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Review

Lipid turnover during senescence

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ABSTRACT

Rapid turnover of stored triacylglycerol occurs after seed germination, releasing fatty acids that provide carbon and energy for seedling establishment. Glycerolipid and fatty acid turnover that occurs at other times in the plant life cycle, including senescence is less studied. Although the entire pathway of β-oxidation is induced during senescence, Arabidopsis leaf fatty acids turnover at rates 50 fold lower than in seedlings. Major unknowns in lipid turnover include the identity of lipases responsible for degradation of the wide diversity of galactolipid, phospholipid, and other lipid class structures. Also unknown is the relative flux of the acetyl-CoA product of β-oxidation into alternative metabolic pathways. We present an overview of senescence-related glycerolipid turnover and discuss its function(s) and speculate about how it might be controlled to increase the energy density and nutritional content of crops. To better understand regulation of lipid turnover, we developed a database that compiles and plots transcript expression of lipid-related genes during natural leaf senescence of Arabidopsis. The database allowed identification of coordinated patterns of down-regulation of lipid biosynthesis genes and the contrasting groups of genes that increase, including 68 putative lipases.

Contents

1. Introduction

Although lipid turnover associated with senescence occurs in most plant tissues, this mini-review will focus primarily on leaves. Other reviews cover, for example, flowers [1]. In addition, we focus on the process of 'natural' senescence that occurs during the normal aging or ontogenic development of a plant rather than senescence that can be induced by a number of treatments such as darkness [2], nitrogen deprivation [3], or other environmental factors such as day length and biotic/abiotic stresses [4]. Senescence that occurs under 'induced' conditions results in substantially different transcriptional responses. For example, the number of 'regulated' genes during natural senescence is about two-fold higher than in dark-induced senescence [5].

Abbreviations: TAG, triacylglycerol; DGAT, diacylglycerol acyl transferase; LACS, long-chain acyl-CoA synthetase; KAT, 3-ketoacyl-CoA thiolase.

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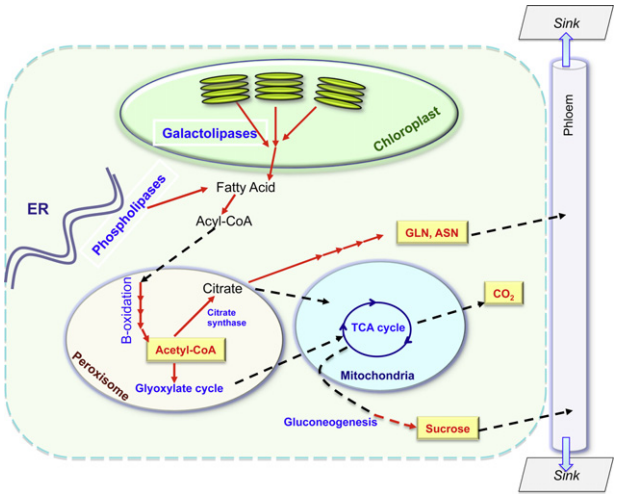

Fig. 1. Lipid turnover during leaf senescence: alternative pathways for acetyl-CoA metabolism. A simplified scheme of pathways involved in turnover of membrane lipids during leaf senescence. Galactolipases and phospholipases release fatty acids from glycerolipids in the chloroplast, ER or other membranes. After activation to acyl-CoA, β-oxidation in the peroxisome produces the central intermediate, acetyl-CoA. Three possible fates of the acetyl-CoA carbon are indicated: (1) entry into TCA cycle (via citrate) and oxidation to release CO_2. (2) Entry into the glyoxylate cycle with eventual metabolism by gluconeogenesis to produce sucrose for export to phloem and sink tissues. (3) Conversion to citrate (citrate synthase) and then amino acids (primarily GLN) and export to phloem and sink tissues. Other alternatives, such as export of carbon as organic acids (e.g. malate) are not shown. Enzyme reactions are represented by blue text and red arrows; transport or diffusion by black arrows.

The major result of leaf senescence is that carbon, nitrogen and other nutrients are 'mobilized' so that they can be transported via the phloem to be used for growth of new tissues or for storage in sink tissues. The developing seed is a predominant sink which at maturity represents 50% or more of the entire above-ground plant mass. Much of this seed biomass is accumulated from carbon (largely sucrose) supplied by leaves (and other photosynthetic tissues) before senescence. Still, a substantial contribution is also derived by breakdown and redistribution of resources from vegetative tissues during senescence [6]. Likewise, root crops, and perennials, including many grasses, also redistribute biomass through a major translocation of carbon, nitrogen and minerals to the roots to provide resources for growth in the spring. Although we are not aware of data on turnover of lipids of tree leaves/needles during senescence, there is a seasonal accumulation of triacylglycerol in the cambium as part of winter acclimation in some trees [7].

The redistribution of resources from source leaves to sink tissues during senescence requires the breakdown and mobilization of a major proportion of diverse cellular components. Because lipids are present in all organelle membranes, their turnover requires major trafficking of acyl chains and headgroups to the sites of degradation. Whether this occurs by mechanisms also used for membrane synthesis is unknown. In the case of lipids, breakdown begins with lipolytic reactions that release fatty acids from membrane lipids which then enter the β-oxidation pathway in peroxisomes (Fig. 1). The total fatty acid content of Arabidopsis, *Brachypodium* and switchgrass leaves decreases at least 80% during senescence [8]. Nitrogen (primarily from proteins) also decreases over 80% in senescing Arabidopsis leaves [9] in large part through protease induction, breakdown of cellular protein and export of amino acids [10]. Chlorophyll, soluble carbohydrates, polysaccharides and several elements (K, Mo, P, etc.) decline by 50% or more [9]. Thus, the breakdown of lipid is one part of the overall coordinated mobilization and redistribution of resources from leaves to seeds (and/or roots/tubers). By the end of senescence, the dry weight of leaves is reduced approximately 50% for Arabidopsis, *Brachypodium* and switchgrass [8]. Most of the remaining biomass is in the form of cell wall polysaccharides and lignin; components outside the cell that are resistant to breakdown. Likewise, cuticular lipid on the surface of plants is not degraded during senescence [8].

Information on lipid metabolism during plant senescence has been presented in earlier reviews [11,12]. In addition to highlighting some recent studies, this mini-review focuses on changes in the expression of Arabidopsis genes related to lipid metabolism, taking advantage of recent microarray analyses during natural leaf senescence [5,13,14] and of recent compilations and annotations of >600 genes associated with acyl-lipid metabolism [15].

2. Fatty acid turnover occurs continually during leaf growth

In addition to the turnover of 80% of leaf fatty acid noted above, it is important to recognize that fatty acid turnover does not occur only during senescence, but is likely a constitutive process that occurs throughout leaf growth. Although the fatty acid content of Arabidopsis leaves as a percent of dry weight is approximately constant throughout leaf expansion, during leaf senescence (50–90 days), leaves have a net decrease of at least 1.6% of total fatty acids per day, resulting in the loss of most fatty acid of fully senescent leaves [8]. Unsaturated fatty acids disappeared at a higher rate than saturated. This overall rate of turnover represents the net change in fatty acid levels and therefore does not tell us how degradation rates may be balanced or exceeded by rates of fatty acid synthesis. Pulse chase radiolabeling experiments with $^{14}CO_2$ [16] indicate that turnover of fatty acids also occurs in young Arabidopsis leaves, at up to 2% per day. Combining these studies suggests that the rate of fatty acid turnover by β-oxidation may not be greatly different in young and old leaves. Prior to senescence, turnover is balanced (or exceeded) by the rate of new fatty acid synthesis. Thus, the net disappearance of fatty acids that occurs later as leaves age can at least partly be attributed to declining synthesis of fatty acids in senescent leaves in addition to an increase in the rate of their degradation. Senescence is clearly associated with increased expression of transcripts encoding enzymes of β-oxidation (see below), and this 'induction' presumably provides additional capacity over a basal level of 'constitutive' turnover. This capacity may provide flexibility in response to increasing utilization by sink tissues. It is also important to note that the rate of breakdown of fatty acids in leaves is much lower than occurs in seedlings after germination. For comparison, the rate of loss of fatty acid in the first 5 days after Arabidopsis seed germination is at least 50-fold higher per dry weight than in senescing leaves [8] likely reflecting the very high synthesis rates of membrane lipid and other cell structures needed for rapid seedling establishment.

3. Accumulation of triacylglycerol is sometimes associated with leaf senescence and may be a transient 'buffer' for fatty acids released from membranes

TAG has been reported to increase during natural senescence of leaves of Arabidopsis [17,18]. This increase was associated with increased levels of diacylglycerol acyl transferase (DGAT) transcripts and protein (by Western blot) [17] during leaf senescence. Although DGAT is most often considered to be ER localized [19], there is also evidence for chloroplast association [17] and that TAG accumulation in Arabidopsis leaves occurs in plastid lipid bodies (plastoglobuli). Nitrogen starvation results in accumulation in plastoglobuli of TAG and phytyl-esters of plastid fatty acids. Both of these lipids can be synthesized by *PHYTYL ESTER SYNTHASE*

which is induced during senescence [18]. TAG accumulation is also observed in cytosolic lipid droplets [20,21] but the relative distribution of TAG within and outside of the plastid has not been quantitatively determined in any study.

Although high TAG levels in senescent leaves have been reported [17], most studies have measured much lower TAG in leaves. For example, TAG levels less than 0.05% of dry weight were reported in senescent Arabidopsis leaves [21]. Likewise, an increase in TAG during natural senescence of Arabidopsis, *Brachypodium* or switchgrass leaves was not observed and this lipid represented less than 0.02% of dry weight and less than 0.5% of the total fatty acid [8]. Since stress induces TAG production in leaves, the different results cited above might be attributed to variations in growth conditions. Difficulties in accurate measurement of low levels of TAG in leaves likely also contribute to results of different studies [8].

Despite the very low level of TAG in leaves, radiolabeling indicates even young leaves have the capacity to synthesize TAG. For example, TAG was observed as the most highly labeled product 30 min after addition of ^{14}C-lauric acid to leaves [22]. The labeling of TAG was transient and the acyl groups appeared to be redistributed to phosphatidylcholine and other membrane lipids. Although TAG is often considered as an end product, these radiolabeling results and recent evidence in animals and yeast reveal a dynamic and reversible metabolism of TAG, which suggests a role as a buffer for storage of acyl chains when they are in net excess [23]. During senescence, if the rate of release of fatty acid from membrane lipids exceeds the capacity of β-oxidation, TAG may be a transient intermediate that prevents detrimental levels of free fatty acid accumulation, or depletion of the free CoA-SH pool.

4. A database of gene expression during natural leaf senescence

There have been several hundred studies of natural or induced plant senescence since 2000, approximately half on Arabidopsis, and more than a dozen that include microarray analyses that evaluate changes in gene expression [24]. In general, these studies included only brief discussion of genes of lipid metabolism. We have selected three robust studies [5,13,14] that provide comprehensive Arabidopsis gene expression data during natural leaf senescence and we have analyzed the data to provide a more lipid-focused understanding. (Although the 'landmark' microarray study of AtGenExpress [14] was not specifically directed at senescence, it includes data on transcripts of leaves at a range of development including senescence.) From these studies we prepared a user-friendly database in Excel to allow comparisons between datasets. The database allows dynamic plotting of expression patterns and simultaneous comparisons between the three datasets (Supplement 1). Comparison of data across three studies increases confidence that changes in gene expression can be attributed to senescence, rather than to differences in growth conditions or to 'hypervariable' gene expression [25]. This database has allowed us to make a number of the observations and conclusions below. The Supplement Excel file includes data for >1000 'lipid-annotated' and related genes that are represented on the three microarray studies. More than 600 of these genes are organized in pathways based on ARALIP (http://ARALIP.plantbiology.msu.edu/). In addition, other genes annotated as lipases at TAIR10.0 are included in the database.

5. Transcripts for fatty acid but not glycerolipid assembly decline markedly as leaves age

Previous radiolabeling studies indicate that fatty acid synthesis is highest in young, expanding leaves and declines as leaves age [26]. This decline could be due to a number of factors, including lack of precursor substrate supply, post-translational down-regulation, protein turnover of lipid biosynthetic proteins, or lower gene expression. The three microarray studies indicate there is a major and coordinated decline in transcript levels for almost all the core fatty acid biosynthetic genes, consistent with earlier metabolic and protein expression observations. The transcripts for more than 15 proteins involved in reactions from pyruvate to long-chain acyl-ACP, decrease 10 fold or more during the transition between young leaves and senescence (Fig. 2; Supplement 1). The oleate, linoleate and other membrane bound desaturases also similarly decrease. The coordinated decrease in transcripts for fatty acid synthesis and desaturation and the labeling/biochemical data, together argue that the leaf slows or stops the production of new fatty acids through transcriptional down-regulation, likely combined with protein turnover. However, this decline in transcripts does not occur for the acyl-ACP thioesterase B, and some isoforms of acyl-ACP desaturase. In addition, a number of genes involved in glycerolipid assembly reactions do not follow the pattern of fatty acid biosynthesis genes (Fig. 2). For example, in addition to DGAT, transcripts for the first two acyl transferases for de novo glycerolipid synthesis, the 'putative' glycerol-3-phosphate acyltransferase-9 and 1-acylglycerol-3-phosphate acyltransferase-2 and lysophosphatidylcholine acyltransferase increase at the same time those for fatty acid synthesis are in decline. One interpretation for these increases is that these acyltransferase activities must be increased or maintained because they are involved in acyl chain 'remodeling' that is required as a response to senescence or other stress. A similar 'disconnect' in the regulation of transcripts for fatty acid supply, compared to those for downstream enzymes of glycerolipid assembly was observed during oilseed development [27]. Although as noted above, TAG accumulation is associated with leaf senescence, the transcripts level of oleosins and most other characteristic seed oilbody proteins is 100–1000 fold lower in senescent leaves than in seeds. One member of the caleosin family, At2g33380, is an exception, with 5–10 fold higher expression in senescent leaf than in seeds.

6. Over 60 'lipases' increase during senescence. Which of these function in fatty acid and glycerolipid turnover?

Fatty acid degradation begins with the removal of fatty acids from the glycerolipid backbone by enzymes that hydrolyze the acyl ester linkage. There are over 270 genes in the Arabidopsis genome with 'lipase' in their annotations (TAIR10.0), of which more than 100 are members of a large gene family referred to as 'GDSL lipases' (so named because of the GlyAspSerLeu motif). It is striking that the number of putative lipase genes that may be involved in lipid catabolism is much greater than the number of membrane or storage glycerolipid structure types.

Based on the lipid substrates hydrolyzed, lipases are grouped into galactolipases, phospholipases, TAG lipases, and lipolytic acyl hydrolases. Galactolipids, the most abundant lipid class of leaves, are thought to be de-esterified by galactolipases, galactosidases and lipolytic acyl hydrolases during leaf senescence. A patatin-like protein [28] and an ethylene-induced DAD-like homologue [29] are examples of lipases that are up-regulated during leaf senescence and potentially involved in galactolipid turnover.

Phospholipases can be grouped into types that hydrolyze head group ester bonds (phospholipase D, phospholipase C, and phosphatidic phosphatase) and those that remove acyl chains esterified to glycerol (phospholipase A1 and phospholipase A2 and phospholipase B). Upregulation of phospholipase D activity is observed in senescing tissues and its key role is implied by delayed senescence in antisense plants and accelerated senescence upon over-expression [30]. Six other members of the large phospholipase

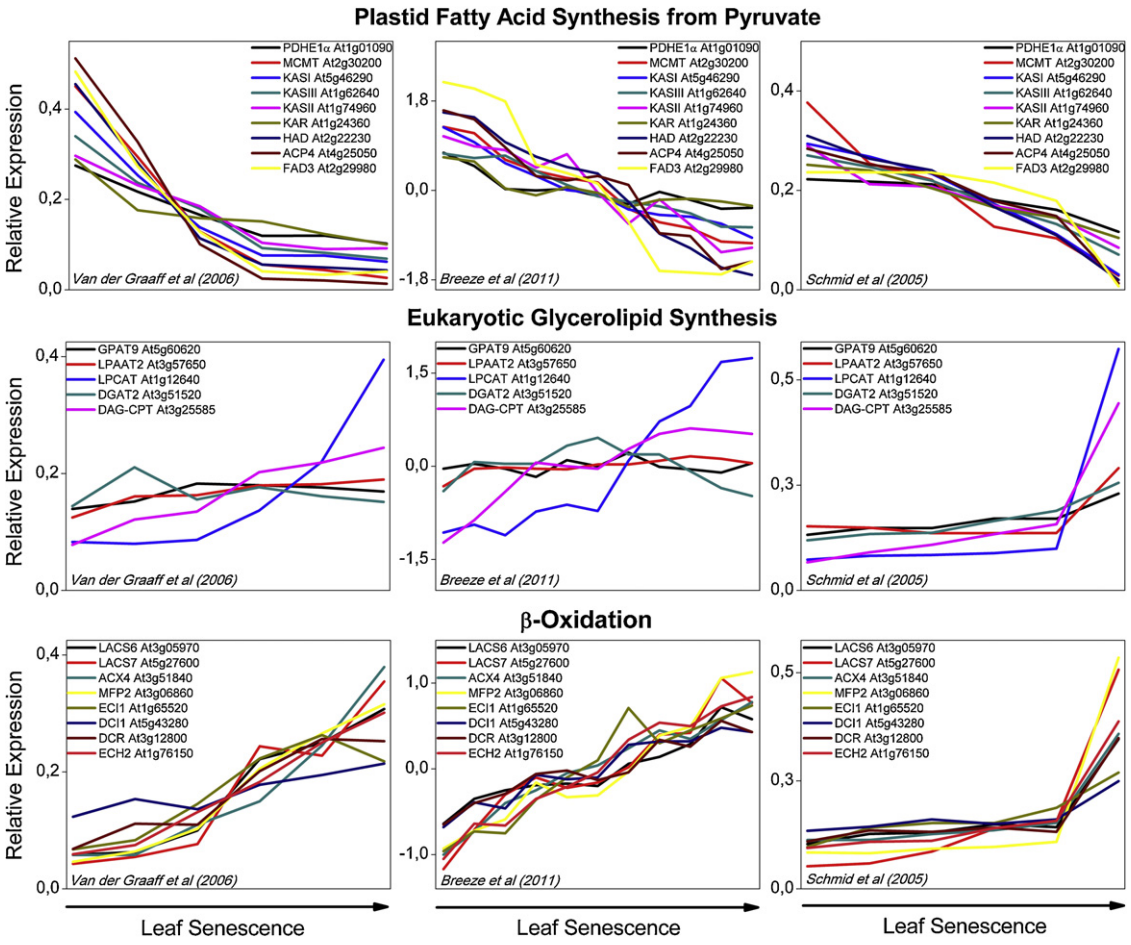

Fig. 2. Expression of genes for selected pathways of lipid metabolism. Data from three microarray analyses of leaf senescence are presented. For clarity, only a subset of genes is presented that displayed similar expression in all three studies. Detailed annotations and data for the genes are presented in the Supplement Database. PDH-E1a, pyruvate dehydrogenase α subunit, E1α component of pyruvate dehydrogenase complex; MCMT, malonyl-CoA: ACP malonyltransferase; KASI, ketoacyl-ACP Synthase I; KASIII, ketoacyl-ACP synthase III; KASII, ketoacyl-ACP synthase II; KAR, ketoacyl-ACP reductase; HAD, hydroxyacyl-ACP dehydrase; ACP4, ACYL carrier protein; FAD3, linoleate desaturase; GPAT9, glycerol-3-phosphate acyltransferase; LPAAT2, 1-acylglycerol-3-phosphate acyltransferase; LPCAT, 1-acylglycerol-3-phosphocholine acyltransferase; DGAT2, Acyl-CoA: diacylglycerol acyltransferase; DAG-CPT, diacylglycerol cholinephosphotransferase; LACS6, long-chain acyl-CoA Synthetase (peroxisomal); LACS7, long-chain acyl-CoA synthetase (peroxisomal); ACX4 acyl-CoA oxidase; MFP2, multifunctional protein; ECI1, enoyl CoA isomerase (peroxisomal); DCI1, dienoyl CoA isomerase; DCR, dienoyl-CoA reductase; ECH2, peroxisomal enoyl-CoA hydratase 2.

D family also increase during senescence. In addition, 11 phospholipase C, two phosphatidate phosphatase members, and one phospholipase A, are induced during senescence. Supplement Table 2 provides a summary of these and other lipase classes.

More than 30 genes putatively encoding TAG or monoacylglycerol lipases are annotated in the Arabidopsis genome, but only a few of them have been characterized. SUGAR-DEPENDENT1 is a major lipase essential for metabolism of TAG during seedling development [31]. This lipase may also have a role during leaf senescence because its transcripts increase markedly during natural leaf senescence. An Arabidopsis homolog of CGI-58, a protein associated with lipodystrophy in humans, also has TAG lipase activity [32]. Its transcripts increase in all 3 microarray studies, and mutants in this protein have increased TAG levels in leaves [33]. Another gene, At2g31690, whose transcripts and protein (by Western blot) are induced in senescence encodes a protein with TAG lipase activity after recombinant expression [34]. The protein is targeted to plastids and co-localizes with plastoglobuli and thus potentially is involved in the degradation of plastid TAG in senescing leaves. Suppressed expression of At2g31690 delayed senescence and also altered chloroplast and thylakoid morphology. SAG101 (At5g14930), a leaf **S**enescence-**A**ssociated **G**ene that is strongly up-regulated during leaf senescence has lipolytic acyl hydrolase activity when expressed in *Escherichia coli* [35]. SAG101 antisense also delayed, and its overexpression accelerated senescence. Unfortunately, most studies on delayed senescence did not determine if lipid levels remained high in the leaves. Also, it is not clear yet if the pleiotropic phenotypes observed when expression of lipases is altered are directly due to their lipase activity on bulk lipids, or more indirectly to a more specific influence on signaling or other pathways.

In addition to the examples above, analysis of the three microarray studies identified 68 'lipase' genes with increased expression (in at least 2 of 3 studies). This represents 25% of all lipase-annotated genes of Arabidopsis, and this number perhaps reflects the importance of recovery of carbon and high energy of lipids for export to sink tissues. How many of these are directly involved in or required for lipid turnover during senescence is a major unknown. When considering this large list, it is important to note that the 'lipase' annotation can include proteins with many different enzyme activities related to ester bonds, and in fact, proteins annotated as lipase may carry out reactions unrelated to lipids. As noted above, there is experimental evidence confirming the activity or role of only a few of these enzymes [15]. Demonstration of lipase activity after expression in a heterologous host (e.g. yeast)

provides an initial indication of enzyme function, but this must be confirmed by determining the metabolic consequences of knock-out and/or over-expression experiments *in planta*. The first TAG lipase with confirmed *in planta* functions was only recently identified after extensive in vitro and in vivo analysis [31]. It is also important to recognize that some enzymes catalyzing the hydrolysis of esters can also catalyze transesterification reactions [36] and may thus have anabolic rather than catabolic roles in vivo. As examples, At1g54570 and At3g26840 had been annotated as "esterase/lipase/thioesterase" but are now known to have acyltransferase activities [18]. A gene annotated as a GDSL lipase was recently shown to be an acyltransferase involved in cutin biosynthesis [37]. A converse example is that an enzyme previously annotated as an acyltransferase has recently been identified as a phospholipase A [38].

7. Transcripts encoding the complete pathway of β-oxidation are coordinately induced during senescence

Within two days after seed germination, genes involved in the oxidative degradation of fatty acids released from TAG are coordinately up-regulated, together with those of the glyoxylate cycle and gluconeogenesis [39]. As shown in Fig. 2, a similar coordinate up-regulation of genes encoding all key enzymes of β-oxidation was observed during natural senescence. Three core proteins (acyl-CoA oxidase, multifunctional protein and 3-ketoacyl-CoA thiolase) are involved in each round of acetate unit release from saturated fatty acids [40]. Transcripts for 5 of the 6 acyl-CoA oxidase isoforms and both isoforms of the multifunctional protein also increase expression as leaves age. Of three ketoacyl-CoA thiolase isoforms, transcripts for *KAT2*, essential for lipid catabolism in seedlings [40] were strongly increased in senescent leaves, consistent with the impact of the *kat2* mutation in delaying leaf senescence [41]. This involvement of *KAT2* in senescence may primarily be through its requirement for jasmonic acid signaling [41], which is consistent with the lack of *kat2* influence on leaf fatty acid turnover [8].

Prior to β-oxidation, fatty acids must be transported into the peroxisomes and activated to acyl-CoA. The peroxisomal ABC transporter that is essential for seed oil mobilization, is also related to β-oxidation in leaves [2] and its pattern of expression increases during senescence. The activation of fatty acids to acyl-CoAs is catalyzed by long-chain acyl-CoA synthetase (LACS). Two peroxisomal LACS isoforms are involved in fatty acid degradation [42] and transcripts for both are up regulated. However, in a double mutant of both isoforms no major differences in fatty acid turnover during senescence were observed in comparison to wild-type leaves [8], suggesting perhaps other LACS isoforms can compensate. Triple (or higher) mutants will likely be necessary to understand overlapping functions of the acyl-CoA synthetase multi-gene family. Besides the core group of β-oxidation genes, the participation of auxiliary enzymes is required for the complete catabolism of fatty acids that contain unsaturated double bonds in the *cis*-configuration at even- and odd-numbered carbons. Transcripts encoding enoyl CoA isomerase, dienoyl CoA isomerase, dienoyl-CoA reductase and peroxisomal enoyl-CoA hydratase 2 are up-regulated in coordination with those that encode the central β-oxidation pathway enzymes (Fig. 2). Thus, the coordinated up-regulation of β-oxidation genes that are required for fatty acid turnover during early seedling development also occurs during leaf senescence. This might imply control by a common transcription or other regulatory factor; however, little is known about transcription factors that are specifically involved in regulation of β-oxidation.

8. Alternative metabolism of the acetyl-CoA product of β-oxidation

There is no evidence that fatty acids released during lipid turnover are transported from one plant organ to another (as occurs in animals). Instead, β-oxidation in the peroxisome is their major fate. Acetyl-CoA and NADH are the carbon and energy products of β-oxidation [40]. During seedling development, the acetyl-CoA product of β-oxidation enters the glyoxylate cycle allowing its carbon to enter gluconeogenesis, and eventually providing carbohydrate for export to the rapidly growing seedling. A similar metabolism would transform carbon from fatty acids of senescing leaves to carbohydrate that is exported via the phloem to seeds, roots or other sink tissues. Reports on the involvement of the glyoxylate cycle during senescence differ between studies [43,44]. Malate synthase protein (by Western blot) increases in pumpkin leaves during senescence [43]. In contrast, there was no malate synthase expression in Arabidopsis dark induced leaf senescence and it was concluded that "...the fate of lipid broken down under conditions of dark starvation and senescence differs in different plant species" [44]. In the three microarray studies, in support of these results, isocitrate lyase (At3g21720) and malate synthase (At5g03860) transcript expression was very low and not induced.

In addition to species differences in glyoxylate cycle association with senescence, we speculate that its contribution may depend on energy available to the leaf from alternative stores (e.g. starch) or remaining photosynthetic capacity. During dark induced senescence, energy from light is not available and therefore, respiration of acetyl-CoA to CO_2 via the tricarboxylic acid cycle would be required to provide the ATP and reductant for cell maintenance or for active export of amino acids and carbohydrates to the phloem. In natural senescence (in sunlight rather than the typically low laboratory light levels), sufficient cofactors can be provided by light to allow the glyoxylate cycle to recycle carbon for export to sink tissues as carbohydrate. A third route for acetyl-CoA utilization may be via peroxisomal citrate synthase. Of two citrate synthase isoforms, At2g42790 clearly increases during senescence. Citrate export from the peroxisome can provide the 5-carbon precursor for glutamine synthesis and for its export from source to sink tissues as a carrier of both carbon and nitrogen. How can flux into these three alternative pathways for acetyl-CoA metabolism be distinguished? We suggest that young leaves be pre-labeled with ^{14}C-fatty acids [22]. A systematic tracking of the movement of ^{14}C-carbon from leaf fatty acids into CO_2, compared to ^{14}C export from leaf as carbohydrate and/or amino acids would distinguish between these alternative pathways. These experiments should be conducted under a variety of senescence and light regimes, which likely will alter the balance between the different catabolic and anabolic fates for acetyl-CoA.

9. Can lipid turnover be blocked to increase the energy density of plant biomass?

It is frequently suggested that accumulation of oil or other hydrocarbons in leaves and stems of biomass crops could increase their energy density and value as biofuels, including bioelectricity [21,33,45–47]. The glycerolipid content of non-senescent leaves is approximately 4–5% of dry weight. Because of the two-fold higher energy content of lipids, up to 10% of leaf chemical energy is represented by the fatty acids. Thus, even if no additional lipids are produced, retention rather than turnover of fatty acids that exist in non-senescent leaves (or stems) could provide an important improvement in energy recovery from biomass crops. In addition, the chemical energy of lipids is more easily recovered from biomass than the complex process of sugar release from lignocellulose followed by its fermentation to ethanol or other liquid fuels. Although

polar lipids are less easily extracted from biomass than TAG, their conversion to free fatty acids during acid treatment of biomass provides more easily recoverable structures.

Key evidence is now available that blocking fatty acid turnover can result in higher leaf TAG levels in Arabidopsis leaves [21]. A mutant blocked in the ABC transporter for fatty acid movement into peroxisomes accumulated TAG levels up to 1.8% of dry weight under conditions of natural senescence, a level almost 50 fold higher than wild type. TAG levels also were increased several fold in non-senescent leaves of a *cgi-58* mutant [33]. Although the levels of TAG achieved so far are modest and may not provide commercial value, they represent an important 'proof-of-concept'. A major goal for lipid research related to bioenergy is to increase leaf/stem TAG levels and to extend this to future biomass crops such as perennial grasses.

So far, the major rationale for increasing lipids in biomass crops, or reducing their breakdown, has been to replace petroleum based liquid transportation fuels with biobased liquids (biodiesel, jet fuel, etc.) [45]. There are at least two other potential advantages of increasing vegetative lipid levels that might have equal or greater value. First, production of bioelectricity from biomass combustion is almost carbon neutral and when used for electric vehicles can provide more than 2-fold more transportation miles (and petroleum displacement) than conversions of lignocellulose biomass to liquid fuels [48]. Second, increasing or retaining the fatty acid content of biomass and other crops may also be an important future strategy for enhancing the calorific value of crops used for animal feed [49]. Since much of plant biomass is poorly digested, adding even 10% of easily digested lipid in fodder would significantly improve its nutritional and commercial value.

10. Questions, unknowns and directions for future research

The biochemistry of fatty acid breakdown via β-oxidation is well understood, but the identity of 'lipase' enzymes responsible for release of fatty acids from glycerolipids during senescence is much more uncertain. As indicated above and in the supplement Table 2, there are transcripts for at least 68 genes annotated as lipases that increase during senescence. Only a few of these are confirmed to have lipase activity, and fewer to have a role essential for lipid breakdown during senescence. Since insertion mutants are available for most, and delayed senescence can be easily detected by a visual screen, a comprehensive survey of these mutants should be undertaken. Concurrent analysis of the fatty acid level of these lines can be rapidly accomplished by direct transmethylation of leaf lipids and gas chromatography. In addition to more detailed characterization, those lines that are identified could be transformed with *WRINKLED1* transcription factor, *DGAT1*, and other genes and gene combinations that are now known to increase lipid accumulation in leaves [46]. This combination of increasing TAG synthesis and the successful approach of reducing β-oxidation [21] will likely prove a key strategy for the accumulation of lipids that remain stable during extended natural senescence under field conditions. Identification and down-regulation of key lipases may also have advantages if it can avoid more pleiotropic effects of β-oxidation knock-down (e.g. on JA metabolism) that impact biomass accumulation.

Perennial grasses that do not flower are among the highest in dry matter production per hectare, with lowest input requirements. Will knockout or over-expression of genes to produce or retain more lipid lead to compromises in vegetative growth and thereby reduce biomass recovered per hectare? It would be surprising if this did not occur. An obvious strategy to mitigate such negative impacts is the use of senescence promoters so that lipid accumulation only occurs after most biomass has accumulated. Although achieving no negative impacts may be unrealistic, adding 5–10% TAG and/or preventing breakdown of membrane glycerolipids in a dry biomass crop would more than compensate for a 5% yield decrease. If dry biomass leaves/stems contain 10% fatty acid this could increase the energy recovered as liquid fuel by 30–40% when compared to lignocellulose fermentation. Alternatively, just as oats with 10% oil are a high energy and premium-value grain for animal feed, 10% lipid in a fodder crop such as alfalfa could substantially increase the digestible calories and thereby its food value. Thus, there will be important applications for research that provides understanding of the mechanisms that control fatty acid and glycerolipid turnover in plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.01.004.

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