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Engineering starch accumulation by manipulation of phosphate metabolism of starch

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Summary

A new understanding of leaf starch degradation has emerged in the last 10 years. It has been shown that starch phosphorylation and dephosphorylation are critical components of this process. Glucan, water dikinase (GWD) (and phosphoglucan, water dikinase) adds phosphate to starch, and phosphoglucan phosphatase (SEX4) removes these phosphates. To explore the use of this metabolism to manipulate starch accumulation, Arabidopsis (*Arabidopsis thaliana*) plants were engineered by introducing RNAi constructs designed to reduce expression of *AtGWD* and *AtSEX4*. The timing of starch build-up was altered with ethanol-inducible and senescence-induced gene promoters. Ethanol induction of RNAi lines reduced transcript for *AtGWD* and *AtSEX4* by 50%. The transgenic lines had seven times more starch than wild type at the end of the dark period but similar growth rates and total biomass. Elevated leaf starch content in maize leaves was engineered by making an RNAi construct against a gene in maize that appeared to be homologous to *AtGWD*. The RNAi construct was expressed using the constitutive ubiquitin promoter. Leaf starch content at the end of a night period in engineered maize plants was 20-fold higher than in untransformed plants with no impact on total plant biomass. We conclude that plants can be engineered to accumulate starch in the leaves with little impact on vegetative biomass.

Introduction

There have been significant advances in understanding differences between leaf starch metabolism and grain starch metabolism over the past decade (Kötting *et al.*, 2009; Nittylä *et al.*, 2004; Ritte *et al.*, 2002; Weise *et al.*, 2004; Yu *et al.*, 2005), which have opened up new approaches to engineering leaf starch. Phosphate has emerged as a critical component of transitory leaf starch metabolism (Blennow and Engelsen, 2010; Blennow *et al.*, 2002; Hejazi *et al.*, 2009; Kötting *et al.*, 2009). Leaf starch differs from grain starch in having a small amount of phosphate, while grain starch has almost none. This phosphate is added by a glucan, water dikinase (GWD). The GWD enzyme is coded for by the gene that is lost in the Arabidopsis mutant called Starch Excess 1 (SEX1) (Ritte *et al.*, 2002). A phosphoglucan phosphatase coded for by the gene *SEX4* is also needed for the breakdown of leaf starch. The phosphoglucan phosphatase removes the phosphate groups added to the starch by the GWD (Kötting *et al.*, 2009). When GWD or the phosphoglucan phosphatase is absent, leaf starch contents are increased relative to wild types. Some of the highest starch contents are found in plants in which transitory (leaf) starch phosphate metabolism has been disrupted. Some of these mutants can accumulate up to 50% of the dry weight of their leaves as starch (Messerli *et al.*, 2007). These plants often have stunted growth and grow poorly under physiologically relevant photoperiods and light levels.

Our aim was to explore the use of phosphate metabolism of transitory starch to cause increased starch contents in leaves. We chose two different model organisms: Arabidopsis because the leaf starch degradation pathway is well understood and maize because of its higher photosynthetic rate and relevance to potential biofuel crops. In Arabidopsis, it is known that when starch phosphate metabolism is disrupted by mutagenesis of either *AtGWD* or *AtSEX4*, growth is stunted (Caspar *et al.*, 1991; Zeeman *et al.*, 1998). It is thought that the growth reduction observed in starch excess mutants occurs because effective investment of sugars in productive leaf biomass is compromised in starch excess mutants. If so, the reduction in growth might be reduced or eliminated by delaying starch accumulation until late in development.

In maize, the pathway of leaf starch degradation is less well characterized (Weise *et al.*, 2011). Maize is a C_4 plant, and photosynthesis is split between the bundle sheath and mesophyll cells. During the day, starch normally accumulates in the bundle sheath cells where most Benson–Calvin cycle activity occurs (Rhoades and Carvalho, 1944; Spilatro and Preiss, 1987; Zirkle, 1929). Known mutants of maize that accumulate large amounts of starch that persist through the night are the result of blocks in the export of carbohydrates to the phloem (Ma *et al.*, 2009; Russin *et al.*, 1996; Slewinski and Braun, 2010). These mutants exhibit severe phenotypes characterized by chlorosis and anthocyanin accumulation in regions of the leaves where carbohydrates accumulate. Starch accumulation is observed not only in the bundle sheath cells but in mesophyll cells as well.

We tested whether starch contents of leaves could be controlled with little or no yield penalty by manipulating the

expression of the genes involved in starch phosphate metabolism. To alter the timing of starch accumulation, RNAi constructs were expressed on conditional promoters in Arabidopsis. An ethanol-inducible promoter was used to control the timing of starch accumulation, and a senescence-induced promoter was used to cause starch to increase late in the plant's life cycle. In maize, RNAi targeting putative starch breakdown genes was expressed on a ubiquitin promoter. The genes for GWD and SEX4 are known in Arabidopsis, and we used bioinformatics techniques to identify a putative *GWD* and *SEX4* from maize. It was found that by altering the expression of these genes in Arabidopsis or maize, leaf starch contents could be increased with little impact on vegetative biomass.

Results

Arabidopsis RNAi constructs

The RNAi constructs were designed using a 400-bp sequence from the 5'UTR and coding regions of the target gene. A 779-bp-long pyruvate dehydrogenase kinase intron (Pdk) followed by the inverse complement of the first 400 bp was added. The RNAi to reduce expression of *AtGWD* (At1g10760) was designed using 85 bp of DNA sequence directly upstream of the ATG start site followed by the first 230 bp of genomic sequence followed by an 85-bp sequence that was taken starting 569 bp upstream of the ATG start site. The last 85 bp of the discontinuous sequence was chosen to avoid homology with, and possible silencing of, the *PWD* gene (At5g26570), a phosphoglucan, water dikinase with some similarity to *AtGWD*. The RNAi to reduce expression of *AtSEX4* (At3g52180) was designed using 253 bp of DNA sequence directly upstream of the ATG start site followed by the first 165 bp downstream of the ATG start site, of genomic sequence. The inverted repeats were regulated by either a senescence-induced gene promoter or an ethanol-inducible promoter with an octopine synthase terminator (3'OCS).

Pattern of starch accumulation in Arabidopsis rosettes

To determine appropriate leaves for the experiment of starch accumulation, plants lacking *AtGWD* (SALK_077211) or *AtSEX4* (SALK_102564) were grown for 10 weeks. Pairs of leaves were labelled by tying different coloured thread around the petiole of the leaves to keep track of each leaf as the plant grew. After 3 weeks of growth, plants were harvested weekly just before the lights came on in the growth chamber. Starch levels were measured in separate pairs of leaves. Starch accumulated to higher levels in the older leaves (leaves 3–12) compared to younger leaves (leaves 13–28) of plants lacking *AtGWD* (Figure S1). In plants lacking *AtSEX4*, starch accumulated in all leaves tested (Figure S2). On the basis of these results, only leaves 3–10 were harvested for starch determinations in WT and transgenic lines containing the RNAi constructs.

Inducible starch accumulation in Arabidopsis

Plants lacking *AtGWD* (SALK_077211) grew more slowly than wild type (Figure 1a,b) as has been reported before (Caspar *et al.*, 1991; Zeeman *et al.*, 1998). However, RNAi *AtGWD* plants with a senescence-induced gene promoter grew to the same size (Figure 1c) even though they had significantly more starch at midday (Figure 1d,e).

Wild-type and transgenic lines containing the senescence-induced *AtGWD* and *AtSEX4* RNAi were grown for 13 weeks. Whole rosettes were assayed for starch in the morning [the time when leaf starch is typically as a minimum (Lu *et al.* 2005)] at the end of 11 and 13 weeks of growth. After 11 weeks, wild-type and RNAi lines had similar starch contents, but after 13 weeks, leaf starch contents were elevated in all four *AtGWD* RNAi lines (Figure 2). We did not observe starch accumulation in senescence-inducible *AtSEX4* RNAi lines (data not shown). Starch levels after 13 weeks were more variable in all lines, and this may have been caused by the variability in senescence of the tissue after 13 weeks of growth.

To provide a system for controlling the time of starch accumulation, we used an ethanol-inducible system. Wild-type, azygous and empty-vector lines containing only the ethanol-inducible promoter were grown for 3 weeks without ethanol. Plants were then grown for an additional 3 weeks while being sprayed daily with 3% ethanol. At the end of 6 weeks, all lines were harvested and assayed for starch. All lines showed similar levels of starch (Figure S3), confirming all were suitable controls.

Wild-type and transgenic lines containing the ethanol-inducible RNAi targeting *AtGWD* or *AtSEX4* were grown for 5 weeks. Plants were then sprayed for 1 week with 3% v/v ethanol/water. After the 1-week induction, leaves for starch determination were harvested just before the lights came on the growth chamber, often the time of day when leaves have least starch (Lu *et al.* 2005). All transgenic lines accumulated more starch than WT (Figure 3). The highest starch-accumulating lines had six times more starch at the end of the night than WT. Equal amounts of starch were seen in the *AtGWD* and *AtSEX4* RNAi lines.

Transcript levels were examined in the higher starch-accumulating lines by qRT-PCR using the primers listed in Table S1. Leaf material for transcript analysis was taken the night after leaf material was taken for starch determinations shown in Figure 4. Transcripts for *AtGWD* or *AtSEX4* were reduced by approximately 50% in all ethanol-inducible transgenic lines tested (Figure 4).

Because the accumulation of leaf starch is often correlated with a reduction in vegetative biomass (e.g. Figure 1a,b), the total biomass of the RNAi lines was measured. An azygous control line, two transgenic RNAi lines against the GWD gene and plants in which GWD was knocked out by tDNA were grown for 3 weeks. Plants were then sprayed with 3% ethanol daily, and whole plants were harvested weekly during the exponential phase of growth and dried to determine total dry biomass. Growth rates of azygous and transgenic lines were similar as determined by an ANOVA/Bonferroni test ($P < 0.05$); however, the plants lacking *AtGWD* grew much slower (Figure 5) as had been seen in the experiment reported in Figure 1.

Maize RNAi constructs

The maize EST database (http://www.ncbi.nlm.nih.gov/) was searched using the last 500 bp of the Arabidopsis *GWD* cDNA sequence. A region on an EST, GenBank accession CD973834, was found that that was 74% identical using 98% of our *GWD* query sequence. The 400 bp of sequence with similarity to the *GWD* gene aligns to three regions in the http://www.maizeGDB.org database B73 RefGen_v2 located on chromosome six between positions 110706215 and 110707015. The three regions are at the end of a putative gene locus GRMZM2G412611. The gene is annotated as an unknown protein with GO prediction of phosphorylation, kinase activity and

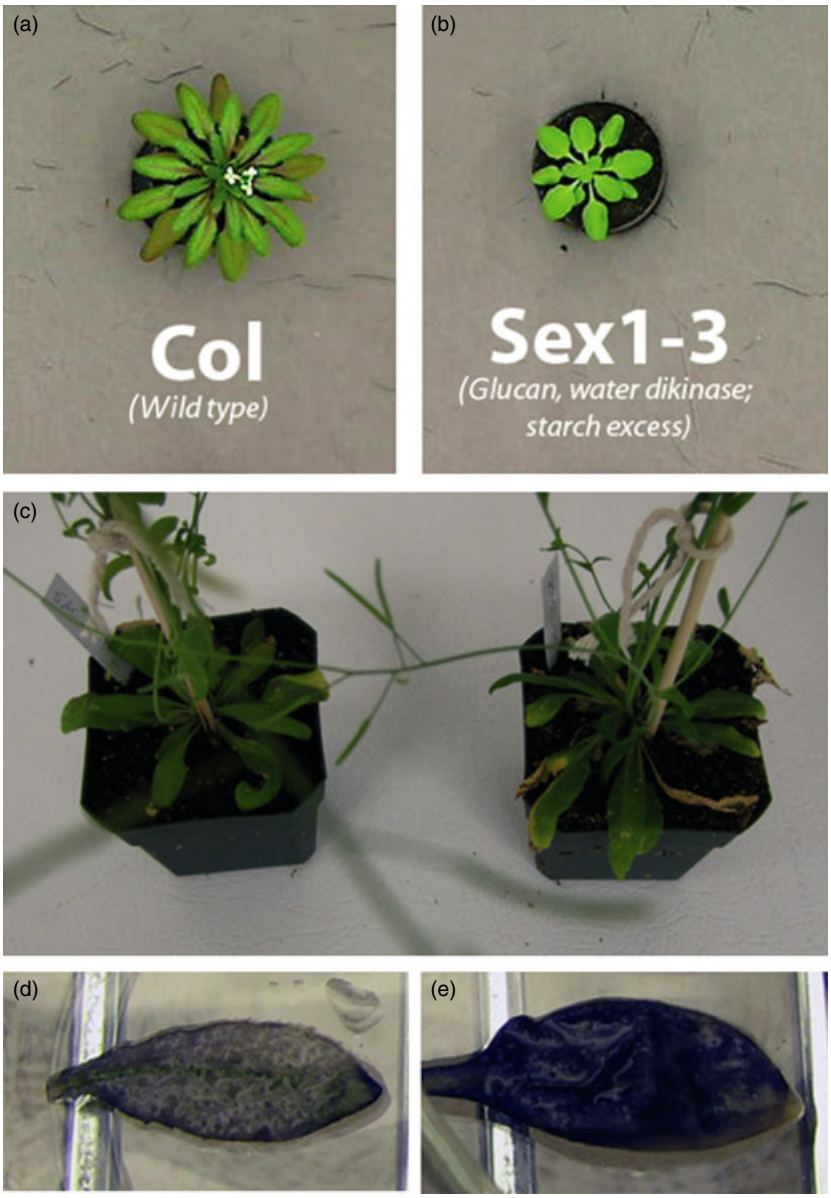

Figure 1 Iodine Staining of leaves from WT and transgenic lines with senescence inducible *AtGWD* RNAi. WT and mutant lacking glucan, water dikinase (GWD) were photographed after 9 weeks of growth (a) and (b). WT and a transgenic line containing senescence inducible *AtGWD* RNAi were photographed after 13 weeks of growth (c). Leaves from 13 week old WT (d) and a senescene inducible *AtGWD* RNAi line (e) were stained for starch using one-quarter-strength Lugol solution after 8 h in the light. Plants were grown in a 12 h photoperiod.

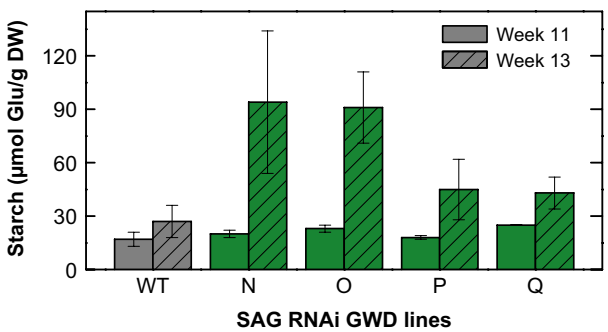

Figure 2 Starch levels in senescence inducible RNAi lines against the gene for glucan, water dikinase. Plant material was harvested after 11 or 13 weeks of growth. Values are mean ± SE (n = 5).

ATP binding. An EMBOSS Needle pairwise alignment between the amino acid sequences of the Arabidopsis GWD and the maize putative GWD-like protein found 61.4% identity and 73.6% similarity (http://www.ebi.ac.uk). By comparison, the maize putative GWD-like protein has 18% identity and 29% similarity with the Arabidopsis PWD (At5g26570) and 43% identity and 60% similarity with the Arabidopsis GWD3 (At4g24450). The RNAi construct to target the putative *ZmGWD* was designed using 371 bp of DNA upstream of the predicted TAG stop codon and 28 bp downstream of the TAG stop codon.

The maize EST database (http://www.ncbi.nlm.nih.gov/) was searched using the first 500 bp of the Arabidopsis *SEX4* cDNA sequence. A region on an EST, GenBank accession BI543017.1, was found that was 78% identical using 39% of the *SEX4*

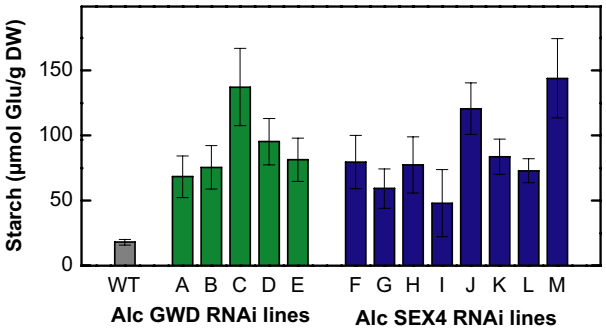

Figure 3 Starch levels in Arabidopsis ethanol inducible RNAi lines against the gene for glucan, water dikinase or phosphoglucan phosphatase. Leaf material was harvested after 1 week of being sprayed with 3% v/v ethanol. Values are mean ± SE (n = 5).

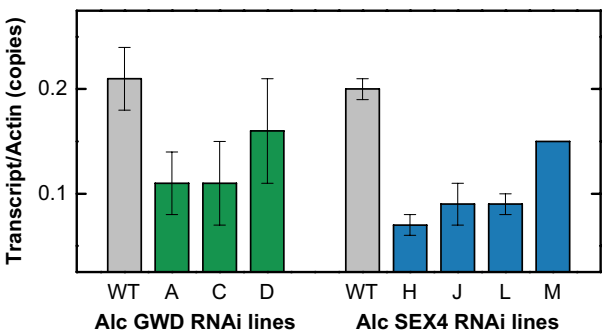

Figure 4 Transcript levels for glucan, water dikinase (GWD) and *SEX4* in their respective RNAi line relative to the transcript level for the Actin2 gene. Plant material was harvested just after the lights went off in the growth chamber when GWD and *SEX4* transcript level have been shown to be at their peak. Values are mean ± SE (n = 5).

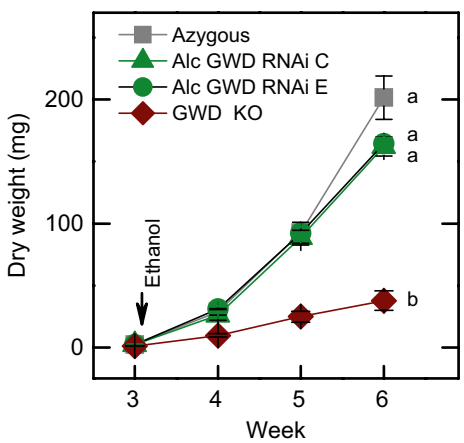

Figure 5 Total above ground dry biomass during the exponential phase of growth. Azygous control lines (squares), two transgenic RNAi lines against the gene for glucan, water dikinase (GWD); line C (triangles) and line E (circles), and a GWD KO line (diamonds) were sprayed daily with 3% ethanol immediately following the initial biomass measurement made at week 3. Values are mean ± SE (n = 6). Values followed by a different letter at week 6 are significantly different as determined by an ANOVA/Bonferroni test (P < 0.05).

query sequence. RNAi constructs were made using 400 bp of sequence that span from position 143 to 543 on EST CD973834 and 400 bp of sequence that span from position 24 to 424 on EST BI543017.1. The 400 bp of sequence with similarity to the *AtSEX4* gene aligns to four regions in the http://www.maizeGDB.org database B73 RefGen_v2 located on chromosome one between positions 2543867 and 2544661. These four regions are at the beginning of a putative gene locus GRMZM2G052546. The gene is annotated as an unknown protein with GO prediction of tyrosine/serine/threonine phosphatase activity and protein amino acid dephosphorylation. An EMBOSS Needle pairwise alignment between the amino acid sequences of the Arabidopsis SEX4 and the maize SEX4-like putative protein found 50.4% identity and 59.6% similarity (http://www.ebi.ac.uk). The RNAi construct was designed using 151 bp upstream of the predicted ATG start codon and 249 bp downstream of the ATG start codon.

Both RNAi constructs were controlled by the constitutively expressed ubiquitin promoter and used the same Pdk intron as in the Arabidopsis constructs. The RNAi inverted repeat was followed by an octopine synthase terminator.

Starch accumulation in maize leaves

Two maize RNAi lines targeting the putative *ZmGWD* and two targeting the putative *ZmSEX4* gene were grown in a growth chamber. Starch levels in the fourth oldest leaf (from the bottom) were elevated 22- to 42-fold in the *ZmGWD* RNAi lines over the empty-vector control line when the plants were 6 weeks old (Figure 6). Starch accumulated to 15%–26% of dry weight of leaves. Starch did not accumulate in any of the putative *ZmSEX4* RNAi lines (data not shown).

Transcript levels were examined in the highest starch-accumulating line by qRT-PCR using the primers listed in Table S2. Leaf material for transcript analysis was taken the night after leaf material was taken for starch determinations shown in Figure 6. Transcript for the putative *ZmGWD* gene was reduced by 80% in the RNAi line (Figure 7).

Microscopic analysis was performed to determine whether starch accumulated in the bundle sheath cells, mesophyll cells or both in the maize RNAi lines. Leaf material was taken from the top fully expanded leaves from 9-week-old plants growing

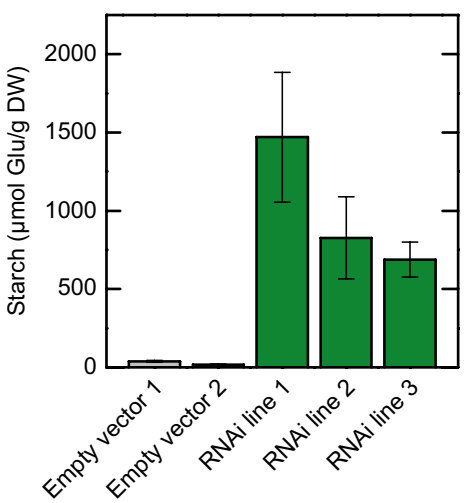

Figure 6 Starch levels in transgenic maize RNAi lines against the GWD like gene. Values are mean ± SE (n = 5). GWD, glucan, water dikinase.

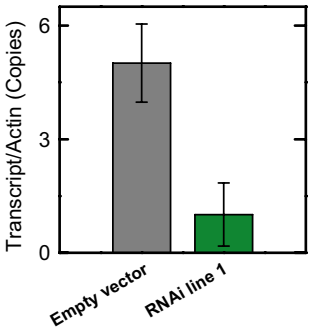

Figure 7 Transcript levels for the GWD like gene in maize relative to the transcript level for the α-Actin gene. Plant material was harvested from the fourth oldest leaf (from the bottom) when the plants were 6 weeks old just after the lights went off in the growth chamber. Values are mean ± SE (n = 4). GWD, glucan, water dikinase.

in a greenhouse. Leaf material was taken approximately 6 h after sunrise. After the leaf material was fixed, embedded and sectioned, sections were stained using one-quarter-strength Lugol's iodine solution and photographed using standard light microscopy. We found starch accumulation exclusively in the bundle sheath cells (Figure 8).

We did not observe any qualitative differences in growth rates or overall biomass in empty-vector control lines and RNAi lines. Total above ground biomass was measured in 9-week-old plants that were grown in growth chambers. We found no differences in fresh (data not shown) or dry weight between our control line and RNAi line (Figure 9).

Discussion

This work demonstrates that it is possible to accumulate leaf starch without a significant decrease in vegetative biomass. Possible explanations for why the starch-accumulating plants were not smaller than the controls include the following: (i) a later onset of starch accumulation may have allowed exponential growth to take place before starch accumulation and concomitant night starvation occurred (Usadel *et al.*, 2008); (ii) the RNAi did not completely block starch degradation allowing some starch to be degraded, sensed and used at night. The milder phenotype of plants lacking *AtSEX4*, compared to plants lacking *AtGWD*, supports the second explanation. Arabidopsis T-DNA insertion lines lacking *AtSEX4* exhibit a diel increase and decrease in leaf starch and accumulate only 50% of the starch of plants lacking *AtGWD* (Zeeman *et al.*, 1998). While plants lacking *AtSEX4* are on average smaller than WT, growth is not nearly as stunted as plants missing *AtGWD* (Caspar *et al.*, 1991; Zeeman *et al.*, 1998).

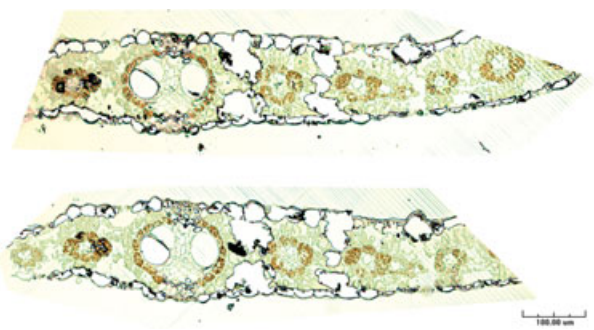

Figure 8 Leaf cross sections of maize RNAi line 1 against the GWD like gene. Starch was stained a brown color using one-quarter-strength Lugol solution. Starch accumulation was observed only in the bundle sheath cells. Empty pockets in section are due to tearing of section as a result of older leaf material used. It was necessary to use older material to localize starch in transgenic plants that had sufficient developmental time to accumulate high starch levels. Bar = 100 μm. GWD, glucan, water dikinase.

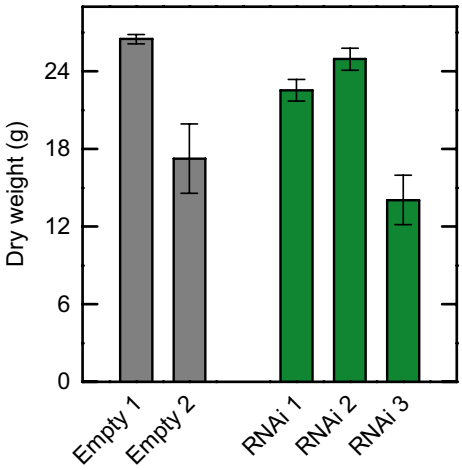

Figure 9 Total above ground biomass of transgenic maize lines. Empty lines contained the vector with only the ubiquitin promoter and Basta resistance marker. RNAi lines contained the RNAi construct against a homologous gene to the GWD gene in Arabidopsis. Plants were harvested after 9 weeks of growth just as they started to flower. The variability within a treatment (empty vector or RNAi) exceeded that between the two treatments (n = 5). GWD, glucan, water dikinase.

Other starch-accumulating mutants such as plants lacking the maltose transporter MEX1 (Nittylä *et al.*, 2004) or the cytosolic amylomaltase DPE2 (Chia *et al.*, 2004; Lu and Sharkey, 2004) accumulate less starch than plants lacking *SEX4* but have reduced yields. However, both MEX1 and the DPE2 are further downstream in the starch degradation pathway (Fettke *et al.*, 2009) than GWD or SEX4, and therefore *mex1* plants and *dpe2* plants accumulate maltose and glucose (Lu *et al.*, 2006), both of which are reducing sugars and both of which may be sensed by signalling proteins (Pego *et al.*, 2000; Rolland and Sheen, 2006). The dwarf phenotype of these mutants may be more a result of maltose and/or glucose toxicity (Stettler *et al.*, 2009) and less a result of absolute carbon starvation from starch accumulation.

The results with *ZmGWD* RNAi plants are also consistent with the hypothesis that the small amount of starch turnover allowed by RNAi constructs was enough to avoid severe growth effects. The RNAi construct was under the ubiquitin promoter control and was therefore expressed during entire life cycle. Leaf starch levels were similar to those in leaves of Arabidopsis plants lacking *GWD*. It was surprising that in maize there was not a correlation between leaf starch accumulation and growth (grain yield was not determined). The situation in the maize RNAi lines could be similar to Arabidopsis plants lacking *AtSEX4* in that enough starch can be metabolized to sustain

metabolism or at least satisfy sensing mechanisms. Twenty per cent of the empty-vector control line transcript level for *ZmGWD* is still present in our RNAi line, so it is not a complete block (Figure 7).

There are several possible reasons why maize with RNAi designed to interfere with expression of the putative *ZmSEX4* did not have higher starch levels in the leaves. (i) The reduction in RNA did not reduce the protein sufficiently. (ii) The lowered level of the enzyme was still enough to allow starch breakdown at sufficient rates to prevent build-up. (iii) Maize starch breakdown may not be as dependent on this phosphatase as is starch breakdown in Arabidopsis. (iv) The gene we found is not the correct *ZmSEX4*. In Arabidopsis, there are two genes with sequence similarity to *SEX4* (Fordham-Skelton *et al.*, 2002). Of these, only one has been shown to cause modest starch accumulation (Comparot-Moss *et al.*, 2010). If there are multiple *SEX4*-like genes in maize, a gene that is not critical for starch degradation may have been silenced.

The use of the ethanol-inducible promoter system demonstrated that it is possible to control the timing of starch build-up. These results indicate that Arabidopsis GWD and SEX4 proteins turn over relatively rapidly and so are significantly depleted soon after inducing the RNAi transgenes.

While starch levels observed in Arabidopsis RNAi lines were six times higher than WT (Figure 3), they were 17 and four times lower than the highest starch-accumulating leaves in the equivalent *GWD* or *SEX*4 T-DNA knockout lines (Figures S1 and S2). The starch levels measured in the RNAi lines occurred over a much shorter time period compared with the T-DNA insert lines. The lower levels of starch in RNAi lines compared with T-DNA insert lines may also reflect the incomplete nature of the RNAi silencing. However, a sixfold increase in starch is proof of concept that manipulation of the kinase and phosphatase expression can be used to engineer starch accumulation with no apparent effect on vegetative biomass. If such a system were to be used for agronomic production of high leaf starch plants, a chemical induction system would not be practical. A more practical solution would be the use of an inducible promoter triggered by an endogenous signal that is turned on at an appropriate time.

The promoter of the SAG12 gene is activated by senescence and causes expression late in the life cycle of the plant (Noh and Amasino, 1999). It had been shown that when the SAG12 promoter was used in tomato to cause cytokinin biosynthesis, leaf senescence was delayed (Swartzberg *et al.*, 2006). Arabidopsis engineered with an RNAi construct against the *GWD* gene and the SAG12 promoter did not exhibit a starch build-up until 11 weeks of growth, leaving only a short period for starch accumulation. A promoter such as SAG29 with stronger expression earlier in the development of the leaf may be more appropriate (Quirino *et al.*, 1999).

In maize, starch accumulated to high levels in the *ZmGWD* RNAi lines. Like WT plants (Rhoades and Carvalho, 1944; Zirkle, 1929), starch accumulation was limited to the bundle sheath cells (Figure 8). The similarity between the putative *ZmGWD* and the *AtGWD* combined with the high level of starch and its localization to the bundle sheath cells indicates that the putative *ZmGWD* likely encodes a GWD.

Previously reported maize mutants accumulating leaf starch have defects in carbohydrate export and accumulate starch in both the bundle sheath and mesophyll cells. The sucrose export defective (sxd1) mutant lacks VTE1 and reduced formation of a 'specific class of plasmodesmata, blocking sucrose export (Porfirova *et al.*, 2002; Russin *et al.*, 1996; Sattler *et al.*, 2003). Phloem loading of sucrose is also disrupted in the sucrose transporter (sut1) mutant as well as the tie dyed 1 (tdy1) mutant (Ma *et al.*, 2009; Slewinski *et al.*, 2009). Cold girdling to block sucrose export also has been studied (Slewinski *et al.*, 2009). The most recently identified maize mutant line accumulating leaf starch is the psychedelic (psc) mutant, which is deficient in an unknown protein involved in carbohydrate partitioning and export but is independent of tdy1 or sxd1 (Slewinski and Braun, 2010). Cold girdling and the psc, tdy1 and sxd1 mutants have stunted growth, and leaf regions in which carbohydrate transport is altered are chlorotic or accumulate high levels of anthocyanins.

The dwarfing and severity of phenotypes observed in the tdy1 and sxd1 mutants may result from the build-up of reducing sugars causing sugar toxicity or sensing issues. The high starch and lack of phenotype observed in both Arabidopsis and maize RNAi lines suggest that when engineering plants for elevated leaf starch, targeting the very beginning of the transitory starch degradation pathway is preferable and can avoid secondary effects from free sugar accumulation on yield and plant health.

The engineering and control of leaf starch could have an important role in biofuel production. Cellulose, hemicellulose and lignin are the largest stores of carbohydrate in non-food plant parts and therefore are the most obvious targets for biofuel conversion that does not compete with the food price and supply. However, any additional carbon that can be stored and retained in the leaf as an easily digestible polysaccharide, such as starch, has the potential to increase the yield of biofuel. In addition, the possibility of using biomass for co-firing in conventional power plants has gained increasing traction in recent years and interest in this area may grow as a method to cap net carbon dioxide emissions (Ohlrogge *et al.*, 2009). For biofuel production or for biomass burning, increasing the energy content of above ground biomass is a highly desirable goal and increased leaf starch is one way to increase energy content. Increasing starch content should also improve fermentability because starch is easily and quickly broken down to glucose and the immediate availability of free glucose has been shown to significantly improve cellulose fermentation (Robinson *et al.*, 2003).

Control of GWD in maize has important implications beyond biofuels. Maize silage, the feedstuff derived from the anaerobic fermentation of maize forage, is a major dietary component of ruminant feed, especially for dairy production. Silage is abundantly available and composed of a large proportion of slowly digestible cellulosic material. However, silage-based diets are supplemented with various grains to provide rapidly digestible starch. The use of leaf starch would be advantageous in eliminating the cost of grain added to the silage and eliminating the zein protein coat that limits the digestibility of the grain starch in the rumen gut (Allen *et al.*, 2003). The phosphate content of starch is important for many industrial applications. By engineering GWD, the phosphate content of leaf starch can be reduced to be similar to that of grain starch. Many industrial uses of starch depend upon the phosphate content. For example, the properties of bio-based plastics depend upon the phosphate content of starch. Bioplastics made from high phosphate starch will degrade faster than those made from low phosphate starch (http://www.bpf.co.uk). This could allow a further diversification of this commodity in a volatile biofuels marketplace.

Experimental procedures

RNAi construction

Arabidopsis

A senescence- or ethanol-inducible promoter system was used for transgenic Arabidospsis RNAi lines. The senescence-inducible promoter is the promoter of the senescence-associated gene 12 (*SAG12*) in Arabidopsis At5g45890 (GenBank accession number U37336) (Noh and Amasino, 1999). The ethanol-inducible promoter was from the fungus *Aspergillus nidulans* (Felenbok, 1991). The Alc promoter system consists of two parts. The first part, AlcR (Genbank AF496548.1), encodes an ethanol binding protein and was connected to a single 35S full-length gene promoter. The second part, the AlcA promoter, was generated by synthesizing the promoter region upstream of the Alcohol dehydrogenase I gene from *A. nidulans* (GenBank M16196.1). The AlcA promoter responds to the AlcR protein when ethanol is present. The AlcA promoter was used to control expression of the RNAi constructs.

DNA for the RNAi inverted repeat against *AtGWD* and *AtSEX4* genes, the 35S promoter, the Alc promoter system and the 3′OCS terminator were synthesized by Bio Basic Inc. (Markham, ON, Canada) and placed in the pUC57 plasmid vector. The plasmid vector was amplified in competent *Escherichia coli* strain DH5α (18265-017; Invitrogen, Carlsbad, CA). DH5α was transformed using a heat shock of 42 °C for 20 s. Plasmid was isolated using QIAprep miniprep kit (Quiagen, Valencia, CA) according to the manufacturer's directions. Once a sufficient quantity of vector plasmid was obtained, promoter-RNAi-terminator constructs were assembled in the pUC57 vector. Two micrograms of DNA of the plasmid containing either the *AtGWD* or *AtSEX4* targeted RNAi inverted repeat sequence and 2 μg of the vector containing either the *SAG12* or Alc promoter, each with the 3′OCS terminator were digested with 2 units each BbvCI and NcoI in buffer number four (New England Biolabs, Ipswich, MA) for 4 h at 37 °C. The vector with promoter and RNAi inverted repeat were gel purified in 0.7% agarose and extracted using a Quiagen MinElute gel extraction kit according to the manufacturer's directions. The RNAi inverted repeat was ligated into the vector between the promoter and terminator for 3 days at 4 °C using 1 unit T4 ligase (Invitrogen). The ligation was performed in a total volume of 10 μL using three times the amount of insert to vector. The fully assembled promoter-RNAi-terminator constructs were then placed in the pART27 destination vector (Gleave, 1992) for Arabidopsis transformation. Both vector and insert were digested with 6 units NotI in buffer number 3 (New England Biolabs) for 3 h at 37 °C. The pART27 vector was dephosphorylated using 10% v/v of 1 U/μL cow intestine alkaline phosphatase (Roch, Nutley, NJ) for 30 min at 37 °C. The vector and inserts were gel purified in 0.7% agarose and extracted using a Quiagen MinElute gel extraction kit according to the manufacturer's directions. The promoter-RNAi-terminator constructs were ligated into the destination vector for 2 days at 4 °C using one unit T4 ligase (Invitrogen). The ligation was performed in a total volume of 10 μL using three times the amount of insert to vector. Confirmation of the vector assembly detailed above was done by PCR using custom designed primers for the pART27 vector and different parts of our promoter RNAi construct. Vector with RNAi constructs (30 ng) was transformed into 50 μL of a near saturated culture of *Agrobacterium* strain GV3101 by electroporation. *Agrobacterium* was grown to near saturation in liquid culture containing 100 μg/mL spectinomycin and 150 μg/mL rifampicin. *Arabidopsis thaliana* Col-0 were transformed using the floral dip method (Clough and Bent, 1998), and six independent transformation events per construct were produced.

Seed from primary transformants were selected on plates containing 0.8% Phytoblend (Caisson Laboratories, North Logan, UT), 4.3 g/L MS salts (MSP C0130; Caisson Laboratories), 1% sucrose, 2.5 mM MES and 50 μg/mL kanamycin using a rapid selection method to generate T_1 plants (Harrison *et al.*, 2006). T_1 plants were grown on soil and allowed to self-pollinate. Approximately 100 seeds from T_1 plants were plated on kanamycin selection plates, and those with a 3 : 1 ratio of resistant plants to susceptible plants were selected and grown to maturity on soil (T_2 generation). Approximately 100 seeds from each plant selected in the T_2 generation were planted and those plants with 100% resistance were selected and used for all further experiments (T_3 generation). Transgene insertion was confirmed by PCR. Azygous plants were found by plating seeds from primary transformants on plates as above without kanamycin. Lack of transgene was confirmed by PCR.

Maize

The RNAi construct for maize was made as described for Arabidopsis. The pMCG1005 vector was used as the destination vector for maize transformation. This vector was provided by Dr. Heidi Kaeppler at the University of Wisconsin-Madison. Once constructs were completed, the pMCG1005 vector with the RNAi cassette was isolated using a Quiagen miniprep kit according to the manufacturer's directions. The vector assembly detailed above was confirmed by PCR using custom designed primers for the pMCG1005 vector and different parts of our promoter RNAi construct. Plasmid was then lyophilized and sent to Dr. Heidi Kaeppler's maize transformation facility. Transformation into line B73 was carried out using *Agrobacterium*, and primary transformants were back-crossed to wild-type B73 plants.

Progeny from this cross containing our transgene (T_1) were selected for when plants were 4 weeks old by painting the tip of third oldest leaf from the bottom with 0.1% w/v glufosinate. Glufosinate was prepared from commercially available Finale™ containing 11.33% glufosinate (Bayer Crop Science, Monheim, Germany). After 1 week, plants were scored and resistant plants were confirmed to contain the construct using PCR.

Plant material and growth conditions

Arabidopsis

Wild-type (Col-0), azygous and tDNA-insert lines lacking *GWD* Sex1-3 (Yu *et al.*, 2001) and (SALK_077211) or *SEX4* (SALK_102564), T_3 homozygous lines containing the senescence or ethanol-inducible RNAi against the GWD or the *SEX4*, and T_3 homozygous empty-vector lines containing the ethanol-inducible promoter but without the inverted repeat RNAi construct were used. Seeds were cold treated at 4 °C in distilled water for 3 days to ensure uniform germination. Seeds were then germinated and grown in 6-cm-diameter plastic pots using Sun Gro Redi-earth plug and seedling mix (Sun Gro, Bellevue, WA). Plants were watered with deionized water for the first 4 weeks and then quarter-strength Hoagland's solution thereafter. Plants were grown in a Percival AR4 growth chamber (Percival, Perry, IA) with a 12-h photoperiod. Day temperature was 22 °C and night temperature was 18 °C. The quantum flux, measured at

leaf level, was 120 μmol/m^2/s. Humidity was maintained at a minimum of 60% RH.

To induce ethanol-inducible lines, all plants were sprayed with 3% ethanol in water. Plants were sprayed once each day 4 h before the lights went off in the growth chamber. Plant material for starch analysis was harvested in the morning just before the lights came on the growth chamber. Leaf material for transcript analysis was harvested in the evening just after the lights went off when *AtGWD* and *AtSEX4* transcript levels have been found to be at their maximum (Smith *et al.*, 2004; Usadel *et al.*, 2008).

Maize

Seeds of T_1 maize lines containing RNAi construct against the GWD-like gene and empty-vector lines containing the ubiquitin promoter but without the inverted repeat RNAi construct were germinated and grown in 8-cm-diameter plastic pots using Baccto professional planting mix (Michigan Peat Company, Houston, TX). When plants were approximately 6 weeks old, they were transferred to 16-cm-diameter plastic pots. Plants were grown in a Bigfoot GC-20 growth chamber (Biochambers Inc, Winnipeg, MB, Canada) illuminated by fluorescent tubes until they were 9 weeks old. Plants were then potted into larger 25-cm-diameter plastic pots and moved to a greenhouse on Michigan State University's campus in East Lansing MI during the months of July, August and September. Plants were watered with tap water containing Miracle-Gro™ tomato plant food 18-18-21 (Scotts Miracle-Gro Products Inc., Marysville, OH) at the manufacturer's recommended concentration of 2.5 mL per 4 L of water. Plant material for quantitative starch analysis was harvested in the morning. Plant material for transcript analysis was harvested in the evening just after the lights went out in the growth chamber or about an hour after sunset for greenhouse grown plants.

Starch analysis

Harvested leaf material was placed in pre-weighed 2-mL microfuge tubes or 50-mL plastic tubes depending on the amount of tissue being harvested. Tubes were then quickly weighed to obtain a fresh weight. Tubes were opened and placed in a drying oven at 70 °C overnight. Tubes were weighed again after drying. If material was harvested in a 50-mL tube, a glass marble was placed in the tube and the tube was shaken to break up the plant material. A known amount of plant material was then aliquoted into a 2-mL tube to obtain a smaller representative sample. One 4-mm silicone carbide particle, #6 grit (11079140sc; BioSpec Inc, Bartlesville, OK), was placed in the 2-mL tube. Leaf material was ground at room temperature in a Retsch MM301 ball mill (Retsch, Newtown, PA) at a frequency of 30 Hz for 30 s using a 24 position Qiagen tissue lyser adapter.

To denature enzymes and remove soluble carbohydrates, 1 mL of 80% ethanol was added to ground leaf material and incubated at 80 °C for 20 min. Following incubation, samples were centrifuged at 20 000 ***g*** for 5 min. The supernatant was discarded and the ethanol incubation was repeated. After the second ethanol incubation, the sample was washed with ethanol up to three additional times to remove colour. Following the ethanol wash, the tubes with pellets were placed in a Speed Vac at low heat for 30 min to remove residual ethanol. The pellet was then resuspended in 250–1000 μL of 200 mM KOH and incubated with tube locks in a dry bath at 95 °C for 30 min to gelatinize the starch. The tubes were then allowed to cool at room temperature for 5 min, and 1 M acetic acid was added to each tube to bring the pH to 5. Starch in the sample was broken down to glucose by adding 5 μL of an enzyme cocktail containing 5 units α-amylase (E-ANAAM Megazyme; Bray, Wicklow, Ireland) and 6.6 units amylogucosidase (E-AMGDF Megazyme) in 200 mM sodium acetate pH 4.8. Samples were incubated at room temperature with starch-degrading enzymes for 2 days. Following starch digestion, samples were centrifuged at 20 000 ***g*** for at 4 °C for 20 min and the supernatant was transferred to a fresh microfuge tube.

The resulting glucose was assayed on a 96-well plate in a Spectra Max M2 plate reader (MDS Analytical Technologies, Sunnyvale, CA) at 340 nm using an NADP(H)-linked assay. Each well that was used was filled with 200 μL of 150 mM Hepes buffer pH 7.2 containing 15 mM $MgCl_2$, 3 mM EDTA, 500 nmol NADP, 500 nmol ATP and 0.4 units glucose-6-phosphate dehydrogenase (G8529; Sigma, St. Louis, MO). Five microlitres of sample was added to each well, and the reaction was started by adding 0.5 units of hexokinase (H4502; Sigma). Absolute glucose amounts were determined using an extinction coefficient of 6220 L/mol/cm for NADPH at 340 nm (Lowry and Passonneau, 1972).

Transcript analysis

RNA was extracted using a Qiagen RNeasy Plant Mini Kit according to the manufacturer's directions. Once RNA was isolated, cDNA was synthesized using 300 ng of total RNA from Arabidopsis or 500 ng of RNA from maize. Super Script II reverse transcriptase (18064; Invitrogen) was used according to the manufacturer's directions. cDNA was stored at −80 °C until used for qPCR.

For Arabidopsis, 2 μL of cDNA was diluted into 198 μL of RNase-free water and 2 μL of the resulting dilution was used for qPCR analysis. For maize, 2 μL of resulting cDNA was used directly. An Eppendorf Mastercycler ep Realplex qPCR thermocycler with a 96 position silver block with SYBR green PCR master mix (4309155; Applied Biosystems, Carlsbad, CA) was used according to the manufacturer's directions. The thermal profile was 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min during which fluoresce was measured. This was followed by a melting curve of: 95 °C for 15 s, 60 °C for 15 s, a ramp from 60 to 95 °C over a 20-min period during which time fluorescence was monitored, and 95 °C for 15 s. Transcript amounts were normalized using the Actin2 housekeeping gene transcript, At3G18780 for Arabidopsis or the Alpha Actin housekeeping gene from maize (La Paz *et al.*, 2010). All primers used in qPCR reactions were used at a concentration of 0.5 μM in the PCR tube. Sequences for primers used are listed in Tables S1 and S2. A slightly larger fragment of each target sequence had previously been amplified from a reverse-transcribed RNA extract, and the resulting DNA was quantitated. Dilutions of these, containing known numbers of copies of the target sequences, were used to prepare standard curves that were used to determine the copy numbers of the plant samples. Primer sequences used to generate templates used in quantification are listed in Tables S3 and S4.

Statistics

Statistical analyses were performed on dry weight data used in Figure 5. Analyses were performed with the program Origin Pro 8 (Origin Lab Corporation, Northhampton, MA) by using a one-way ANOVA followed by a multiple comparison test using a Bonferroni correction.

Microscopy

Leaf material from maize RNAi lines was taken from fully mature 9-week-old plants late in the day. Fully expanded leaves from the top, fully lit part of the plant were chosen. Leaf sections 1–2 mm by 10 mm were placed in a 7-mL vial with 2 mL of 2.5% glutaraldehyde/formaldehyde in 100 mM cacodylate buffer. Microwave-assisted fixation was carried out with an EMS-9000 precision pulsed laboratory microwave oven (Electron Microscopy Sciences, Hatfield, PA). Samples were fixed for 2 min at 15% power and a temperature of 30 °C. Samples were post-fixed in 2 mL of 1% OsO_4 in 100 mM cacodylate buffer. Samples were post-fixed for 10 min at 15% power and a temperature of 30 °C. Following post-fixation, samples were dehydrated in the microwave at 15% power at 30 °C for 10 min at each step using a progressively more concentrated acetone series of 50%, 70%, 80%, 90% and 100%. Dehydrated samples were fixed in EPON resin in the microwave at 15% power at 45 °C for 20 min at each step using an EPON/acetone series of 1 : 1, 3 : 1, 100%. The embedded samples were placed in moulds and polymerized at 60 °C overnight. Samples were sectioned using a microtome and diamond knife to a thickness of 500 nm. Sections were transferred to a glass microscope slide and stained for 5 min using one-quarter-strength Lugols solution (Fluka 62650; Sigma-Aldrich, St. Louis, MO). Sections were viewed and photographed using a standard bright field light microscope.

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References

Allen, M.S., Coors, J.G. and Roth, G.W. (2003) *Corn Silage*. Madison, WI: American Society of Agronomy, Inc.

Blennow, A. and Engelsen, S.B. (2010) Helix-breaking news: fighting crystalline starch energy depostis in the cell. *Trends Plant Sci.* **15**, 236–240.

Blennow, A., Nielsen, T.H., Baunsgaard, L., Mikkelsen, R. and Engelsen, S.B. (2002) Starch phosphorylation: a new front line in starch research. *Trends Plant Sci.* **7**, 445–450.

Caspar, T., Lin, T.P., Kakefuda, G., Benbow, L., Preiss, J. and Somerville, C. (1991) Mutants of Arabidopsis with altered regulation of starch degradation. *Plant Physiol.* **95**, 1181–1188.

Chia, T., Thorneycraft, D., Chapple, A., Messerli, G., Chen, J., Zeeman, S., Smith, S.M. and Smith, A.M. (2004) A cytosolic glycosyltransferase is required for conversion of starch to sucrose in Arabidopsis leaves at night. *Plant J.* **37**, 853–863.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Comparot-Moss, S., Kotting, O., Stettler, M., Edner, C., Graf, A., Weise, S.E., Streb, S., Lue, W.L., MacLean, D., Mahlow, S., Ritte, G., Steup, M., Chen, J., Zeeman, S.C. and Smith, A.M. (2010) A putative phosphatase, LSF1, is required for normal starch turnover in Arabidopsis leaves. *Plant Physiol.* **152**, 685–697.

Felenbok, B. (1991) The ethanol utilization regulon of *Aspergillus nidulans*: the alcA-alcR system as a tool for the expression of recombinant proteins. *J. Biotechnol.* **17**, 11–18.

Fettke, J., Hejazi, M., Smirnova, J., Höchel, E., Stage, M. and Steup, M. (2009) Eukaryotic starch degradation: integration of plastidial and cytosolic pathways. *J. Exp. Bot.* **60**, 2907–2922.

Fordham-Skelton, A.P., Chilley, P., Lumbreras, V., Reignoux, S., Fenton, T.R., Dahm, C.C., Pages, M. and Gatehouse, J.A. (2002) A novel higher plant protein tyrosine phosphatase interacts with SNF1-related protein kinases via a KIS (kinase interaction sequence) domain. *Plant J.* **29**, 705–715.

Gleave, A.P. (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20**, 1203–1207.

Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C. and Cottage, A.C. (2006) A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. *Plant Methods*, **2**, 19.

Hejazi, M., Fettke, J., Kötting, O., Zeeman, S.C. and Steup, M. (2009) The laforin-like dual-specificity phosphatase SEX4 from Arabidopsis hydrolyzes both C6- and C3-phosphate esters introduced by starch-related dikinases and therby affects phase trasition of α-glucans. *Plant Physiol.* **152**, 711–722.

Kötting, O., Santelia, D., Edner, C., Eicke, S., Marthaler, T., Gentry, M.S., Comparot-Moss, S., Chen, J., Smith, A.M., Steup, M., Ritte, G. and Zeeman, S. (2009) Starch-Excess4 is a laforin-like phosphoglucan phosphatase required for starch degradation in *Arabidopsis thaliana*. *Plant Cell*, **21**, 334–346.

La Paz, J.L., Vicient, C., PuigdomŠnech, P. and Pla, M. (2010) Characterization of polyadenylated cryIA(b) transcripts in maize MON810 commercial varieties. *Anal. Bioanal. Chem.* **396**, 2125–2133.

Lowry, O.H. and Passonneau, J.V. (1972) *A Flexible System of Enzymatic Analysis*. Orlando, FL: Academic Press.

Lu, Y. and Sharkey, T.D. (2004) The role of amylomaltase in maltose metabolism in the cytosol of photosynthetic cells. *Planta*, **218**, 466–473.

Lu, Y., Gehan, J.P. and Sharkey, T.D. (2005) Daylength and circadian effects on starch degradation and maltose metabolism. *Plant Physiol.* **138**, 2280–2291.

Lu, Y., Steichen, J.M., Weise, S.E. and Sharkey, T.D. (2006) Cellular and organ level localization of maltose in maltose-excess *Arabidopsis* mutants. *Planta*, **224**, 935–943.

Ma, Y., Slewinski, T.L., Baker, R.F. and Braun, D.M. (2009) Tie-dyed1 encodes a novel, phloem-expressed transmembrane protein that functions in carbohydrate partitionin. *Plant Physiol.* **149**, 181–194.

Messerli, G., Nia, V.P., Trevisan, M., Kolbe, A., Schauer, N., Geigenberger, P., Chen, J., Davison, A.C., Fernie, A.R. and Zeeman, S. (2007) Rapid classification of phenotypic mutants of Arabidopsis via metabolite fingerprinting. *Plant Physiol.* **143**, 1484–1492.

Nittylä, T., Messerli, G., Trevisan, M., Chen, J., Smith, A.M. and Zeeman, S.C. (2004) A novel maltose transporter is essential for starch degradation in leaves. *Science*, **303**, 87–89.

Noh, Y.-S. and Amasino, R.M. (1999) Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant Mol. Biol.* **41**, 181–194.

Ohlrogge, J., Allen, D., Berguson, B., DellaPenna, D., Shachar-Hill, Y. and Stymne, S. (2009) Driving on biomass. *Science*, **324**, 1019–1020.

Pego, J.V., Kortstee, A.J., Huijser, G. and Smeekens, S.G.M. (2000) Photosynthesis, sugars and the regulation of gene expression. *J. Exp. Bot.* **51**, 407–416.

Porfirova, S., Bergmuller, E., Tropf, S., Lemke, R. and Dörmann, P. (2002) Isolation of an Arabidopsis mutant lacking vitamin E and identification of a cyclase essential for all tocopherol biosynthesis. *Proc. Natl. Acad. Sci. USA*, **99**, 12495–12500.

Quirino, B.F., Normanly, J. and Amasino, R.M. (1999) Diverse range of gene activity during *Arabidopsis thaliana* leaf senescence includes pathogen-independent induction of defense-related genes. *Plant Mol. Biol.* **40**, 267–278.

Rhoades, M.M. and Carvalho, A. (1944) The function and structure of the parenchyma sheath plastids of the maize leaf. *Bull. Torrey Bot. Club*, **71**, 335–346.

Ritte, G., Lloyd, J.R., Eckermann, N., Rottmann, A., Kossmann, J. and Steup, M. (2002) The starch-related R1 protein is an a-glucan, water dikinase. *Proc. Natl. Acad. Sci. USA*, **99**, 7166–7171.

Robinson, J., Keating, J.D., Mansfield, S.D. and Saddler, J.N. (2003) The fermentability of concentrated softwood-derived hemicellulose fractions with and without supplemental cellulose hydrolysates. *Enzyme Microb. Technol.* **33**, 757–765.

Rolland, F. and Sheen, J. (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.* **57**, 675–709.

Russin, W.A., Evert, R.F., Vanderveer, P.J., Sharkey, T.D. and Briggs, S.P. (1996) Modification of a specific class of plasmodesmata and loss of sucrose export ability in the sucrose export defective1 maize mutant. *Plant Cell*, **8**, 645–658.

Sattler, S.E., Cahoon, E.B., Coughlan, S.J. and DellaPenna, D. (2003) Characterization of tocopherol cyclases from higher plants and cyanobacteria. Evolutionary implications for tocopherol synthesis and function. *Plant Physiol.* **132**, 2184–2195.

Slewinski, T.L. and Braun, D.M. (2010) The *Psychedelic* genes of maize redundantly promote carbohydrate export from leaves. *Genetics*, **185**, 221–232.

Slewinski, T.L., Meeley, R. and Braun, D.M. (2009) *Sucrose transporter1* functions in phloem loading in maize leaves. *J. Exp. Bot.* **60**, 881–892.

Smith, S.M., Fulton, D.C., Chia, T., Thorneycroft, D., Chapple, A., Dunstan, H., Hylton, C., Zeeman, S.C. and Smith, A.M. (2004) Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in Arabidopsis leaves. *Plant Physiol.* **136**, 2687–2699.

Spilatro, S.R. and Preiss, J. (1987) Regulation of starch synthesis in the bundle sheath and mesophyll of *Zea Mays* L. *Plant Physiol.* **83**, 621–627.

Stettler, M., Eicke, S., Mettler, T., Messerli, G., Hortensteiner, S. and Zeeman, S.C. (2009) Blocking the metabolism of starch breakdown products in Arabidopsis leaves triggers chloroplast degradation. *Mol. Plant*, **2**, 1233–1246.

Swartzberg, D., Dai, N., Gan, S., Amasino, R. and Granot, D. (2006) Effects of cytokinin production under two SAG promoters on senescence and development of tomato plants. *Plant Biol.* **8**, 579–586.

Usadel, B., Bläsing, O.E., Gibon, Y., Retzlaff, K., Höhne, M., Günther, M. and Stitt, M. (2008) Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in Arabidopsis rosettes. *Plant Physiol.* **146**, 1834–1861.

Weise, S.E., Weber, A. and Sharkey, T.D. (2004) Maltose is the major form of carbon exported from the chloroplast at night. *Planta*, **218**, 474–482.

Weise, S.E., van Wijk, K.J. and Sharkey, T.D. (2011) The role of transitory starch in C3, CAM, and C4 metabolism and opportunities for engineering leaf starch accumulation. *J. Exp. Bot.* **62**, 3109–3118.

Yu, T.S., Kofler, H., Häusler, R.E., Hille, D., Flügge, U.I., Zeeman, S.C., Smith, A.M., Kossmann, J., Lloyd, J., Ritte, G., Steup, M., Lue, W.L., Chen, J.C. and Weber, A. (2001) The Arabidopsis sex1 mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. *Plant Cell*, **13**, 1907–1918.

Yu, T.S., Zeeman, S.C., Thorneycroft, D., Fulton, D.C., Dunstan, H., Lue, W.L., Hegemann, B., Tung, S.Y., Umemoto, T., Chapple, A., Tsai, D.L., Wang, S.M., Smith, A.M., Chen, J. and Smith, S.M. (2005) alpha-Amylase is not required for breakdown of transitory starch in Arabidopsis leaves. *J. Biol. Chem.* **280**, 9773–9779.

Zeeman, S.C., Northrop, F., Smith, A.M. and ap Rees, T. (1998) A starch-accumulating mutant of *Arabidopsis thaliana* deficient in a chloroplastic starch-hydrolyzing enzyme. *Plant J.* **15**, 357–385.

Zirkle, C. (1929) Development of normal and divergent plastid types in Zea Mays. *Bot. Gaz.* **88**, 186–203.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Starch levels in leaves of an Arabidopsis tDNA-insert line lacking GWD.
Figure S2 Starch levels in leaves of an Arabidopsis tDNA-insert line lacking SEX4.
Figure S3 Starch levels in WT, azygous, and ethanol inducible empty-vector control lines.
Table S1 Primers used for Arabidopsis qPCR.
Table S2 Primers used for maize qPCR.
Table S3 Primers used for Arabidopsis template generation for qPCR standards.
Table S4 Primers used for maize template generation for qPCR standards.

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