Running Title: Transcriptomic analysis of senescence in maize

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Transcriptional and metabolic analysis of senescence induced by preventing pollination in maize

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Financial Support: DOE Great Lakes Bioenergy Research Center (DOE BER Office of Science DE-FC02-07ER64494)

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ABSTRACT

Transcriptional and metabolic changes were evaluated during senescence induced by preventing pollination in the B73 genotype of maize (*Zea mays* L.). Accumulation of free glucose and starch, and loss of chlorophyll in leaf was manifested early at 12 days after anthesis (DAA) while global transcriptional and phenotypic changes were evident only at 24 DAA. Internodes exhibited major transcriptomic changes only at 30 DAA. Overlaying expression data onto metabolic pathways revealed involvement of many novel pathways including those involved in cell wall biosynthesis. To investigate the overlap between induced and natural senescence, transcriptional data from induced senescence in maize was compared with that reported for Arabidopsis (*Arabidopsis thaliana*) undergoing natural and sugar-induced senescence. Notable similarities with natural senescence in Arabidopsis included up-regulation of senescence-associated genes (SAGs), ethylene and jasmonic acid biosynthetic genes, and APETALA2, ethylene-responsive element binding protein and no apical meristem transcription factors. However, differences from natural senescence were highlighted by unaltered expression of a subset of the SAGs, cytokinin, abscisic acid, and salicylic acid biosynthesis genes. Key genes up-regulated during sugar-induced senescence in Arabidopsis, including a cysteine protease (*SAG12*) and three flavonoid biosynthesis genes (*PAP1, PAP2* and *LDOX*), were also induced suggesting similarities in senescence induced by pollination prevention and sugar application. Coexpression analysis revealed networks involving known senescence-related genes and novel candidates; 82 of these were shared between leaf and internode networks highlighting similarities in induced senescence in these tissues. Insights from this study will be valuable in systems biology of senescence in maize and other grasses.
INTRODUCTION

Senescence is a highly regulated degradative process that results in the death of a cell, organ, or organism. In monocarpic species such as maize, the onset of flowering results in cessation of vegetative growth and, as grain filling nears completion, leaf senescence is triggered by mechanisms that are not completely understood. These include a complex interplay of metabolic changes, hormonal signaling, and environmental cues (Lim et al., 2007). Leaf senescence is a nutrient recycling process wherein complex macromolecules such as proteins, lipids, and nucleic acids are broken down and transported to the developing sink (Himelblau and Amasino, 2001; Lim et al., 2007). Since leaf senescence results in loss of photosynthetic activity, delaying senescence can potentially enhance carbon assimilation and, as a result, grain and biomass yield. Higher accumulation of photosynthates in vegetative plant parts will produce value-added feedstocks with increased energy density for biofuel production.

Elucidation of the molecular mechanisms underlying senescence has been facilitated by the cloning and characterization of senescence-related genes isolated from forward and reverse genetic approaches (Buchanan-Wollaston et al., 2003; reviewed by Lim et al., 2007). Results from these investigations, however, have been limited to a handful of genes. With advancements in genome sequencing and global gene expression profiling tools, several studies have evaluated global transcriptomic reprogramming during natural and induced senescence (Guo et al., 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Breeze et al., 2011). Such studies have generated a wealth of information and led to the identification of senescence-associated genes (SAGs) that are up-regulated during senescence. A recent study mined public databases and annotated over 1,300 SAGs from 21 plant species, the majority (over 1,000) of these were identified in Arabidopsis (Arabidopsis thaliana) (Liu et al., 2011). These SAGs encode a wide variety of proteins with diverse functions including hormone response pathways,
macromolecule breakdown, nutrient recycling, and transcriptional regulation. Functional characterization of some of these SAGs has provided useful insights into the mechanistic details underlying senescence (Guo and Gan, 2005; Lim and Nam, 2005; Lim et al., 2007). However, a complete understanding of complex interactions between multiple metabolic pathways involved in senescence and underlying regulatory genes is lacking. Furthermore, such transcriptional analyses have not been done for grasses such as maize (Zea mays L.), a C4 annual, which may have distinct molecular mechanisms underlying senescence.

The onset and progression of senescence is controlled by several developmental and environmental factors. Disease pressure, shading, extreme temperatures, oxidative stress, and nutrient starvation are known to induce senescence (Buchanan-Wollaston et al., 2003; Lim et al., 2007). Likewise, internal factors including changes in hormone and sugar levels as well as source-sink relationships have also been shown to affect the senescence program (Quirino et al., 2000; Pourtau et al., 2006; Lim et al., 2007; Wingler and Roitsch, 2008; Wingler et al., 2009). In Arabidopsis, for instance, direct application of sugars to the leaf cells resulted in premature senescence (Pourtau et al., 2006). In barley (Hordeum vulgare), hyper-accumulation of carbohydrates in the leaves due to stem-girdling resulted in early senescence (Parrott et al., 2007). Manipulations of source-sink ratios by removing the developing seed/fruit sink often delay senescence as seen in many species including sunflower (Ho et al., 1987), soybean (Crafts-Brandner et al., 1984), cowpea (Khanna-Chopra and Reddy, 1988), and wheat (Biswas and Mandal, 1986). On the contrary, lack of sink has been shown to trigger and/or accelerate senescence in certain species including maize (Christensen et al., 1981; Crafts-Brandner et al., 1984; Ceppi et al., 1987), barley (Mandahar and Garg, 1975), and pepper (Hall, 1977). Interestingly, the effect of sink removal on senescence in maize is genotype dependent as sink-deficient plants of certain genotypes undergo early senescence while others senesce at a normal rate (Ceppi et al., 1987). Manipulation of senescence through these mechanisms provides a
useful experimental system to study the underlying molecular and biochemical processes.

The goal of the current study was to explore global gene expression and metabolic changes during the onset and progression of induced senescence in maize. Early senescence was induced by preventing pollination in inbred B73, the reference maize genotype (Schnable et al., 2009). Gene expression changes in leaf and internode tissues were studied using a custom NimbleGen microarray (Sekhon et al., 2011) and coexpression networks were constructed to annotate novel senescence-related genes. To help visualize diverse biological processes affected during senescence, we adapted the MapMan tools (Usadel et al., 2005). Metabolite analysis of selected structural and storage carbohydrates and their correlation with sucrose/starch pathway genes is presented. For comparison of induced and natural senescence, expression patterns of maize gene orthologs implicated in senescence in other species (Liu et al., 2011) were documented. To further explore the role of sugars in senescence induced by pollination prevention, we compared the transcriptome data with those obtained from sugar-induced senescence (Pourtau et al., 2006) and sugar hyper-accumulation in leaves (Lloyd and Zakhleniuk, 2004) in Arabidopsis. This study provides an enhanced understanding of the complex molecular processes associated with senescence in maize.

RESULTS AND DISCUSSION

Phenotypic, metabolic, and transcriptomic indicators of induced senescence in the B73 genotype of maize

The maize B73 genotype has been previously reported to develop visual symptoms of premature senescence in the absence of a seed sink (Ceppi et al., 1987). We
utilized this observation as the basis for characterizing metabolomic and transcriptomic changes underlying the senescence process. In this study, senescence was induced by covering the silks (female inflorescence) with glassine bags to prevent pollination. Induced senescence resulted in phenotypic differences relative to the control pollinated plants including reduction in green color, accumulation of red pigments, and eventually desiccation of the leaves. The symptoms were first visible on the leaf at 21 days after anthesis (DAA), and were well developed by 24 DAA (Figure 1A). At 30 DAA, the entire ear leaf blade had senesced in the non-pollinated plants (NONPOL), while leaves in pollinated plants (POL) remained green. No obvious phenotypic effect of lack of pollination on internodes was observed in this study (data not shown).

To study transcriptional changes associated with induced senescence, we performed microarray analysis using a custom NimbleGen microarray (Sekhon et al., 2011). Two biological replicates each for the leaf originating from the uppermost (primary) ear-bearing node and for the internode above the primary ear-bearing node from pollinated and non-pollinated plants at 0, 6, 12, 18, 24, and 30 DAA were used for the analysis (see Materials and Methods for details). Pearson’s correlation coefficient between biological replicates ranged between 0.980-0.995 indicating high reproducibility of the biological replicates and the array platform (Table S1). Nearly 80% of the probe sets were expressed in at least one of the samples indicating substantial coverage of the transcriptome. The proportion of probe sets expressed was similar among individual samples and ranged between 65.8 and 68.2% indicating that plant tissues were not transcriptionally quiescent even at the later stages of the experiment (Figure S1).

A snapshot of the transcriptome during induced senescence was generated by clustering all samples using principle component analysis (PCA). Consistent with their distinct developmental programs, the leaf and internode samples clustered separately (Figure 1B). PCA indicated that transcription was similar in
leaves of POL and NONPOL plants through 18 DAA, but began to diverge at 24 DAA. This is consistent with the appearance of visual senescence symptoms at 24 DAA in leaves. In the internodes, divergence of transcription was observed at 30 DAA. Internodes are expected to senesce later than leaves as those provide mechanical strength and act as transportation channels for efficient incorporation of the breakdown products to the sink. Furthermore, in annual plants such as maize, selection against lodging has likely contributed to this developmental timing.

To investigate the relationship between induced senescence and sugar partitioning, we measured the levels of major storage carbohydrates in both leaves and internodes. Starch content was significantly higher in the NONPOL leaves at 12 DAA and afterwards (Figure 1C). Starch hyper-accumulation was delayed in NONPOL internodes, reaching at significantly higher levels only at 30 DAA (Figure 1D). Likewise, levels of free glucose were higher in NONPOL leaves starting at 12 DAA and remained significantly higher from 18 DAA onwards (Figure 1E). However, no differences were observed for free glucose in internodes (Figure 1F). No significant effect of induced senescence was observed on sucrose levels in the leaves and internodes (data not shown).

Chlorophyll degradation is another major process associated with senescence (Lim et al., 2007). In the current study, total chlorophyll levels in NONPOL leaves declined significantly faster than controls with significant reduction first visible at 12 DAA (Figure 1G). More dramatic loss of chlorophyll was recorded at 24 and 30 DAA in the NONPOL plants. A similar trend was observed for chlorophyll $a$ and $b$, and total carotenoids (Figure S2). Interestingly, while endogenous levels of chlorophyll in NONPOL plants dropped significantly during later stages of the experiment, levels of free glucose and starch increased during this period. A steady decline in total chlorophyll levels was also observed during normal development even though free glucose and starch levels registered an increase at 30 DAA (Figure 1G).
To summarize, hyper-accumulation of free glucose and starch and degradation of chlorophyll are the earliest events during induced senescence, observed a week after anthesis, while phenotypic and global transcriptional changes occur much later (24 and 30 DAA). Thus, after the early change in sugar and chlorophyll status, there exists a window of time before the onset of broader reprogramming of the plant transcriptome.

Overlaying transcriptional data onto metabolic pathways indicates modulation of diverse cellular pathways during induced senescence

To evaluate specific cellular processes associated with induced senescence, we overlaid differentially expressed genes onto metabolic networks using MapMan (Usadel et al., 2005). A cursory overview of the selected metabolic pathways revealed that most of these pathways remain unchanged in leaf up to 18 DAA (Figure S3). However, a major change in transcriptional activity was observed at 24 DAA (Figure 2A). Most notable changes were observed for photosynthesis, carbohydrate (CHO) metabolism, and cell wall-related processes, which are discussed in detail in the following sections. Consistent with breakdown of membrane lipids being a hallmark of senescence (Thompson et al., 1998), several genes involved in lipid degradation were up-regulated. Genes involved in synthesis of secondary metabolites including flavonoids, phenylpropanoids, and phenolics were also up-regulated. Secondary metabolites including flavonoids and carotenoids play an important role in maintaining redox homeostasis in the cells by quenching reactive oxygen species (ROS) (Dietz, 2003; Heber et al., 2006) thus ensuring metabolic functioning. At 30 DAA, transcription in most pathways represented in MapMan was down-regulated in the early senescing plants (Figure 2B). A few processes, including those involved in breakdown of starch and celluloses and in fermentation, were still transcriptionally active in senescing plants denoting the macromolecular breakdown associated with plant death.
While physiological and/or molecular aspects of leaf senescence have been studied in many plant species, less is known about internodes. Consistent with PCA, notable global changes were not observed through the 24 DAA stage (Figure S4). At 30 DAA, however, we observed substantial alterations of several metabolic pathways including major and minor CHO metabolism, cell wall related processes, lipid metabolism, secondary metabolism, and amino acid metabolism in internode tissue (Figure 3). Many of these processes parallel the leaf tissues albeit occurring at a later stage, and signify that macromolecular breakdown and stress response, first observed in leaves, is later manifested in the internode.

Transcriptional and metabolic analyses reveal substantial changes in sugar synthesis and metabolism during induced senescence

Storage and free sugars

According to metabolic data, differential partitioning of sugars and starch due to lack of sink tissue is an early event during induced senescence. Transcriptional profile of the sugar pathway genes was, however, more complex. In NONPOL leaves, one extracellular invertase was up-regulated at 24 DAA while another was consistently down-regulated (Figure S5A-B). Extracellular invertases regulate sugar partitioning by controlling phloem loading due to their positive association with sink strength (Roitsch and Gonzalez, 2004). Two putative sucrose synthases also had contrasting expression profiles; while one was up-regulated in NONPOL leaves, the other was down-regulated (Figure S5C-D). A role of sucrose synthases in senescence has been proposed earlier (Subbaiah et al., 2007). Contrasting expression patterns suggest functional divergence of the role of these enzymes in senescence.

Storage of excess sugars as starch is preferred over the free sugars as it is water insoluble and therefore not expected to affect the osmotic environment of the
cells. Consistent with increased starch content in leaves, a putative starch synthase and a putative starch branching enzyme were up-regulated in NONPOL leaves (Figure S5E-F). Starch accumulation, especially in internodes, is also potentially desirable for improving biomass quality and density as a value-added feedstock for biofuels.

**Structural and cell wall bound sugars**

One of the most striking observations from the MapMan analysis was the extent of transcriptional activity in the cell wall-related pathways in NONPOL leaf at 24 and 30 DAA (Figure 2). Up-regulation of genes involved in cellulose and mannan/xylose degradation is consistent with substantial macromolecule degradation associated with senescence. Surprisingly, genes involved in cellulose and hemicellulose synthesis, and in cell expansion (expansins) were also up-regulated in NONPOL leaf suggesting incorporation of sugars into cell walls. For example, a cellulose synthase orthologous to Arabidopsis *KOR* (Nicol et al., 1998) and *roothairless3*, which encodes a COBRA-like protein implicated in cellulose synthesis (Hochholdinger et al., 2008), were up-regulated at 18 DAA (Figure S6A-B). Changes in the amount of structural carbohydrates were, however, subtle. Metabolic analyses revealed higher levels of cell wall-bound glucose in the leaves of NONPOL 12 and 18 DAA but the levels were reversed at 30 DAA (Figure 4 A-B). Crystalline cellulose content did not change during induced senescence in either tissue type (data not shown). These observations could be a manifestation of lower plant biomass resulting from loss of photosynthetic activity in NONPOL plants, as also reported in an earlier study (Ceppi et al., 1987). Lower net accumulation of structural carbohydrates could also be due to simultaneous breakdown as suggested by hyperactivity of degradative processes.

Increased abundance of hemicellulosic polysaccharides was also suggested by gene expression analysis. For instance, a putative ortholog of Arabidopsis mannan synthase *ATCSLA9* (Davis et al., 2010) was up-regulated in NONPOL
leaves (Figure S6C). A putative glucuronyltransferases similar to PGSIP3 (Oikawa et al., 2010) was also up-regulated suggesting increased glucuronoxylan synthesis (Figure S6D). Concomitantly, higher amounts of xylose, a major component of hemicellulose (Reiter, 2002) accumulated in the cell walls of NONPOL leaves and internodes at 30 DAA (Figure 4 C-D). Other components of hemicellulose, however, did not show sizeable changes in the senescing tissues (not shown). Thus, there appears to be an opportunity to divert excess photosynthates to plant cell wall in the form of pentose and hexose sugars. While levels of such sugars were low, improvements are possible through identification of underlying genes and selection of favorable alleles.

Minor sugars

Among minor CHO's, notable changes included up-regulation of myo-inositol oxygenases and raffinose synthases in leaves (Figure 2). In Arabidopsis, up-regulation of myo-inositol oxygenases have been associated with increased synthesis of ascorbic acid, a major antioxidant, while raffinoses are osmoprotectants which also prevent oxidative damage (Lorence et al., 2004; Nishizawa et al., 2008). Thus, it appears that these sugars, which exist at relatively low levels in non-senescing cells, may have specialized functions during senescence.

Effect of induced senescence on photosynthesis

Degradation of chlorophyll, followed by subsequent loss of photosynthetic activity, is one of the earliest visible signs of leaf senescence. Significantly accelerated loss of chlorophyll in NONPOL leaves started at 12 DAA (Figure 1G). However, a sizeable decrease in transcriptional activity of photosynthesis-related genes was not observed until 24 DAA (Figure 2). A majority of the genes involved in the light reactions and the Calvin cycle were suppressed in NONPOL plants. In contrast,
several genes involved in chlorophyll degradation including, for example, a gene encoding stay-green protein SGR1 and a chlorophyll b reductase were up-regulated after 18 DAA (Figure S7A-B). These genes are involved in the initial steps of chlorophyll degradation (Hortensteiner, 2009), thus the timing of their up-regulation indicates the initiation of chlorophyll catabolism. Two other genes important for chlorophyll catabolism, SGR2 and pheophorbide a oxygenase (lls1/ACD1), were also up-regulated albeit below the 3-fold cutoff (Figure S7C-D). Intriguingly, expression profile of lls1 agreed with the chlorophyll content in the leaves and showed increased expression at 12 DAA followed by lower expression at 18DAA. Several genes in the glutathione-ascorbate cycle were up-regulated consistent with an increased need for the cells to quench ROS produced due to chlorophyll catabolism.

**Modifications in phytohormone pathways during induced senescence**

The role of hormones has been investigated in normal and induced senescence in Arabidopsis (Quirino et al., 2000; Lim et al., 2007; Breeze et al., 2011) but not in senescence induction due to lack of a sink in maize. Ethylene is known to enhance leaf and flower senescence (Lim et al., 2007). Many of the genes involved in ethylene synthesis and signaling pathways were up-regulated in senescing leaves (Figure S8). For instance, two ethylene biosynthetic pathway genes, 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), were up-regulated (Figure S8A-B). Both, ACS and ACO are also induced during senescence in Arabidopsis (van der Graaff et al., 2006). Among the ethylene receptors, a putative ortholog of the Arabidopsis ethylene receptor ETR1 (Bleecker et al., 1988) was down-regulated (Figure S8C) while a putative ortholog of ethylene-responsive element binding protein EREBP-4 (Leubner-Metzger et al., 1998) was up-regulated (Figure S8D). Ethylene receptors were also found to be unchanged during natural and induced senescence in
Arabidopsis (van der Graaff et al., 2006). Thus, increased ethylene synthesis in leaves is consistent with its role in senescence; however, ethylene signaling during induced senescence likely uses alternative signaling components.

Jasmonic acid (JA) promotes senescence primarily through degradation of chlorophyll thus retarding the photosystem II activity (Reinbothe et al., 2009). Consistent with this observation, a putative lipoxygenase similar to Arabidopsis LOX1 was up-regulated in early senescing leaves (Figure S8E). Another gene encoding a putative allene oxide cyclase (AOC) similar to Arabidopsis AOC3 was also up-regulated (Figure S8F). Lipoxygenases are involved in the first step of JA synthesis (Gfeller et al., 2010), while AOCs has been shown to catalyze JA biosynthesis during the wound response (Stenzel et al., 2003). This observation suggests a role of JA in induced senescence in maize. This is in contrast to dark induced senescence in Arabidopsis wherein JA is not essential (van der Graaff et al., 2006). No notable expression differences were observed for genes involved in the synthesis, degradation or signaling of cytokinins, abscisic acid (ABA) and salicylic acid (SA) (data not shown) suggesting against a major role of these in sink-deficient senescence in maize.

**Induced senescence is associated with sizeable change in transcriptional regulation**

A comprehensive list of 2,516 maize transcription factors belonging to 47 families is available at GRASSIUS (http://grassius.org) and described elsewhere (Yilmaz et al., 2009). We found that 270 transcription factors belonging to 40 families were differentially expressed in leaf during induced senescence, accounting for 6.3% of the total differentially expressed genes (Table S2). K-means clustering showed that these genes have three predominant expression profiles (Figure 5). The first cluster, consisting of 90 genes, had comparable expression in the POL and NONPOL leaves up to 18 DAA but showed a substantial up-regulation in NONPOL
leaves at 24 and 30 DAA (Figure 5A). Two highly represented families in this cluster include the APETALA 2-ethylene response element binding (AP2-EREBP) and the NAC (NAM (no apical meristem), ATAF, CUC (cup-shaped cotyledon)) transcription factors. These two categories are also predominant during onset of natural senescence in Arabidopsis (Guo et al., 2004; Buchanan-Wollaston et al., 2005; Breeze et al., 2011). Over 200 AP2-EREBPs are present within the maize genome, accounting for 8.4% of all transcription factors. Members of this group have been shown to be up-regulated due to higher cellular glucose levels (Cheng et al., 2002) and likely mediate sugar sensing through interactions with ABA and ethylene (Dietz et al., 2010). Members of this group have also been implicated in ensuring redox homeostasis (Khandelwal et al., 2008) which in turn is crucial for maintaining metabolic activities in the cell. An AP2/EREBP protein has also been proposed to regulate biosynthesis of carotenoids (Welsch et al., 2007) and may be important for protection against oxidative stress.

NAC transcription factors constitute a large group of 134 members representing 5.3% of all transcription factors in maize. NAC proteins are involved in a wide array of developmental processes including secondary cell wall formation (Zhong et al., 2006) and programmed cell death (Ohashi-Ito et al., 2010; Yamaguchi et al., 2011). An ABA-responsive NAC (VND-INTERACTING2) protein was shown to relay ABA-mediated abiotic stress signals to induce leaf senescence (Yang et al., 2011). In Arabidopsis, an NAC (ORE1) protein has been shown to induce age-dependent senescence (Kim et al., 2009). The putative maize ORE1 ortholog (GRMZM2G009892_T01) also showed substantial up-regulation at later stages of induced senescence in leaves (not shown). Members of several other classes of transcription factors including bZIP, WRKY, MADS, and heat shock factors (HSF) were also over expressed during induced senescence.

Interestingly, two clusters of transcription factors were down-regulated during induced senescence (Figure 5B-C). These likely regulate the normal metabolic processes and are suppressed in early senescing plants. While
substantial expression reprogramming of transcription factors reported in this and earlier studies (Guo et al., 2004; Buchanan-Wollaston et al., 2005; Breeze et al., 2011) underscore the complexities of the underlying transcriptional program, only a handful of these have been experimentally substantiated. Thus, these genes are valuable targets for further investigations of regulatory changes associated with senescence.

**Senescence induced by preventing pollination in maize has commonalities and differences with natural senescence in other species**

Induced senescence in maize offers an attractive approach to initiate a comparative time-course study of the transcriptional and metabolic events occurring during senescence. However, the extent of similarity between the molecular mechanisms underlying natural and induced senescence in maize is not known. To address this question, we examined the transcriptional changes in genes previously implicated in natural senescence. In Arabidopsis, a group of SAGs that show enhanced expression during senescence have been reported (Buchanan-Wollaston et al., 2005). Recently, a leaf senescence database documented over 1,300 SAGs from several plant species (Liu et al., 2011). Based on this database, we annotated the maize orthologs and paralogs of the SAGs and identified 1,618 genes belonging to 615 orthologous groups (see Materials and Methods). Of these, 170 genes belonging to 126 distinct orthologous groups were differentially expressed in the current experiment (Table S3). However, only 77 of these genes were up-regulated at 18 DAA or later (Figure S9A) as would be expected for this group of genes if they responded similarly as previous investigations of natural senescence have indicated. Some of the major categories of genes in this group included those involved in protein degradation, transcriptional regulation, secondary metabolism, transport, and hormone metabolisms (Group A, Table S3). This set included maize orthologs of SAG12, a reliable marker that is induced during developmental senescence (Lohman et al.,
1994) but not due to stress (Weaver et al., 1998; Miller et al., 1999). Interestingly, 93 of the genes were down-regulated during induced senescence; 26 of these showed an early suppression starting 18 DAA (Figure S9B) while another 67 showed lower expression at 24 DAA (Figure S9C). Genes showing early suppression included those involved in transcriptional regulation, signaling (receptor kinases), and cell organization (Group B, Table S3). Genes showing loss of expression later in the experiment included those related to transcriptional regulation, hormone metabolism, signaling (receptor kinases), and transport (Group C, Table S3).

From this analysis, similarities and differences between induced senescence in maize and natural senescence in other species are evident. As the SAGs were originally identified due to their over-expression during natural senescence (Liu et al., 2011), up-regulation of their orthologs in maize is consistent with similarities between induced senescence and natural senescence. However, the homologs of previously identified SAG genes that are down-regulated, especially during early stages of the experiment (after 18 DAA), are intriguing. While these could be due to differences in induced and natural senescence, it is also possible that the senescence program of maize, a C4 annual monocot, differs considerably from that of Arabidopsis. Transcriptomic analysis of maize leaves during natural senescence will help address these questions.

**Senescence induced by pollination prevention in maize has transcriptional similarities with sugar-induced senescence in Arabidopsis**

Several studies have investigated the genome-wide transcriptional consequences of sugar accumulation. In barley, hyper-accumulation of sugars due to stem-girdling of leaves accelerated senescence, and transcriptional analysis revealed up-regulation of several senescence-related genes (Parrott et al., 2007). In Arabidopsis, transcriptome analysis of *pho3*, a mutant which lacks sucrose...
transporter (SUC2) and hyper-accumulates soluble sugars and starch in the leaves (Gottwald et al., 2000), showed induction of several senescence-related and flavonoid biosynthesis genes (Lloyd and Zakhleniuk, 2004). Application of sugar coupled with low nitrogen lead to early senescence and the associated transcriptional changes mimicked those observed during natural senescence (Pourtau et al., 2006). Interestingly, putative maize orthologs of seven key genes induced during sugar-induced senescence (Pourtau et al., 2006) were also up-regulated in leaf tissue during early senescence induced by pollination prevention (Figure 6, Table S4). Expression of a maize gene putatively encoding SAG12, showed a four-fold up-regulation. One notable contradiction was APL3, a gene encoding the large subunit of glucose-1-phosphate adenylyltransferase, that was up-regulated in Arabidopsis down-regulated in NONPOL leaves at 30 DAA (data not shown). This is consistent with lower starch accumulation in NONPOL leaves at 30 DAA this stage likely due to loss of primary metabolism at later stages of the experiment. Of 104 Arabidopsis genes induced by glucose irrespective of N status (Pourtau et al., 2006) for which a maize orthologs could be identified, 24 (23%) showed two-fold or more up-regulation in our study (Table S5). Thus, while there are similarities in expression of selected genes, differences exist between the two studies.

Similar to the early senescing pho3 mutants in Arabidopsis (Lloyd and Zakhleniuk, 2004), most known genes involved in flavonoid biosynthesis were up-regulated during later (24 and 30 DAA) stages of induced senescence in our study. This included two myb transcription regulators of anthocyanin biosynthesis, PAP1 (GRMZM2G051256_T01) and PAP2 (GRMZM2G048295_T01), a gene encoding leucoanthocyanidin dioxygenase (LDOX; GRMZM2G345717_T01), and most additional genes mentioned in pho3 study (Table S4). Incidentally, expression of PAP2 and LDOX and accumulation of anthocyanins are also induced by higher sugar content (Solfanelli et al., 2006; Loreti et al., 2008). Flavonoids, particularly anthocyanins, reduce risk of photo-oxidative damage to leaf cells during
senescence and thereby help in nutrient retrieval from senescing leaves (Feild et al., 2001).

Induction of senescence by sugar accumulation or sugar starvation is debated and evidence presented for both scenarios is not conclusive (van Doorn, 2008; Wingler et al., 2009). While our data do not provide conclusive evidence to support a causative role of sugars in induced senescence in maize, hyper-accumulation of free sugars in the leaves, and similarities in gene expression changes with sugar-induced senescence in Arabidopsis (Pourtau et al., 2006) is consistent with such a role.

Certain genotypes do not undergo early senescence upon pollination prevention despite higher sugar accumulation in leaf tissue indicating existence of natural variation (Crafts-Brandner et al., 1984; Ceppi et al., 1987). Molecular and physiological basis of such differences in the threshold of sugar tolerances are unknown. Possible explanations include differential subcellular localization of sugars in leaf cells and genetic differences for pathways that incorporate signals from sugar or other cues associated with the absence of a seed sink. Interestingly, such genotypic differences were also observed in soybeans, which generally exhibit delayed senescence upon deflowering, and directly correlated with the rate of Rubisco degradation and loss of photosynthetic ability (Crafts-Brandner and Egli, 1987). A study involving two maize genotypes with contrasting responses to lack of pollination reported that such induction of senescence is likely controlled by a single dominant trait (Ceppi et al., 1987). Further experiments using mutants defective in sugar transport and metabolism, and genomic approaches to explore natural variation and identification of underlying allelic variants in maize will provide further insights.

In this study, senescence was induced by removal of seed, a major carbon sink. It is conceivable that reduction in seed sink upon completion of grain filling at maturity could similarly act as a trigger for the onset of natural senescence. Indeed higher leaf glucose and starch levels are observed in the POL plants at later stages
Sugar levels also increase during the period coinciding with the onset of natural senescence in maize (Crafts-Brandner et al., 1984) and Arabidopsis (Quirino et al., 2001; Diaz et al., 2005; Pourtau et al., 2006). There is mounting evidence that sugar accumulation in aging leaves either triggers or enhances the rate of senescence (Pourtau et al., 2006; Parrott et al., 2007; Wingler and Roitsch, 2008). Conversely, the availability of an alternative sink after completion of grain filling could potentially slow down such sugar accumulation and delay the onset of senescence. In grain crops like maize, delaying senescence and storing of excess sugars in metabolically stable forms like starch, hemicellulose, and perhaps oil, offers great potential for developing value added biofuel feedstocks.

Gene coexpression network analysis reveals known and putative novel genes involved in senescence

Coexpression network analysis utilizes large-scale gene expression studies for grouping of novel genes involved in specific processes (Eisen et al., 1998) to predict biologically meaningful gene interactions (Borghi et al., 2010). We used Weighted Gene Correlation Network Analysis (WGCNA) (Zhang and Horvath, 2005) to build coexpression networks from a subset of genes that passed a coefficient of variation (CV) cutoff criteria (see Materials and Methods). Of the 2,548 genes from leaf tissues that passed the CV filter, 2,054 were placed into four distinct gene modules by the WGCNA procedure (Table S6). Genes within each module showed very similar expression patterns in the POL and NONPOL leaves up to 18 DAA (Figure 7A). This is also evident from a heat map generated by summarization of all genes within a module into eigengenes (Langfelder and Horvath, 2007) (Figure S10A). However, substantial differences were evident at 24 and 30 DAA, consistent with trends observed in PCA clustering and MapMan analysis. For instance, genes in module A2 were substantially down-regulated in NONPOL leaves at 30 DAA compared to POL leaves. Module A3 is particularly
interesting as it contains genes that showed substantial up-regulation in the NONPOL leaves at 24 DAA and sustained higher levels at 30 DAA. Of 1,103 genes in this module, 71 were transcription factors (Yilmaz et al., 2009) dominated by the NAC and AP2/EBERP families that are known to be involved in senescence (see above). This module contains several other senescence-related genes. For instance, an SGR (senescence-inducible chloroplast stay-green protein 1) is present; SGRs are involved in the early steps of chlorophyll catabolism and mark the onset of senescence (Hortensteiner, 2009). Three SAGs (Senescence-associated protein 5, 6, and 15) were also represented in this group. Interestingly, several members of the flavonoid biosynthetic pathway including \( a1 \) (dihydroflavonol-4-reductase), \( bz2 \) (bronze2), and \( fht1 \) (flavanone 3-hydroxylase1) were also represented. Flavonoids protect against stress and are likely important for maintaining redox in the cell during active chlorophyll breakdown and ROS production. The presence of biologically meaningful genes in the coexpression network supports the relevance of the networks in finding novel senescence-related genes.

For internodes, fewer (1,273) genes met the CV cutoff criteria and WGCNA analysis classified 950 of these genes into three modules (Table S7). Expression profiles within each of the modules were nearly identical between the POL and NONPOL tissues through 24 DAA (Figure 7B, Figure S10B), suggesting lack of major transcription changes until later stages of development. However, genes in the NONPOL samples in module B2 showed a dramatic increase at 30 DAA. While it is generally believed that the onset of leaf senescence is developmentally coordinated with that of other plant organs, molecular aspects of senescence of the non-leaf tissues are not well understood. Interestingly, of the 142 genes in module B2, 83 were also present in the leaf module A2 suggesting substantial overlap between senescence programs in the two organs. Except for three AP2/EBERP transcription factors, none of these common genes are known to be involved in senescence and could represent novel genes specific to internode senescence.
MATERIALS AND METHODS

Plant materials, growing conditions, and sampling details

B73 inbred plants were grown at the West Madison Agricultural Research Station, Verona, WI during summer of 2009. The soil type of the experimental field is Plano silt loam with pH 6.8, organic matter 3%, phosphorus 93 ppm, and potassium 240 ppm. During field preparation, 200 kg/acre of Urea (46-0-0) was applied. The herbicides Callisto (Syngenta, Greensboro, NC; 142 g per acre), Dual II (Syngenta, Greensboro, NC; 710 ml per acre) and Simazine (Agrisolutions, Brighton, IL; 227 g per acre) were applied one day after planting. Plants were hand planted in 2.9 meters long rows with row and plant spacing of 76 cm and 24 cm, respectively.

The primary and all subsequent ears of all plants were covered with shoot bags before silk emergence. Upon silk emergence in at least 80% of the plants in each row, shoot bags from plants in alternating rows were removed on the same day to allow open pollination of the control plants. In these plants, supplemental pollinations were performed the same day to ensure maximum seed set. In the remainder of the rows, primary and secondary ears were kept completely covered to prevent pollination and subsequent seed set. This was considered as 0 DAA. Only those pollinated and non-pollinated plants that showed silk emergence at 0 DAA were included in the experiment.

The leaf and internode tissues from two biological replicates were collected from pollinated and non-pollinated plants at 0, 6, 12, 18, 24, and 30 DAA. For each biological replicate, both tissues were collected from two competitive (non-border and well-spaced from neighbors) randomly chosen plants and pooled for further analysis. From the leaf at the primary ear-bearing node, a 30 cm section of leaf
blade and mid-rib, excluding ligule and sheath, was collected. For internode tissues, the internode above the primary ear-bearing node was harvested and the nodes on both ends were completely removed leaving approximately 10cm of internode that did not include any leaf or sheath tissue. Samples were chopped into small pieces, flash frozen in liquid nitrogen, and stored at -80ºC. All the transcriptional and biochemical analyses were performed on two biological replicates.

**Microarray analysis, present calls and identification of differentially expressed genes**

RNA extraction, microarray design, hybridization, data extraction, and analysis were performed as described previously (Sekhon et al., 2011). A gene with a RMA-normalized linear expression value of \( \geq 200 \) in at least in one of the stages/treatment was considered to be expressed. The expression cut-off was an arbitrarily chosen conservative value that was over 2.5 times the mean normalized signal from 165 random sequences spotted on each slide, which had a mean of 76 across all 48 slides used for the experiment. From the set of genes expressed above the minimum expression cutoff, we selected those genes that showed at least 3-fold change in either treatment (pollinated or non-pollinated) at each stage. This resulted in identification of 4,299 and 624 differentially expressed transcripts in leaves and internodes, respectively (Table S8a-b).

**Clustering**

PCA was performed on RMA-normalized \( \log_2 \)-transformed expression values of 30,892 probe sets by first grouping the genes in 1,000 clusters using k-mean statistics followed by PCA using the Spotfire DecisionSite for Functional Genomics (DSFG) package (http://spotfire.tibco.com/).

**MapMan analysis**
MapMan software (Usadel et al., 2005) was obtained from [http://mapman.gabipd.org](http://mapman.gabipd.org). The mapping used for the analysis was based on the sequences provided in the file obtained from http://ftp.maizesequence.org/current/filtered-set/ZmB73_5b_FGS_cds.fasta.gz. To generate the mapping, the sequences were processed by the Mercator annotation pipeline ([http://mapman.gabipd.org/web/guest/app/mercator](http://mapman.gabipd.org/web/guest/app/mercator)) that combines search results from BLAST results (bit score cut-off of 80) against Arabidopsis TAIR9 proteins, rice (*Oryza sativa*) TIGR5 proteins, protein sequences from the SwissProt plant protein annotation project; RPSBLAST against the conserved domain database (CDD) and the clusters of orthologous genes database (KOG), and a full InterProScan analysis (version 4.7). To compute a MapMan bin for the input sequences, the bin classifications for each search match were retrieved from the reference mappings and a MapMan bin was computed based on the bit scores of the hits. For the MapMan analysis, log₂ ratio of fold change in expression (pollinated tissue/non-pollinated tissues) was calculated for each stage of sampling. Only those genes meeting the expression cutoff criteria of at least 3-fold change in expression in either treatment were used for MapMan analysis. The resulting data table along with the mapping file was used for analysis.

**Orthology analysis for annotation of maize senescence-associated genes**

Orthologous and paralogous gene relationships were identified using OrthoMCL (version 1.4, Li et al., 2003). All proteins from Arabidopsis (TAIR10, Swarbreck et al., 2008), *Medicago truncatula* (version 3.5; Medicago Genome Sequence Consortium, [http://medicago.jcvi.org/cgi-bin/medicago/download.cgi](http://medicago.jcvi.org/cgi-bin/medicago/download.cgi)), rice (version 6.1, Ouyang et al., 2007), and maize (AGPv1, Schnable et al., 2009) were used for orthology analysis. All-vs-all BLASTP alignments were performed with a minimum e-value requirement of 1e-10 (Altschul et al., 1990). Blast results and FASTA sequence files were processed by custom Perl scripts to generate bpo and gg intermediate files that were then used as input to OrthoMCL. The ortholog groups
were used to identify maize senescence-related genes by identifying those ortholog groups that contained Arabidopsis, rice, and *M. truncatula* proteins from a list of 1,326 previously identified leaf senescence genes ([http://psd.cbi.pku.edu.cn/](http://psd.cbi.pku.edu.cn/)) (Liu et al., 2011).

**Coexpression network analysis**

Before performing coexpression analysis, coefficient of variation (CV = σ/µ) calculations were performed on the log2 transformed expression values for all genes. Untransformed expression values less than one were transformed to zero. Genes with CV values less than 0.1 were removed to reduce the number of genes with low expression variance which would indicate little change in expression across treatments/sampling time points. WGCNA was used to identify modules of genes that have highly correlated expression (Zhang and Horvath, 2005). Log2 expression data were processed using the WGCNA R-package (Langfelder and Horvath, 2008). The WGCNA β and treecut parameters were set to 30 and 0.8, respectively. All other parameters were used with their default values, and the blockwiseModules function was used for module identification. Heatmaps were constructed that depict the eigengenes from each identified module. Eigengenes are the eigenvector from the first principle component analysis of the log2 transformed matrix of expression values for all genes in a cluster (Langfelder and Horvath, 2007). Eigengenes represent a centroid measure of the expression levels of all genes in a cluster.

**Metabolic analysis**

*Grinding and Dispensing Samples*: An aliquot of the frozen leaf and internode samples was lyophilized and then finely ground on a custom-designed robot, iWALL (Santoro et al., 2010). For each sample, six 1.4 mL tubes (MP52607PK, Micronic North America, McMurray, PA) containing 1.3-1.7 mg of the ground tissue were prepared, three each for digestibility and fractionation assay (below).
Digestibility Assays: The samples were pre-treated as described earlier (Santoro et al., 2010). For digestion, 50 μL solution containing 0.5 μL Accellerase 1000 (Genencor, Rochester, NY), 1 M citrate buffer (pH 4.5), and 0.01% sodium azide was added to all tubes to a final volume of 0.8 ml and final enzyme concentration of 50 mg protein/g glucan. Samples were incubated at 50°C for 20 h with end-over-end rotation followed by centrifugation at 1,500×g for 3 min. Supernatants were transferred into 96-well plates and subsequently transferred in quadruplicate into two 384-well plates each for measuring glucose (Glc) and pentose (Pent). The Glc content was assayed using the glucose oxidase/peroxidase (GOPOD) method (K-GLUC, Megazyme, Ireland) following manufacturer’s instructions with some modifications as described (Santoro et al., 2010). Each digestion assay was quantitated for Glc and Pent in quadruplicate. Glc and Pent yield refers to the amount of each sugar released relative to the total dry mass dispensed into each tube.

Sugar fractionation assays: For assaying free Glc, 750 μL of distilled water was pipetted to the tubes prepared earlier using the automated workstation. Next, 50 μL of a solution containing 1 M citrate buffer (pH 4.5) and 0.01% sodium azide was added resulting in a final volume of 0.8 mL. Subsequent sample incubation and processing was identical to that used for digestibility assay. Glc was assayed using GOPOD method described above.

Determination of Starch: To the pre-prepared sample tubes, 150 μL of 2M KOH was added followed by incubation at 90°C for 1 h and cooling on ice. Next, 600 μL 1.2M NaOAc buffer (pH 3.8) was added to each Micronic tube to neutralize the solution. For starch hydrolysis, 60 μL of a solution containing 5 μL amylglucosidase (K-TSTA, Megazyme, Ireland), 5 μL α-amylase (K-TSTA, Megazyme), distilled water, and 0.01% sodium azide was added. The rest of the processing of these samples was identical to that used for digestibility assay. The heating before starch hydrolysis is sufficient to degrade all pre-existing free glucose
in the samples, thus, glucose detected after the hydrolysis step is derived from starch. Data was recorded as explained for digestibility assay.

Matrix polysaccharide composition analysis: A 60 mg aliquot was ball milled using the iWall grinding and feeding robot as described above. The alcohol insoluble residue was prepared from the ball-milled material followed by an amylase and pullulanase treatment for starch removal (Foster et al., 2010). The isolated lignocellulosic cell wall material was dried and weighed into three technical replicates of 2 mg each to assay the matrix polysaccharide composition and crystalline cellulose content. The polysaccharide composition was analyzed via GC-MS after a 2M trifluoroacetic acid (TFA) hydrolysis and subsequent alditol acetate derivatization of the neutral monosaccharides present the hydrolysate. The remaining residue left from the TFA hydrolysis, composted of mainly crystalline cellulose, was stripped of any residual lignin, amorphous glucose, and hemicelluloses using the updegraff reagent and then subsequently hydrolyzed with sulfuric acid. The resulting monosaccharides in the hydrolysate were quantified using the colorimetric anthrone assay.

Quantification of chlorophylls and carotenoids: Chlorophyll \( a \), chlorophyll \( b \), total chlorophylls, and total carotenoids were measure following a previously published protocol (Lichtenthaler, 1987) with minor modifications. Briefly, freeze-dried leaf tissue was ground to 1 mm particle size and 10 mg aliquot was used for extraction. Compounds were extracted with a mixture containing 96% ethanol and 4% of 0.1 M ammonium hydroxide. Absorbance values were recorded at 649, 654, 665, and 750 nanometers. Each sample had four technical replicates and the values were averaged from the technical replicates. Quantification of each compound was performed following the published equations (Lichtenthaler, 1987).
ACKNOWLEDGEMENTS

Authors thank Karl Haro von Mogel and Emily Rothfusz for help in collection and processing of samples, Dr. Richard Amasino for helpful discussions and suggestions, and James Johnson for critically reading the manuscript. Authors also thank Marc Lohse and the MapMan team for help with generation of the mapping file for the maize genome. Authors also thank anonymous reviewers for their helpful suggestions to improve the manuscript. This work was supported by the U. S. Department of Energy Great Lakes Bioenergy Research Center (Department of Energy Office of Science BER DE-FC02-07ER64494).
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FIGURE LEGENDS

Figure 1: Chronology of phenotypic, transcriptomic, and metabolic changes during induced senescence. A. Phenotypic changes in leaf at primary ear-bearing node due to induced senescence. B. Clustering based on principle component analysis performed for all samples. Developmental stages (days after anthesis) and tissues are mentioned in the callout boxes; Int, Internode. Pollinated and non-pollinated tissues are denoted by blue and red shapes, respectively. C-D. Accumulation of starch in leaves (C) and internodes (D). E-F. Accumulation of free glucose (Glc) in leaves (E) and internodes (F). G. Total chlorophyll content in leaves. For graphs C-G), mean values of two biological replicates are presented and the error bars at each data point indicate standard error for the two replicates. One, two, and three asterisks indicate FDR-corrected Student’s t-test p-value below 0.05, 0.01, and 0.001, respectively. DAA, days after anthesis.

Figure 2: Metabolism overview showing transcriptional profile during induced senescence at 24 (A) and 30 (B) days after anthesis in the leaf tissue. Log2 ratios of average NimbleGen microarray expression in pollinated and non-pollinated tissues were calculated and the resulting file was loaded onto MapMan (http://mapman.gabipd.org/) to generate the overview. Thus, the log2 ratio value of 1, 2, and 3 denotes 2, 4, and 8 fold change, respectively. Positive log2 values represented by blue color indicate up-regulation in the pollinated plants and signify that those transcripts are suppressed during early senescence, while negative values shown by red color denote up-regulation of the transcripts during early senescence. Color saturates at 8-fold change. Each square represents a differentially expressed transcript (See Materials and Methods).

Figure 3. Metabolism overview showing transcriptional profile during induced senescence in internode 30 DAA stage. Details of the figure are same as described for Figure 2.
Figure 4. Accumulation of major structural carbohydrates during induced senescence. A-B. Cell wall-bound (CW) glucose (Glc) in leaf (A) and internode (B). C-D. Xylose in leaf (C) and internode (Int) (D) tissue. Developmental stages (days after anthesis, DAA) are shown on x-axis of each graph. The error bars at each data point indicate standard error obtained from two biological replicates.

Figure 5. Differential expression patterns of transcription factors during induced senescence. Differentially expressed genes annotated as transcription factors (Yilmaz et al., 2009) were separated into three groups based on k-means clustering. Log₂ ratios of expression in pollinated and non-pollinated plants were used for this analysis. Representative expression trend of each group is shown as line graphs. Number and name of individual transcription factor family belonging to each group are shown as pie chart below the line graph. DAA, days after anthesis.

Figure 6: Expression patterns key genes upregulated during sugar-induced senescence in Arabidopsis (Pourtau et al., 2006) in the current study. Log₂ ratios of expression in pollinated and non-pollinated plants were used to make this heatmap. Blue color indicates higher expression in pollinated tissues while red color signifies higher expression in non-pollinated tissue. Developmental stages are mentioned on top while the genes and annotations are mentioned on right. Asterisks indicate the genes that were also upregulated in pho3 mutants lacking sucrose transporter SUT2 (Lloyd and Zakhleniuk, 2004) (see text for details).

Figure 7: Normalized expression patterns of genes belonging to distinct modules in pollinated and non-pollinated leaf (A) and internode (B) tissues. Weighted Gene Coexpression Network Analysis was used to identify modules of genes with highly correlated expression (See Materials and Methods). Normalized expression values (Z scores) are shown.
SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Number of probe sets expressed at each stage in leaf (A) and internode (B) of pollinated and non-pollinated plants. Stages (days after anthesis) are shown on x-axis while number of genes is shown on y-axis. For a gene to be considered as expressed, an arbitrary cutoff of linear expression value more than 200 was used (see Materials and Methods for details).

Figure S2: Change in the contents of chlorophyll a (A), chlorophyll b (B), and total carotenoids (C) during induced senescence. Error bars are based on standard error calculated from two biological replicates. One, two, and three asterisks indicate FDR-corrected Student’s t-test p-value below 0.05, 0.01, and 0.001, respectively.

Figure S3. Metabolism overview showing transcriptional profile during early senescence at 0 (A), 6 (B), 12 (C) and 18 (D) days after anthesis in leaf tissue. Log$_2$ ratios for average expression in pollinated (normal development) and non-pollinated plants were loaded onto MapMan (http://mapman.gabipd.org/) to generate the overview. Each square represents a differentially expressed transcript (See Materials and Methods). On the log$_2$ scale ranging between 3 and -3, dark blue color represents 8-fold or more up-regulation in the pollinated plants and signifies that those transcripts are suppressed during early senescence, while dark red color signifies 8-fold or more up-regulation of the transcripts during early senescence.

Figure S4. Metabolism overview showing transcriptional profile during early senescence at 0 (A), 6 (B), 12 (C), 18 (D), and 24 (E) days after anthesis in the internode tissue. Log$_2$ ratios for average expression in pollinated and non-pollinated plants were loaded onto MapMan (http://mapman.gabipd.org/) to generate the overview. Each square represents a differentially expressed transcript.
(See Materials and Methods). On the log₂ scale ranging between 3 and -3, dark blue color represents 8-fold or more up-regulation in the pollinated plants and signifies that those transcripts are suppressed during early senescence, while dark red color signifies 8-fold or more up-regulation of the transcripts during early senescence.

Figure S5: Expression patterns of selected genes related to synthesis and metabolisms of free and storage sugars in leaves. (A-F) Expression profiles of selected genes mentioned in the text. Developmental stages are shown on x-axis while the linear expression value is shown on y-axis. Name of the gene is listed at the top of the chart. Red lines represent non-pollinated sample while blue line denotes pollinated sample.

Figure S6: Expression patterns of selected genes related to synthesis of structural and cell wall-bound sugars. (A-D) Expression profiles of selected genes mentioned in the text. Developmental stages are shown on x-axis while the linear expression value is shown on y-axis. Name of the gene is mentioned at the top of the chart. Red lines represent non-pollinated sample while blue line denotes pollinated sample.

Figure S7: Expression patterns of selected genes related to chlorophyll degradation. (A-D) Expression profiles of selected genes mentioned in the text. Developmental stages are shown on x-axis while the linear expression value is shown on y-axis. Name of the gene is mentioned at the top of the chart. Red lines represent non-pollinated sample while blue line denotes pollinated sample.

Figure S8: Expression patterns of selected genes related to hormone biosynthesis or signaling. (A-F) Expression profiles of selected genes mentioned in the text. Developmental stages are shown on x-axis while the linear expression value is shown on y-axis. Name of the gene is mentioned at the top of the chart. Red lines represent non-pollinated sample while blue line denotes pollinated sample.
Figure S9: Differential expression patterns of senescence-associated genes in leaves during induced senescence. Log₂ ratios of expression in pollinated and non-pollinated plants were used to make this heatmap. Shown here are the genes that were upregulated (A) or down-regulated early (B) or late (C) during induced senescence. Blue color indicates higher expression of transcript in pollinated tissues while red color signifies higher expression of the transcript on non-pollinated tissue. Sampling stages (days after anthesis) are mentioned on top while the genes are mentioned on right. Tissues were clustered using hierarchical clustering (see Materials and Methods).

Figure S10: Heatmap showing the coexpression network modules in leaf (A) and internodes (B). Modules are showed as rows in the heatmap and mentioned on left. Samples are shown as columns indicated below the heatmap. Each sample in module is represented by an eigengene (See Materials and Methods for details). Color scale shown on right indicates the value of each eigengene which reflect the relative expression levels of all genes in the module.
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