Sugar Potentiation of Fatty Acid and Triacylglycerol Accumulation

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Photosynthetically derived sugar provides carbon skeletons for lipid biosynthesis. We used mutants of Arabidopsis (Arabidopsis thaliana) and the expression of oleogenic factors to investigate relationships among sugar availability, lipid synthesis, and the accumulation of triacylglycerol (TAG) in leaf tissue. The adg1 mutation disables the small subunit of ADP-glucose pyrophosphorylase, the first step in starch synthesis, and the suc2 mutation disables a sucrose/proton symporter that facilitates sucrose loading from leaves into phloem. The adg1suc2 double mutant increases glucose plus sucrose content in leaves 80-fold relative to the wild type, total fatty acid (FA) content 1.8-fold to 8.3% dry weight, and TAG more than 10-fold to 1.2% dry weight. The WRINKLED1 transcription factor also accumulates to higher levels in these leaves, and the rate of FA synthesis increases by 58%. Adding tt4, which disables chalcone synthase, had little effect, but adding the tgd1 mutation, which disables an import of lipids into plastids to create adg1suc2tgd1, increased total leaf FA to 13.5% dry weight and TAG to 3.8% dry weight, demonstrating a synergistic effect upon combining these mutations. Combining adg1suc2 with the sdp1 mutation, deficient in the predominant TAG lipase, had little effect on total FA content but increased the TAG accumulation by 66% to 2% dry weight. Expression of the WRINKLED1 transcription factor, along with DIACYLGLYCEROL ACYLTRANSFERASE1 and the OLEOSIN oil body-associated protein, in the adg1suc2 mutant doubled leaf FA content and increased TAG content to 2.3% dry weight, a level 4.6-fold higher than that resulting from expression of the same factors in the wild type.

Sugars are the energy currency of the cell, fueling growth and development. Therefore, it is critical for the cell to have the capacity to sense its sugar/energy status and to respond to maintain an appropriate physiological status (Sheen, 2014). When sugar and energy are plentiful, anabolic metabolism predominates over catabolism, enabling growth and development. Conversely, when sugar and energy are limiting, growth slows due to a shift from anabolism to catabolism. Sugar signaling is a highly complex process involving multiple partially redundant regulatory networks that are principally mediated by three multifunctional sensor complexes: hexokinase, a Snf1-related protein kinase (SnRK1), and target of rapamycin (Li and Sheen, 2016). SnRK1 is a key protein kinase that, under low-sugar conditions, phosphorylates more than 1,000 target proteins, resulting in a stimulation of catabolism and a simultaneous repression of anabolism, thereby promoting sugar availability (Price et al., 2004). Conversely, when sugar is abundant, SnRK1 becomes inhibited, resulting in a shift from catabolism to anabolism, thereby mediating sugar homeostasis.

Fatty acid (FA) synthesis is highly dependent on the supply of photosynthetically derived sugar as a source of carbon skeletons, ATP, and reductant. Many factors have been reported to influence the accumulation of triacylglycerol (TAG) in vegetative tissues (Xu and Shanklin, 2016). Factors that increase lipid synthesis and accumulation have been categorized as push, pull, and protect (Vanhercke et al., 2014). Examples of push factors include WRINKLED1 (WR1), an APETALA2 transcriptional factor that induces the expression of more than 20 genes involved in glycolysis and FA synthesis (Cernac and Benning, 2004; Maeo et al., 2009), and TRIGALACTOSYLDIACYLGLYCEROL1 (TGD1), a permease-like protein involved in lipid reimport from the endoplasmic reticulum to the plastid that, when mutated, stimulates FA synthesis and TAG accumulation (Xu et al., 2005). Pull factors include various acyltransferases such as DIACYLGLYCEROL ACYLTRANSFERASE1 (DGAT1), which catalyzes the formation of TAG from diacylglycerol and acyl-CoA (Bouvier-Navé et al., 2000), and PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE (PDAT), which catalyzes the formation of TAG by an acyl transfer from the sn-2 position.
of phospholipids to diacylglycerols (Dahlqvist et al., 2000). Protect factors includes ÖLEOSIN1 (OLE1), which is an amphipathic lipid body-associated protein that influences oil body size (Miquel et al., 2014) and physically protects TAG from hydrolysis by lipases such as SUGAR-DEPENDENT1 (SDP1), the predominant TAG lipase in Arabidopsis (Arabidopsis thaliana; Kelly et al., 2013).

Despite the close dependence of FA and TAG synthesis on sugar availability, until recently little was known about the interrelationship between the regulation and coordination of these processes. However, in recent years, several links between sugar signaling and coordination of these processes have emerged. For example, a putative SnRK1 target site at Ser-197 was identified in nasturtium (Tropaeolum majus) DGAT1, mutation of which to Ala increased DGAT activity, and overexpression of the mutant in Arabidopsis seed resulted in increased oil accumulation (Xu et al., 2008). Sugar has been reported to potentiate the oleogenic effects of the WRI1 transcription factor in vegetative tissues (Cernac and Benning, 2004; Sanjay, et al., 2011). We recently reported a mechanistic explanation for the sugar potentiation of WRI1 (i.e. that SnRK1, encoded by KIN10 and KIN11 in Arabidopsis [Williams et al., 2014], phosphorylates two previously unidentified SnRK1 target sites within WRI1 that marks it for proteasomal degradation [Zhai et al., 2017]). According to the model, under low-sugar conditions, when SnRK1 is active, WRI1 is degraded and lipid synthesis is repressed. Conversely, when sugar is abundant, SnRK1 is repressed, WRI1 is stabilized, and lipid synthesis is stimulated.

In photosynthesizing leaves, Suc is synthesized primarily in mesophyll cells, from where it is transported to phloem in a two-step process. SWEET proteins mediate the passive release of Suc from photosynthetic cells to the apoplast (Chen et al., 2012). Suc is loaded subsequently from the apoplast into the phloem by the strongly expressed SUC-PROTON SYMPORTER2 (SUC2; Gottwald et al., 2000). Several Arabidopsis lines with mutations in SUC2 that result in impaired Suc loading into the phloem have been reported (Srivastava et al., 2008). The decrease in Suc export from source leaves starves nonphotosynthetic sink tissues and results in a severely stunted growth phenotype. Relative to wild-type Arabidopsis, suc2-4 shows a dramatic, 20-fold increase in the level of transient carbohydrate (i.e. the sum of Glc, Fru, Suc, and starch). The majority of transient carbohydrate in suc2-4 leaves accumulates as starch (Srivastava et al., 2008), the synthesis of which competes with lipid synthesis for carbon skeletons. Elevated sugar in suc2-4 also results in the induction of chalcone synthase (TT4), a polyketide synthase type III enzyme that mediates the first committed step in flavonoid synthesis that ultimately leads to anthocyanins (Ohito et al., 2001).

The first committed step in starch synthesis is ATPα-Glc-1-P adenylyl transferase, also known as ADP-Glc pyrophosphorylase (ADGase; Preiss, 1996). ADGase catalyzes the formation of ADP-Glc from Glc-1-P and ATP. ADGase is composed of small and large subunits encoded by ADG1 and ADG2, respectively. In adg1 mutant plants, neither ADG1 nor ADG2 accumulates, and the plants are devoid of ADGase activity (Wang et al., 1998).

In this work, we exploit the availability of various Arabidopsis mutant backgrounds to probe the consequences of elevated levels of sugar on TAG accumulation in leaves by analyzing the total FA and TAG levels in various single and multiple mutants with an initial focus on adg1 and suc2, which we combined to create the adg1suc2 double mutant. We show that increased levels of sugar correlate with increased accumulation of total FA and TAG by increasing the rate of FA synthesis. The adg1suc2 double mutant accumulated more than 80-fold higher leaf sugar (Glc and Suc) and approximately 10-fold more TAG than wild-type leaves. The double mutant also showed a significant increase in the accumulation of the WRI1 transcription factor. The triple mutant adg1suc2sdp1 resulted in a further increase in TAG, and the quadruple mutant adg1suc2sdp1tgd1 resulted in a doubling of TAG accumulation relative to the triple mutant to 3.8% (dry weight). That the total FA in this line accumulated to 13.5% (dry weight) points to a bottleneck in the assembly of TAG from FA. Overexpression of WRI1, DGAT1, and OLE1 in the adg1suc2 background led to significantly higher levels of TAG than expression in wild-type plants.

RESULTS
Introducing adg1 into the suc2 Mutant Background Partially Rescues Its Growth Retardation

The suc2-4 mutant (Salk_038124; henceforth referred as suc2) is a T-DNA insertion mutant line disrupted in the second intron of AtSUC2 that results in a severely stunted growth phenotype relative to the wild type (Fig. 1A). It was crossed with the starchless mutant adg1-1, which shows similar growth to that of the wild type, to generate the adg1-1suc2-4 double mutant (referred to herein as adg1suc2). Combining adg1 with suc2 partially reversed the severe growth retardation phenotype of the suc2 mutant such that leaves of the double mutant expanded in a more normal manner (Fig. 1A). During seed germination, adg1suc2 mutants also demonstrated more robust primary root elongation than suc2. Primary roots of adg1suc2 were 8.4-fold longer than those of suc2 at 2 weeks after germination and growth on one-half-strength Murashige and Skoog (1/2MS) medium without Suc (Fig. 1B). However, when cultured on 1/2MS medium supplemented with 1% Suc, there was little difference in primary root elongation between adg1suc2 and suc2 (Supplemental Fig. S1).

High Sugar Accumulation in the adg1suc2 Mutant

The primary phenotype of the adg1 mutant is the lack of starch in leaves at the end of the light period, as visualized by iodine staining (Fig. 2A). Similarly, the adg1suc2 double mutant failed to accumulate starch at the end of the day (Fig. 2A). The combined content of Glc and Suc in the suc2 plant is 21-fold that of the wild
type, whereas that of adg1suc2 is 3.8-fold higher than that of suc2 and 80-fold higher than that of the wild type (Fig. 2B). Leaves of adg1suc2 plants display a visibly reddish hue consistent with the accumulation of anthocyanins. Analysis of these leaves revealed that they accumulated 218-fold more anthocyanin than wild-type plants, corresponding to approximately 1.5% of dry weight. We next investigated the expression levels of two genes that had been demonstrated previously to be induced upon incubation with Suc (i.e., TT4, a key enzyme involved in the biosynthesis of flavonoids, and a class II trehalose-6-phosphatase synthase [TPS5], which is not catalytically active in producing TREHALOSE-6-PHOSPHATE; Schluepmann et al., 2004; Solfanelli et al., 2006). To test whether TT4 and TPS5 also are induced by endogenous Suc accumulation, qRT-PCR was used to quantify their expression in leaves of the wild type and the adg1suc2 double mutant. The expression of TT4 in adg1suc2 was approximately 6-fold higher than in suc2 and approximately 1,000-fold higher than in the wild type (Fig. 3B). The expression of TPS5 also was up-regulated significantly in adg1suc2 and suc2 mutants, although to a lower degree than that of TT4 (Fig. 3C). Previous studies showed that elevated sugar accumulation results in altered accumulation of TMT1/2 and SUC4, which influence the distribution of Suc between the cytosol and the vacuole. However, our qRT-PCR data showed that the expression of TMT1/2 and SUC4 in adg1suc2 does not differ significantly from that in the wild type (Fig. 3D), showing that their transcription is not regulated by Suc availability.

**TAG Accumulation in Leaves of the adg1suc2 Double Mutant**

The accumulation of significant amounts of Glc and Suc in adg1suc2 leaves makes it an ideal background in which to study TAG biosynthesis in leaf tissues with elevated sugar content. Compared with the wild type, adg1suc2 shows an abundance of lipid droplets in leaves when visualized with the nonpolar lipid-selective fluorescent dye BODIPY 493/503 (Invitrogen; Fig. 4A). TAG quantification of mature leaves of 5-week-old plants showed that adg1suc2 accumulated 1.2% TAG (dry weight), which was approximately 12-fold higher than that of the wild type (Fig. 4B). Correspondingly, leaf total FA content in adg1suc2 was 1.8-fold higher than in the wild type and reached 8.3% (dry weight; Fig. 4C). A short-duration (30 min) [1-14C]acetate labeling experiment was used to test if high endogenous sugar increases de novo FA synthesis in a similar manner to that observed upon the coexpression of OLE1, WRI1, and DGAT in the wild type, a combination of genes indicated with different letters above histogram bars are significantly different (Student’s test for all pairs of genotypes, n = 10, P < 0.05). The experiment was repeated three times, and data from one representative experiment are presented.

Figure 1. The severely stunted phenotype of suc2 is partially rescued in adg1suc2. A, Phenotypes of the shoots of the wild type (WT), adg1, suc2, and adg1suc2. Plants were grown on soil for 6 weeks. B, Root growth of 2-week-old seedlings of the indicated genotypes grown on a 1/2MS plate (top). The lengths of primary roots of plants from the top are shown at bottom. Values are means ± so for each genotype. Levels.
previously shown to increase TAG accumulation that we use here as a positive control. The relative rate of FA synthesis in leaves of adg1suc2 was 58% higher than that of the wild type but was not significantly different from that of suc2 (Fig. 4D).

Previous studies showed that sugar potentiates the accumulation of lipids when WRI1 is ectopically expressed (Cernac and Benning, 2004; Sanjaya et al., 2011; Kelly et al., 2013). In other work, it has been reported that the transcription of WRI1 can be mildly up-regulated in the presence of very high sugar levels (Masaki et al., 2005). To test whether high endogenous Suc also affects WRI1 in adg1suc2, the gene expression and protein levels of WRI1 were quantified by qRT-PCR and immunoblotting, respectively. The results show that the gene expression level was not significantly different between the wild type and adg1suc2 (Fig. 5A); however, while the WRI1 protein was barely detectable in wild-type leaves, its levels were substantially higher in leaves of adg1suc2 (Fig. 5B). For comparison, three oleogenic genes, Cys-OLE1, WRI1, and DGAT1, were assembled into a single construct we refer to as OWD. Equal loading was demonstrated by performing duplicate western analysis using the constitutive histone H3; however, we note that the presumptive Rubisco band appears somewhat stronger in the double mutant and OWD. To visualize expression, cyan fluorescent protein (CFP) was fused at the N terminus of WRI1 while GFP was fused at the C terminus of Cys-OLE1, generating a Cys-OLE-GFP, CFP-WRI1, and DGAT1 (OWD) construct (Supplemental Fig. S2A). For this experiment, the ectopic expression of WRI1 is used as a positive control for the WRI1 western blot. The pattern of expression of WRI1 polypeptide in adg1suc2 is similar to that of OWD lacking fluorescent tags in the wild type. Together, these data indicate that the WRI1 polypeptide is stabilized in adg1suc2 and that the increased abundance of WRI1 is associated with increased FA synthesis and increased TAG accumulation.

Stacking Additional Gene Mutations with adg1suc2
Further Increases the Accumulation of TAG and Total FA

As shown in Figure 3, adg1suc2 had dramatically up-regulated gene expression of TT4 and accumulated 1.5% (dry weight) of anthocyanins. TT4 catalyzes the conversion of 4-coumaroyl-CoA and malonyl-CoA to naringenin chalcone in the cytosol. Malonyl-CoA plays a key role in chain elongation in FA biosynthesis in chloroplasts. Although it remains unclear whether there is an exchange of malonyl-CoA pools between the cytosol and chloroplast, we tested whether blocking anthocyanin synthesis by combining a TT4 mutant with adg1suc2 would result in any increase in FA synthesis. To achieve this, adg1suc2 was crossed with tt4 to generate the adg1suc2tt4 triple mutant, which, as expected, lost the reddish hue associated with anthocyanin accumulation (Fig. 6A). Combining adg1suc2 with tt4 resulted in a significant increase in its total FA to 10% dry weight from 8% dry weight in the adg1suc2 double mutant (Fig. 6B), and a similar increase in TAG accumulation was observed (Fig. 6B).

Next, we tested whether suppressing TAG turnover by introducing a mutation in the SDP1 TAG lipase would further enhance TAG accumulation in the leaves of adg1suc2 (Fig. 6A). adg1suc2sdp1 increased TAG content by 66% over that accumulated by adg1suc2, while total FA content was not altered significantly (Fig. 6B).

In this study, leaves of the 5-week-old tgd1 mutant accumulated TAG to approximately 0.4% of leaf dry weight. To determine whether the TGD1 mutation would provide a synergistic effect in the context of the adg1suc2tt4 triple mutant background, we generated the adg1suc2tt4tgd1 mutant.
The growth and development of the adg1suc2tt4tg1 quadruple mutant mirrored that of adg1suc2 and adg1suc2tt4 (Fig. 6A). The adg1suc2tt4tg1 quadruple mutant accumulated 3.8% TAG in 5-week-old leaves, which represents increases of 3.4- and 50-fold over that of adg1suc2tt4 triple mutant and wild-type leaves, respectively. The levels of total FA in leaves of adg1suc2tt4tg1 reached 13.5% dry weight (Fig. 6B). That leaf TAG content in adg1suc2tt4tg1 of 3.8% is much higher than that of the combined TAG of tgd1 plus adg1suc2tt4 at 1.5% (i.e. 0.4% + 1.1%, respectively) indicates a positive synergistic effect on TAG synthesis for this combination of mutations.

Ectopic Expression of WRI1, DGAT1, and OLE1 in the adg1suc2 Double Mutant Enhances TAG and Total FA Accumulation

That elevated leaf sugar levels correlate with the increased accumulation of both FA and TAG prompted us to explore the effects of overexpressing several genes that have been shown to enhance lipid accumulation in tobacco (Nicotiana tabacum; Vanhercke et al., 2014). Specifically, this would address whether a high-sugar background would further enhance the lipogenic effects of overexpressing OWD relative to their expression in wild-type leaves. The OWD construct (described above) was first tested in tobacco leaves. WRI and Cys-OLE showed strong expression after a 2-d infiltration, as visualized by fluorescent signals localized in the nucleus and oil drops, respectively (Supplemental Fig. S2B). After 4 d of infiltration, TAG accumulation in OWD-infiltrated leaves reached 3.3% dry weight, representing a 60-fold increase compared with leaves infiltrated with an empty vector control (Supplemental Fig. S2C). Having confirmed its functionality, OWD was transformed into wild-type plants. The gene transformation efficiency of the wild type of the OWD construct was much lower than for other constructs. Only three independent transgenic lines were obtained from one transformation experiment. We observed that ectopic
overexpression of OWD resulted in a negative growth impact when expressed in the wild type. All three OWD transgenic lines were small and exhibited reduced fecundity compared with wild-type plants. The highest TAG accumulation observed in mature leaves of 5-week-old OWD transgenic plants was only 0.5% (Fig. 7A). It has been shown that an exogenous supply of Suc can increase vegetative TAG content in WRI1- or Fis3-transformed plants (Kelly et al., 2013; Zhang et al., 2016). To test the hypothesis that OWD can increase TAG biosynthesis in the presence of elevated sugar, we generated OWD/adg1suc2 transgenic plants by crossing OWD/wild-type transgenics with adg1suc2. TAG and total FA contents in OWD/adg1suc2 were further elevated to 2.3% and 11% dry weight, compared with 1% and 8.3% TAG and total FA contents in adg1suc2, respectively (Fig. 7B). These increases in TAG content in OWD/adg1suc2 are significantly larger than expected by the addition of their individual contributions. The FA composition of mature leaves of the wild type, adg1suc2, adg1suc2sdp1, OWD/adg1suc2, and adg1suc2tt4tgd1 shows that, as TAG content increases from 0.08% in the wild type to 3.8% in adg1suc2tt4tgd1, the levels of 18:3 declined as 18:2 became more abundant, suggesting that the desaturation capacity for 18:2 becomes limiting as FA accumulation increases. This is

Figure 4. TAG accumulates in leaves of adg1-suc2. A, Lipid droplets are abundant in leaves of adg1suc2. Representative confocal fluorescence micrographs show mesophyll tissue from the wild type (WT) and adg1suc2 after 6 weeks of growth on soil after staining with BODIPY 493/503. DIC, Differential interference contrast image. Bars = 50 μm. B and C, TAG (B) and total FA (C) were quantified from 6-week-old leaves of soil-grown plants as indicated. D, [1-14C] Acetate incorporation into fatty acyl products by leaf strips after 30 min of labeling. OWD, the line coexpressing WRI1, DGAT1, and OLE1, was used as a positive control for the labeling assay. DW, Dry weight; FW, fresh weight. Values are means ± SD for each genotype. Levels indicated with different letters above histogram bars are significantly different (Student’s t test for all pairs of genotypes, n = 5, P < 0.05). Each experiment was repeated three times, and data from a single representative experiment are presented.
consistent with previous reports that the capacity of FAD3 desaturase is limiting to the level of 18:3 accumulation (Lemieux et al., 1990; Arondel et al., 1992). The level of 18:0 increased significantly in the FA pool of OW/D/adv1suc2 compared with adv1suc2 (Fig. 7C).

**DISCUSSION**

**Partial Rescue of suc2 by Introducing adv1**

The severe dwarf phenotype of suc2 is attributed to its inability to export Suc to developing shoots and roots, which severely restricts the carbon available for growth and development. Combining adv1 with suc2 partially mitigates the dwarf phenotype with respect to both shoot size and primary root elongation, suggesting that more Suc is available to shoots and roots in the adv1suc2 double mutant. This view is supported by the observation that adv1suc2 and suc2 seeds that germinate on 1/2MS medium supplemented with Suc show equivalent primary root elongation. One possible explanation for the partial rescue of suc2 by introducing adv1 is that inactivation of the competing starch biosynthesis resulted in a higher intracellular Suc concentration. This would increase the Suc gradient across the membranes of phloem companion cells in adv1suc2 relative to that of suc2, thereby driving passive sugar efflux. However, at this time, we cannot discount the possibility that high levels of sugar could activate, or induce, the accumulation of other phloem-loading Suc transporters to facilitate increased Suc transport to shoots and roots.

**Sugar Accumulation in adv1suc2 Leaves**

Leaves do not normally accumulate high levels of photosynthetically fixed sugars because they are either converted to starch, the predominant short-term storage carbohydrate in leaves (Komor, 2000), or loaded directly into phloem and transported to sink tissues. Environmental conditions such as high light or low temperatures can favor Suc synthesis and accumulation. Low temperatures decrease carbon utilization and allow Suc concentrations to rise (Pollock, 1990). High concentrations of Suc are found naturally only in specialized plant storage organs such as the stems of sugarcane (Saccharum officinarum). These specialized organs have efficient sugar transporters and sugar-metabolizing enzymes, such as Suc synthases and invertases, that facilitate phloem unloading and sugar compartmentalization into the vacuoles of storage parenchyma (Zhu et al., 2000; Carson and Botha, 2002; Carson et al., 2002; Casu et al., 2003). Here, we demonstrate an 80-fold increase of sugars in mature Arabidopsis leaves by decreasing Suc export and blocking starch synthesis simultaneously in the adv1suc2 double mutant. Unlike Suc storage organs, which have efficient sugar transporters and sugar-metabolizing enzymes that compartmentalize Suc into vacuoles, elevated levels of Suc accumulate in the cytosol of adv1suc2. This is consistent with the observation that adv1suc2 and the wild type have similar expression levels of TMT1/2, which encodes the proton-coupled antiporter capable of high-capacity vacuolar Glc and Suc loading (Schulz et al., 2011), and similar levels of SUC4, a proton-coupled symporter that exports Suc from the vacuole (Schneider et al., 2012).

**Suc Favors TAG Biosynthesis**

It has been demonstrated that Suc has effects on various aspects of plant metabolism. For example, Suc induces fructan (i.e., polymers of Fru) synthesis in grasses (Nagaraj et al., 2001; Noël et al., 2001). Suc also acts as a signaling molecule that initiates/activates starch synthesis by inducing the gene expression of AGPase large subunits, starch synthase (GBSS1) and β-amylase (Nakamura et al., 1991; Harn et al., 2000; Wang et al., 2001; Nagata et al., 2012), and by posttranslationally activating the AGPase enzyme by redox modification (Tiessen et al., 2002). The down-regulation of photosynthetic CO2 fixation by Suc is a widely observed phenomenon (Van Oosten and Besford, 1994), as is the induction of genes
related to anthocyanin and flavonoid biosynthesis upon Suc feeding (Solfanelli et al., 2006). Consistent with exogenous Suc feeding, the strongly elevated levels of intracellular Suc in suc2adg1 resulted in dramatic increases in chalcone synthase mRNA and significant accumulation of anthocyanin.

The TAG content of 4-week-old Arabidopsis roots cultured on agar supplemented with 3% Suc was significantly

Figure 6. Introduction of additional mutations, tt4, sdp1, and tgd1, further increases TAG and/or total FA content when combined with adg1suc2. A. Phenotypes of the shoots of 4-week-old plants of the indicated genotypes. Bars = 20 mm. B. TAG was quantified in leaves of the wild type (WT), double mutant adg1suc2, triple mutants adg1suc2tt4 and adg1suc2sdp1, and quadruple mutant adg1suc2tt4tg1d1 (left). Total FA also was quantified in corresponding leaves of the indicated genotypes (right). DW, Dry weight. Values are means ± SD for each genotype. Levels indicated with different letters above histogram bars are significantly different (Student’s t test for all pairs of genotypes, n = 5, P < 0.05). Each experiment was repeated three times, and representative data from a single experiment are presented.
higher than that of roots grown on agar without supplementation, because sugar provides carbon skeleton, ATP, and reductant for TAG synthesis (Kelly et al., 2013). In this study, we show that the adg1suc2 mutant, which accumulates significantly more Suc, accumulates 10-fold more TAG than wild-type plants. It also has been reported that Glc supplementation is necessary for TAG accumulation in seedlings overexpressing...
CONCLUSION

A series of Arabidopsis lines with increased leaf sugar accumulation were created and analyzed for the amount of total FA and TAG. Increased levels of sugar correlated with increased accumulation of total FA and TAG along with increased rates of FA synthesis. This likely results from a combination of two mechanisms: (1) through increasing the supply of carbon skeletons, ATP, and reductant (Sanjaya et al., 2011); and (2) via inhibiting KIN10-dependent phosphorylation that predisposes WRI1 to degradation. That WRI1 expression did not differ significantly between adg1suc2 and the wild type supports this view. The data presented here, that elevated sugar correlates with increased accumulation of WRI1, is consistent with the previously proposed model of WRI1 stabilization upon Suc-mediated inhibition of KIN10 (Zhai et al., 2017). This also supports our model for a dual role for sugar in providing carbon skeletons for FA synthesis and through inhibiting KIN10 activity and, thereby, stabilizing WRI1.

In the storage tissues of some varieties of sugarcane, sugar levels can approach 650 mM (Welbaum and Meinzer, 1990) without stimulating TAG accumulation in stems (Zale et al., 2016). One possible explanation is that the majority of Suc is compartmentalized in vacuoles, resulting in relatively low levels of Suc in the cytoplasm that are available as carbon skeletons for FA synthesis. It is also possible that oleogenic transcription factors such as WRI1, or some of the enzymes of TAG synthesis, are poorly expressed when sugarcane parenchyma cells are accumulating high levels of sugar.

Sugar Potentiation of TAG Accumulation

WRI1 (Cernac and Benning, 2004). WRI1 overexpression studies show that FA synthesis is potentiated by exogenous Suc treatment (Masaki et al., 2005) or by increasing intracellular Suc resulting from the down-regulation of starch synthesis by APG RNA interference (Sanjaya et al., 2011). These studies suggested that Suc plays a role as a signaling molecule in regulating the expression of WRI1 target genes. In this study, we observed a substantial increase in the level of WRI1 polypeptide in adg1suc2 relative to the wild type, which most likely results from the suppression of posttranscriptional KIN10-dependent phosphorylation that predisposes WRI1 to degradation. That WRI1 expression did not differ significantly between adg1suc2 and the wild type supports this view. The data presented here, that elevated sugar correlates with increased accumulation of WRI1, is consistent with the previously proposed model of WRI1 stabilization upon Suc-mediated inhibition of KIN10 (Zhai et al., 2017). This also supports our model for a dual role for sugar in providing carbon skeletons for FA synthesis and through inhibiting KIN10 activity and, thereby, stabilizing WRI1.

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MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 (the wild type) and tt4-3 (Zhang et al., 2010) were obtained from the Arabidopsis Biological Resource Center. The suc2-4 mutant was provided by Brian Ayre (University of North Texas). In addition, the following mutant lines were used in this study: suc2-4 (Srivastava et al., 2008), adg1-1 (Lin et al., 1988), sdp1-5 (Kelly et al., 2013), and fdl (Xu et al., 2005). Homozygous double, triple, or quadruple mutants were generated by crossing the corresponding mutant lines. The genotyping primers used to identify homozygous mutants are listed in Table I. For overexpression of OLE1, DGAT1, and WRI1 in adg1suc2suc2-4, adg1suc2suc2-4 was crossed with an OWD/wild type (OLE1, DGAT1, and WRI1 overexpression line in the wild-type background). All plants were grown with a light/dark cycle of 16 h/8 h, day/night temperatures of 23°C/19°C, a photosynthetic photon flux density of 250 μmol m⁻² s⁻¹, and 75% relative humidity.

RNA Isolation and qRT-PCR

To quantify the gene expression of TT4, TPSS, SUC4, TMT1/2, WRI1, DGAT1, and PDAT1, total RNAs were isolated from the wild-type and adg1suc2suc2 and treated with DNaseI. cDNA was prepared using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). qRT-PCR was performed with the CFX96 qPCR Detection System (Bio-Rad) and gene-specific primers for TT4, TPSS, WRI1, DGAT1, PDAT1, F-box (ATSG1571), and Express1 (AT4G33880), among which F-box and Express1 were used as reference genes (Table I). Statistical analysis of qRT-PCR data was carried out with REST2009 (Pfaffl et al., 2002).

Leaf Iodine Staining

For leaf starch iodine staining, fresh leaves sampled from the wild type, suc2-4, and adg1suc2suc2-4 were decolored by incubating with absolute ethanol at 70°C for 5 min before staining with diluted Lugol’s solution (Tsai et al., 2009).

Leaf Total Anthocyanin, Suc, and Glc Quantification

Total leaf anthocyanin levels were quantified by the methods described by Fuleki and Francis (1968). For the quantification of Suc and Glc of leaves, assays were performed according to the manufacturer’s instructions for the Sucrose Assay Kit and the Glucose (GO) Assay Kit (Sigma).

TAG and Total FA Quantification

Total lipids (TAG and polar lipids) were isolated from 100 mg of fresh leaf tissue with 700 μL of methanol:chloroform:formic acid (2:1:0.1, v/v/v) and mixed by vigorous vortexing for 30 min. After the addition of 1 mL KCl and 0.2 mL H₃PO₄, samples were vortexed and clarified by centrifugation at 2,000g for 10 min; total lipids were collected as the lower phase. For TAG quantification, 60 μL of total lipids was fractionated by thin-layer chromatography using hexane:diethyl ether:acetic acid (70:30:1, v/v/v) as the mobile phase on Silica Gel 60 plates (Merck) and visualized after spraying with 0.05% primuline (in 80% acetone). TAG fractions identified under UV light were isolated from the plate and transmethylated to FA methyl esters by incubation with the addition of 1 mL of boron trichloride-methanol and incubation at 80°C to 85°C for 40 min. For total FA quantification, 10 μL of total lipids was directly transmethylated with boron trichloride-methanol. For both assays, 5 μg of C17:0 was added as an internal standard prior to transmethylaion. FA methyl esters were extracted into hexane and dried under a stream of dry nitrogen before dissolving in 100 μL of hexane, followed by analysis using the Agilent Technologies 7890A

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with 200 containing 0.01% (w/v) Tween 20 as wetting agent under a photon was determined by liquid scintillation counting.

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were purchased from Agrisera. A total of 50 mg of mature leaves of 4-week-old (DFMFDDGKHECLNLENLDC) corresponding to residues 299 to 317 of the WRI1 fresh tissue was incubated with a solution containing 100 plants were cut into approximately 8–mm pieces. For BODIPY staining, (2004) Genes expressed in sugarcane maturing inter- nal laser scanning microscope with its excitation wavelength set at 488 nm. The BODIPY-stained lipid droplets were imaged using a Leica SP5 con- 

LITERATURE CITED

ACKNOWLEDGMENTS

We thank Brian Ayre (University of North Texas) for the gift of suc-2 and Dr. F.W. Studier (Brookhaven National Laboratory) for critically reading the article.

Received June 19, 2017; accepted August 22, 2017; published August 25, 2017.

In Vivo [1-14C]Acetate Labeling

[1-14C]Acetate labeling was performed according to the method of Koo et al. (2004). In brief, half leaves were incubated in 25 mM MES (pH 5.7) buffer containing 0.01% (w/v) Tween 20 as wetting agent under a photon flux of 180 μmol photons m−2 s−1 at 25°C. Labeling was initiated by the addition of 10 μCi of sodium [1-14C]acetate solution (58 mCi mmol−1; American Radiolabeled Chemicals). Labeling was terminated by removal of the medium, after which the leaf material was washed three times with water. Total lipids were extracted and separated as described above. Radioactivity associated with total lipids was determined by liquid scintillation counting.

Lipid Staining

Mature leaves from 6-week-old soil-grown wild-type and adj1-1ssuc2-4 plants were cut into approximately 8–× 2-mm pieces. For BODIPY staining, fresh tissue was incubated with a solution containing 100 μg mL−1 BODIPY 493/503 (excitation/emission wavelengths; Invitrogen) in 0.1% Triton X-100 (by dilution from a 10 mg mL−1 DMSO stock solution). Vacuum was applied for 10 min, before washing twice with phosphate-buffered saline to remove excess stain. The BODIPY-stained lipid droplets were imaged using a Leica SLP confocal laser scanning microscope with its excitation wavelength set at 488 nm. Lipid droplets were visualized at ×63 magnification, with ≥2 of 824 and 1,200 for BODIPY stain and chlorophyll, respectively.

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource or GenBank database under the following accession numbers: SUC2 (AT1G22710), ADG1 (AT5G48300), SDP1 (AT5G40400), TGD1 (AT1G19800), DGAT1 (AT2G19450), TTD1 (AT1G31930), TPS3 (AT1G17770), PLAT1 (AT5G30600), TMT1 (AT2G8860), TMT2 (AT4G35500), SUC4 (AT1G09680), BCCP2 (AT5G15530), KAS1 (AT5G46290), PKPα1 (AT5G52920), OLE1 (AT1G25140), and WRJ1 (AT5G34320).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phenotyping the root growth of 2-week-old seedlings of the indicated genotypes grown on vertically oriented 1/2MS plates supplemented with 1% Suc.

Supplemental Figure S2. Transient expression of OWD in tobacco leaves.

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GC System equipped with a 5975C mass selective detector and a 60-m × 0.25-mm i.d. DB23 column (Supelco).

Antibody and Immunoblotting

Anti-WRI and anti-histone H3 antibodies were used in this study. Anti-WRI1 polyclonal antibodies were raised in rabbits immunized with a synthetic peptide (DFMFDDGKHECLNLENLDC) corresponding to residues 299 to 317 of the WRI1 amino acid sequence (Pierce, Thermo Fisher). Histone H3 polyclonal antibodies were purchased from Agrisera. A total of 50 mg of mature leaves of 4-week-old wild-type and adj1ssuc2 plants was ground under liquid nitrogen and then mixed with 200 μL of preheat protein extraction buffer (8 M urea, 2% SDS, 0.1 M DTT, 20% glycerol, 0.1 M Tris-HCl [pH 6.8], and 0.004% Bromophenol Blue). After clarification by centrifugation at 16,000 × g, 30 μL of supernatant was loaded into each and resolved by SDS-PAGE before transfer to PVDF membranes for immunoblot analysis. Immunoblots of target proteins were visualized using alkaline phosphatase-conjugated secondary antibodies and the substrates 5-bromo-4-chloro-3-indoly phosphate/nitroblue tetrazolium (Bio-Rad).


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