>
<td>OPDA/16:3 PG</td>
<td>2.7</td>
</tr>
<tr>
<td>TAG 50:4</td>
<td>2.5</td>
</tr>
<tr>
<td>OPDA/18:2 DGDG</td>
<td>2.5</td>
</tr>
<tr>
<td>4MTB_Met-GLS</td>
<td>2.4</td>
</tr>
<tr>
<td>OPDA/OPDA DGDG</td>
<td>2.3</td>
</tr>
<tr>
<td>TAG 58:1</td>
<td>2.1</td>
</tr>
<tr>
<td>Ile</td>
<td>2.0</td>
</tr>
<tr>
<td>OPDA/16:3 MGDG</td>
<td>MGDG 18:3/dnOPDA</td>
</tr>
</tbody>
</table>

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Figure 2. (Continued.) 7-methylsulfinylheptyl-GLS, and 8-methylsulfinyloctyl-GLS, respectively; PC, phosphatidylcholine; PE, phosphatidyethanolamine; Sin-Glc, sinapoyl-Glc; Sin-Mal, sinapoyl-malate; SQDG, sulfoquinovosyldiacylglycerol.
Figure 3. Heat map of metabolite changes during leaf expansion and early senescence. A, Schematic representation of samples used for metabolite profiling. Plants were grown under short-day conditions. A single leaf (13th or 14th leaf emerging after germination) was harvested at three developmental stages. Upon harvesting, the leaf was divided into three parts of equivalent lengths termed tip (T), middle (M), and base (B). B, SAG12 expression level. Transcript abundance of the gene is presented as...
documents that Glc, Fru, Xyl, Ara, and maltose contents were decreased during leaf expansion; however, during early senescence, Fru and to a lesser extent Glc and maltose started to increase at T3 (Fig. 5A; Supplemental Fig. S1B). Suc levels were increased at T3 but displayed no reduction during leaf expansion (Fig. 5A). Other metabolites in sugar metabolism (i.e. trehalose and arabinose) were highly increased during expansion but unaltered at T3 (Fig. 3D; Supplemental Fig. S1B). Within sugar metabolism, galactinol and raffinose displayed a rather unique pattern of high accumulation at B2 and B3, in contrast to the high accumulation of other sugar metabolites in the tip (Fig. 5B).

For amino acids, complex accumulation patterns were observed. During early senescence, significant increases at T3 were observed in the three BCAAs (Leu, Ile, and Val) and two of the three AAAs (Trp and Phe; Fig. 5, C and D); however, the BCAAs and AAAs showed different changes in the second stage. The three BCAAs showed similar patterns to each other with no difference in distributions between each leaf part at the second stage, but the AAAs Trp and Phe already started increasing at T2, while Tyr increased during expansion but was unaltered during early senescence. A similar pattern of increase to that observed for Trp and Phe was also observed for the stress response amino acids β-Ala and Pro (Fig. 5E). By contrast, significant decreases during expansion and early senescence were observed for Met-GLSs (Fig. 3D; Supplemental Fig. S1B). Figure 5F shows one of the Met-GLSs, 4MSOB. The levels of all other Met-GLSs displayed a similar pattern to that of 4MSOB, with the exception of 7-methylsulfanylheptyl-GLS, which showed a slight increase at the third stage. Changes in kaempferol glycosides showed similarity to those of the Met-GLSs, with the exception that they displayed a smaller reduction at T3 (Fig. 3D; Supplemental Fig. S1B). Moreover, indole-GLSs displayed mixed patterns of changes in metabolite levels on a fresh weight basis (Fig. 3D). However, the two indole-GLSs (1MI3M and 4MI3M) displayed a specific distribution with a high accumulation in the base of the leaf (Figs. 4B and 5G). Particularly, 1MI3M resembled the pattern of accumulation of galactinol and raffinose (Fig. 5, B and G).

A comparison of the levels of the TCA cycle intermediates between stages revealed complex changes (Fig. 5H), perhaps suggestive of the cycle operating in different modes (Sweetlove et al., 2010) during the progression of senescence. However, when comparing their distributions within a single leaf, they displayed similar distributions to one another at the second and third stages (Figs. 4B and 5H), being of high abundance in the leaf tip and of low abundance in the leaf base. 2-Oxoglutaric acid and succinic acid displayed unique changes, exhibiting decreased levels in the leaf base and increased levels in the leaf tip at the second and third stages when compared with the corresponding leaf parts at the first stage of development. The spatially dependent levels of nutrient ions are shown in Figure 5I. Sulfate and nitrate were increased across the leaf gradient, while phosphate was decreased. That said, distinct changes in sulfate and nitrate were observed between stages. With increasing senescence, sulfate accumulated at the tip region and stayed constant at the base, nitrate levels progressively accumulated at the leaf base, and phosphate progressively declined in all stages, especially in the tip region, indicating mobilization and reallocation of nitrate and phosphate. The amino acids Gln and Glu, as well as Asn and Asp, showed opposite accumulation patterns at various stages and leaf parts (Fig. 5I). Furthermore, the Asn/Asp ratio increased from the first to the third stage, and the Gln/Glu ratio increased in the leaf tip of stage 3. Thus, with increasing age, the nitrogen-rich amino acids Asn and Gln accumulated, with a concomitant decrease of Asp and Glu, respectively.

**Overview of Metabolite Changes during Plant Senescence**

In order to obtain a global view of the metabolic changes during plant senescence, principal component analysis (PCA) of the approximately 260 annotated metabolites detected in the whole-plant and single-leaf experiments was conducted (Fig. 6). In the whole-plant experiment (Fig. 6A), the first principal component (PC1), accounting for 47% of the total variance, resolved the tissue specificities between leaves and siliques. The metabolites in silique tissues were completely separated from those of rosette leaves and upper leaves. The second principal component (PC2), accounting for 28% of the total variance, resolved the time series of leaf senescence. Metabolite changes in the upper leaves showed similar patterns to those in rosette leaves, despite a shift in their onset of change corresponding to standard senescence markers such as chlorophyll or SAGs. In the single-leaf experiment (Fig. 6B), PC1, accounting for 62% of the total variance, resolved the time...

**Figure 3.** (Continued.)

Mean fold changes from B1. Expression values were normalized to relative levels of PDF2. Data represent mean values of two biological replicates for each time point, each measured in two technical replicates. Error bars represent SD. Different letters represent statistically significant differences (P < 0.05) using Tukey’s test. F.W., Fresh weight. C and D, Lipids, pigments, and quinones (C) and primary and secondary metabolites (D) displayed on a metabolic pathway representation. Log, ratios of fold changes from the average at the first stage (the average value of T1, M1, and B1) are given by shades of red or blue colors according to the scale bar. Data represent mean values of three to five biological replicates for each time point. Statistical analysis was performed using Tukey’s test (Supplemental Table S4). ND, Not determined. For abbreviations of metabolite names, see Figure 2.
series of leaf development and early senescence. In particular, the metabolites of the first stage were resolved from the other stages in PC1. PC2, accounting for 12% of the total variance, resolved the physiological phase changes, displaying earlier senescence in the tips of leaves and a basipetal progression of senescence. At the third stage, the senescent tip part (T3) was clearly separated from middle (M3) and base (B3) parts by this phase changes, displaying earlier senescence in the tips of leaves and a basipetal progression of senescence. At the third stage, the senescent tip part (T3) was clearly separated from middle (M3) and base (B3) parts by this
component. Thus, PCA corroborates gross changes between various stages and displays sequential changes indicating coordinated processes.

**DISCUSSION**

Leaf senescence is an essential developmental process accompanied by changes in the expression of thousands of genes and many metabolic and signaling pathways (Breeze et al., 2011). Although many SDGs and SAGs have been characterized (Miao et al., 2004; Guo and Gan, 2006; Kim et al., 2009; Balazadeh et al., 2010), there is little information on the distinctive spatiotemporal metabolic changes occurring during the senescence process. In order to study senescence-associated metabolites and metabolic remobilization during senescence, profiling of metabolites, including pigments, lipids, sugars, amino acids, organic acids, and secondary metabolites (in total, approximately 260 annotated metabolites), was performed using samples obtained from leaves and siliques at different stages of development.

![Figure 5](image-url)

**Figure 5.** Changes of the selected metabolites in the tip (T), middle (M), and base (B) regions of single leaves during expansion and early senescence. A, Sugars. B, Galactinol and raffinose. C, BCAAs. D, AAAs. E, Stress response amino acids (AAs). F, Met-GLS. G, Indole-GLSs. H, TCA cycle metabolites. I, Nutrient ions. J, Gln, Glu, Asn, and Asp. Peak height for A, B, E, and H in GC-TOF-MS analyses and peak area for F and G in LC-electrospray ionization-MS analyses were normalized to sample fresh weight. The unit for C, D, and J in HPLC analyses is nmol g⁻¹ fresh weight (FW). The unit for I in ion analysis is μmol g⁻¹ fresh weight. Data represent mean values of three to five bulked samples for each leaf region at each time point. Error bars represent SD. Different letters represent statistically significant differences (P < 0.05) using Tukey’s test (Supplemental Table S4). For abbreviations of metabolite names, see Figure 2.
senescence stages in whole-plant and single-leaf studies throughout the progression of senescence.

Specific Spatiotemporal Changes of Chloroplast Lipids, Ceramides, and Storage Lipids in Leaves during Developmental Senescence

The decrease of chloroplast lipids (MGDGs, DGDGs, and PGs) has been used as a senescence marker for a long time because this decrease is highly correlated with chlorophyll loss in senescent leaves (Ferguson and Simon, 1973; Fong and Heath, 1977; Koiwai et al., 1981; Harwood et al., 1982; Yamauchi et al., 1986; Wanner et al., 1991). Comprehensive transcriptome analysis of a "single" leaf during developmental senescence also revealed that chlorophyll biosynthesis and photosynthesis genes were down-regulated and chlorophyll degradation genes were up-regulated orderly during leaf development and senescence (Breeze et al., 2011). The results reported here are consistent with these studies, since a continuous decrease of chloroplast lipids was observed during leaf development and senescence in both whole-plant and single-leaf experiments (Figs. 2A and 3C). Within single leaves, however, no gradient of these lipids along the basipetal axis (tip to base) was established (Fig. 4A), not even between yellowing and still green parts at later developmental stages irrespective of the established chlorophyll gradient (Fig. 3B). This result indicates that the decrease of chloroplast lipids occurs across the whole leaf and precedes the actual decrease of chlorophyll that results in leaf yellowing. Whether it is due to reduced production or increased degradation cannot be deduced here, but as the CAB genes also are concomitantly reduced, excess chlorophyll cannot be bound and protected and thus is degraded (Höfgen et al., 1994). The decrease of chloroplast lipids was primarily age dependent rather than due to chlorophyll loss per se.

In contrast to the insignificant change of chloroplast lipids in T3, a significant increase of ceramides and lysolipids was observed in the T3 samples (i.e. the tip of senescing leaves; Fig. 3C), suggesting that the specific accumulation of these lipids was senescence dependent and, hence, provides a diagnostic marker. Reports that ceramide induces programmed cell death in Arabidopsis (Leipelt et al., 2001; Liang et al., 2003) and that lysolipids can activate phospholipase A, which generates fatty acids recognized as second messengers in the senescence process (Paul et al., 1998), support the importance of a rapid increase of these lipids in the yellowing regions for mediating the progression from senescence to cell death. Similar to the changes in ceramides and lysolipids, plastoquinone and ubiquinone also showed a senescence-dependent increase (Figs. 2A and 3C). These results fit well within the context of leaf senescence, since the redox states of these species have been reported to be involved in the protection of plant cells against reactive oxygen species that are generated by lipid degradation (Alleva et al., 2001; Bhattacharjee, 2005). TAGs are known to be the major storage lipids that accumulate in developing seeds, flower petals, pollen grains, fruits, and senescent leaves (Stymne and Stobart, 1987; Kaup et al., 2002; Murphy, 2005). The accumulation of TAGs during leaf senescence was consistent with the results of this study (Figs. 2A and 3C) and has been reported to be likely involved in the mobilization of membrane lipid carbon to phloem-mobile Suc (Kaup et al., 2002). The distributions of TAGs in green single leaves at the first stage clearly showed an opposite pattern compared with chlorophyll or other chloroplast lipids in the single-leaf experiment (Fig. 4A). Low contents of TAGs in greener tip regions (T1) with high chlorophyll contents at the first stage (Fig. 4A) might be caused by a high proportion of recycling of fatty acids for energy and membrane synthesis.

Metabolism of Amino Acids and Mineral Nutrients during Developmental Senescence

While chlorophyll contents decreased during leaf senescence, we observed an increase of the contents of
the three BCAAs and two AAAs (Phe and Trp, not Tyr; Figs. 2B and 3D). A dramatic increase of AAAs and BCAAs was also observed during dark-induced senescence (Gibon et al., 2006; Fahnenstich et al., 2007; Araújo et al., 2010, 2011) and senescence promoted by nitrate limitation (Diaz et al., 2005). The accumulation of BCAAs has been proposed to be critical to support alternative respiration under dark-induced senescence (Araújo et al., 2010, 2011), while the accumulation of AAAs has been suggested to be an important source of precursors to support the synthesis of a large number of secondary metabolites including flavonoids for protecting cells during senescence (Radwanski and Last, 1995; Wittstock and Halkier, 2002; Pichersky et al., 2006; Korkina, 2007). This was consistent with the result in the whole-plant experiment, where the accumulation of anthocyanin contents in addition to Phe was observed during leaf senescence (Fig. 2B). Usually, this increase in amino acid content has been thought to be caused by protein degradation. However, steady-state levels of the amino acids might depend not only on the rate of their release from protein degradation but also on the relative rate of their anabolism and catabolism and likely also is a function of their rate of transport. During mineral nutrient deficiency-induced senescence, a common phenotype was the perturbation of amino acid metabolism accompanied by a loss of chlorophyll and protein contents; however, the pattern of amino acid changes differed between respective nutrient-deficient conditions, for example, sulfate, nitrate, and phosphate deficiency (Watanabe et al., 2010). Indeed, the accumulation of BCAAs and AAAs was not always observed in plants under different nutrient deficiencies (Obata and Fernie, 2012). This suggests that the flux and transport of these amino acids may vary under nutrient stress conditions in order to compensate each affected metabolic pathway for the specific nutrient deficiency that it faces.

Pro, β-Ala, and GABA are often documented to be stress-induced osmoprotectants (Skirycz et al., 2010; Obata and Fernie, 2012). Their high accumulations in the leaf tip region (Fig. 5E) were in sharp contrast to the high accumulations of galactinol and raffinose in the base region, which are also stress-induced osmoprotectants (Fig. 5B). This observation may suggest that Pro, β-Ala, and GABA function as osmoprotectants in the exact senescent or stressed region; however, further experimentation will be needed to confirm this hypothesis. Asn and Gln are major long-distance transport forms of both carbon and nitrogen and, as such, the most abundant amino acids in xylem and phloem (Lea and Miflin, 1980; Urquhart and Joy, 1981). Gln, Glu, Asn, and Asp and their conversion products have been long documented to be key for nitrogen transport. An increase of Gln/Glu and Asn/Asp ratios was observed here, both in the whole-plant and single-leaf experiments (Fig. 5; Supplemental Fig. S2, A and B), despite the fact that the absolute concentrations of Gln, Glu, Asn, and Asp themselves decreased in senescent leaves in comparison with RL1 in the whole-leaf experiment (Fig. 2B). Thus, during the senescence process, their rates of interconversion appear to be more tightly regulated than their concentrations in the plant, especially as export to the sinks can be expected. With increasing senescence, we observed a decrease of nitrate toward the tip of the leaf, with a concomitant increase at the base (Fig. 5I). Phosphate showed a similar behavior to nitrate, but additionally, total amounts were reduced, suggesting active export already at these stages (Fig. 5I). The whole-plant experiment suggests an export of nitrate and phosphate at later stages of senescence (Fig. 2B). However, sulfate contents rather displayed an increase during senescence, especially toward the leaf tip in the single-leaf experiment (Fig. 5I) and also at the whole-plant level (Fig. 2B). This observation is not without precedence, since a negative correlation between nitrate and sulfate contents has previously been reported (Blomzandstra and Lampe, 1983; Diaz et al., 2005). The interaction between nutrients for uptake and the imbalance caused by the deficiency of one or more minerals are well described (Marschner and Marschner, 1995; Kant et al., 2011). The continuous decrease of nitrate in whole-plant senescence and the low levels of this ion in yellow tip regions, most progressed in senescence in single leaves, could be caused by the reduction of nitrate to ammonium, as is required for conversions of Glu and Asp to Gln and Asn, respectively, or its transport to sinks. The decreases of nitrate and phosphate and the increase of sulfate during senescence are consistent with their general nutrient mobilities, with nitrogen and phosphorus reported to be highly mobile nutrients whereas sulfur is relatively immobile (Bennett, 1993; Marschner et al., 1996). This is known from the fact that sulfur deficiency causes head chlorosis of young leaves as existing sulfate is not sufficiently mobilized, while nitrogen starvation causes chlorosis of old leaves and green young leaves due to efficient mobilization and transport. This, though, is in stark contrast to the ability of Arabidopsis to mobilize sulfate during sulfate starvation (Nikiforova et al., 2003, 2004) and suggests distinct processes during nutrient starvation and developmental senescence.

The ratio of Gly to Ser is used as a classical indicator of photosynthetic activity (Wingler et al., 1999, 2000). It has furthermore been suggested to be a possible senescence marker, since early-senescing recombinant inbred lines selected from the Arabidopsis Bay-0 × Shahdara recombinant inbred line population displayed a considerably higher Gly/Ser ratio than late-senescing recombinant inbred lines (Diaz et al., 2005). In this study, opposing changes in Gly and Ser levels were observed between the whole-plant (wherein they decreased; Fig. 2B) and single-leaf (wherein they increased; Fig. 3D) experiments during leaf senescence. However, the Gly/Ser ratio displayed a similar pattern in both instances (Supplemental Fig. S2C), namely, a higher ratio at RL1 in the whole-leaf experiment and in
all regions of leaves at the first stage in the single-leaf experiment, indicating higher photosynthetic activity in younger vegetative leaves paralleling high photosynthetic activity. This observation is consistent with the theory that photosynthesis plays a key protective role with respect to the photosynthetic apparatus. A high Gly/Ser ratio was also observed at T3, where photosynthesis activity is low due to senescence in the single leaf. It is conceivable that this might be caused by some stress response such as water loss in leaves during the senescence process, since photosynthesis has been reported to play a beneficial role during stresses such as heat and drought (Osmond and Grace, 1995; Wingler et al., 2000; Foyer and Noctor, 2009). However, a direct support for this hypothesis in the context of our results is currently lacking. That said, a relationship between senescence and photosynthesis has been suggested in several studies of photosynthesis-deficient mutants (Somerville, 1986; Blackwell et al., 1988; Leegood et al., 1995). However, differences in light conditions (long days in the whole-plant experiment and short days in the single-leaf experiment) for plant growth are another possible explanation for the differences in Gly and Ser levels observed here. The biosynthesis and conversion of Gly and Ser are highly affected by light (Ho et al., 1999a, 1999b). These opposite changes in absolute sugar levels between experiments (Figs. 2B and 3D) were also observed in one of the detected phenylpropanoids (i.e. sinapoyl-Glc). Interestingly, the biosynthesis of this secondary metabolite has been documented as being light regulated (Strack et al., 1986).

**Carbon Metabolism during Developmental Senescence**

Sugars have been discussed as positive regulators of plant senescence (Rolland et al., 2002). However, their roles in senescence remain controversial (Noodén, 1980; Yoshida, 2003; van Doorn, 2004, 2008; Wingler et al., 2006, 2009), since both sugar starvation and addition can induce SAG expression (Gan and Amasino, 1997; Fujiki et al., 2000, 2001; van Doorn, 2004). Furthermore, although the decline of photosynthesis that occurs during senescence should lead to a decrease of sugar levels, an increase of sugars during senescence was reported in several plant species, including Arabidopsis (Masclaux et al., 2000; Quirino et al., 2001; Stessman et al., 2002; Diaz et al., 2005; Masclaux-Daubresse et al., 2005, 2007; Pourtau et al., 2006; Wingler et al., 2006, 2012). These data are in accordance with the results obtained here in the whole-plant experiment, where we observed an increase of sugar levels in leaves during developmental senescence (Fig. 2B). Of particular interest might be the accumulation of trehalose during leaf senescence. In plants, trehalose is produced in a two-step biochemical reaction involving trehalose 6-phosphate (T6P) synthase, which converts UDP-Glc and Glc-6-P to T6P, and T6P phosphatase, which hydrolyzes T6P to trehalose (Eastmond and Graham, 2003; Paul et al., 2008; Schluepmann and Paul, 2009). T6P has been identified as a signaling molecule for high carbon availability and to be involved in the regulation of a number of developmental processes in plants (Paul et al., 2008; Schluepmann et al., 2012). Of note, a strong (approximately 30-fold) increase of T6P content was recently reported during leaf development and senescence in Arabidopsis leaves; overexpressing a bacterial T6P phosphatase to reduce T6P level in transgenic plants was accompanied by a delay of senescence (Wingler et al., 2012). Sugar levels (Glc, Fru, and to some extent Suc) increased during senescence in both the Columbia-0 wild type and T6P phosphatase overexpressors. Thus, leaf senescence was slowed upon T6P phosphatase overexpression, despite the fact that sugar levels increased, indicating that sugar accumulation alone is not sufficient for the initiation of senescence; instead, sugar signaling during senescence requires T6P (Wingler et al., 2012). This conclusion is supported by the observation that senescence was delayed compared with the wild type when, in T6P phosphatase overexpressors were challenged by external sugars. Although T6P levels were not determined in our study, a report by Veyres et al. (2008) revealed accelerated senescence in the Arabidopsis sweetie mutant that contains elevated levels of T6P, trehalose, and starch (Veyres et al., 2008). Currently, however, the precise relation of T6P and trehalose during senescence remains open.

In contrast to the whole-plant studies, our analysis of single leaves revealed that sugars such as Glc, Fru, Xyl, Ara, and maltose decreased during the expansion phase, although Fru started to increase significantly at T3 during early senescence (Figs. 3D and 5A). This decrease of sugars during leaf expansion (Fig. 3D) might be consistent with observations made in tobacco (Nicotiana tabacum) leaves, where changes of sugar levels (Glu and Fru) showed a biphasic behavior. More specifically, Glu and Fru levels decreased from the youngest leaves (no. 30) to older leaves (no. 25), increased in older leaves (nos. 15 and 20) when chlorophyll content started to decline, and then strongly increased again in older leaves (toward leaf no. 9; Masclaux et al., 2000), suggesting that a transient sugar accumulation occurs during the sink-to-source transition at a particular leaf stage. Similarly, the decrease of sugars in the single-leaf experiment during leaf expansion (Fig. 3D) might indicate a breakpoint with respect to metabolic activity that indicates the transition of young (sink) leaves to older (source) leaves. However, currently, it cannot be excluded that the differences in light conditions (long days in the whole-plant experiment versus short days in the single-leaf experiment) additionally affected sugar metabolism, resulting in a decrease of sugars during leaf expansion (Fig. 3D). Within single leaves, galactinol and raffinose displayed unique diagnostic spatiotemporal patterning, with high accumulation observed in the basal region of the leaf (Fig. 5B). Increases of both metabolites as osmoconcentrates under stress conditions such as...
cold, heat, salt, and drought have been well documented (Taji et al., 2002; Kaplan et al., 2004, 2007; Nishizawa et al., 2008; Sanchez et al., 2008). The high accumulation of these metabolites might be needed to protect the plant cells in the basal region to allow the progression of senescence, presumably to sustain functionality (e.g. for export processes in this region). This unique pattern further suggests the existence of specific spatial regulatory mechanisms of nutrient synthesis or transport even within single leaves. The clear distribution (high in tip, low in base) of TCA cycle intermediates observed at the second and third stages in single leaves (Fig. 3D) is also in keeping with this assumption. The metabolic status of the TCA cycle in different parts of the leaf was already altered at the fully expanded stage, as if anticipating the sequential senescence process. This suggests that different modalities of the TCA cycle (Sweetlove et al., 2010) may be important for the initiation and completion of the senescence process from tip to base, which occur prior to the establishment of major senescence syndromes such as chlorosis.

**Carbon-Based Secondary Metabolites under the Regulation of Nutrient Balance and Light during Senescence**

Flavonoids and phenylpropanoids are carbon-based secondary metabolites. Their concentrations fluctuate greatly in response to cellular circumstances such as high-light stress, especially UV-B light, and biotic stress during the defense against herbivores and pathogens (Harborne and Williams, 2000; Peters and Constabel, 2002; Agati and Tattini, 2010; Lovdal et al., 2010; Samanta et al., 2011; Wu et al., 2012) or abiotic stresses such as mineral nutrient depletion (Watanabe et al., 2010). Anthocyanin accumulation occurs in senescing leaves prior to chlorophyll breakdown (Feild et al., 2001). This is thus a likely protective mechanism, since the absence of chlorophyll affects the susceptibility to light-induced reactive oxygen damage in leaf cells caused by decreasing the photosynthetic capacity (Baker and Hardwick, 1973; Lee et al., 1987; Nooden et al., 1996). In the whole-plant experiment, elevation of anthocyanin contents was observed with increasing senescence stages and chlorophyll degradation (Figs. 1B and 2B) and in parallel to the accumulation of osmoprotectants such as Pro, β-Ala, and GABA (Fig. 2B) and further antioxidants such as ubiquinone and plastoquinone (Fig. 2A). In comparison with anthocyanin accumulation in senescing leaves, flavonol glycosides were decreased (Fig. 2B). This is in keeping with the observed reduction of kaempferol glycosides levels in the anthocyanin overaccumulator line (pap1-D; Tohge et al., 2005). In addition to the important roles of flavonoids and phenylpropanoids under light stress, nutrient conditions such as nitrogen, sulfur, and phosphorus deficiency as well as high-carbon stress can also cause anthocyanin synthesis (Do and Cormier, 1991; Rajendran et al., 1992; Bonguebartelsman and Phillips, 1995; Nooden et al., 1996; Stewart et al., 2001; Nikiforova et al., 2004; Park et al., 2007). Many studies concerning the relationship between nitrogen and the rate of CO₂ assimilation and photosynthesis have suggested that anthocyanin accumulation is a metabolic marker for carbon/nitrogen imbalance under such stress conditions (Smart, 1994; Zheng et al., 2009). However, despite the fact that anthocyanin levels were clearly elevated during developmental senescence in leaves in the whole-plant experiment (Fig. 2B), such a correlation was not observed in the single-leaf experiment (Fig. 3D). This might be related to the strong decrease of sugar levels during the expansion phase, since even at early senescence stages (Fig. 3D) the increase of sugars in the tip region might not be enough to result in carbon/nitrogen imbalances.

**Sulfur-Containing Secondary Metabolites**

GLSs, which are sulfur-containing secondary metabolites, have been implicated in plant defense against various insects and pathogens (Halkier, 1999; Rask et al., 2000) and are a major sink for reduced sulfur in Arabidopsis (Chen et al., 2001; Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006). In our secondary metabolite profiling, Met-GLSs and indole-GLSs displayed differences in the spatiotemporal patterning of metabolites during senescence. Met-GLSs highly accumulated in younger green leaves and in siliques, while the indole-GLSs, 1MI3M and 4MI3M, accumulated in older senescing leaves. I3M showed similar changes to the Met-GLSs, but the accumulation of 13M appeared to be affected by the production level of 1MI3M or 4MI3M (Figs. 2B and 3D). These different accumulation patterns suggest the existence of different regulatory mechanisms for their synthesis or different metabolic fluxes as well as different physiological roles during senescence in plants. Reports suggesting that indole-GLSs, especially 1MI3M and 4MI3M, were more responsive to induction than Met-GLSs by wounding, insects, and JAs (Bodnaryk, 1992, 1994; Doughty et al., 1995) fit with the high accumulation of 1MI3M and 4MI3M in senescent leaves, where cellular damage and JA accumulation occur (Buchanan-Wollaston et al., 2003). Although the temporal accumulation patterns of Met-GLSs and indole-GLSs were different, they were similarly distributed within a single leaf. In contrast to almost all primary metabolites, which accumulated largely in the tip region, Met-GLSs and indole-GLSs accumulated in the basal leaf regions (Figs. 4B and 5, F and G). The high accumulation of these metabolites might protect the basal region and petiole in a manner similar to that of raffinose and galactinol (Fig. 5B). A speculative hypothesis put forward here might be that high accumulation of the GLSs in the base region of leaves might form a barrier against opportunist necrotrophic fungi, which are able to colonize senescent and dying leaf tissues as GLSs, while the isothiocyanate compounds activated...
Sink-Source Relationships

Nutrient remobilization from leaves to seeds during plant senescence is important for establishing seed yield and quality in crops and, therefore, has been studied extensively (Schiltz et al., 2005; Howarth et al., 2008; Munier-Jolain et al., 2008; Slewinski, 2012). Leaf-seed balance has also commonly been thought to be an important factor in monocarpic plants for the regulation of leaf senescence, given that sink size and the metabolism in sinks affect the reallocation of nutrients from the senescing leaves, even though this control does not seem to occur in all monocarpic plants, including Arabidopsis (Killingbeck, 1996; Noodén and Penney, 2001; van Doorn, 2004). Nevertheless, the high concentration of Met-GLSs in siliques containing developing seeds (Fig. 2B) seems to be in accordance with the transport of high amounts of Met-GLSs from leaves, the major site of GLS synthesis, to developing seeds by the GLS transporters GTR1 and GTR2 (Nour-Eldin et al., 2012). The high concentration of TCA cycle metabolites and sugars in siliques (Fig. 2B) suggests that there is a high production of amino acids and fatty acids for the synthesis of storage seed protein and storage lipids such as TAGs in siliques with developing seeds. Furthermore, several osmoprotectants such as maltose, sorbitol, and galactinol displayed an increase during silique development (Table 1), suggesting that these metabolites play an important role during silique and seed maturation. As indicated through the PCA analysis (Fig. 6A), siliques with developing seeds as sinks display a distinctively different metabolite composition than leaves, corroborating their specific physiological program. Similarly, Wagstaff et al. (2009) reported that even the silique wall, which is a photosynthetic organ during early stages of development, shows transcriptional features during senescence different from those in senescing leaves (Wagstaff et al., 2009). Thus, both studies reveal features unique to senescence occurring in siliques compared with senescence in rosette leaves.

The concept of the sink-source relationship can also be applied to leaves, with younger leaves acting as sinks and half expanded to mature leaves as source. This transition from sink to source in leaves is important for plant development, because nutrients are translocated from source leaves to sink leaves in addition to seeds. Within a leaf, the sink-source transition occurs in a basipetal manner from tip to base. When the tip region of a leaf starts to become a source, the base region behaves as a sink. The translocation of phloem-mobile nutrients such as sugars and amino acids during the sink-source transition has been well studied (Jones and Eagles, 1962; Fellows and Geiger, 1974; Pitcher and Daie, 1991; Roberts et al., 1997; Imlau et al., 1999; Jeong et al., 2004; Howarth et al., 2008). Even though steady-state metabolite analyses do not fully represent metabolite translocation fluxes in leaves, the metabolite concentration changes observed in senescing leaves may be expected to result from both flux changes and changes of metabolism caused by the sink-source transition. In the whole-plant experiment, rosette leaves and upper leaves, which were supposed to be source tissues and sink tissues, respectively, were analyzed separately (Figs. 1 and 2). The PCA plot of the whole-plant experiment (Fig. 6A) displayed a shift of the timing of metabolite changes between rosette leaves and upper leaves corresponding to a shift of the timing of chlorophyll loss (Fig. 1B). This result suggested that upper leaves can also behave as source tissues in the same way as rosette leaves when they mature and are subdued to the sink-source transition. In the single-leaf experiment, two different sink-source relationships of metabolite changes were observed in the PCA plot (Fig. 6B). First, there is a significant change between 50% expanded leaves and fully expanded leaves (younger leaf-older leaf relationship), potentially representing different metabolic states. The 50% expanded leaves are still growing while the fully expanded leaves have stopped growing. Second, there is the significant change at the senescing tip region (T3) at the yellowing leaf stage (younger base of leaf-older tip of leaf relationship). In the 10% to 20% yellow leaves, a metabolite gradient was observed in a basipetal manner from tip to base on the PCA plot (Fig. 6B).

CONCLUSION

This study of the spatiotemporal metabolite patterns of Arabidopsis during developmental senescence provides an unprecedented rich catalog of metabolites in relation to leaf and silique development. The complex response pattern was cataloged over various defined stages of leaf development from expansion to senescence, siliques, and even subparts of single leaves during the plant life cycle. This provides a helpful database and blueprint to be correlated to future studies (e.g., transcriptome analyses or nutrient depletion studies). In addition to corroborating previous studies, we could identify a matrix of stage-specific diagnostic markers and link changes in metabolite contents to physiological processes, such as nutrient ion mobilization, GLS synthesis, and the formation of osmoprotective and oxidative stress-protective metabolite patterns with distinct spatial or temporal distribution. The plant needs to find a compromise between catabolic processes, maintaining functionality as long as possible, and the need to transport metabolites from source to sink organs before the final senescence stage of massive cell death and organ abandonment is reached in order to ensure optimal seed development and, if applied to agricultural plants,
crop yield. The developmental metabolome presented here is the result of a highly ordered and regulated process, as a result of transcriptional changes, anabolic and catabolic reactions, and transport processes. We analyzed and compared multiple stages here, but a more detailed analysis of the fluxes will still be necessary, as will employing the strategy on crop plant species and under agricultural conditions. We could show, though, that even within regions of a single leaf, specific metabolite gradients are established at various developmental stages until senescence.

The physiology of these patterns can be partly linked to the breakdown of photosynthesis and catabolic processes, protection against oxidative stresses, and transport processes for the mobilization of nutrients, but they are partially not explainable at present (e.g. why glucosinolates accumulate at the basal leaf part, or why some osmoprotectants show a distinct patterning within a single leaf along the sink-source-transition trajectory). This metabolome study could potentially act as a master blueprint with which various senescence process studies can be compared in the future (e.g. when analyzing mutant lines, mineral nutrient depletion, or dark-induced senescence or other senescence-inducing stresses such as drought, salt, or temperature stress). Beyond that, senescence programs and the effect of adverse abiotic and biotic conditions are tightly linked to crop productivity (for review, see Gregersen et al., 2013), and we provide here, to our knowledge, the first full catalog of the developmental senescence metabolome of a plant. The linkage between senescence and genotype and environmental conditions is complex and dependent on the respective crop species or the environmental conditions. While delayed senescence in stay-green mutants of maize (Zea mays) and sorghum (Sorghum bicolor) increased yield, due to prolonged accumulation of carbohydrates, this is not true for all cereal crop plants, as stay-green wheat varieties do not show yield advantages, as this phenotype is probably interfering with the fast programmed translocation of metabolites from the leaves to the seeds after anthesis (Sykorová et al., 2008). Crops have usually been selected under optimal mineral nutrient conditions; thus, for example, nitrogen-depleted wheat matures faster and senesces earlier, producing lower yields and poorer seed protein quality (Gregersen, 2011). Understanding such processes will be helpful in analyzing the respective strategies on crop plants.

Quantitative Reverse Transcription-PCR Analysis

Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen), and complementary DNA was synthesized with SuperScript III RNase H reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The primers used for quantitative reverse transcription (qRT)-PCR are listed in Supplemental Table S2. SYBR Green Master Mix reagent (Applied Biosystems) was used for amplification according to the protocols provided by the supplier. qRT-PCR was conducted on an optical 384-well plate with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The nuclear gene PROTODERMAL FACTOR2 (PDF2; AT1G13320) was used as a reference for determining relative transcript abundance; among several others, this gene has previously been tested in qRT-PCR experiments (Czechowski et al., 2005). In addition to PDF2, we analyzed other reference genes, namely UBQ10 (At4g05320), ELONGATION FACTOR1a (At5g60390), and GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (At1g13440). Among them, PDF2 showed the most stable expression in this study.

Determination of Chorophyll Contents

Frozen ground material (10 mg) was homogenized in 1 mL of absolute ethanol. After centrifugation at 2,500g for 5 min, the chlorophyll in the supernatant was quantified by spectrophotometric measurement at 652 nm (Munoz, 1961).

Determination of Soluble Protein Contents

Frozen ground material (10 mg) was homogenized in 100 μL of extraction buffer containing 250 mM potassium phosphate, pH 8.0, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol. The soluble protein amount was measured with the Bio-Rad Bradford reagent (Bio-Rad Laboratories) according to the manufacturer’s instructions.

Determination of Starch Contents

Starch content was measured as described previously (Hendriks et al., 2003). Frozen ground material (20 mg) was homogenized twice in 1 mL of 80% (v/v) ethanol at 80°C for 1 h. For starch determination, the pellet from the ethanol extraction was solubilized by heating it to 95°C in 0.2 mL NaOH for 30 min. After acidification to pH 4.9 with HCl/sodium acetate, pH 4.9, part of the suspension was digested overnight with amylglucosidase and α-amylase. The G1c content of the supernatant was then used to assess the starch content of the sample. The G1c content was detected as described by Stitt et al. (1989) with volumes adapted to a microplate format.

Determination of Ion Contents

Frozen ground material (20 mg) was homogenized in 200 μL of 0.1 mM HCl. Samples were centrifuged for 5 min at 14,000g and 4°C. The supernatant was transferred to an Ultrafree MC 5000 M NMLF Filter Unit (Millipore) and centrifuged for 90 min at 5,000g and 4°C. After filtration, samples were diluted 10 times with deionized water and analyzed by the Dionex ICS-2000 system with a KOH gradient (Dionex).

Determination of Cys and GSH Contents

Quantitative analyses of the reduced forms of Cys and GSH were performed by a combination of monomobromobimane fluorescent labeling and
HPLC (Anderson, 1985; Fahey and Newton, 1987). Frozen ground material (20 mg) was homogenized in 60 μL of 0.1 M HCl (fresh weight basis) with a mixer mill. A mixture of 20 μL of extract and 40 μL of 25 μM N-acetyl-Cys (as the internal standard) was reacted with 5 μL of 30 μM monobromobimane in acetonitrile and 10 μL of 8.5 mM N-ethylmorpholine for 20 min at 37°C in the dark. The labeling reaction was terminated by the addition of 10 μL of acetic acid, and the resulting solution was then subjected to HPLC analysis. HPLC was carried out as described previously (Saito et al., 1994).

**Determination of OAS and Amino Acid Contents**

OAS and amino acids were determined following a protocol modified from Kim et al. (1997). Frozen ground material (20 mg) was homogenized in 200 μL of 80% (v/v) aqueous ethanol, 200 μL of 50% (v/v) aqueous ethanol (buffered with 2.5 mM HEPES/KOH, pH 6.2), and 100 μL of 80% (v/v) aqueous ethanol. Ethanol/water extracts were subjected to HPLC analysis using a Hyperclone C18 (ODS; octadecyl silane) column (Phenomenex) connected to an HPLC system (Dionex). OAS and amino acids were measured by precolumn online derivatization with 0-phthalaldehyde in combination with fluorescence detection (Lindroth and Mopper, 1979; Kim et al., 1997).

**Measurement of Primary Metabolite by GC-Time of Flight-MS Analysis**

Metabolite profiling by GC-time of flight (TOF)-MS was performed as described previously (Liese et al., 2006; Erban et al., 2007). Frozen ground material (60 mg for the whole-plant experiment and 30 mg for the single-leaf experiment) was homogenized in 300 μL of methanol at 70°C for 15 min and then 200 μL of chloroform at 37°C for 5 min. The polar fraction was prepared by liquid partitioning into 400 μL of water. The polar fraction was derivatized by methoxyamination and subsequent trimethylsilylation. Samples were analyzed using GC-TOF-MS (ChromATOF software, Pegasus driver 1.61; LEICO). The chromatograms and mass spectra were evaluated using TagFinder software (Luedemann et al., 2008) and NIST05 software (http://www.nist.gov/srd/molist.cfm). Metabolite identification was manually supervised using the mass spectral and retention index collection of the Golm Metabolome Database (Kopka et al., 2005; Hummel et al., 2010). Peak heights of the mass fragments were normalized on the basis of the fresh weight of the sample and the added amount of an internal standard (ribitol for the whole-plant experiment and [13C]sorbitol for the single-leaf experiment).

**Measurement of Secondary Metabolites by LC-Electrospray Ionization-MS**

Metabolite profiling by LC-electrospray ionization-MS was performed as described previously (Tohge and Fernie, 2010). Frozen ground material (20 mg) was homogenized in 100 μL of 80% methanol. All data were processed using Xcalibur 2.1 software (Thermo Fisher Scientific).

**Measurement of Lipophilic Metabolites by UPLC-MS**

Frozen ground material (50 mg) was homogenized as described previously (Giavalisco et al., 2011; Hummel et al., 2011) in 1 mL of a cold 13 (v/v) methanol:methyl-tert-butyl-ether solution that was subjected to constant shaking and sonication. Following phase separation, extract aliquots of the upper organic phase (lipids) were dried in a Speed-Vac before resuspending them in an acetonitrile:isopropanol (7:3, v/v) mixture. UPLC separations were performed by liquid partitioning into 400 μL of chloroform at 37°C for 5 min. The polar fraction was prepared by liquid partitioning into 80% (v/v) aqueous ethanol, 200 μL of 8.5 mM N-ethylmorpholine (buffered with 2.5 mM HEPES/KOH, pH 6.2), and 100 μL of 80% (v/v) aqueous ethanol. Ethanol/water extracts were subjected to HPLC analysis using a Hyperclone C18 (ODS; octadecyl silane) column (Phenomenex) connected to an HPLC system (Dionex). OAS and amino acids were measured by precolumn online derivatization with 0-phthalaldehyde in combination with fluorescence detection (Lindroth and Mopper, 1979; Kim et al., 1997).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Heat map of metabolite changes during early leaf senescence.

**Supplemental Figure S2.** Ratios of Glu/Gln, Asn/Asp, and Gly/Ser.

**Supplemental Table S1.** Metabolic changes in different tissues during development senescence.

**Supplemental Table S2.** Primers of housekeeping genes, SDGs, and SAGs used for qRT-PCR analysis.

**Supplemental Table S3.** Raw data and statistical analysis of metabolite profiles in whole-plant experiments.

**Supplemental Table S4.** Raw data and statistical analysis of metabolite profiles in single-leaf experiments.

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Metabolite Changes during Developmental Senescence


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