

Determining the Role of *Tie-dyed1* in Starch Metabolism: Epistasis Analysis with a Maize ADP-Glucose Pyrophosphorylase Mutant Lacking Leaf Starch

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In regions of their leaves, *tdy1-R* mutants hyperaccumulate starch. We propose 2 alternative hypotheses to account for the data, that *Tdy1* functions in starch catabolism or that *Tdy1* promotes sucrose export from leaves. To determine whether *Tdy1* might function in starch breakdown, we exposed plants to extended darkness. We found that the *tdy1-R* mutant leaves retain large amounts of starch on prolonged dark treatment, consistent with a defect in starch catabolism. To further test this hypothesis, we identified a mutant allele of the leaf expressed small subunit of ADP-glucose pyrophosphorylase (*agps-m1*), an enzyme required for starch synthesis. We determined that the *agps-m1* mutant allele is a molecular null and that plants homozygous for the mutation lack transitory leaf starch. Epistasis analysis of *tdy1-R*; *agps-m1* double mutants demonstrates that *Tdy1* function is independent of starch metabolism. These data suggest that *Tdy1* may function in sucrose export from leaves.

Key words: ADP-glucose pyrophosphorylase, maize, *Tie-dyed1*

Carbon partitioning is the process whereby photoassimilates are allocated from their site of synthesis in leaves to the rest of the plant. As photoassimilates accumulate in leaves during daylight, excess carbon is temporarily stored as transitory starch in the chloroplasts (Dinges et al. 2003; Lu and Sharkey 2006; Zeeman et al. 2007). In maize (*Zea mays*), transitory starch occurs almost entirely in the bundle sheath chloroplasts (Rhoades and Carvalho 1944). The first committed step in starch synthesis is catalyzed by ADP-glucose pyrophosphorylase (AGPase) (James et al. 2003; Hannah 2005). AGPase is a heterotetrameric enzyme composed of 2 small and 2 large subunits (James et al.

2003; Hannah 2005; Georgelis et al. 2007). Mutations in principally leaf expressed genes encoding AGPase result in reduced or no transitory starch synthesis (Lin et al. 1988a, 1988b; Wang et al. 1998; Rosti et al. 2006; Lee et al. 2007; Rosti et al. 2007). Conversely, mutations in genes functioning in transitory starch catabolism result in excess starch accumulation in leaves (Zeeman et al. 1998; Blauth et al. 2001; Critchley et al. 2001; Yu et al. 2001; Dinges et al. 2003; Chia et al. 2004; Lu and Sharkey 2004; Niittyta et al. 2004). Most of these mutants display reduced growth and delayed flowering presumably due to decreased transport of assimilates to the growing sink tissues and in some cases exhibit leaf chlorosis.

To understand the genetic control of carbon partitioning, we are characterizing maize mutants that hyperaccumulate carbohydrates in their leaves. A variegated, recessive mutant, *tie-dyed1-Reference* (*tdy1-R*) contains ~16-fold higher starch and ~3- to 10-fold higher soluble sugar levels in chlorotic leaf regions relative to wild type (Figure 1A–D) (Braun et al. 2006). The *tdy1-R* mutants also show reduced growth and delayed flowering likely due to the retention of carbohydrates within the leaves (Braun et al. 2006). Through a genetic mosaic analysis, we localized the site of *Tdy1* function to the innermost tissue layer of leaves containing the interveinal mesophyll, bundle sheath, and vascular cells (Baker and Braun 2007). One hypothesis for *Tdy1* function is that it controls sucrose export into the veins (Braun et al. 2006). If *tdy1-R* mutants have reduced sucrose export capacity, soluble sugars would accumulate in mutant leaves and result in greater amounts of carbon being partitioned to starch. However, an alternative hypothesis is that *Tdy1* functions in the starch catabolism pathway in the photosynthetic cells. In this case, in *tdy1-R* mutant leaves,

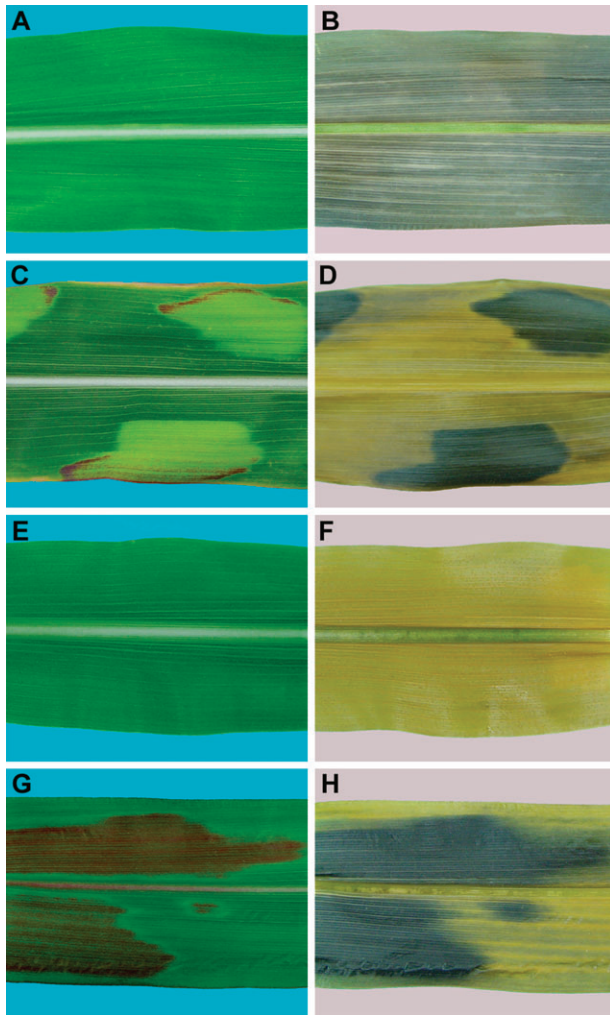


Figure 1. Excess starch is retained in *tdy1-R* chlorotic leaf regions after prolonged dark exposure. (A, B, E, and F) show wild-type leaves; (C, D, G, and H) show *tdy1-R* leaves. (A, C, E, and G) are leaf photographs; (B, D, F, and H) show the same leaves, cleared of photosynthetic pigments and starch stained with IKI. (A–D) Leaves collected at the end of the day; (E–H) leaves collected after 72 h of darkness. Note that the leaf in (B) was stained longer than the leaf in (D) to demonstrate starch in wild type. The leaves in (F) and (H) were stained equivalently. In (C) and (G), the anthocyanin accumulation within *tdy1-R* chlorotic regions is likely induced in response to osmotic stress (Chalker-Scott 1999; Braun et al. 2006).

reduced starch degradation activity would produce the observed overaccumulation of starch. Sugars would then presumably hyperaccumulate due to feedback inhibition of starch synthesis in the chloroplasts.

In this report, we distinguish between these hypotheses. To do so, we characterize a maize mutation in the gene encoding the leaf expressed small subunit of AGPase. Both the large and small subunits of AGPase are encoded by small gene families in maize with different members showing overlapping but preferential expression within

a tissue (Giroux and Hannah 1994; Prioul et al. 1994; Giroux et al. 1995; Hannah et al. 2001; Georgelis et al. 2007; Rosti and Denyer 2007; Cossegal et al. 2008). In maize leaves, the predominantly expressed small subunit gene is *Agpslz μ* (Prioul et al. 1994; Hannah et al. 2001; Rosti and Denyer 2007; Cossegal et al. 2008). Here, we show that plants homozygous for a mutation in *Agpslz μ* (*agps-m1*) lack leaf transitory starch synthesis. Furthermore, we utilize this mutant to test the hypothesized function of *Tdy1* in starch metabolism.

Materials and Methods

Plant Growth Conditions and Genetic Materials

Plants were grown in the summer nursery in the Rock Springs Agronomy Farm, Pennsylvania State University. For the dark shift experiment, plants were initially grown in a greenhouse supplemented with sodium vapor and metal halide lamps under a 12-h day (30 °C)/12-h night (20 °C) prior to being moved into a dark room maintained at 25 °C. The *tdy1-R* allele introgressed into B73 has been described (Braun et al. 2006). The *agps-m1* allele was isolated using the Trait Utility System for Corn (TUSC, Pioneer Hi-Bred International, Johnston, IA) (Bensen et al. 1995). Plants containing the *agps-m1* allele were outcrossed to *Mu Killer* (Slotkin et al. 2003; Slotkin et al. 2005) to silence *Mu* activity and then introgressed three times into the B73 genetic background prior to analyses. Plants carrying the *agps-m1* allele were identified by polymerase chain reaction (PCR) genotyping using an *Agpslz μ* primer GTACAATCTAGTCCCAGC-ACCACC and a primer designed to the terminal inverted repeat of *Mu* elements AACGCCTCCATTTCGTCGAATCC with PCR conditions of 94 °C 30 s, 60 °C 30 s, 72 °C 30 s for 35 cycles. The wild-type *Agpslz μ* allele was genotyped using the above gene primer with a second *Agpslz μ* primer GCCTT-GCCTCTGATCCGTCGGGTTG using the same PCR conditions except with a 45-s extension time. Plants from F₂ families segregating *agps-m1* and *tdy1-R* were PCR genotyped for *Agpslz μ* and *agps-m1* and were visually scored for the *tdy1-R* variegated leaf phenotype. Plants scored as homozygous for *agps-m1* were confirmed to lack leaf starch by starch staining.

Morphometric and Biochemical Analyses

Iodine–potassium iodide (IKI) staining of leaf starch, soluble sugars, and chlorophyll quantifications and morphological analyses were done as described in Braun et al. (2006), Baker and Braun (2007), Baker and Braun (2008), and Ma et al. (2008). For leaf number, plant height, days to flowering, and ear length $n = 7$ –14 plants; for chlorophyll content $n = 30$; for total kernel number per ear $n = 12$; and kernel weight is the average of 100 kernels per ear from $n = 10$. Sugar content was determined from adult leaves of greenhouse grown plants collected at the end of the day. For *tdy1-R* single mutant and *tdy1-R; agps-m1* leaves, chlorotic regions were assayed. The in-gel AGPase activity assay was performed according to Wang et al. (1998). After detecting

AGPase activity, the gel was fixed and stained with Coomassie Blue followed by destaining with ethanol/acetic acid to visualize equal protein loading.

Expression Analyses

RNA was isolated from mature leaves of field grown plants using the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA) and cDNA synthesized with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturers' instructions. The PCR primer sequences used to monitor

expression of *Agpslz_m* were CCGACGGCGCGGCCGGGC-GA and GCTCTTGAGAGGTGACGGTTGAG. The PCR conditions were 95 °C 15 s, 60 °C 15 s, and 72 °C 15 s for 35 cycles with a final concentration of 5% dimethyl sulfoxide (DMSO) and 1.5% glycerol in the reaction. The PCR primer sequences for glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) were AGATCAAGATCGGAATCAACG and GAAGCGTCTTGGAGTCCTTGA. The PCR conditions were as above omitting the DMSO and glycerol.

Results and Discussion

One possibility to account for the elevated starch levels observed in *tdy1-R* leaves is that *Tdy1* functions to promote starch turn over. To test if *tdy1-R* had altered starch breakdown, we exposed wild-type and *tdy1-R* mutant plants to extended periods of darkness and stained leaves with IKI to monitor starch depletion. After 24 h of darkness, wild-type leaves contained only a trace amount of starch indicating that the great majority of starch was metabolized, whereas *tdy1-R* mutant leaves had high levels of starch (data not shown). After 72 h of darkness, wild-type leaves were completely devoid of starch, yet *tdy1-R* mutants still contained high starch levels (Figure 1E–H). Hence, even after 3 days without photosynthesis, *tdy1-R* mutants retained abundant starch in their leaves. These data are consistent with the hypothesis that *Tdy1* could function in transitory starch breakdown.

To distinguish between the 2 hypothesized functions of *Tdy1*, we identified a maize mutant unable to synthesize transitory starch. If *Tdy1* functions in starch catabolism, we reasoned that plants lacking leaf starch would have no substrate for TDY1 to act on (directly or indirectly). Therefore, placing *tdy1-R* in a genetic background lacking leaf starch is predicted to suppress the *tdy1-R* phenotype. Conversely, if *Tdy1* functions in sucrose export, starch synthesis mutants should show no effect on *tdy1-R* phenotypic expression. To identify mutations in *Agpslz_m*

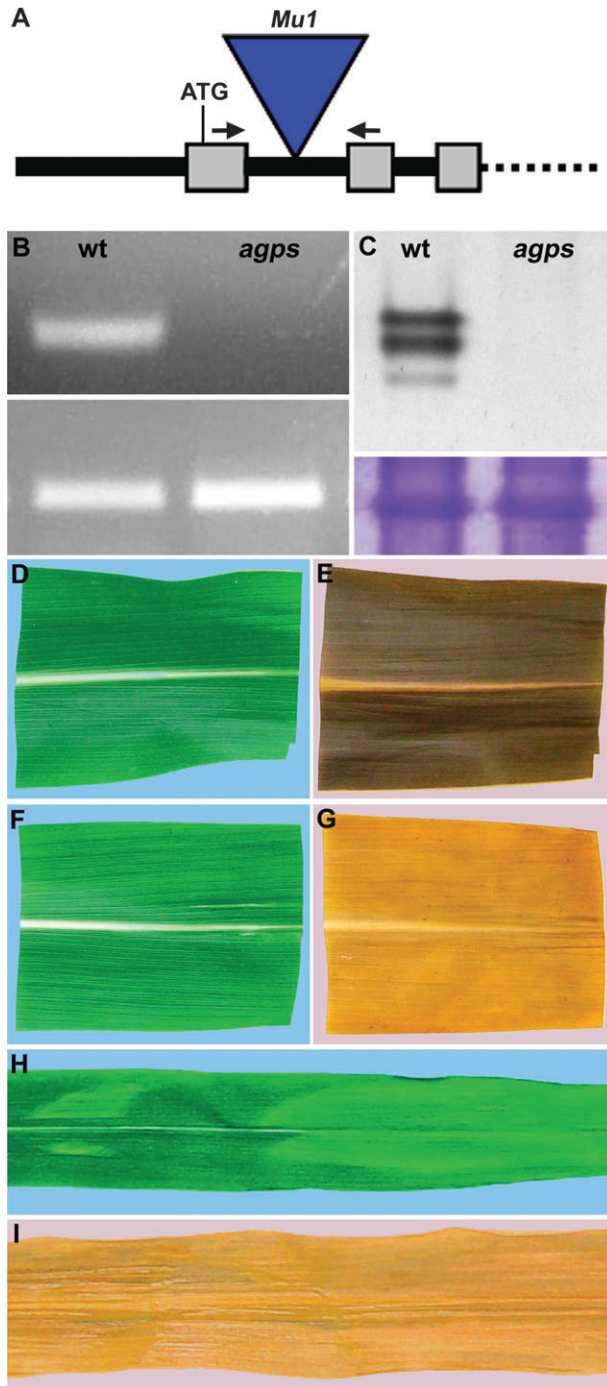


Figure 2. Mutant characterization of *agps-m1* and epistasis analysis with *tdy1-R*. (A) Model of 5' portion of *agps-m1* allele showing *Mu1* insertion in intron 1. Exons are shown as boxes, introns and 5' untranslated region are shown as lines (not drawn to scale). Arrows indicate locations of primers used for reverse transcription–PCR (RT-PCR). (B) RT-PCR shows wild-type leaves express *Agpslz_m* but no RNA is detected in *agps-m1* mutant leaves. Bottom panel shows expression of *GAPDH* in both wild-type and mutant leaves. In (B) and (C), wt, wild type and *agps*, *agps-m1* mutant. (C) In-gel activity assay detects active AGPase in wild type but not in *agps-m1* mutant leaf extracts. Bottom panel is the same gel after Coomassie Blue staining showing equal amounts of protein loaded per lane. (D, F, and H) Photograph of leaves collected at the end of the day. (E, G, and I) Corresponding leaves after IKI staining. (D, E) Wild type. (F, G) *agps-m1*. (H, I) *tdy1-R*; *agps-m1* double mutant. The leaves in (E), (G), and (I) were stained equivalently.

Table 1. Mutants of *agps-m1* have normal growth

Growth parameters	Wild type	<i>agps-m1</i> mutant
Leaf number	21.3 ± 0.2	21.2 ± 0.4
Leaf chlorophyll content	56.9 ± 0.9	54.6 ± 0.8
Plant height	199.7 ± 3.9	187.3 ± 7.1
Days to anthesis	68.1 ± 0.5	68.3 ± 1.2
Days to silking	70.9 ± 0.7	72.0 ± 1.4
Ear length	117.8 ± 4.3	111.9 ± 4.4
Kernel weight	165 ± 18	140 ± 10
Kernel number	428 ± 19	419 ± 20

Plants were grown in the summer nursery. Values are means ± standard error. Chlorophyll content (relative units), plant height (cm), time to flowering (days after planting), ear length (cm), and kernel weight (mg) are indicated. Mutants were not statistically different from wild type using the Student's *t*-test at $P < 0.05$.

and thereby potentially generate plants without leaf starch, we utilized TUSC to isolate a *Mutator* (*Mu*) transposable element insertion into *Agpslz η* (Figure 2A). We recovered a *Mu1* transposon inserted into the first intron and refer to the mutant allele as *agpslz η -m1::Mu1*, here abbreviated *agps-m1*.

Characterization of *agps-m1* mutant plants demonstrated that the insertion eliminated gene function. Reverse transcription-PCR analysis on leaf RNA showed that although the *Mu1* element inserted into an intron, no RNA was detected (Figure 2B). We confirmed the allele is a null by assessing the AGPase activity in wild-type and mutant leaf extracts and determined that the mutant produced no functional protein (Figure 2C). Wild-type leaf extracts produce 2 strong bands and a faster migrating weaker intensity band on the activity gels. All 3 forms are missing in the *agps-m1* mutant extracts (Figure 2C), indicating that the small subunit function is required to produce all leaf isoforms. Lastly, we examined leaf starch accumulation at the end of the day by IKI staining and determined that *agps-m1* mutant leaves lack starch (Figure 2D–G). Together, these analyses demonstrate that the *Agpslz η* gene provides the small subunit of AGPase responsible for transitory starch synthesis in maize leaves. This finding is consistent with a recent report suggesting that although an alternatively spliced RNA of the endosperm functioning small subunit of AGPase, *Brittle2*, is expressed in leaves, it does not appreciably contribute to the AGPase activity in leaves (Rosti and Denyer 2007).

Under field conditions, the growth of *agps-m1* mutant plants may have slightly diminished vigor (Table 1).

Table 2. Sugar quantification in wild type, single and double mutant leaves

Leaf tissue	Sucrose	Percentage of wild type	Glucose	Percentage of wild type	Fructose	Percentage of wild type
Wild type	11.2 ^a ± 0.5	100	1.6 ^a ± 0.2	100	0.4 ^a ± 0.1	100
<i>agps-m1</i>	20.6 ^b ± 1.3	184	1.4 ^a ± 0.1	87.5	0.7 ^b ± 0.1	175
<i>tdy1-R</i>	38.9 ^c ± 2.4	347	10.0 ^b ± 1.1	625	1.5 ^c ± 0.2	375
<i>tdy1-R; agps-m1</i>	36.1 ^c ± 2.4	322	5.7 ^c ± 0.5	356	1.4 ^c ± 0.2	350

Data represent means from 4 samples ± standard error, and the units are milligram/gram fresh weight. Different letters in superscript within a column indicate statistically significant differences between samples determined using the Student's *t*-test at $P \leq 0.05$.

However, it was not significantly different from wild-type siblings, similar to descriptions of rice and barley AGPase mutants (Rosti et al. 2006; Lee et al. 2007; Rosti et al. 2007). This was a somewhat surprising finding because maize partitions a greater amount of carbon to starch than does rice or barley (Kalt-Torres et al. 1987; Hussain et al. 1999; Cairns et al. 2002). It is possible that in the field conditions in the summer nursery, sufficient sucrose is produced to compensate for the loss of carbon transiently stored as starch. In support of this idea, we found that sucrose levels in *agps-m1* leaves were approximately twice that of wild type (Table 2). A similar observation was reported for a mutation in the maize leaf pullulanase enzyme (Dinges et al. 2003). In this case, the inability to completely degrade transitory starch at night resulted in a reduced synthesis of starch the following day and a greater partitioning of fixed carbon to sucrose, but no morphological phenotypes were evident (Dinges et al. 2003).

To test the hypothesis that *Tdy1* functions in starch catabolism, we analyzed *tdy1-R; agps-m1* double mutant plants. If *Tdy1* functioned in starch breakdown, we would expect *agps-m1* mutants to suppress the *tdy1-R* phenotype. Instead, double mutant leaves were variegated and expressed the *tdy1-R* mutant phenotype (Figure 2H). IKI staining revealed that the double mutant leaves contained no detectable starch (Figure 2I). Furthermore, the soluble sugar levels in the double mutants were similar to the *tdy1-R* single mutants (Table 2). Thus, *agps-m1* is not epistatic to *tdy1-R*. These data argue against the hypothesis that *Tdy1* functions in starch breakdown because expression of the *tdy1-R* phenotype was independent of the presence or absence of starch. We interpret these data to support the conclusion that *Tdy1* and *Agpslz η* function in independent pathways. Therefore, we favor the hypothesis that *Tdy1* functions to promote the sucrose export capacity of leaves. Future work will test this hypothesis by characterizing the molecular function of TDY1 and determining its role in phloem transport.

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