Regulation of carbon partitioning is essential for plant growth and development. To gain insight into genes controlling carbon allocation in leaves, we identified mutants that hyperaccumulate carbohydrates. *Tie-dyed2 (tdy2)* is a recessive mutant of maize (*Zea mays*) with variegated, nonclonal, chlorotic leaf sectors containing excess starch and soluble sugars. Consistent with a defect in carbon export, we found that a by-product of functional chloroplasts, likely a sugar, induces *tdy2* phenotypic expression. Based on the phenotypic similarities between *tdy2* and two other maize mutants with leaf carbon accumulation defects, *tdy1* and sucrose export defective1 (*sxd1*), we investigated whether *Tdy2* functioned in the same pathway as *Tdy1* or *Sxd1*. Cytological and genetic studies demonstrate that *Tdy2* and *Sxd1* function independently. However, in *tdy1/+*; *tdy2/+* F1 plants, we observed a moderate chlorotic sectored phenotype, suggesting that the two genes are dosage sensitive and have a related function. This type of genetic interaction is referred to as second site noncomplementation and has often, though not exclusively, been found in cases where the two encoded proteins physically interact. Moreover, *tdy1*; *tdy2* double mutants display a synergistic interaction supporting this hypothesis. Additionally, we determined that cell walls of chlorotic leaf tissues in *tdy* mutants contain increased cellulose; thus, *tdy* mutants potentially represent enhanced feedstocks for biofuels production. From our phenotypic and genetic characterizations, we propose a model whereby TDY1 and TDY2 function together in a single genetic pathway, possibly in homo- and heteromeric complexes, to promote carbon export from leaves.

All aspects of plant growth and development are dependent upon proper control of carbon allocation. For carbon to be correctly partitioned, photoassimilates fixed in source leaf tissues must be distributed through the veins to carbon-importing sink tissues (Turgenev, 1989, 2006). Maize (*Zea mays*) leaves have three different types of longitudinally arranged veins, small, intermediate (collectively referred to as minor), and large (lateral), which perform different roles in carbon distribution (Russell and Evert, 1985). Minor veins principally function to load fixed carbon from the photosynthetic cells (Fritz et al., 1983). These veins intergrade into the lateral veins, which primarily function in the long-distance transport of assimilates (Fritz et al., 1989). Maize is a C4 plant with two distinct photosynthetic cell types, mesophyll and bundle sheath cells, that cooperatively carry out carbon assimilation. Mesophyll cells encircle the bundle sheath cells, which surround the veins (Esau, 1977). Maize is an apoplastic phloem-loading species (Evert et al., 1978). Suc synthesized in mesophyll cells diffuses along the symplastic transport pathway through plasmodesmata into bundle sheath cells and then into vascular parenchyma cells (Rusin et al., 1996). From there, Suc is exported to the apoplast prior to loading into the phloem. Although the translocation path of fixed carbon in maize leaves was described nearly 40 years ago (Hofstra and Nelson, 1969), little is known concerning the mechanisms that regulate this process.

In most plant species, Suc is the major form of fixed carbon translocated, and it is loaded into the companion cells and/or sieve elements of the phloem by Suc transporters (SUTs; Lalonde et al., 2004; Sauer, 2007). It is also known that Suc acts as a signal to regulate SUT transcript and protein levels (Chiou and Bush, 1998; Vaughn et al., 2002; Ransom-Hodgkins et al., 2003), but the components of the signaling cascade have not been elucidated. Evidence demonstrating that SUTs are essential for phloem loading comes from mutational studies (Riesmeier et al., 1994; Kuhn et al., 1996; Burkle et al., 1998; Gottwald et al., 2000). Plants with decreased SUT function from either antisense transgenic expression or a T-DNA insertion accumulate carbohydrates in their leaves. The excess photoassimilates cause feedback down-regulation of photosynthetic gene expression and chlorosis (Sheen, 1990; Goldschmidt and Huber, 1992; Krapp and Stitt, 1995; Koch, 1996; Jeanette et al., 2000; Rolland et al., 2006). SUT mutant plants also show diminished shoot growth, delayed flowering, and decreased yield due to the reduced export of carbohydrates to the growing sink tissues.
In maize, two recessive mutants have been described with carbon-accumulating leaf phenotypes resembling SUT loss-of-function mutations. Unlike SUT mutants that have uniform chlorotic phenotypes, *tie-dyed1* (*tdy1*) and *sucrose export defective1* (*sxd1*) mutant leaves develop variegated chlorotic sectors that hyperaccumulate carbohydrates (Russin et al., 1996; Provencher et al., 2001; Braun et al., 2006). The formation of these nonclonal sectors cannot be explained by the clonal basis of maize leaf development (Poethig and Szymkowiak, 1995; Baker and Braun, 2007), suggesting that a mobile signal, potentially a sugar, coordinates their development. Additionally, both *tdy1* and *sxd1* plants display reduced growth, are slow to flower, and have decreased yield. However, despite the similarities in their phenotypes, each mutant has unique properties. *Sxd1* encodes tocopherol cyclase, and *sxd1* mutants lack tocopherols (Sattler et al., 2003). In *sxd1* mutants, callose is deposited over the plasmodesmata of the bundle sheath-vascular parenchyma cell interface in leaf minor veins (Botha et al., 2000). This blockage prevents Suc movement along the symplastic transport pathway. The resultant *sxd1* chlorotic sectors progressively develop, initiating at the leaf tip and spreading toward the base. On the other hand, *tdy1* mutant leaves have normal tocopherol levels and do not exhibit ectopic callose deposits or any alterations in plasmodesmatal ultrastructure along the symplastic pathway (Ma et al., 2008). Further, *tdy1* chlorotic sectors appear shortly after leaves emerge from the whorl, are stable once visible, and tend to be bounded by lateral veins (Braun et al., 2006; Baker and Braun, 2007). From genetic and cytological investigations, we previously determined that *Tdy1* and *Sxd1* function in separate genetic pathways and that *Tdy1* likely functions within the veins to limit carbohydrate accumulation in leaves (Baker and Braun, 2007; Ma et al., 2008).

The combination of leaf variegation and carbon hyperaccumulation phenotypes seen in *tdy1* and *sxd1* mutants is novel. Other carbon accumulation mutants have been reported, but they affect the entire leaf and are not variegated. For example, in addition to the SUT mutants described above, mutations in starch catabolism result in the build-up of excess starch throughout the chlorotic tissues (Baker and Braun, 2007; Ma et al., 2008). Additionally, both *tdy1* and *sxd1* appear to be unique and may offer insights into the regulation of carbon accumulation in leaves. Incorporating these phenotypes and other data into a threshold model led us to propose that *Tdy1* acts as an osmotic stress or sugar sensor to induce SUT function (Braun et al., 2006).

SUT induction would promote Suc export and thereby decrease carbohydrate content in leaf tissues below a threshold necessary to produce a chlorotic sector.

We have taken a genetic approach to identify additional genes regulating carbon accumulation in maize leaves. In analyzing a large collection of mutants with variegated nonclonal sectors in their leaves, we discovered several new mutants that hyperaccumulate carbohydrates within the chlorotic tissues. In this article, we report the characterization of the third maize mutant affecting leaf carbon partitioning, *tdy2*. *tdy2* mutant plants develop stable, nonclonal, chlorotic leaf sectors with greatly elevated carbohydrate content. We determined that *tdy2* chlorotic sectors are frequently bounded by lateral veins and that they are induced by a chloroplast-derived signal, likely a sugar. Similar to *tdy1* and *sxd1* plants, *tdy2* mutants have reduced stature, delayed flowering, and decreased seed yield, presumably due to the retention of carbohydrates in leaves. However, phenotypic, cytological, and genetic analyses demonstrate that *Tdy1* and *Tdy2* function independently of *Sxd1*.

To determine if *Tdy2* acts in the same genetic pathway as *Tdy1*, we constructed double mutant families. Intriguingly, *F1* plants doubly heterozygous for *tdy1* and *tdy2* exhibited a moderately sectored phenotype. This type of genetic interaction whereby two recessive loci show a phenotype in the *F1* generation is called second site noncomplementation (SSNC, also referred to as nonallelic noncomplementation; Badano and Katsanis, 2002; Hawley and Gilliland, 2006). SSNC has been documented in animals, yeast, and in a couple of instances in plants (Stearns and Botstein, 1988; Hays et al., 1989; Harris and Juriloff, 1998; Yook et al., 2001; Folkers et al., 2002; Kaplinsky and Freeling, 2003). In many, though not all cases, SSNC is indicative of a physical association between the proteins encoded by the two loci and suggests that *TDY1* and *TDY2* may function in the same genetic pathway and physically interact. Double mutant plants show a synergistic interaction of severely chlorotic leaves consistent with this hypothesis. Models for the *TDY1*-*TDY2* interaction and function are presented.

Finally, because of the carbohydrated hyperaccumulation in *tdy* mutant leaves, we examined whether there was an increase in carbon deposited into the cell wall as a terminal storage site. We found that *tdy* mutant leaves have increased cellulose levels, suggesting that these stocks may serve as enhanced feedstocks for the production of biofuels. The utility of carbon hyperaccumulating mutants is discussed.

**RESULTS**

*tdy2* Is a Variegated Leaf Mutant That Hyperaccumulates Carbohydrates in Chlorotic Sectors

To characterize additional genes promoting carbon export from maize leaves, we screened for nonclonal...
sectoring mutants resembling tdy1. We identified a new mutant, tdy2, that forms chlorotic leaf sectors similar in appearance to tdy1 (Fig. 1, A–C). tdy2 plants were reciprocally crossed with inbred lines, and the F1 progeny were wild type, indicating that the mutation is not maternally inherited. The F1 plants were self-fertilized, and the F2 segregation ratio indicated that tdy2 is a recessive mutation (149 wild type:51 tdy2; x₀² = 0.03, P > 0.05). A series of crosses using B-A translocation stocks was performed to map tdy2 (Beckett, 1994). Only progeny from the chromosome 5 L cross uncovered the tdy2 mutation; however, the phenotype of the 5 L hypoploid individuals (two mutant copies of tdy2 over a deletion) was mild compared to tdy2 homozygotes (Fig. 1D). These data suggest that tdy2 is a hypomorphic mutation that still retains some function, i.e. it is not a null allele (Muller, 1932). We fine mapped tdy2 to the distal end of the long arm of chromosome 5 in bin 5.09 using molecular markers (four recombinants/214 chromosomes with IDP217), indicating that tdy2 is a distinct locus from tdy1 (mapped to chromosome 6) and sxd1 (located near the centromere of chromosome 5, bin 5.04). Similar to tdy1 but different than sxd1, tdy2 chlorotic sectors are stable once formed (Fig. 1E; Supplemental Fig. S1).

tdy1 and sxd1 mutants hyperaccumulate carbohydrates in chlorotic leaf tissues (Russin et al., 1996; Braun et al., 2006). To determine if tdy2 mutants also contain high levels of carbohydrates, we stained leaves with iodine-potassium iodide to visualize starch. tdy2 chlorotic tissues stained much more strongly than green leaf tissues, indicating the chlorotic sectors contain higher levels of starch (Fig. 2, A–D). We quantified the amount of Suc, Glc, Fru, and starch in tissues from tdy2 chlorotic regions, tdy2 green regions, and wild-type leaves. tdy2 chlorotic regions contained approximately 3- to 8-fold higher levels of carbohydrates compared to green leaf tissues from the mutant or wild type (Fig. 2, E–H). Hence, tdy2 mutants hyperaccumulate carbohydrates in the chlorotic leaf tissues. In addition, similar to tdy1 and sxd1 mutants, tdy2 plants display a reduction in plant height, delayed time to flowering, diminished inflorescence size, and decreased kernel number, most likely due to the retention of carbohydrates in vegetative tissues (Supplemental Fig. S2).

Both tdy1 and sxd1 mutants exhibit similar levels of chlorosis associated with hyperaccumulation of carbohydrates (Ma et al., 2008). However, pigment levels in the green tissues differ between the two mutants. Green tissues in tdy1 mutants have photosynthetic pigment levels comparable to wild type, whereas sxd1 green leaf tissues show mild chlorosis and contain only approximately 70% of wild-type pigment levels (Ma et al., 2008). To characterize the photosynthetic pigment levels in tdy2 tissues, we measured their abundance. The chlorophyll a, chlorophyll b, and total carotenoid levels in tdy2 chlorotic regions were 50% to 70% of those in wild type, while the chlorophyll a/b ratio in chlorotic regions was elevated (Table I). In contrast, pigment levels in green tdy2 tissues were not statistically different from wild type. In this regard, the tdy2 phenotype is similar to tdy1 and distinct from sxd1.

Starch Accumulation Precedes Chlorosis in tdy2 Leaves

Excess accumulation of carbohydrates in leaves is known to down-regulate photosynthetic gene expression and result in chlorosis (Sheen, 1990; Goldschmidt and Huber, 1992; Krapp and Stitt, 1995; Koch, 1996; Jeannette et al., 2000; Rolland et al., 2006). To investigate whether tdy2 leaves accumulate carbohydrates prior to chlorosis, we stained emerging leaves for starch before visible sector formation. Wild-type leaves did not show any regions that differentially accumulated starch (Fig. 3). However, tdy2 leaves that visibly lacked chlorotic regions displayed leaf areas that preferentially accumulated starch. These starch-accumulating regions would presumably have developed into chlorotic tissues. Because carbohydrate accumulation pre-
cedes chlorosis, it suggests that the primary defect may be in carbon export rather than in plastid development. The discrete development of *tdy2* starch-accumulating regions and chlorotic sectors parallels events in *tdy1* mutant leaves (Supplemental Fig. S1; Braun et al., 2006) but contrasts with the continuous expansion of sectors seen in *sxd1* leaves (Russin et al., 1996; Provencher et al., 2001).

**Lateral Veins Often Coincide with *tdy2* Sector Boundaries**

*tdy2* mutant leaves often exhibited a very sharp boundary between chlorotic and green tissues (Fig. 4A; Supplemental Table S1). Cross sections through these regions determined that the sharp boundaries frequently corresponded to lateral veins. Under reflected light, it can be seen that the bundle sheath cells on either side of the lateral vein differ in their chlorophyll pigmentation (Fig. 4B). As shown by chlorophyll autofluorescence upon UV illumination, a significant reduction in chlorophyll is seen in the bundle sheath and mesophyll cells from the chlorotic tissue relative to the green tissue (Fig. 4C). Furthermore, after starch staining, it is evident that bundle sheath cells on the chlorotic side of the lateral vein hyperaccumulate starch, while those on the opposite side contain much less starch (Fig. 4D). In addition, similar to *tdy1* and *sxd1* chlorotic tissues, we observed that mesophyll cells in *tdy2* chlorotic tissue stain strongly for starch, whereas mesophyll cells in *tdy2* green tissue do not (Fig. 4D). Hence, lateral veins frequently delineate *tdy2* chlorotic regions. Lateral veins also tend to be located at the sharp boundaries between *tdy1* chlorotic and green tissues (Baker and Braun, 2007). However, in *sxd1* mutants, it is the minor, not lateral, veins that are affected (Russin et al., 1996). As lateral veins primarily function in the long-distance transport of assimilates (Fritz et al., 1989), it suggests that the transport of Suc through this class of veins may limit expansion of a *tdy2* chlorotic region.

**A Chloroplast By-Product Induces *tdy2* Phenotypic Expression**

In our studies of the *tdy1* mutant, we found that functional chloroplasts are required to produce a fusible compound, potentially a sugar, that induces the *tdy1* sectored phenotype (Baker and Braun, 2007). To test whether functional chloroplasts were likewise required for expression of the *tdy2* chlorotic phenotype, we analyzed double mutants between *tdy2* and *iojap1* (*ij1*). *ij1* is a recessive, nuclear mutation that conditions defective chloroplasts that lack ribosomes and results in albino longitudinal stripes in an otherwise largely green leaf (Fig. 5A; Walbot and Coe, 1979; Coe et al., 1988). Because it is not possible to visualize a change in chlorophyll pigmentation in white tissue, we crossed *tdy2* into a genetic stock that accumulates anthocyanins exclusively in the *tdy2* starch-accumulating sectors (Fig. 5B). We utilized this anthocyanin accumulation to specifically mark *tdy2* phenotypic regions and detect their presence within albino tissues. In examining *tdy2; ij1* double mutant leaves, we observed that albino cells expressed the *tdy2* anthocyanin accumulation phenotype only if contiguous to green leaf tissue expressing a *tdy2* sector (Fig. 5C). Starch staining the double mutant leaves demonstrated that starch and anthocyanin accumulation...
perfectly coincided and that the albino cells expressed the tdy2 starch accumulation phenotype (Fig. 5D). Histological examinations verified this finding (Fig. 5, E–P). ijl albino tissue not expressing the tdy2 anthocyanin phenotype contained no detectable starch, consistent with previous data that ijl albino cells lack starch (Fig. 5M; Rhoades and Carvalho, 1944). However, albino cells expressing anthocyanins in tdy2: ij1 double mutant leaves accumulated starch in the mesophyll and bundle sheath cells as in the tdy2 chlorotic regions but at a reduced level, presumably due to the heterotrophic cells metabolizing a portion of the imported sugar (Fig. 5, J and P). These data are in agreement with previous data showing that albino tissues accumulate starch if fed surplus sugar (Cox and Dickinson, 1971) and that tdy1: ij1 albino cells can accumulate starch (Baker and Braun, 2007). These data suggest that functional chloroplasts are not required to express the tdy2 phenotype, and that a mobile chloroplast by-product, possibly Suc, induces the tdy2 phenotype.

### Table 1. Photosynthetic pigment levels in wild-type and tdy2 leaves

Data represent means from six samples ± se.

<table>
<thead>
<tr>
<th>Leaf Tissue</th>
<th>Chlorophyll a µg/g fresh weight</th>
<th>Percentage of Wild Type</th>
<th>Chlorophyll b µg/g fresh weight</th>
<th>Percentage of Wild Type</th>
<th>Chlorophyll a/b</th>
<th>Total Carotenoids µg/g fresh weight</th>
<th>Percentage of Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1,278.6 ± 111.8</td>
<td>100</td>
<td>369.5 ± 21.5</td>
<td>100</td>
<td>3.46</td>
<td>320.9 ± 4.2</td>
<td>100</td>
</tr>
<tr>
<td>tdy2 green</td>
<td>1,242.2 ± 43.5</td>
<td>97.2</td>
<td>363.5 ± 19.5</td>
<td>98.4</td>
<td>3.42</td>
<td>308.9 ± 10.1</td>
<td>96.2</td>
</tr>
<tr>
<td>tdy2 chlorotic</td>
<td>829.9 ± 67.8</td>
<td>65.0</td>
<td>192.2 ± 17.8</td>
<td>52.0</td>
<td>4.32</td>
<td>220.5 ± 7.0</td>
<td>68.7</td>
</tr>
</tbody>
</table>

*Value is significantly different from wild type at P ≤ 0.01 using the Student’s t test.

### Plasmodesmata along the Symplastic Pathway Appear Unobstructed in tdy2 Leaves

sxl1 mutants accumulate carbohydrates in their leaves due to a blockage along the Suc symplastic pathway (Russin et al., 1996; Provencher et al., 2001). Specifically, callose is deposited over the plasmodesmata between the bundle sheath and vascular parenchyma cell interface, thereby preventing Suc from being exported to the apoplast (Botha et al., 2000). tdy1 mutants do not deposit callose over this cellular interface, suggesting that they accumulate carbohydrates by a different mechanism (Ma et al., 2008). To determine whether tdy2 mutants contain callose deposits occluding the symplastic pathway as observed in sxl1 leaf minor veins, we performed aniline blue fluorescence microscopy. By gently scraping away the abaxial epidermis, mesophyll, and bundle sheath cells, we exposed the bundle sheath-vascular parenchyma cell interface and then stained the tissue with aniline blue (Fig. 6A). Aniline blue binds callose and emits blue-white light on UV excitation. As shown in para-dermal sections of leaf minor veins, callose is found in wild-type tissues only in the sieve plates between sieve elements (Fig. 6B). In contrast, in sxl1 mutants, many ectopic callose deposits are detected at the bundle sheath-vascular parenchyma cell interface (Fig. 6C). No ectopic callose deposits were observed in either green or chlorotic leaf tissues from tdy2 mutants (Fig. 6, D and E). These data suggest that tdy2 mutants do not have callose deposits precluding symplastic Suc movement as found in sxl1 mutants.

Though we did not observe callose deposits blocking the symplastic pathway in tdy2 mutants, it is possible that another plasmodesmatal structural defect could account for the carbohydrate accumulation. To test this hypothesis, we performed transmission electron microscopy (TEM) on wild-type and tdy2 leaves (Fig. 7). In wild-type leaves, starch predominantly accumulates in bundle sheath cell chloroplasts but is largely absent from mesophyll cell chloroplasts (Fig. 7A; Rhoades and Carvalho, 1944). However, in tdy2 chlorotic tissues, starch hyperaccumulates in both bundle sheath and mesophyll cell chloroplasts (Fig. 7B). Inspecting the bundle sheath-vascular parenchyma cell interface, we did not detect any structural alterations to, or deposits over, the plasmodesmata in wild-type or tdy2 leaves (Fig. 7). In wild-type leaves, starch predominately accumulates in bundle sheath cell chloroplasts but is largely absent from mesophyll cell chloroplasts (Fig. 7A; Rhoades and Carvalho, 1944). However, in tdy2 chlorotic tissues, starch hyperaccumulates in both bundle sheath and mesophyll cell chloroplasts (Fig. 7B). Inspecting the bundle sheath-vascular parenchyma cell interface, we did not detect any structural alterations to, or deposits over, the plasmodesmata in wild-type or tdy2 leaves (Fig. 7, C and D). We similarly investigated the appearance of plasmodesmata at all cellular interfaces along the symplastic loading pathway. In every case, the tdy2 samples appeared indistinguishable from wild type (Supplemental Fig. S3). Collectively, these data suggest that tdy2 mutants do not accumulate carbohydrates due to physical blockage of the plasmodesmata as seen in sxl1 plants.
Figure 4. Lateral veins frequently are found at sharp boundaries between tdy2 chlorotic and green tissues. A, Photograph of tdy2 leaf segment with sharp boundary between the green and the chlorotic region (arrow). B, Leaf cross section spanning the sharp boundary viewed in reflected light shows that a lateral vein (arrowhead) separates chlorotic and green tissues. C, Same section viewed in UV light shows reduced chlorophyll autofluorescence (red color) in the chlorotic tissue compared with the green tissue. Note that bundle sheath cells on either side of the lateral vein contain differing levels of chlorophyll. D, Same section starch stained and viewed in bright-field shows that mesophyll cells in chlorotic tissue contain starch (arrows). Additionally, bundle sheath cells on the chlorotic side of the lateral vein contain more starch than bundle sheath cells on the green side of the vein. Scale bars = 100 μm.

tdy2 and tdy1 Display SSNC and a Synergistic Interaction

In addition to the cytological investigations showing tdy2 plants accumulate carbohydrates due to a different mechanism than sxd1 mutants, we observed an additive genetic interaction in tdy2; sxd1 double mutants (data not shown). Together, these data indicate that the two genes function in distinct pathways. Similarly, we previously determined that tdy1 and sxd1 also define separate genetic pathways (Ma et al., 2008). Hence, we tested whether Tdy2 acts in the same genetic pathway as Tdy1. Most surprisingly, in the F1 generation, the doubly heterozygous tdy1/+; tdy2/+ individuals, which would be expected to display a wild-type phenotype (Fig. 8A), exhibited a tdy sectored leaf phenotype indicative of SSNC (Fig. 8C; Supplemental Fig. S4). Cases of SSNC have been reported previously in plants, for example, between genes functioning in Arabidopsis trichome morphogenesis (Folkers et al., 2002), and in maize inflorescence development (Kaplinsky and Freeling, 2003). To determine if the tdy chlorotic sectors observed in tdy1/+; tdy2/+ F1 plants accumulated carbohydrates, we starch stained the sectored leaves. Similar to tdy1 or tdy2 chlorotic sectors, the chlorotic tissues of F1 plants hyperaccumulated starch relative to wild type or green regions of F1 leaves (Fig. 8, B and D). These data indicate that Tdy1 and Tdy2 are dosage sensitive and may act in the same process.

To further characterize the interaction between Tdy1 and Tdy2, F2 families segregating both mutants were analyzed. Remarkably, we found that leaves of tdy1; tdy2 double mutants were virtually completely chlorotic (Fig. 8E). The reduction in chlorophyll in the double mutant leaves was comparable to that seen in the chlorotic regions of single mutants, and all of the chlorotic tissues displayed similar down-regulation of photosynthesis (Supplemental Table S2). Starch staining the double mutant chlorotic leaf blades showed that they hyperaccumulated starch throughout the tissue (Fig. 8F). By quantifying soluble sugar accumulation in the double mutant plants relative to wild type, we determined that the chlorotic tissue contained greatly elevated levels of carbohydrates (Table II). In addition, the double mutant plants had a stronger reduction in plant height compared with either single mutant or wild-type siblings (Fig. 8G). Based on the nearly identical phenotypes, the F1 SSNC effect and the synergistic genetic interaction observed in the double mutants, we conclude that Tdy1 and Tdy2 function similarly to promote carbohydrate export from leaves. Further, these data also suggest that Tdy1 and Tdy2 may act in the same genetic pathway, potentially in a complex.

tdy Mutants Have Increased Cellulose in Their Cell Walls

Because tdy1 and tdy2 mutants build up very high concentrations of carbohydrates within chlorotic regions of leaf tissue, we investigated whether the surplus carbon can be shunted into the cell wall as a permanent carbon storage reserve. To examine the cellulose content of the cell wall, we performed calcofluor white fluorescence microscopy. Calcofluor white binds cellulose and fluoresces upon UV light exposure. In comparing wild type to chlorotic tissues from tdy1, tdy2, or tdy1; tdy2 double mutant leaves, we determined that the single and double mutants fluoresce more strongly than wild-type siblings (Fig. 9). No differences were detected among the single and double mutants. These data suggest that the mutants have greater cellulose content in their cell walls compared with wild type. Quantifying cellulose levels in tdy2 chlorotic regions and wild-type leaves supported these findings (Supplemental Table S3). Additionally, it has been reported that carbon availability derived
from starch turnover and sugar abundance positively influences lignin deposition in Arabidopsis (Rogers et al., 2005). As tdy mutants contain excess starch and soluble sugars, we analyzed whether the increased carbon pools in the chlorotic tissues were associated with increased lignin deposition by phloroglucinol staining. In contrast to the results in Arabidopsis, we did not observe changes in lignin levels in tdy chlorotic regions compared with wild type (Supplemental Fig. S5). Together, these data suggest that the tdy mutants have increased cellulose content in their leaves and that the mechanisms controlling lignin biosynthesis may differ between maize and Arabidopsis.

DISCUSSION

In this article, we identified and characterized tdy2, a mutant that produces variegated leaves with chlorotic regions that hyperaccumulate carbohydrates. The tdy2 mutant phenotype strongly resembles the tdy1 mutant, but the new mutant maps to a different chromosomal location. This indicates that tdy2 is an independent locus from tdy1 or sxd1. Further, the chromosomal segments where the three genes reside are not syntenous, indicating that Tdy2 does not represent an ancient duplication of Tdy1 or Sxd1 (Gale and Devos, 1998). The carbohydrate accumulation within tdy2 chlorotic regions suggests that the mutation leads to a defect in Suc export from leaves. Moreover, the starch accumulation in tdy2 mesophyll cells appears similar to leaves blocked in phloem export (Russin et al., 1996; Jeannette et al., 2000). However, cytological investigations show that tdy2 mutants do not display similar physical obstruction of plasmodesmata along the Suc symplastic pathway as found in sxd1 mutants. From genetic analyses, we conclude that Tdy2 acts in the same pathway as Tdy1 and that both genes function independently from Sxd1. From our phenotypic characterization and genetic studies, we propose that Tdy2 functions with Tdy1 to promote Suc export from leaves.

tdy2 chlorotic sectors appear shortly after the leaf blade emerges from the whorl and are stable in shape and size once formed. At this time, cell differentiation is complete and the phenotype is irreversible. Interestingly, the chlorophyll a/b ratio in tdy2 chlorotic regions is greater than in wild type or green tissue from mutant leaves. This may be an acclimation response similar to that observed in leaves that develop in high light, whereby the amount of energy harvested is reduced to prevent photodamage (Lichtenthaler et al., 1981). The altered ratio may also be due to the excess carbohydrates having a greater effect on mesophyll cells than bundle sheath cells. For example, mesophyll cells contain grana stacks that are the principal site of the chlorophyll a/b-binding protein LHCII, whereas bundle sheath cells contain mostly agranal thylakoids and lack LHCII (Kirchanski, 1975; Schuster et al., 1985). In tdy2 chlorotic tissues, the relative increase in carbohydrate accumulation and disruption of grana stacks may be much greater in mesophyll cell chloroplasts than in bundle sheath cell plastids, which normally accumulate starch. This would result in reduced LHCII levels, the greater decrease in chlorophyll b, and the increased chlorophyll a/b ratio observed in the chlorotic regions.

Down-regulation of photosynthetic gene expression and chlorosis is known to occur under conditions of high carbohydrate accumulation. Accordingly, we determined that carbohydrates accumulate in tdy2 leaf blades prior to visible chlorosis. This suggests that chlorosis is a secondary effect and that the primary defect may be in carbon export. In agreement with this idea, lateral veins frequently were found at boundaries between green and chlorotic tdy2 regions. The association of lateral veins with tdy2 sector boundaries suggests that failure to load Suc into the veins may account for the build up of carbohydrates within the photosynthetic cells and the subsequent chlorosis. This hyperaccumulation of carbohydrates in leaf tissues is correlated with decreased transport to and reduction in the size of sink tissues (Russin et al., 1996; Burkle et al., 1998; Gottwald et al., 2000; Niittyla et al., 2004) and likely accounts for the diminished inflorescence size and yield seen in tdy2 mutants. The fact that both tdy1 and tdy2 chlorotic sector boundaries predominantly occur at lateral veins is consistent with the hypothesis that they have similar functions and act within the same pathway. In addition, because albino tissues express the tdy2 phenotype only if adjacent to chlorotic regions within green tissue, it implies that a mobile chloroplast by-product, potentially a sugar, induces the phenotype. Together, these data suggest that tdy2 chlorotic regions result from a failure to load Suc into the veins rather than from a defect in chloroplast function.

Models for Tdy1-Tdy2 Interaction

Phenotypic similarities and genetic studies suggest that Tdy1 and Tdy2 function in the same pathway to promote carbohydrate export from leaves. The SSNC interaction observed in tdy1/+; tdy2/+ F1 plants indicates that these genes may encode proteins that physically interact (Stearns and Botstein, 1988; Hawley and Gilliland, 2006), although examples are known whereby two loci display SSNC and the corresponding proteins do not physically associate but still function in the same pathway (Yook et al., 2001). Further, this interaction is specific for tdy1 and tdy2 because we did not observe any phenotype in F1 progeny from either tdy1 or tdy2 crossed with sxd1. These data, along with the double mutant characterization, suggest that the Tdy1-Tdy2 pathway is distinct from the action of Sxd1. In addition, tdy1; tdy2 double mutants showed a strong enhancement of the chlorotic phenotype, indicating that the two mutations have a synergistic interaction. These data also indicate that neither single mutant completely abrogates the biological process(es)
TDY1 and TDY2 functions in. Therefore, we propose a model in which TDY1 and TDY2 form homo- and heteromeric protein complexes that function to promote Suc export.

Based on dosage analysis, tdy2 is a hypomorphic allele. This suggests tdy2 is not a null mutation and potentially produces a gene product with partially reduced function. If the tdy2 mutant allele encodes a “poison” protein with reduced function, it may be able to physically compete with wild-type TDY2 protein for binding to interacting proteins, such as TDY1. Incorporating the poison TDY2 protein into a complex is hypothesized to reduce the number of functional TDY1-TDY2 and TDY2-TDY2 protein complexes. This could account for the phenotype we observe in doubly heterozygous tdy1/+;tdy2/+ F1 plants. Similarly, in the hypoploids, by decreasing the dosage of tdy2 from two to one, the amount of the poison TDY2 protein is presumably reduced. This would sequester less of the TDY1 protein into nonfunctional TDY1-TDY2 complexes, allowing more TDY1-TDY1 functional homomeric complexes to form. This would be consistent with the milder phenotype detected.

Three models have been proposed to explain the molecular basis of SSNC (Badano and Katsanis, 2002; Hawley and Gilliland, 2006). In the first case, the two proteins may function in a protein complex. Mutation in one copy of either gene does not lead to a phenotype. However, mutation of one copy of both genes prevents formation of a functional complex and leads to a phenotype. The second model is that the two genes function in a linear pathway. A hypomorphic mutation in one gene reduces the flux of product through the pathway but does not eliminate it com-

**Figure 5.** A mobile chloroplast by-product induces tdy2 phenotypic expression. A, ij1 leaf with longitudinal white stripes. B, tdy2 in anthocyanin expressing genetic background with red pigments accumulated solely in chlorotic regions. C, tdy2; ij1 double mutant leaf shows anthocyanin accumulation in albino cells adjacent to chlorotic, anthocyanin-accumulating tissues (arrow). D, Cleared, starch-stained leaf from C shows starch accumulates in albino, anthocyanin-accumulating cells (arrow). E to P, Leaf cross sections of each single mutant and tdy2; ij1 double mutant viewed in bright-field (E, H, K, and N) and under UV light (F, I, L, and O). G, J, M, and P show starch-stained sections in bright-field. Arrows in J and P show starch accumulation in mesophyll cells. E to G, tdy2 green tissue. H and J, tdy2 chlorotic tissue. K to M, ij1 albino tissue. N to P, tdy2; ij1 albino tissue accumulating anthocyanin. Dark color in N corresponds to air bubbles. Scale bars = 50 μm.
completely. A second hypomorphic mutation in another gene in the pathway additionally reduces the flux through the pathway, effectively eliminating production of the final product and leading to a phenotype. The third model is that the two genes function in redundant parallel pathways. Again, mutation of one copy of either gene has no phenotype. However, mutation of one copy of both genes lowers flux through both pathways sufficiently to produce a phenotype.

From additional genetic studies, we have been able to rule out the second model to explain the *tdy1-tdy2* SSNC. In an effort to clone *Tdy1*, we performed a transposon mutagenesis and identified new alleles of *tdy1* with variegated leaf phenotypes indistinguishable from the reference allele. One of these alleles was found to contain a large deletion that removed the *Tdy1* gene entirely and is therefore a null allele (Golubovskaya et al., 2006). The *tdy1-deletion/+; tdy2/+* F$_1$ individuals exhibited SSNC and a sectored pheno-

type (D. Braun, unpublished data). Thus, our genetic analysis supports either the first or third SSNC models for the *tdy1-tdy2* F$_1$ interaction but excludes the second, as the interaction we observe between *tdy1* and *tdy2* is not dependent on both alleles being hypomorphic mutations. Future work characterizing the molecular nature of the gene products and whether they reside in a complex will distinguish between these models.

We previously proposed that the *tdy1* leaf variegation can be explained by a threshold model (Braun et al., 2006, and refs. therein). We hypothesized that Suc accumulation above the threshold induces a chlorotic sector, while a Suc concentration below the threshold produces normal-appearing green tissue. Sensitivity to the threshold must occur for only a limited developmental window prior to or up to the time that sector formation is first visible, because, after this time, the phenotype of green mutant tissue is stable and appears similar to wild type. Perhaps local variation in photoassimilate production due to different light levels or decreased rate of Suc movement from photosynthetic cells into the phloem may account for the initiation of a chlorotic sector. Upon accumulation, Suc likely diffuses into neighboring cells where the elevated concentration induces phenotypic expression. Hence, we hypothesized that *Tdy1*...
functions as an osmotic stress or sugar sensor to regulate SUT activity to promote Suc export (Braun et al., 2006). In the absence of TDY1, improper SUT function may lead to the failure to export Suc into the vein, the overaccumulation of carbohydrates in photosynthetic cells, and ultimately to the formation of a chlorotic sector. tdy2 mutants have a variegated phenotype strongly resembling tdy1 mutants; therefore, we propose a similar threshold model to explain the tdy2 variegation. Furthermore, the dosage sensitivity we observe in F1 plants and the synergistic interaction in the double mutants suggests that the two proteins may interact. We hypothesize that TDY1 and TDY2 form multimeric protein complexes controlling Suc export, either directly, by physically interacting with a SUT, or indirectly, perhaps by functioning in a Suc-sensing signal transduction pathway that regulates SUT abundance (Chiou and Bush, 1998; Vaughn et al., 2002; Ransom-Hodgkins et al., 2003). Mutation of one copy of either Tdy1 or Tdy2 reduces the amount of functional protein complexes but does not result in a phenotype. A second mutation in the other gene reduces the amount of functional protein complexes to the point that Suc export ability may be compromised. tdy1 and tdy2 mutant phenotypes are dependent on high light conditions likely for the synthesis of sufficient photoassimilate levels (Supplemental Fig. S6; Braun et al., 2006). In high light environments where high Suc flux is expected, these conditions reveal the limited Suc export capacity under which tdy chlorotic sectors form. As Tdy1-Tdy2 function is reduced, we suggest that the regulation of SUT activity becomes severely hampered. This would cause the Suc concentration to build up, exceed the threshold, and induce a chlorotic phenotype. In tdy1; tdy2 double mutants, the above scenario leads to an almost entirely chlorotic leaf blade that accumulates excess carbohydrates throughout.

Precedence for our model for TDY1 and TDY2 interacting in protein complexes is provided by the characterization of variegated mutants in Arabidopsis. Mutations in the VARIEGATED1 (VAR1) and VAR2 loci produce plants with green and white sectored leaves. var1; var2 double mutant plants display a synergistic interaction of severely albino leaves with few green regions (Sakamoto et al., 2002; Zaltsman et al., 2005). VAR1 and VAR2 encode similar FtsH proteins that form homomeric and heteromeric protein complexes (Chen et al., 2000; Sakamoto et al., 2002, 2003; Yu et al., 2004, 2005). Further, mutations in one gene lead to a decrease in protein abundance of the other, likely due to failure to form an active protein complex and degradation of nonassembled subunits (Sakamoto et al., 2003; Yu et al., 2004, 2005). Based on the genetic and molecular interactions, a threshold model has been proposed to explain the variegation in var1 and var2 mutants (Yu et al., 2004, 2005, 2007). Hence, this paradigm may explain our observations for the tdy1 and tdy2 genetic interaction.

Table II. Carbohydrate quantification in wild-type versus chlorotic tdy1; tdy2 leaves

Data represent means from six samples ± s.d. Values for double mutant tissue were statistically different than wild type as determined by the Student’s t test at P ≤ 0.001.

<table>
<thead>
<tr>
<th>Leaf Tissue</th>
<th>Suc Percentage of Wild Type</th>
<th>Glc Percentage of Wild Type</th>
<th>Fru Percentage of Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>17.1 ± 3.4 mg/g fresh weight</td>
<td>1.8 ± 0.3 mg/g fresh weight</td>
<td>1.1 ± 0.3 mg/g fresh weight</td>
</tr>
<tr>
<td>tdy1; tdy2</td>
<td>46.8 ± 1.7 mg/g fresh weight</td>
<td>11.3 ± 1.1 mg/g fresh weight</td>
<td>11.5 ± 0.8 mg/g fresh weight</td>
</tr>
</tbody>
</table>

Figure 8. Genetic interaction between tdy1 and tdy2. A, C, and E, Leaves before staining. B, D, and F, Same leaves cleared and starch stained. A and B, Wild type. C and D, tdy1/+; tdy2/+. F, leaf with chlorotic sectors. E and F, tdy1; tdy2 double mutant leaf displays complete chlorosis and starch hyperaccumulation throughout. G, Left to right, wild type, tdy2 single mutant, tdy1 single mutant, tdy1; tdy2 double mutant. [See online article for color version of this figure.]
Another example of the parallels between the proposed role of the Tdy loci and an Arabidopsis variegated mutant is evinced by the chlorophyll a/b binding protein gene underexpressed1 (cuel) mutant (Li et al., 1995). cue1 mutants have aberrant mesophyll cell plastids leading to a pale phenotype, while the bundle sheath cell plastids appear normal and green, giving the leaf a reticulate pigmentation pattern. cue1 is defective in the chloroplast phosphoenolpyruvate/phosphate translocator (Streatfield et al., 1999). Mutations in this transporter lead to altered mesophyll cell gene expression, plastid function, and morphology, but not to the overaccumulation of carbohydrates. The cue1 phenotype has some resemblance to the tdy mutants in affecting mesophyll cell function and in the proposed roles of TDY1 and TDY2 regulating a transporter. However, there are also differences between the cue1 and tdy phenotypes in that tdy mutants show bundle sheath cell defects and carbohydrate accumulation in photosynthetic cells that are not seen in cue1 mutants.

It is worthwhile to mention that tdy1; tdy2 double mutants with severely chlorotic leaves produce tassels that shed pollen. This observation indicates that the chlorotic leaves are capable of sufficient Suc export to support development of the inflorescence sink tissue. Therefore, as reported for tdy1; sxd1 double mutant leaves (Ma et al., 2008), tdy1; tdy2 double mutant leaves do not retain sink identity but must be source leaves with reduced export capacity. Furthermore, these data also suggest that there are additional genes that control carbon export from leaves. Future work characterizing other variegated nonclonal sectoring mutants that hyperaccumulate carbohydrates may identify these genes.

tdy Mutants as Enhanced Feedstocks for Biofuels Production

tdy mutants hyperaccumulate starch and soluble sugars in chlorotic leaf sectors. In addition, we found that excess sugars are shunted to the cell wall and deposited as cellulose without an increase in lignin. These data indicate that tdy mutant leaves have elevated cellulose levels and suggest the possibility that tdy mutants may have applications in the production of biofuels. For example, the yield of sugars that could be fermented from tdy carbon hyperaccumulating chlorotic leaf tissues is expected to be increased compared with that of nonmutants. We are currently exploring this possibility. Future work combining the tdy mutants with other maize mutants with reduced lignin deposition, the brown midrib mutants (Barriere et al., 2004), will potentially create lines with greater cellulose to lignin ratios that may serve as superior stocks for biofuels production. Lastly, we note that our data showing that there is no increase in lignin deposition in tdy mutants with excess carbon pools differs from the case in Arabidopsis. Hence, this suggests that the mechanisms regulating lignin deposition may not be conserved between Arabidopsis and grasses, and that it will be necessary to characterize cell wall biosynthesis pathways in maize and other grasses for bioenergy applications utilizing these species (Bush and Leach, 2007; Lawrence and Walbot, 2007).

MATERIALS AND METHODS

Growth Conditions and Genetic Stocks

Maize (Zea mays) plants were grown in the summer at the Pennsylvania State University Rock Springs Agronomy Farm. For the sectoring developmental time course, sector boundary analysis, photosynthesis measurements, and starch staining emerging leaves experiments, plants were grown in a greenhouse supplemented with sodium vapor and mercury halide lamps at 1,400 μmol m⁻² s⁻¹ light under a 12-h day (30°C)/12-h night (20°C). Plants were grown under low light 100 μmol m⁻² s⁻¹ in a growth chamber under a 12-h day (30°C)/12-h night (20°C). The tdy2-Reference allele (hereafter tdy2) was isolated from an ethyl methylsulfonate mutagenized F2 population. tdy2 mutants were backcrossed to the B73 inbred line three or more times prior to analyses. The tdy1-Reference (tdy1), B-A translocations, anthocyanin-accumulating genetic background, ij1, and sxd1 stocks used have been described previously (Braun et al., 2006; Baker and Braun, 2007; Ma et al., 2008). For fine mapping, tdy2 introgressed five times into B73 was outcrossed to Mo17, the F2 plants self-fertilized, and F3 progeny analyzed.

Quantitative and Double Mutant Analyses

Morphometrics, photosynthetic pigments, photosynthesis, vein boundary, starch staining, and carbohydrates measurements were performed as previously described (Braun et al., 2006; Baker and Braun, 2007). Aniline blue staining of callose and TEM investigations of plasmodesmata were performed as described by Ma et al. (2008). For examining cellulose abundance, free-hand leaf cross sections were stained on the slide with aqueous 0.1 mg mL⁻¹ calcofluor white for 2 min, rinsed, and mounted (Ruzin, 1999). Samples were examined under UV illumination with a 340-380-nm excitation filter and a 435-485-nm bandpass emission filter on a Nikon Eclipse 80i fluorescent microscope with a 100-W mercury lamp. All images within a Figure were captured using identical conditions by a Nikon DMD1200F digital camera. To inspect lignin accumulation, leaf cross sections were photographed under UV light, then stained with acidified phloroglucinol according to Ruzin (1999) and photographed in bright-field.

Microscopy

Free-hand leaf cross sections were examined under reflected light, UV light, bright-field, and starch stained as described (Braun et al., 2006; Baker and Braun, 2007). Aniline blue staining of callose and TEM investigations of plasmodesmata were performed as described by Ma et al. (2008). For examining cellulose abundance, free-hand leaf cross sections were stained on the slide with aqueous 0.1 mg mL⁻¹ calcofluor white for 2 min, rinsed, and mounted (Ruzin, 1999). Samples were examined under UV illumination with a 340-380-nm excitation filter and a 435-485-nm bandpass emission filter on a Nikon Eclipse 80i fluorescent microscope with a 100-W mercury lamp. All images within a Figure were captured using identical conditions by a Nikon DMD1200F digital camera. To inspect lignin accumulation, leaf cross sections were photographed under UV light, then stained with acidified phloroglucinol according to Ruzin (1999) and photographed in bright-field.
Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Developmental time course for tdy2 sector formation.

Supplemental Figure S2. Morphometric analyses of wild type and tdy2 mutants.

Supplemental Figure S3. Plasmodesmata ultrastructure in wild type and tdy2 mutants.

Supplemental Figure S4. SSNC phenotypes of tdy1/+; tdy2/+ F1 leaves.

Supplemental Figure S5. Phloroglucinol-stained wild-type and mutant leaves.

Supplemental Figure S6. Wild-type and tdy2 leaves grown under low light.

Supplemental Table S1. tdy2 chlorotic sector boundary analysis.

Supplemental Table S2. Chlorophyll levels and photosynthesis rates of wild-type and mutant leaves.

Supplemental Table S3. Cellulose quantification in wild-type and tdy2 chlorotic leaves.

ACKNOWLEDGMENTS
We thank Tony Omei and Scott Harkcom for excellent plant care. We thank Lisa Harper for performing the mutagenesis that generated the tdy2 allele and Mike Muszynski for help fine mapping. We are grateful to Tom Siewinski for performing the photosynthesis measurements, Sarah Nilsen for expert advice, and Sally Assmann for the use of the LICOR 6400. We also thank Dan Cosgrove and Nick Kaplinsky for advice on the cellullose assays. We appreciate the suggestions of two anonymous reviewers, Mike Muszynski and Surinder Chopra for critically reading the paper, and members of the Braun and McSteen labs for discussion of the data and comments on the manuscript.

Received October 22, 2007; accepted January 14, 2008; published January 24, 2008.

LITERATURE CITED


Botha CEJ, Cross RHOM, van Bel AJE, Peter CI (2000) Phloem loading in the sucrose-export-defective (SXD-1) mutant maize is limited by callose deposition at plasmodesmata in bundle sheath-vascular parenchyma interface. Protoplasma 214: 65–72


Haywle RS, Gilliland WD (2006) Sometimes the result is not the answer: the truths and the lies that come from using the complementation test. Genetics 174: 5–15


Walbot V, Coe EH (1979) Nuclear gene 1ioj conditions a programmed change to ribosome-less plastids in Zea mays. Proc Natl Acad Sci USA 76: 2760–2764


Supplemental Figure S1. *tdy2* chlorotic sectors appear shortly after leaf emergence and are stable once formed. A-E show the same *tdy2* leaf photographed over successive days. A. On day 2 after complete emergence of the leaf blade, no sign of a chlorotic sector is evident. Dotted oval corresponds to area where sector will form in subsequent images. B. By day 3, a slight contrast is visible between the chlorotic and green tissue (arrow). C. Day 4 shows a stronger contrast between green and chlorotic tissue (arrow). D. Day 5. E. Day 6. Note that the sector boundary does not change.
**Supplemental Figure S2.** *tdy2* plants display reduced height, delayed time to flowering and diminished yield. **A-E.** Wild type is shown in gray bars and *tdy2* mutants in white bars. Values are averages ± SE. For plant height and flowering time, n = 13; for tassel length, n = 14; for ear length, n = 18, and for kernel number per ear, n = 10. Asterisks indicate values statistically different from wild type determined using the Student’s *t* test at *P* < 0.05. **A.** Plant height is measured in cm. **B.** Anthesis and silking dates are measured in days after planting. **C, D.** Tassel and ear length are measured in cm. **E.** Kernel number per ear. **F, G.** Photographs of wild type (left) and *tdy2* (right) tassels and ears.
**Supplemental Figure S3.** Plasmodesmata appear unobstructed along the Suc symplastic pathway in *tdy2* chlorotic tissue. TEM images of wild type (A,C,E) and *tdy2* (B,D,F) cellular interfaces. M, mesophyll. BS, bundle sheath. Scale bars = 500 nm.
**Supplemental Figure S4.** SSNC phenotypes of \textit{tdy1/+; tdy2/+} \textit{F}_1 leaves. 

\textbf{A, B}. Leaves of \textit{F}_1 plants from a cross of \textit{tdy1} in the anthocyanin accumulating genetic background crossed with \textit{tdy2} in a B73 background. Anthocyanins accumulate in chlorotic sectors. 

\textbf{C, D}. \textit{tdy1/+; tdy2/+} \textit{F}_1 leaves display chlorotic regions. Both mutations were backcrossed to B73 five times prior to crossing together.
Supplemental Figure S5. *tdy* mutants do not show any change in lignin deposition in chlorotic leaf tissues. A,B,E,F show leaf cross sections under UV light. C,D,G,H show same leaves stained with phloroglucinol and photographed in bright-field. Lignin is stained magenta. A,C. wild type. B,D. *tdy2*. E,G. *tdy1*. F,H. *tdy1; tdy2* double mutants. Scale bars = 100 μm.
Supplemental Figure S6. *tdy2* mutants grown under low light do not sector or differentially accumulate starch. **A, C** show wild type and *tdy2* leaves from low light grown plants, respectively. **B, D** show same leaves, cleared and starch stained.
Supplemental Table S1. *tdy2* chlorotic sector boundary quantification

<table>
<thead>
<tr>
<th></th>
<th>Lateral veins</th>
<th>Intermediate veins</th>
<th>Other&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
<td>59</td>
<td>9</td>
<td>32</td>
</tr>
</tbody>
</table>

136 sharp boundaries from 25 chlorotic sectors on 12 leaves were examined. Frequency is the percentage of a particular class among all samples. <sup>a</sup> indicates that the boundary did not correspond to an identifiable structure. No sharp boundaries were observed at either small or transverse veins.
**Supplemental Table S2.** Chlorophyll and photosynthesis rate quantification

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Chlorophyll</th>
<th>% of wild type</th>
<th>Photosynthesis</th>
<th>% of wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>37.1(^a) ± 0.61</td>
<td>100</td>
<td>28.9(^a) ± 0.33</td>
<td>100</td>
</tr>
<tr>
<td>tdy1 green</td>
<td>37.9(^a) ± 1.06</td>
<td>102.2</td>
<td>23.2(^b) ± 1.56</td>
<td>80.3</td>
</tr>
<tr>
<td>tdy2 green</td>
<td>37.1(^a) ± 0.43</td>
<td>100</td>
<td>23.6(^b) ± 1.16</td>
<td>81.7</td>
</tr>
<tr>
<td>tdy1 chlorotic</td>
<td>18.7(^b) ± 0.21</td>
<td>50.4</td>
<td>1.4(^c) ± 0.39</td>
<td>4.8</td>
</tr>
<tr>
<td>tdy2 chlorotic</td>
<td>19.2(^b) ± 0.49</td>
<td>51.8</td>
<td>1.2(^c) ± 0.08</td>
<td>4.2</td>
</tr>
<tr>
<td>tdy1; tdy2 chlorotic</td>
<td>18.9(^b) ± 0.44</td>
<td>50.9</td>
<td>1.4(^c) ± 0.35</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Chlorophyll levels were measured with a SPAD 502 meter and photosynthesis rates were determined with a LICOR 6400 in different phenotypic (green or chlorotic) leaf tissue from specified genotypes. Values represent the means ± SE in relative units for chlorophyll (n = 12) and in \(\mu\)mol CO\(_2\) fixed m\(^{-2}\) s\(^{-1}\) for photosynthesis (n = 4). Statistical significance was determined using the Student’s \(t\)-test and indicated by the different lettered superscripts within a column. The experiment was performed three times, with similar results obtained for each replicate. Data from one replicate is presented.
**Supplemental Table S3.** Cellulose quantification in wild type and *tdy2* chlorotic leaves.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Cellulose</th>
<th>% of wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>719.4 ± 28.9</td>
<td>100</td>
</tr>
<tr>
<td><em>tdy2</em> chlorotic</td>
<td>820.8 ± 17.9</td>
<td>114</td>
</tr>
</tbody>
</table>

Cellulose content was assayed in leaf tissue from specified genotypes. Values represent the means ± SE in nmol glucose/mg dry weight for cellulose (n = 6). Statistical significance was determined using the Student’s *t*-test and indicated by the different lettered superscripts. The experiment was performed three times, with similar results for each replicate. Data from one replicate is presented.