CHARACTERIZATION AND CLONING OF TIE-DYED1, A NOVEL GENE

REGULATING CARBOHYDRATE PARTITIONING IN MAIZE LEAVES

A Dissertation in

Integrative Biosciences

by

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ABSTRACT

Acquisition of cell identity requires communication among neighboring cells. To dissect the genetic pathways regulating cell signaling in later leaf development, a screen was performed to identify mutants with sectors that violate cell lineage boundaries in maize (Zea mays) leaves. We identified a recessive mutant, tie-dyed1 (tdy1), which develops stable, nonclonal variegated yellow and green leaf sectors. Sector formation requires high light, occurs during a limited developmental time, and is restricted to leaf blade tissue. Yellow tdy1 sectors accumulate excess soluble sugars and starch, whereas green sectors appear unaffected. Significantly, starch accumulation precedes chlorosis in cells that will become a yellow sector. Retention of carbohydrates in tdy1 leaves is associated with a delay in reproductive maturity, decreased stature, and reduced yield. To explain the tdy1 sectoring pattern, we propose a threshold model that incorporates the light requirement and the hyperaccumulation of photoassimilates.

tdy1 and sucrose export defective1 (sxd1) are the only recessive mutants known with nonclonal chlorotic leaf sectors that hyperaccumulate starch and soluble sugars. Based on their similar mutant phenotypes, we investigated whether tdy1 and sxd1 function in the same pathway. Using aniline blue staining for callose and TEM to inspect plasmodesmal ultrastructure, we determined that tdy1 does not have any physical blockage or alteration along the symplastic transport pathway as found in sxd1 mutants. To test whether the two genes function in the same genetic pathway, we constructed F2 families segregating both mutations. Double mutant plants showed an additive
interaction for growth related phenotypes and soluble sugar accumulation, and expressed
the leaf variegation pattern of both single mutants indicating that \(Tdy1\) and \(Sxd1\) act in
separate genetic pathways. Although \(sxd1\) mutants lack tocopherols, we determined that
\(tdy1\) mutants have wild type tocopherol levels, indicating that \(Tdy1\) does not function in
the same biochemical pathway as \(Sxd1\). From these and other data we conclude that \(Tdy1\)
and \(Sxd1\) function independently to regulate carbon export.

To characterize \(Tdy1\)’s function at the molecular and cellular levels, \(Tdy1\) was
cloned using transposon-tagging. \(Tdy1\) encodes a novel protein highly conserved in
grasses with orthologs in rice, sorghum and sugarcane. Though no ortholog was found in
dicot plants, two stretches of amino acid sequences were found to have similarities to
TDY1, which implies the function of TDY1 may be conserved in higher plants. In
addition, maize genomic DNA Southern blotting revealed a duplicate gene for \(Tdy1\), and
multiple related sequences were discovered by BAC filter hybridization suggesting that
\(Tdy1\) belongs to a small gene family. It is possible that the closest duplicate gene
partially compensates for \(Tdy1\) function so that the \(tdy1\) mutant does not show uniform
chlorosis in its leaves. Semi-quantitative RT-PCR showed that \(Tdy1\) is expressed in both
source leaves and young sink tissues. Furthermore, \textit{in situ} hybridization localized \(Tdy1\)
RNA in phloem cells, supporting a potential function in phloem transport. As sucrose
transporters are involved in phloem loading, their expression patterns were examined and
overlapping expression was found for \(ZmSUT2\) and \(ZmSUT4\). The results suggest that
\(Tdy1\) may function in regulating phloem transport of sucrose in maize plants.
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Chapter 1

Introduction

This chapter provides background knowledge about maize leaf development, C₄ carbon assimilation and sucrose transporters in plants.

1.1 Maize leaf development and cell differentiation

The maize leaf is an excellent model system for genetic and developmental studies because of its morphologically simple structure, regular patterns of cell division and stereotypical relationship between cells during development. I will briefly describe the initiation and patterning of a maize leaf.

The group of cells in the vegetative apical meristem that defines the population of future leaf cells are called founder cells (Poethig, 1984). There are around 250 founder cells encircling the shoot apical meristem (Poethig, 1984). These founder cells are meristematic and two or more layers of founder cells divide periclinally to form a leaf primordium (Freeling, 1992; Poethig and Szymkowiak, 1995). Different phytomer segments, i.e., leaf blade, leaf sheath, node and internode, are initiated by founder cell division occurring within the primordium (Sylvester et al., 1996). The boundary between the leaf blade and leaf sheath is marked by a linear band of uniquely shaped cells, which will divide periclinally and form the ligule (Sylvester et al., 1996). Subsequent polarized
cell division and expansion gives rise to the lanceolate shaped mature maize leaf. The development of a maize leaf occurs basipetally with differentiation beginning at the tip and the most basal cells dividing and differentiating last. Clonal analysis indicates that the great majority of cell lineages within a maize leaf are arranged longitudinally, which is parallel to the midrib (Poethig and Szymkowiak, 1995). Hence, the cells in a clonal sector are genetically related since they are derived from the same founder cells.

After the identity of leaf parts has been acquired and the primordia have been established, the next step in leaf development is to elaborate how different tissues differentiate into different types. A mature maize leaf can be subdivided into 5 tissue layers in the transverse dimension, the adaxial and abaxial epidermal layers, two subdermal mesophyll cell layers, and the innermost tissue layer comprising the veins, bundle sheath and interveinal mesophyll cells (Nelson et al., 2002). In maize, the leaf epidermis is derived from the L1 layer in the SAM, whereas the internal cell layers are derived from the L2 layer (Poethig, 1984). The leaf margin is distinct from other parts of the maize leaf in that all of the five tissue layers are derived from the L1 layer of the SAM. It has been found that cells generally differentiate based on their final position in the leaf, not according to their clonal history (Nelson and Langdale, 1989; Langdale and Nelson, 1991; Sylvester et al., 1996). However, studies of the Xcl1 and tangled1 maize mutants showed that in these cases cell fate can be determined by cell lineage instead of position (Jankovsky et al., 2001; Kessler et al., 2002).
Leaves perform many physiological functions, primary among which is photosynthesis. The development of photosynthetic capability is intimately related to the differentiation of photosynthetic tissues. Certain cells derived from layers of the SAM undergo photosynthetic differentiation. The L1 layer gives rise to stomatal guard cells in the epidermis and some mesophyll cells at the leaf margin, while the L2 layer gives rise to photosynthetic cells (Nelson and Langdale, 1989).

A series of parallel veins derived from each founder cell in the SAM represent each longitudinal unit of a leaf encircling the shoot (Sharman, 1942; Esau, 1943; Steffensen, 1968; Russell and Evert, 1985). Although overall maize leaf development occurs basipetally, the differentiation of the midvein and the lateral veins is acropetal. These vascular tissues are derived from meristematic regions called procambium. The midvein differentiates first from the procambium towards the tip of the leaf and divides the leaf into halves longitudinally (Langdale and Nelson, 1991). Unlike the major veins, the intermediate and small veins in the leaf blade differentiate basipetally as the space between these major veins is increasing (Nelson and Langdale, 1989; Freeling, 1992). In maize, another set of veins established perpendicularly to the major and intermediate veins is known as transverse veins, subsequently interconnecting the longitudinal veins.

As a typical C₄ plant, maize displays two types of photosynthetic cells, the bundle sheath and the mesophyll cells. Together with the vascular cells, they form a special anatomical pattern called Kranz anatomy (Figure 1.1). Maize leaves contain a single layer of bundle sheath cells encompassing the vein. Bundle sheath cells are more directly
related to the veins rather than neighboring mesophyll cells because they are derived from the same cell lineage (Bosabalidis et al., 1994). An early transverse cell division produces an outer and inner cell. The outer cell becomes the bundle sheath cell and the internal cells encompassed by the future bundle sheath cells become the vascular tissues (Bosabalidis et al., 1994). It is also argued that the lateral bundle sheath cells, which are in the central layer of the leaf between the mesophyll cell and the vascular tissues, are more closely related to the ground meristem cells which give rise to the mesophyll cells, while the adaxial and abaxial bundle sheath cells are more closely related to the primordial cells which give rise to the veins (Langdale et al., 1989; Bosabalidis et al., 1994). These results also showed that cell fate is not determined by cell lineage but by the final position of the cell.

Photosynthetic bundle sheath and mesophyll cells cooperate to fix carbon, but show different chloroplast morphologies. In mesophyll cells, the chloroplasts are oval and contain many grana stacks, whereas the chloroplasts in bundle sheath cells are oblong in shape and have few grana stacks (Kirchanksi, 1975). In addition, starch is normally accumulated during the day in bundle sheath chloroplasts but not in mesophyll cells (Rhoades and Carvalho, 1944). The distinct anatomy of bundle sheath cells and mesophyll cells indicates that they have different functions.
Figure 1.1: Diagram of Kranz anatomy in a maize leaf. M, mesophyll cells; B, bundle sheath cells; V, veins. Both the mesophyll and bundle sheath cells are responsible for photosynthesis. The adjacent veins in a maize leaf are separated by two mesophyll cells and two bundle sheath cells. Other internal white space corresponds to mesophyll cells and air spaces.
1.2 C₄ carbon assimilation

Maize leaves undergo C₄ photosynthesis involving metabolic co-operation of the mesophyll and bundle sheath cells. C₃ plants fix CO₂ into C₃ acids in mesophyll cells and the carboxylation reaction is inhibited by O₂ in photorespiration. However, C₄ plants are able to concentrate CO₂ in the bundle sheath cells to minimize photorespiration and maximize photosynthesis efficiency. In Figure 1.2, it is shown that CO₂ is first fixed in the mesophyll cell by phosphoenolpyruvate carboxylase (PEPCase), which is insensitive to O₂, into the C₄ acid, oxaloacetic acid. There are three variations of C₄ photosynthesis and maize belongs to the NADP⁺-malic acid type (Edwards et al., 2001). In maize, malate is transferred to the bundle sheath cell and is subsequently decarboxylated to release CO₂. Meanwhile, pyruvate is produced and transferred from the bundle sheath cell back to the mesophyll cell, where the pyruvate is converted to the phosphoenolpyruvate acceptor required to enter next cycle. The released CO₂ will be fixed in the Calvin Cycle in the bundle sheath cell.
Figure 1.2: C₄ photosynthesis in maize mesophyll (MC) and bundle sheath cell (BS).
OAA, oxaloacetate; MAL, malate; PYR, pyruvate; PEP, phosphoenolpyruvate; RuBP, ribulose bisphosphate. 1, PEP carboxylase (PEPCase); 2, NADP⁺-malate dehydrogenase; 3, NADP⁺-malate enzyme; 4, pyruvate-orthophosphate dikinase (PPDK); 5, ribulose bisphosphate carboxylase/oxygenase (Rubisco).
The involvement of two cell types in C₄ photosynthesis requires strict compartmentation of photosynthetic enzymes in different cells according to their functions. Ribulose bisphosphate carboxylase/oxygenase (Rubisco) and malic enzyme (ME) are localized in bundle sheath cells, while malate dehydrogenase (MDH), PEPCase and pyruvate phosphate dikinase (PPDK) function in mesophyll cells (Langdale et al., 1989; Langdale and Nelson, 1991; Edwards et al., 2001). It has been reported that C₄ spatial and positional gene expression is light regulated (Nelson and Langdale, 1989; Langdale and Nelson, 1991; Edwards et al., 2001). Rubisco can be detected in both mesophyll and bundle sheath cells in dark-grown maize seedlings, while illumination represses Rubisco expression in mesophyll cells (Sheen and Bogorad, 1985). Langdale et al (1988b) found that under low light conditions, Rubisco is only detected in mesophyll cells while other C₄ enzymes are absent. High light induces Rubisco in bundle sheath cells and results in high levels of cell-specific C₄ enzymes such as PPDK (Nelson and Langdale, 1989). These results suggest that the maize leaf expresses the C₃ photosynthetic program in low light, and in high light, induces the accumulation of transcripts for C₄ enzymes.

Cellular localization studies revealed that Rubisco is restricted to the chloroplasts in the bundle sheath cells of adult maize leaves, while PEPCase is found in the cytoplasm of mesophyll cells (Edwards et al., 2001). The accumulation of C₄ enzymes does not occur concurrently in that bundle sheath specific mRNA accumulates prior to mesophyll cell specific mRNA. It has also been shown that bundle sheath specific mRNAs accumulate in cells before Kranz anatomy has morphologically differentiated, indicating
that Kranz anatomy is not absolutely required for the expression of some C₄ mRNAs (Langdale et al., 1988).

One of the outcomes of photosynthesis is to provide the carbon supply for plant growth and development. Sucrose is the principal end product of photosynthesis in that it is considered to be a nonreducing sugar. Although photosynthesis takes place in leaf chloroplast, sucrose synthesis occurs in the cytosol of mesophyll cells. Since chloroplasts cannot export hexose phosphates directly, hexoses are usually converted to C₃ intermediates, which are transported by the triose phosphate translocator (TPT) located on the chloroplast inner membrane (Buchanan et al., 2000). The exported triose phosphate can be converted into hexose phosphate in the cytosol and vice versa. In C₄ plants, there are other translocators found on the chloroplast membrane similar to TPT that can exchange a broader range of sugar phosphates, including triose phosphate, 3-PGA (3-phosphoglycerate), 2-PGA and phosphoenolpyruvate, while C₃ plants can only transport the first two (Buchanan et al., 2000). After sucrose is synthesized, some of it will be transiently stored during the day in the vacuole. The accumulation of sucrose in the cytosol results in the reduction of sucrose synthesis and increases the hexose phosphate pool. As a feedback mechanism, triose phosphate export is prevented and results in the accumulation of triose phosphate in the chloroplast. Conversely, as the amount of sucrose in the cytosol decreases, the sucrose synthesis pathway is reactivated and carbon flow diverts back to sucrose.
During the day and in high light, the photosynthesis rate exceeds the ability of the tissue to export assimilated sucrose out of the mesophyll cells. Hence, the excess fixed carbon is used to synthesize starch which is transiently stored in the bundle sheath cell chloroplast, and the activation of ADP-glucose pyrophosphorylase (AGP) switches carbon flow from sucrose to starch. Starch is another principal carbohydrate accumulated in plant cells, and it serves as a carbohydrate store supporting plant growth and metabolism during the night when photosynthesis is not available (Smith et al., 2005). The products from the degradation of starch during the night, either glucose or maltose, are exported respectively by a hexose transporter and a maltose transporter located on the chloroplast envelope from the chloroplast to the cytosol for sucrose synthesis, which is transported out of mesophyll cells and into the veins. Evidences of recent studies increasingly support maltose as the principal sugar exported from the chloroplast at night in bean, spinach and Arabidopsis (Niittyla et al., 2004; Weise et al., 2004). The maltose in the cytosol can be degraded by a cytosolic, glucanotransferase-like protein (DPE2) into another glucose molecule besides the one transported directly from the chloroplast (Chia et al., 2004; Lu and Sharkey, 2004; Zeeman et al., 2004). Many mutants having defects in the starch degradation pathway have been discovered, in which starch cannot be catabolized and results in starch accumulation in the leaves (Zeeman et al., 1998; Critchley et al., 2001; Yu et al., 2001; Dinges et al., 2003; Niittyla et al., 2004).

The diurnal cycling of carbohydrates in plant cells underlies the modulation of gene expression by carbohydrates. Based on whether gene expression is enhanced by elevated or reduced amounts of sugar, genes involved in carbohydrate metabolism are
classified into two classes. The first class of genes is influenced by sugar starvation and is called “famine genes”. This class of genes is usually involved in photosynthesis, sucrose mobilization and starch degradation, such as Rubisco, PEPCase, PPDK and ME. The second class of genes is enhanced by sugar abundance and is called “feast genes”. These genes function in starch synthesis including AGP, starch synthase and starch branching enzymes (Koch, 1996; Buchanan et al., 2000).

1.3 Sucrose transport in leaves and phloem loading/unloading

Sucrose serves as the primary long-distance carbon transport compound in most plants. Transport of sucrose can be achieved through two routes (Giaquinta, 1983; van Bel, 1993; Eschrich and Fromm, 1994). One is cell to cell symplastically through plasmodesmata, while the other is across cell membranes with the help of membrane localized transporters utilizing an apoplastic step. In the maize leaf, sucrose diffuses from its site of synthesis in the mesophyll cell cytosol into the bundle sheath cell and then into the vascular parenchyma cell through the plasmodesmata (Fritz et al., 1983). After sucrose diffuses into vascular parenchyma cells, the next step is export to the apoplast which occurs by an unknown mechanism. The uptake of photoassimilate molecules by the sieve elements is known as phloem loading, and minor veins are considered to be the major sites of phloem loading.

Phloem loading can be achieved either apoplastically or symplastically. Most plant species characterized have apoplastic phloem loading (apoplastic loaders). Sucrose
is exported out of vascular parenchyma possibly by a sucrose transporter located on the
plasma membrane of vascular parenchyma to the apoplasm (Fritz et al., 1983; van Bel,
1993). The sucrose in the apoplasm is then imported into the sieve elements and/or
companion cells by sucrose transporters localized on the plasma membranes of these
cells. This process requires the function of a H+-ATPase to generate the proton gradient
driving sucrose co-transport across a membrane. Most plants that translocate principally
sucrose are apoplastic phloem loaders, including most herbaceous plants of temperate
origin and the majority of crops. In maize, sucrose can be directly loaded to thin-walled
sieve elements without the companion cells as intermediates along the transport pathway
and also to thick-walled sieve elements that lack companion cells (Oparka and Turgeon,
1999).

Symplastic loading is accomplished entirely through plasmodesmata connecting
the parenchyma cells and SE-CC complex and is driven by diffusion (Sjolund, 1997;
Oparka and Turgeon, 1999; Lalonde et al., 2003; Minchin and Lacointe, 2005). Many
temperate woody plants are symplastic loaders, including most trees, (Turgeon and
Medville, 1998) and around 20% of putative symplastic loaders are herbaceous species
(Buchanan et al., 2000). Symplastic loaders have numerous plasmodesmata connecting
the bundle sheath cells and the companion cells, which are known as intermediate cells,
and are very large compared to their associated sieve elements. The translocated
carbohydrates are usually larger molecules, such as raffinose family oligosaccharides
(RFOs) and stachyose, while verified apoplastic loaders principally transport sucrose.
Symplastic loaders only accumulate slightly higher levels of sucrose in their phloem than
the mesophyll cells. It was thought that symplastic loaders were insensitive to the sucrose transporter inhibitor, PCMBS ($p$-chloromercuribenzenesulfonic acid). However, using this inhibitor in a putative symplastic loader, *Alonsoa meridionalis*, revealed that sucrose loading is at least partly mediated by a sucrose transporter in this species (Knop et al., 2004).

Plasmodesmata are channels that span neighboring cell walls connecting the cytoplasms between adjacent cells. Plasmodesmata span two types of membranes, the plasma membrane and a portion of modified endoplasmic reticulum (ER). Small soluble molecules can be transported in the cytoplasmic region, called the cytoplasmic sleeve, which is located between the plasma membrane and ER membrane (Zambryski and Crawford, 2000). Plasmodesmata are considered to be fluid, dynamic structures whose structure and function can be modified to fulfill the transport requirements of specific molecules, cells and tissues. Molecules smaller than the basal SEL (size exclusion limit), such as sugars, metabolites, ions and amino acids, diffuse through the plasmodesmata. Molecules larger than the basal SEL require selective transport and the conformation of the plasmodesmata pore to be changed to pass through plasmodesmata (Buchanan et al., 2000). As plasmodesmata are dynamic and capable of transient increases in their SEL, the modifications do not have to be permanent (Roberts and Oparka, 2003). Within a plasmodesma, the appressed ER forms the desmotubule. Studies indicate that the desmotubule is a close strand. However it is thought that molecules soluble in the membrane, such as proteins and ER lipids, could move through it (Grabski et al., 1993; Lazzaro and Thomson, 1996; Cantrill et al., 1999). The movement of molecules in the
ER may be important at the pore-plasmodesmata between sieve element and companion cells because ER and plasmodesmata act to link the sieve element and companion cells as a continuum (Roberts and Oparka, 2003). However, a recent study on transgenic tobacco using a sucrose transporter promoter to express GFP in the phloem cells indicated that the SEL of the plasmodesmata between the sieve tube and companion cell is below 27 kDa (Martens et al., 2006b).

Callose deposition at plasmodesmata has been found in response to wounding and pathogenesis (Roberts and Oparka, 2003). Callose deposits at the neck of plasmodesmata has been thought to be related to hormone balance alterations or changes in the osmotic potential of plant material (Botha and Cross, 2000). A maize mutant sucrose export deficient (sxd1) was characterized to have defects in sugar movement due to the occlusion of plasmodesmata by callose deposition at the bundle sheath-vascular parenchyma cell interface (Russin et al., 1996; Botha et al., 2000a). The blockage of this sugar transport pathway results in the build up of soluble sugars in both the mesophyll and bundle sheath cells. The gene has been cloned and found to encode tocopherol (vitamin E) cyclase. SXD1 has high similarity to Arabidopsis VTE1, which is the orthologous enzyme in tocopherol biosynthesis. Maeda et al (2006) found that Arabidopsis vte1 and vte2 mutants had reduced photoassimilate export and callose deposition in the phloem parenchyma transfer cell walls suggesting a crucial function for tocopherols in low-temperature adaptation and phloem loading. In addition, RNAi-mediated silencing of StSXD1 in transgenic potatoes caused similar starch and soluble
sugar accumulation as well as callose deposition in leaves (Hofius et al., 2004). It is not known how a lack of tocopherol leads to the deposition of callose.

Carbohydrates that enter the sieve elements through phloem loading will be transported in the sieve tubes to developing sink tissues. Individual sieve elements are stacked on each other and separated by sieve pores, which are derived from plasmodesmata in their end walls. The sieve pores facilitate solute flow from cell to cell through the sieve tubes. The differentiation process of converting plasmodesmata into sieve pores forms the sieve tube, a continuous, membrane-lined compartment for nutrient flow, molecule and signal transport throughout the entire plant (Ryals et al., 1996).

It is generally accepted that phloem transport is through bulk flow driven by an osmotically generated hydraulic pressure gradient (Sjolund, 1997; Minchin and Lacointe, 2005). In order to maintain the high pressure gradient along the phloem path, it is essential that sucrose leaked to the surrounding apoplasm is retrieved into the sieve element by sucrose transporters localized to the sieve element and companion cell plasma membrane (Lalonde et al., 2003). Upon reaching sink tissues, such as roots, developing leaves and reproductive tissues, sucrose is unloaded from the phloem. Like phloem loading, phloem unloading could also occur by two pathways, symplastic and apoplastic. It has been published that in many plants, plasmodesmata interconnect protophloem sieve elements in the root apices, sink leaves as well as storage sinks with non-phloem cells (Lalonde et al., 2003). Dye coupling studies have identified a functional symplastic route in Arabidopsis root tips (Oparka et al., 1994; Oparka et al., 1995). In the maize root,
translocated sucrose was found to follow a symplastic unloading route in root cortical cells (Giaquinta et al., 1983a). Recent studies have discovered unloading domains (ULD) in symplastic sinks (Stadler et al., 2005a; Stadler et al., 2005b). The plasmodesmata between the sink phloem sieve elements and the ULD cells have SEL of greater than 67 kDa which allows symplastic movement of sucrose into the ULD cells.

Although the symplastic route is the one found in most sink tissues for sucrose transport, questions have been raised about the capacity of the interconnecting plasmodesmata to support the observed rates of sucrose transport in maize root tips (Bret-Harte and Silk, 1994). It is proposed that there is the possibility of symplastic and apoplastic routes operating in parallel or shifts between these two pathways, which has been observed during sink development of tubers and fruits (Lalonde et al., 2003). In storage roots of sugar beets, a model for phloem unloading was proposed suggesting that sucrose was exported to the apoplasm from the SE-CC complexes, retrieved by the phloem parenchyma and symplastically translocated to storage parenchyma cells (Fieuw and Willenbrink, 1990). However, some studies detected large SEL of plasmodesmata in developing leaves and root apices (Schulz, 1995; Imlau et al., 1999; Oparka et al., 1999), which indicates that the plasmodesmata can account for the high photoassimilate fluxes. In developing maize leaves, it has been reported that phloem unloading could be apoplastic given the symplastic isolation of sieve elements (Evert and Russin, 1993). However, a study in developing barley leaves using carboxyfluorescein, a phloem mobile dye, indicated a symplastic phloem unloading route is more likely into grass sink leaves (Haupt et al., 2001).
Symplastic phloem unloading is the predominant pathway characterized in most plant sink tissues. However, further studies on the role of sucrose transporters and apoplastic unloading in sink tissues is needed to facilitate a more complete understanding of phloem loading and unloading.

**1.4 Plant sucrose transporters**

Plant sucrose transporters (SUTs) play crucial roles in the cell to cell movement and long-distance transport of sucrose. SUTs are membrane-localized proteins with 12 transmembrane α-helices, and they are encoded by multi-gene families (Lalonde et al., 2003; Sauer, 2007). SUTs are proton coupled symporters and expressed in phloem cells. SUTs are responsible for apoplastic loading in leaves, maintaining the high concentration of sucrose in the phloem during long-distance transport, regulating sucrose influx or efflux into temporary storage sinks such as vacuoles, and post-sieve element transport of sucrose in developing seeds or fruits (Bosabalidis et al., 1994; Wang and Fisher, 1994; Patrick and Offler, 1995; Ruan and Patrick, 1995; Patrick and Offler, 1996; Tegeder et al., 1999). In the past decade, genes encoding SUTs have been identified, isolated and functionally studied in both dicot and monocot plants.

Initially, SUTs were suggested to function in phloem loading based on their expression in the vascular tissues of source leaves but not in sink leaves (Riesmeier et al., 1993; Sauer, 2007). Further studies on transgenic potato and tobacco plants expressing antisense StSUT1 and NtSUT1, respectively, as well as Arabidopsis plants with a T-DNA
insertion into the *AtSUT2* gene showed that sucrose transporters function in phloem loading (Riesmeier et al., 1994; Burkle et al., 1998; Gottwald et al., 2000). The mutant plants had defects in sucrose export from source leaves, which resulted in starch accumulation in the leaves. In addition, the accumulation of excess carbohydrates in the leaves caused chlorosis, inhibited sink development and retarded plant growth. Further studies on sucrose transporters have found diverse locations of SUTs’ expression. Besides source leaves, expression of SUTs were also detected in the phloem of stems, roots, developing and germinating seeds and floral organs. Their functions are postulated to retrieve sucrose leaked to the extracellular space or promote sucrose efflux from sink sieve elements (Williams et al., 2000; Sauer, 2007).

As more SUTs have been identified, it was discovered that all plants have small families of SUT genes. Arabidopsis has 9 SUT-like sequences though two have been proposed to be pseudogenes (Sauer, 2007). Rice has 6 SUTs, which are OsSUT1-OsSUT5 and OsSUC4 (Aoki et al., 2003). Phylogenetic analysis classified plant SUTs into four groups (Sauer, 2007): group 1 contains only monocot SUTs, group 2 includes only dicot SUTs, and group 3 and group 4 consists of both monocot and dicot SUTs. For group 1, four SUTs have been functionally characterized, HvSUT1 (Weschke et al., 2000), OsSUT1 (Hirose et al., 1997), ShSUT1 (Rae et al., 2005; Reinders et al., 2006) and ZmSUT1 (Aoki et al., 1999; Carpaneto et al., 2005). In addition, three wheat SUT genes, TaSUT1A, B and D have been cloned and found to be expressed in developing grains, source leaves and stem (Aoki et al., 2002; Aoki et al., 2004). All of these monocot SUT1 genes have also been found to be expressed in sink tissues suggesting
they may also function to transport sucrose into sink cells. Besides rice OsSUT1, OsSUT3 and OsSUT5 also belong to group 1 by phylogenetic analyses. In addition, OsSUT2, OsSUT4 and ZmSUT2 belong to group 3, while OsSUC4, HvSUT2 and ZmSUT4 are classified in group 4 (Sauer, 2007). However, none of these group 3 and 4 genes have been functionally characterized.

Since the rice genome has been sequenced, it has the most identified monocot SUT genes. (Aoki et al., 2003; Sauer, 2007). Five rice SUTs have been found to be expressed in both sink and source tissues (Aoki et al., 2003). In barley, HvSUT1 is expressed preferentially in the cells at the maternal–filial boundary, while HvSUT2 expression is almost equal in different tissues (Weschke et al., 2000). A later study revealed that HvSUT2 is a tonoplast-localized protein (Endler et al., 2006). These data suggest a role of HvSUT1 in regulating sucrose unloading from the maternal tissues and/or loading into the endosperm, whereas HvSUT2 may regulate sucrose influx and efflux into the vacuole. Studies on a sugarcane sucrose transporter, ShSUT1, indicated that it is localized to the periphery of vascular bundles in the stem suggesting a role of ShSUT1 in sucrose partitioning between vascular bundles and storage parenchyma cells of the sugarcane stem internode (Rae et al., 2005). To date, three sucrose transporters have been identified in maize, ZmSUT1, ZmSUT2 and ZmSUT4, while only ZmSUT1 was characterized (Aoki et al., 1999). Expression analyses showed that ZmSUT1 was expressed in mature leaf but not in etiolated leaves, and that ZmSUT1 expression was light regulated (Aoki et al., 1999). It was also shown that ZmSUT1 is expressed in different tissues, such as leaf sheaths, germinating seeds and culms. These data suggest
two physiological functions for ZmSUT1, sucrose loading from source leaf and unloading into sink tissues such as pedicles. Electrophysiological analyses in Xenopus laevis oocytes expressing ZmSUT1 cRNA demonstrated that ZmSUT1 is capable of both sucrose release and uptake (Carpaneto et al., 2005).

SUTs in dicots have been extensively studied, especially in Arabidopsis, potato, tomato and plantago. Most of the SUTs are localized to sieve elements and companion cells, indicating their function in phloem loading and/or unloading. Monocot SUTs have not been studied as intensely and fewer SUT members have been identified or isolated from monocots. Future experiments aimed at protein localization, gene silencing and transport activity analyses of monocot SUTs will provide more information to understand sucrose transport in monocots and how SUTs regulate phloem loading and unloading.

1.5 Objectives of the research

Maize is an ideal system for studying leaf mutants with sectors that violate clonal boundaries and the signals involved in sector formation. So far, only one mutant with nonclonal chlorotic and green sectors in maize leaves, sxd1, has been identified and characterized to have defects in sugar transport in leaves. However, Sxd1 turned out to encode tocopherol cyclase in the tocopherol biosynthesis pathway, and the relationship between carbohydrate accumulation and tocopherol deficiency is unclear. Carbohydrate partitioning in maize has been studied for many years, but the processes controlling and regulating sucrose transport is still not thoroughly understood. A genetic screen was
undertaken to identify additional leaf mutants with nonclonal chlorotic sectors that may function in phloem loading. The focus of my thesis is on characterizing a second maize leaf mutant, *tie-dyed1*, with nonclonal chlorotic (the same meaning as yellow, which is used in Chapter 2) and green sectors. In the thesis, research on the identification and physiological characterization of the mutant is described in Chapter 2. As *sxd1* and *tdy1* mutants have many similarities, I investigated whether the two genes function in the same pathway regulating carbon export, and the findings are presented in Chapter 3. The cloning of *Tdy1*, expression studies on the gene and its possible role in regulating carbohydrate partitioning in maize is discussed in Chapter 4. Finally, in Chapter 5, I discuss the significance of my findings and suggest possible future routes for investigation.
Chapter 2

*tie-dyed1* regulates carbohydrate accumulation in maize leaves

Notes:

1. Fluorescent microscopy and morphometric analyses were performed by Frank Baker.

2. Pigment analyses were performed by David Braun.

3. Photosynthesis and stomatal conductance measurements were performed by Michael G. Muszynski.

4. Electron microscopy was performed by Noriko Inada.

5. Other experiments were performed by Yi Ma and this chapter has been published in Plant Physiology (see Yi Ma’s Vita attached at the end).
Chapter 2

tie-dyed1 regulates carbohydrate accumulation in maize leaves

2.1 INTRODUCTION

The maize leaf has long been an attractive model system to study genes controlling leaf development and cell fate acquisition. It is a lanceolate organ divided into the distal blade and proximal sheath, which wraps around and connects the leaf to the stem. A large number of mutations perturbing various aspects of leaf development have been characterized (Freeling, 1992; Hall and Langdale, 1996). These have defined genes that act early in leaf development to specify regional fates and those that coordinate later spatial patterning events or final differentiation of specific cell types (Sylvester et al., 1990; Sylvester et al., 1996). Whereas mutational analysis and the cloning and characterization of the respective genes have elucidated many of the early leaf-patterning genes, much remains to be understood about the role cell-cell signaling plays in later leaf development.

Genetic studies using clonal mosaic analyses have revealed the clonal lineages comprising maize leaf development and determined that the orientations of cell divisions in growing leaf primordia are mostly transverse to the long axis of the leaf (Poethig, 1984; Poethig and Szymkowiak, 1995). These divisions produce linear cell files arranged in parallel to the long axis of leaves (Figure 2.1A, page 33). Significantly, the great
majority of investigations have found that cell identity is determined by a cell's final position within a leaf rather than its lineage (Sylvester et al., 1996), although exceptions exist (Jankovsky et al., 2001; Kessler et al., 2002). Hence, positional signals communicated from neighboring cells largely control acquisition of cell identity.

Maize uses C₄ carbon assimilation in leaf blade tissue, whereas other green tissues, such as sheath, utilize the C₃ pathway (Langdale and Nelson, 1991). The internal leaf tissue exhibits Kranz anatomy, with two distinct photosynthetic cell types arranged concentrically around the vascular tissue (Esau, 1977a). Mesophyll cells surround bundle sheath cells, which in turn surround the vein. This anatomical arrangement reflects differences in physiological function. For example, almost all starch synthesis and accumulation occurs in the bundle sheath cells, but is largely absent from the mesophyll cells (Rhoades and Carvalho, 1944). Additionally, extensive grana stacking is seen in the mesophyll cell chloroplasts, whereas little or none is present in the bundle sheath cell thylakoid membranes (Kirchanksi, 1975).

To date, two genes responsible for specifying bundle sheath cell differentiation have been characterized. Both bundle sheath defective2 (bsd2) and golden2 (g2) mutants fail to accumulate the bundle sheath cell-specific enzyme Rubisco and have altered bundle sheath cell chloroplast ultrastructure (Langdale and Kidner, 1994; Roth et al., 1996). Neither mutant perturbs leaf blade mesophyll cell identity or function. Both genes were cloned from transposon-induced mutations that display somatic instability and reversion to wild-type function (Hall et al., 1998; Brutnell et al., 1999). Failure of the
revertant wild-type sectors to restore function to the adjacent mutant tissue indicates that both BSD2 and G2 likely function cell autonomously and are not involved in communicating identity or function to nearby cells. Genes that coordinate a change in identity and physiological function at the whole-tissue level in leaves have not been identified.

To identify genes regulating cell fate among neighboring groups of cells, we screened for mutants that produce sectors that violate clonal lineage patterns in maize leaf development. We reasoned that sectors that cross lineage boundaries may result from a mobile signal transmitted between nonclonally related cells. For our screen, we utilized chloroplast pigmentation as an easily scorable marker to visualize nonclonal leaf sectors. In this article, we describe the isolation and characterization of *tie-dyed1* (*tdy1*), the first of a large group of mutants referred to as nonclonal-sectoring mutants. *tdy1* was isolated from an ethyl methanesulfonate (EMS)-mutagenized population and is inherited as a stable, nuclear recessive mutation that conditions variegated yellow and green leaf sectors. *tdy1* sectoring is restricted to a limited time in leaf development and is uniform throughout a sector, implicating cell signaling in sector formation. Additionally, sectors are irreversible and do not progressively expand in size. Whereas *tdy1* green sectors are essentially like wild-type tissue, *tdy1* yellow sectors display excessive accumulation of starch and soluble sugars within the photosynthetic cells, suggesting a defect in carbon export. Carbohydrate accumulation occurs prior to detection of a yellow sector, indicating that the down-regulation of chlorophyll levels (chlorosis) results from the hyperaccumulation of photoassimilates (Sheen, 1990). To our knowledge, no other
mutant has been described that results in discretely variegated leaf sectors containing elevated levels of carbohydrates (Chatterjee et al., 1996; Keddie et al., 1996; Rodermel, 2002; Wang et al., 2004). Based on our investigations, a threshold model is proposed to explain the tdy1 variegation and carbohydrate accumulation phenotypes.

2.2 MATERIALS AND METHODS

2.2.1 Plant Materials and Growth Conditions

Plants were grown in the summer nurseries at the Bay Area Research and Extension Center, University of California at Berkeley (San Jose, CA), and at the Rock Springs Agronomy Farm, Pennsylvania State University (State College, PA). For photosynthesis and stomatal conductance measurements, plants were grown in the summer nursery in Johnston, IA. Plants grown in the greenhouse and growth chamber were supplemented with sodium vapor and mercury halide lamps at 1,400 (high) or 75 (low) µmol m⁻² s⁻¹ light under a 12-h d (30°C)/12-h night (20°C) cycle. For the constant high light experiment, plants were grown in a high light growth chamber under 24-h constant light with a constant temperature of 28°C. For the visible sector time-course experiments, tdy1-segregating families were grown in the greenhouse and newly emerged fourth leaves were observed throughout their development.
2.2.2 Genetic Stocks

Maize (*Zea mays*) EMS-mutagenized F$_2$ families were obtained from J. Hollick (University of California, Berkeley, CA). These populations were derived from mutagenizing pollen from stock 611A obtained from the Maize Genetics Cooperation Stock Center as described by Hollick and Chandler (2001). The anthocyanin-accumulating genetic line is a W23-derived stock containing structural and regulatory alleles necessary for anthocyanin expression in leaves (see Hollick and Chandler, 2001 for description). B73 inbred seed was provided by Pioneer Hi-Bred. The \textit{tdy1-Reference} (\textit{tdy1-R}; hereafter \textit{tdy1}) mutation was introgressed into the B73 genetic background three or more times prior to analyses, except in the experiments performed using the anthocyanin-accumulating background. B-A translocation stocks were obtained from M. Scanlon (Cornell University, Ithaca, NY). \textit{tdy1} was crossed to inbreds B73, Mo17, and W23, and F$_1$ plants self fertilized to create segregating F$_2$ families used for molecular fine mapping.

2.2.3 Pigment Analyses

Chlorophyll and total carotenoid concentrations were quantified according to Lichtenthaler and Wellburn (1983). Leaf discs of field-grown mature leaf tissues were harvested with an 11-mm-diameter cork borer, weighed, frozen in liquid nitrogen, ground to a fine powder in a mortar and pestle, and extracted in acetone. For \textit{tdy1} leaves, samples were collected from the center of large green or yellow sectors to avoid border regions.
Samples were kept on ice in the dark during processing. Extracts were gently rotated at 4°C in the dark for 1 h. Cell debris was removed by centrifugation and the supernatant assayed for pigment concentrations. For each tissue type (tdy1 yellow, tdy1 green, and wild type), 12 different samples were measured. The experiment was performed three times.

2.2.4 Fluorescent and Electron Microscopy

For cytological studies, samples were isolated from mature, field-grown leaves collected at the end of the day. Tissues for leaf histology were processed according to Inada et al. (1998). Briefly, small segments of fresh leaf blade tissue, approximately 2 × 2 mm, were fixed at 4°C overnight in 4% paraformaldehyde buffered with 20 mM sodium cacodylate, pH 7.5, dehydrated in a graded ethanol series, and embedded in Technovit 7100. Thin sections of approximately 1 µm were made with an Ultramicrotome (RMC MT6000; RMC) and stained with 100 µg mL⁻¹ DiOC7 in ethanol. Sections were observed under blue light with a Zeiss Axiophot fluorescent microscope. Additionally, free-hand sections were examined under bright-field and UV illumination using a Nikon Eclipse 80i fluorescent microscope.

To analyze chloroplast ultrastructure, samples were prepared for TEM analysis. In the initial step, segments of leaf blades were dissected with a sharp razor blade and immediately placed into 5% glutaraldehyde, 50 mM sodium cacodylate buffer, pH 7.2, on ice. The remainder of tissue preparation was performed under vacuum using a Pelco 3451
laboratory microwave system (Ted Pella). Tissues were fixed for 4 min, rinsed with 50 mM sodium cacodylate, pH 7.2, for 40 s three times, postfixed in 1% osmium tetroxide for 4 min, and rinsed with water for 40 s three times. Tissues were dehydrated through a graded acetone series (each 40 s twice) and infiltrated with Spurr's epoxy resin (4 min each step; Spurr, 1969). All of the above steps took place at 45°C. Power settings were 1 for fixation and dehydration steps, 2 for the first infiltration step (acetone:resin [2:1]), 3 for the second infiltration step (acetone:resin [1:1]), and 4 for the rest of steps. Ultrathin sections of approximately 100 nm were cut with a glass knife on an ultramicrotome, lifted on to glow-discharged carbon-coated copper grids stained with 0.5% formvar, and observed at 80 kV on a FEI Tecnai 12 transmission electron microscope.

2.2.5 Carbohydrate Quantification

Soluble sugar (Suc, Glc, and Fru) and starch quantities were determined using commercial assay kits according to the manufacturer's instructions (R-Biopharm) as described (Dinges et al., 2003). Mature, adult leaves were collected on three different days from field-grown plants at the end of the photoperiod and six samples were measured for each tissue. The experiment was repeated three times.

To visualize starch in leaf tissues, leaves of wild-type and tdy1 mutants were collected as above, decolorized in boiling 95% ethanol, and stained with IKI according to Ruzin (1999). To visualize starch prior to observing chlorotic sectors, leaf 10 from
greenhouse-grown plants was harvested when the base of the leaf blade was emerging from the whorl, divided into thirds, and similarly analyzed.

2.2.6 Photosynthesis and Stomatal Conductance Measurements

Measurements were taken on adult, fully expanded leaves in midsummer between 1 PM and 3 PM using a LICOR LI-6400 open photosynthesis system. As part of a randomized design, plants were grown in three separate replicate plots. The device maintained the following conditions throughout the assays: an artificial light source with an intensity of 1,800 µmol photosynthetically active radiation m$^{-2}$ s$^{-1}$, leaf temperature of 34°C to 35°C, CO$_2$ concentration of 400 µL L$^{-1}$, air flow of 500 µmol, and relative humidity of 58% to 64%. Three samples were assayed for each tissue type on three different days. Data presented in Table 2.2 are a combined analysis because no differences between the datasets were found, whether compared for a particular day or across all dates.

2.2.7 Morphometric Analyses

For morphometric studies, measurements were made from at least two F$_2$ families with $\geq$ 30 individuals and segregating wild type and $tdyl$ in a 1:1 fashion. Representative data from one family are shown. Plant height was measured from the surface of the soil to the tip of the central spike of the tassel ($n = 13$). Tassel height was measured from the node of the topmost leaf to the tip of the central spike ($n = 18$). Anthesis date was
recorded as the day of first pollen shed \((n = 14)\). Similarly, silking date was the date of first silk emergence \((n = 15)\). Ear length was measured from the base of the bottom row of kernels to the tip of the ear \((n = 12)\). Kernel weight was determined as the average of 100 randomly selected F3 kernels per ear \((n = 12)\).

### 2.2.8 Statistical Analyses

For photosynthetic pigment quantifications, carbohydrate measurements, and the morphometric analyses, statistical significance was determined using Student's two-tailed \(t\) test. For photosynthesis and stomatal conductance assays, statistical significance was determined using ANOVA (Sasaki et al.). Data from each day and combined across all three days showed the same Duncan's groupings.

### 2.3 RESULTS

#### 2.3.1 \(tdy1\) is a variegated mutant displaying nonclonal leaf blade sectors

To identify genes coordinating regional leaf identity, we screened for mutants with sectors that extend laterally beyond clonal lineages. Eight-hundred-forty EMS-mutagenized \(F_2\) families were screened and a family was found to segregate plants with yellow- and green-pigmented leaf sectors that violate clonal lineage relationships (Figure 2.1B). The sectors were stable in both shape and size over the lifespan of the leaves, with no reversion from green-pigmented to yellow-pigmented tissue or vice versa.
Mutant leaves did not become necrotic or senesce any earlier than their wild-type siblings. Sectors often formed regional islands surrounded by the opposite sector type, demonstrating that the external environment alone does not condition a sector because cells adjacent to the sector developed in a nearly identical environment, but formed the opposite sector type (Figure 2.1C, circle). Additionally, formation of a yellow or green sector occurred coordinately over a region of tissue as evidenced by uniform pigmentation and lack of flecking, which would be expected to occur if each cell was independently sectoring. These data suggest that cell-cell signaling orchestrates sector formation in mutant leaves.

When the \textit{tdy1} mutation was introduced into a genetic background capable of anthocyanin production in leaves, anthocyanins accumulated only in the \textit{tdy1} yellow sectors (Figure 2.1C). Hence, in this line, anthocyanins specifically mark \textit{tdy1} yellow sectors. We analyzed \textit{tdy1} plants throughout development to examine when and where sectors formed. Although the degree of sectoring per leaf varied, all leaves were capable of sectoring. Interestingly, \textit{tdy1} yellow/red sectors manifested only in leaf tissue with blade identity, including reduced blade tissue at the tip of husk leaves, the specialized leaves that function to protect the ear (Figure 2.1D). Sectors were not seen in leaf sheaths, in the sheaths of husk leaves, or on the stem, suggesting the \textit{tdy1} defect specifically affects the leaf blade. Sectors could form anywhere in the blade, but with the greatest tendency to form in the tip and midregion, corresponding to the leaf areas receiving maximal incident light.
Figure 2.1: Clonal and nonclonal sectors in maize leaves. A, Arrow indicates clonal sector of white tissue illustrating longitudinal arrangement of cell files in a maize leaf. B, Variegated *tdy1* mutant leaf with yellow and green nonclonal sectors. C, *tdy1* leaf in anthocyanin-accumulating genetic background showing that yellow sectors accumulate red anthocyanins. White circle shows a regional island sector located adjacent to tissue of the opposite sector type. D, *tdy1* red sectors occur only in leaf blade tissues; leaf sheaths and husk leaf sheaths do not develop sectors. White arrow indicates a *tdy1* sector in leaf blade tissue at the tip of an ear husk leaf.
Because mutants displayed a variegated sectoring pattern reminiscent of the style of tie-dyed clothing, the mutation was named \textit{tdy}1. To investigate the inheritance of the \textit{tdy}1 mutation, mutant plants were reciprocally crossed to standard inbred lines of maize. F\textsubscript{1} progeny were all wild type, indicating that the mutation is recessive and not maternally inherited through chloroplasts. F\textsubscript{1} plants were self fertilized to produce a F\textsubscript{2} generation that segregated 3:1 wild type:mutant (215 wild type:61 \textit{tdy}1, \chi^2 [3:1] = 1.24; \( P = 0.27 \)), supporting monofactorial recessive inheritance. The \textit{tdy}1 locus was mapped to the long arm of chromosome six using a series of B-A translocations (Beckett, 1994). Hypoploid \textit{tdy}1/– plants were phenotypically equivalent to the parental euploid \textit{tdy}1/\textit{tdy}1 mutants. This suggests that the \textit{tdy}1 mutation is a genetic null mutation because there is no difference in phenotypic severity between one versus two mutant doses of the gene (Muller, 1932). Molecular markers were used to further fine map \textit{tdy}1. \textit{tdy}1 resides between markers umc1653 (approximately 0.3 cM proximal; one recombinant/320 chromosomes) and asg7 (1.25 cM distal; four recombinants/320 chromosomes).

Intriguingly, although \textit{tdy}1 is a stable recessive mutation and the gene product is defective in every cell, the phenotype is evident only in some portions of the leaf.

2.3.2 \textit{tdy}1 yellow sectors have reduced levels of photosynthetic pigments

\textit{tdy}1 green sectors appeared similar to wild-type leaf tissue with the consequences of the \textit{tdy}1 mutation apparent only in the yellow tissue. To quantify the variation in pigmentation, we measured the amount of chlorophyll \( a/b \) and total carotenoids in mature leaves of \textit{tdy}1 yellow sectors, \textit{tdy}1 green sectors, and wild type (Table 2.1). \textit{tdy}1 yellow
sectors accumulated approximately 19% to 24% of chlorophyll \(a/b\), and 34% to 40% of the total carotenoids compared to wild type. The pigment profile of \(tdy1\) green tissue was not significantly different from wild-type siblings. Hence, the \(tdy1\) mutant yellow sectors fail to accumulate photosynthetic pigments to the same level as the wild-type tissue, yet the green sectors appear equivalent to wild type.

2.3.3 High light during a limited developmental period Is required for sector formation

Because chloroplast pigment accumulation is light regulated (Sestak, 1963) and dramatically reduced in \(tdy1\) yellow sectors, we hypothesized that light intensity plays a role in sector initiation. To test this hypothesis, a family segregating \(tdy1\) was grown under conditions of low or high light. Under a low light regime, \(tdy1\) and wild-type leaves did not sector (Figure 2.2A). In contrast, \(tdy1\) leaves that developed under high light conditions produced sectors, whereas wild-type leaves showed no sectors (Figure 2.2B). These results indicate a high light requirement for sector formation in \(tdy1\) leaves. Consistent with this finding, growing \(tdy1\) plants under constant high light produced strongly yellow-sectored leaves with a few green sectors restricted to leaf undulations and margins.
Figure 2.2: High light is necessary for *tdy1* sector formation. A. Wild type and *tdy1* leaves from plants grown under low light condition (75 μmols / m²•sec). B. Wild type and *tdy1* leaves from plants grown under high light condition (1400 μmols / m²•sec). C. Subsequent leaves generated after wild type and *tdy1* plants were transferred from low light to high light environment.
Table 2.1: Photosynthetic pigment quantification

<table>
<thead>
<tr>
<th>Leaf Tissue</th>
<th>Chlorophyll <em>a</em></th>
<th>% of Wild Type</th>
<th>Chlorophyll <em>b</em></th>
<th>% of Wild Type</th>
<th>Total Carotenoids</th>
<th>% of Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3,294.6 ± 82.0</td>
<td>100</td>
<td>714.9 ± 18.7</td>
<td>100</td>
<td>780.4 ± 23.6</td>
<td>100</td>
</tr>
<tr>
<td><em>tdy1</em> green</td>
<td>3,298.6 ± 69.4</td>
<td>100.1</td>
<td>689.3 ± 14.8</td>
<td>96.4</td>
<td>799.2 ± 14.8</td>
<td>102.4</td>
</tr>
<tr>
<td><em>tdy1</em> yellow</td>
<td>705.5<em>a</em> ± 20.1</td>
<td>21.4</td>
<td>141.6<em>a</em> ± 5.5</td>
<td>19.8</td>
<td>280.9<em>a</em> ± 8.0</td>
<td>36.0</td>
</tr>
</tbody>
</table>

Data represent means from 12 samples ± SE, and the units are µg/g fresh weight.

* Value is significantly different from wild type at *P* ≤ 0.0001 using Student's *t* test.
To examine the developmental competence of mutant plants to respond to a high light signal, we performed a light-shift experiment by transferring the low light-grown plants into the high light environment. After transfer, tdy1 mutants produced new leaves that sectored (Figure 2.2C). However, tdy1 leaves that had matured in the low light environment did not sector. This experiment demonstrates that there is a limited developmental time when sectoring is conditioned. Once past this time point, tdy1 leaves are not competent to respond to the high light signal.

2.3.4 tdy1 sectors appear after leaf emergence

We monitored emerging tdy1 leaves to investigate the timing and location of sector formation (Figure 2.3). Day 1 was defined as the day the blade portion of a leaf was fully emerged from the whorl. Upon emergence and through day 4, the tdy1 blade was a uniform pale green and showed no indication of sectoring (Figure 2.3A). The first sign of sectoring was observed on day 5 as a region of tissue slightly paler than the neighboring light-green tissue (Figure 2.3B). Ten days after emergence, the sector had become more evident against the darker green tissue background (Figure 2.3C). The final form of the sector, as marked with anthocyanins, is shown on day 18 (Figure 2.3D). Comparing the ultimate shape of the sector on day 18 with its shape on the day it is first visible, it can be seen that the boundary is unchanged (compare, Figure 2.3B and D). This indicates that sectoring is not progressive, but occurs within a developmentally limited time frame.
Figure 2.3: *tdy1* sectors are evident 5 d after leaf emergence from whorl. A, Day 1, blade portion of the leaf has fully emerged from the whorl. B, Day 5, faint yellow-green sector is visible. C, Day 10, the sector has a lighter olive-green coloration compared to neighboring green tissue. D, Day 18, the sector is marked by anthocyanin accumulation. Asterisks in B, C, and D mark the tip of the sector.
2.3.5 *tdy1* yellow sectors hyperaccumulate starch and display altered cellular ultrastructure

Because *tdy1* yellow sectors have decreased photosynthetic pigment levels compared to green sectors, we examined whether alterations in cellular and/or plastid morphology were present in mutant leaves. Wild-type and mutant tissue anatomy was visualized using 3,3'-dihexyloxacarbocyanine iodine (DiOC7), a fluorescent dye that stains membranes, as well as by bright-field and UV illumination to inspect chlorophyll accumulation. Under UV illumination, chlorophyll autofluoresces a red color, whereas cell walls autofluoresce blue. In wild-type leaves stained with DiOC7, mesophyll cells show bright-green fluorescence that corresponds to numerous chloroplasts with abundant photosynthetic membranes (Figure 2.4A). Bundle sheath cells, with their less developed chloroplast membrane system, fluoresce less intensely, but also contain black precipitate indicative of starch grains (Figure 2.4A). This precipitate is not observed in mesophyll cells. As seen with bright-field and UV illumination, chlorophyll is abundant in wild-type photosynthetic cells (Figure 2.4, D and G). Photosynthetic cells in green sectors from *tdy1* mutant leaves appear similar to wild type. Notably, mesophyll cells in green *tdy1* sectors display intense membrane staining equivalent to wild type and bundle sheath cells show the same dark precipitate (Fig. 4B). Additionally, chlorophyll abundance and autofluorescence in both photosynthetic cell types is comparable to wild type (Figure 2.4, E and H). In contrast, yellow *tdy1* sectors exhibit strong reduction in membrane staining (Figure 2.4C), chlorophyll accumulation, and autofluorescence (Figure 2.4, F and I). Significantly, both mesophyll and bundle sheath cells in *tdy1* yellow sectors contain
copious amounts of the dark precipitate, suggesting they accumulate excess starch (Figure 2.4C).

To verify that the precipitate was starch and investigate the cellular ultrastructure, we examined wild-type and mutant photosynthetic cells using transmission electron microscopy (TEM). In mature wild-type leaves, mesophyll chloroplasts are round, have many grana membrane stacks, and accumulate little or no starch crystals, whereas bundle sheath chloroplasts are oblong, have few grana stacks, and accumulate multiple starch grains (Figure 2.5A). Chloroplasts from leaves of tdy1 green tissue appear similar to wild-type sibling tissue (Figure 2.5B). However, in tdy1 yellow sectors from the same leaf, both the mesophyll and bundle sheath chloroplasts accumulate large quantities of starch grains that occupy the vast majority of chloroplast volume (Figure 2.5C). In mesophyll cells, almost no other organelles are discernible as the predominant vacuole appresses the chloroplasts and cytoplasm against the cell wall. Additionally, few grana stacks are visible in the chloroplasts. In bundle sheath cells, starch-packed chloroplasts are more round than oblong in shape and hardly any stroma can be seen. We conclude that the large excess of starch accumulating in mesophyll and bundle sheath chloroplasts of tdy1 yellow sectors indeed corresponds to the dark precipitate observed in the fluorescent microscope (Figure 2.4C).
Figure 2.4: *tdy1* yellow sectors have reduced membrane staining and decreased chlorophyll autofluorescence. A to C, Fluorescent micrographs of cross sections of mature leaves stained with DiOC₇, a dye that stains membranes. White arrowheads indicate dark precipitate. A, Wild-type leaf. Bundle sheath (BS) cells surround the veins and mesophyll (M) cells surround the BS cells. Black arrow indicates intensely fluorescent mesophyll cell chloroplast. B, *tdy1* green sector likewise shows bright membrane staining in mesophyll chloroplasts. C, *tdy1* yellow sector displays greatly reduced membrane staining and abundant dark precipitates in both bundle sheath and mesophyll cells. D to F, Bright-field illumination of free-hand cross sections. G to I, UV illumination of free-hand cross sections. D and G, Wild type. E and H, *tdy1* green sector. F and I, *tdy1* yellow sector. Scale bars = 10 µm (A–C); 50 µm (D–I).
Figure 2.5: Photosynthetic cells in *tdy1* yellow sectors hyperaccumulate starch. TEM micrographs show ultrastructure of mesophyll (M) and bundle sheath (BS) cells. Starch grains are visible as white crystals inside chloroplasts indicated with white arrows. Black arrows show grana stacks in mesophyll chloroplasts. A, Wild-type leaf showing starch accumulation in BS cell chloroplasts, but no starch accumulation in M cell chloroplasts. B, *tdy1* green-leaf tissue showing starch accumulation in BS cell chloroplasts, but no starch in M cell chloroplasts. C, *tdy1* yellow sectors hyperaccumulate starch grains in both M and BS cell chloroplasts. Scale bars = 5 µm.
2.3.6 *tdy1* yellow sectors accumulate excess carbohydrates prior to chlorosis

To test whether all photosynthetic cells in a yellow sector were accumulating high levels of starch, we qualitatively examined wild-type and *tdy1* sectored leaves by iodine staining (Figure 2.6). We found that all cells throughout the yellow *tdy1* sectors stained strongly for starch accumulation (Figure 2.6D). *tdy1* green sectors and wild-type tissue were weakly stained, indicating that these tissues contained far less starch (Figure 2.6, B and D).

Because vacuoles are the temporary storage site for excess Suc transiently accumulated during daylight (Kaiser and Heber, 1984), we hypothesized that the predominant vacuoles visible in TEM images of *tdy1* yellow sectors may be due to elevated Suc accumulation in addition to excessive starch. To test this hypothesis, soluble sugar and starch concentrations within *tdy1* and wild-type leaves were quantified. As shown in Figure 2.7, *tdy1* yellow sectors accumulated approximately 3 times as much Suc and approximately 10 to 16 times as much Glc, Fru, and starch as *tdy1* green sectors and wild-type leaves, whereas no significant differences were observed between green *tdy1* sectors and wild type. Hence, *tdy1* yellow sectors contain greatly increased amounts of carbohydrates.
Figure 2.6: *tdy1* yellow sectors accumulate starch throughout the sectors. A and B, Wild-type leaf. C and D, *tdy1*-sectored leaf. A and C, Photographs before staining. B and D, Photographs of cleared, IKI-stained leaves.
Figure 2.7: *tdy1* yellow sectors accumulate excess soluble sugars and starch. Bars represent the mean of six samples and error bars represent the SE. Wild type is represented in dark gray, *tdy1* green sectors in light gray, and *tdy1* yellow sectors in white. A, Suc. B, Glc. C, Fru. D, Starch quantification in leaves. An asterisk indicates that the value is significantly different from wild type at $P \leq 0.0001$ using Student's $t$ test. Units for each image are mg/g fresh weight.
High levels of photoassimilates are known to repress photosynthetic gene expression (Sheen, 1990) and therefore could be responsible for the yellow sectors in *tdy1* mutant leaves. Conversely, impaired plastid development could be the primary defect in *tdy1* yellow sectors and cause chlorosis, which in turn could lead to carbohydrate accumulation. To determine whether carbohydrate accumulation precedes visible sector formation, developing leaves were cleared of pigments and stained with iodine-potassium iodide (IKI) to detect starch. Emerging leaves were divided into thirds corresponding to the tip (oldest), middle, and base (youngest) regions. No region in wild-type leaves preferentially accumulated starch (Figure 2.8, A and B). However, faintly visible yellow sectors in *tdy1* leaf tips already accumulated high levels of starch as shown by iodine staining (Figure 2.8, C and D, arrows). The middle portion of the same leaf lacked visible sectors, yet differentially accumulated starch in cells that would presumably become yellow sectors (Figure 2.8, C and D, arrowhead). Finally, the base region of the blade just emerging from the whorl lacked visible sectors and preferential starch accumulation. Thus, excess carbohydrate accumulation occurs shortly after the tissue emerges from the whorl and prior to visible detection of a chlorotic sector in mutant leaves.
Figure 2.8: *tdy1* yellow sectors accumulate starch prior to chlorosis. A and B, Wild-type leaf. C and D, *tdy1* leaf. Leaves were divided into thirds corresponding to the tip (oldest), middle, and base (youngest) regions. A and C, Photographs before staining. B and D, Photographs of cleared, IKI-stained leaves. Arrows point to faintly visible yellow sector in mutant leaf tip that accumulates excess starch. Arrowhead indicates region of tissue preferentially accumulating starch prior to chlorosis.
2.3.7 Reduced rates of photosynthesis and gas exchange in tdy1 yellow sectors

The large accumulation of photoassimilates in tdy1 yellow sectors was a surprising finding given the reduced photosynthetic pigment levels and the decreased chloroplast membrane abundance within tdy1 yellow sectors (Table 2.1; Figure 2.4C). To test whether the yellow sectors could be directly synthesizing the carbohydrates present in these tissues, we investigated the rate of photosynthesis and gas exchange in mutant and wild-type leaves (Table 2.2). Significantly, tdy1 yellow sectors had approximately 16% of the photosynthetic rate of wild-type siblings, whereas green tdy1 tissues had a photosynthetic rate similar to wild type. Most strikingly, yellow tdy1 leaf sectors displayed approximately 3% of the stomatal conductance compared to wild-type leaves, whereas green sectors were not significantly different from wild type. Together, these results may suggest that the tdy1 yellow sectors are not actively synthesizing the large amounts of carbohydrates present in these tissues.

<table>
<thead>
<tr>
<th>Leaf Tissue</th>
<th>Photosynthesis</th>
<th>% of Wild Type</th>
<th>Stomatal Conductance</th>
<th>% of Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>28.4 ± 1.3</td>
<td>100</td>
<td>0.089 ± 0.01</td>
<td>100</td>
</tr>
<tr>
<td>tdy1 green</td>
<td>24.3 ± 2.4</td>
<td>85.5</td>
<td>0.081 ± 0.02</td>
<td>91.0</td>
</tr>
<tr>
<td>tdy1 yellow</td>
<td>4.5a ± 0.9</td>
<td>15.8</td>
<td>0.003a ± 0.001</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Data represent means of nine samples combined from 3 d ±SE. Units for photosynthesis rate are µmol CO₂ fixed m⁻² s⁻¹ and the units for stomatal conductance measurements are µmol. a Value is significantly different from wild type at P < 0.001 using ANOVA.
2.3.8 Reduced growth in *tdy1* mutants

Because *tdy1* mutants have reduced rates of photosynthesis and contain elevated carbohydrate levels in leaves, we investigated whether plant growth and yield were affected in the mutant. Compared to wild type, plant height was reduced approximately 16% in *tdy1* mutants (Figure 2.9A). In addition, a delay in reproductive maturity and a reduced inflorescence size were evident. Relative to wild type, anthesis in *tdy1* plants was delayed by 4 d and silk emergence by 5 d (Figure 2.9B). Mutant tassels showed a dramatic 33% reduction in size (Figure 2.9C) and branch length and number were also decreased. Last, *tdy1* ears were 20% shorter than wild type and kernel weight was reduced by 25% (Figure 2.9D). These data suggest that retention of carbohydrates in *tdy1* leaves leads to retardation of growth and yield of mutant plants.
Figure 2.9: Reduced growth and yield in *tdy1* mutants. Bars represent the mean and error bars represent the SE. Wild type is represented in dark gray and *tdy1* mutants in white. An asterisk indicates that the value is significantly different from wild type at $P < 0.001$ using Student's *t* test. A, *tdy1* mutants have reduced plant height ($n = 13$). Units are centimeters above the soil surface. B, *tdy1* mutants are delayed in flowering. Anthesis is pollen shed ($n = 14$) and silking is silk emergence ($n = 15$). Units are days after planting (dap). C, *tdy1* mutants have reduced tassel size. D, *tdy1* mutants have reduced ear size. WT, Wild type.
2.4 DISCUSSION

We have identified and characterized a nonclonal sectored mutant, *tdy1*, which causes large yellow- and green-variegated sectors to form in leaf blades. *tdy1* sectors violate established cell lineage relationships in maize leaves. Thus, sector formation cannot be explained by a cellular inheritance-based mechanism, but must involve communication among cells, coordinating a decision to sector. This conclusion is drawn from the observation that all cells within a sector are uniformly pigmented. In addition, we frequently find islands of one sector type surrounding cells of the other sector type (Figure 2.1C). These observations indicate that the external environment alone does not condition a sector because cells laterally adjacent, but of opposite sector type, experience nearly identical stimuli (light, temperature, CO₂ levels, etc.) as they develop. Instead, these data suggest that an endogenous signal is at least partly responsible for orchestrating sector formation. As elaborated below, we propose that Suc is a candidate for this endogenous signal.

The external environment plays an important role in *tdy1* sectoring, however, because we found that it is strongly influenced by the light environment. Induction of sectoring does not occur under a low light regime, but requires high light (Figure 2.2, A and B). A possible explanation is that high light is necessary to produce the endogenous signal that is communicated among neighboring groups of cells and determines their fate. Production of high levels of photoassimilates occurs in high light and is consistent with the proposed role for Suc as the signal. Further, light-shift experiments revealed that there
is a limited time during leaf development when high light induces sector formation. We observed that leaves that matured in the low light environment failed to form sectors when shifted to the high light environment and, hence, had passed this time point in their development. This conclusion is also supported by the fact that green sectors of mature, field-grown *tdy1* leaves that are repeatedly exposed to daily high light levels during the summer never form yellow sectors later in development (Figure 2.1B).

Sectors first become visible 5 d after the leaf blade completely emerges from the whorl (Figure 2.3). Upon emergence, leaf blade tissues are exposed to sufficient light levels to complete chloroplast development and to accumulate high quantities of chlorophylls (Kirchanksi, 1975). Additionally, two other major events occur in leaf development around this time. First, soon after exposure to light, the leaf transitions from a carbon-importing sink tissue to a photosynthetically active, carbon-accumulating and carbon-exporting source tissue (Turgeon, 1989; Evert et al., 1996). Second, development is completed as a basipetal wave of differentiation passes down the leaf and determines cell identity (Sylvester et al., 1990; Sylvester et al., 1996). These are developmental events that occur once in the lifetime of a leaf and may underlie the timing and irreversibility of *tdy1* sector formation.

The *tdy1* mutant phenotype is evident only in leaf blade tissues. Sectors are not seen in leaf sheaths, stems, husk sheaths, or glumes (Figure 2.1D; data not shown). The physiology and anatomy of maize leaf blades differ significantly from these other photosynthetic tissues. With the exception of the first leaf blade, which displays C₃
characteristics, blade tissue utilizes the C₄ carbon assimilation pathway, whereas sheath, husk, glume, and stem tissues all use C₃ carbon assimilation (Crespo et al., 1979; Langdale and Nelson, 1991). Additionally, blade tissue is the principal site of carbon fixation and thereby experiences a much greater osmotic flux compared to C₃ tissues (Hofstra and Nelson, 1969a; Evert et al., 1996). Another difference is that the majority of photoassimilate loading into veins occurs in the blade. Whereas some loading does occur in the veins of these other tissues, their vein systems principally function in long-distance transport rather than in loading of assimilates (Fritz et al., 1989). Some of these distinctions may be responsible for why tdy1 sectors are found only in leaf blades.

Examination of the histology and ultrastructure of tdy1 yellow sectors revealed striking differences compared to green tdy1 sectors and wild-type leaves. tdy1 yellow sectors have greatly reduced photosynthetic membranes in the chloroplasts of mesophyll cells and accumulate excessive starch in both mesophyll and bundle sheath cells. Iodine staining of starch in faintly sectored tdy1 leaves shows that all photosynthetic cells throughout a sector display the starch accumulation phenotype. Furthermore, starch accumulation precedes visible observation of a yellow sector, suggesting that chlorosis is a secondary consequence of a defect in carbohydrate partitioning. Quantifying the carbohydrate levels in tdy1 sectors revealed that yellow tissues accumulate approximately 3- to 16-fold higher levels of Suc, Glc, Fru, and starch compared with tdy1 green sectors or wild-type leaves. Because Suc is transiently stored during the day in the vacuole, the engorged vacuoles observed in yellow sectors (Figure 2.5C) may be at least partly explained by an increased osmotic potential causing water uptake into the vacuoles.
Consistent with this, anthocyanins accumulate exclusively in the yellow sectors of *tdy1* leaves. Epidermal cells, the site of anthocyanin accumulation in the leaf blade, are symplastically connected to underlying mesophyll cells. Therefore, they presumably also contain high carbohydrate levels in *tdy1* yellow sectors and likewise experience osmotic stress, which is known to result in anthocyanin accumulation (Chalker-Scott, 1999).

Mesophyll cell chloroplasts do not normally accumulate starch unless plants have experienced severe stress, such as removal of the developing ear or blockage of phloem loading (Allison and Weinmann, 1970; Jeannette et al., 2000b). The large quantities of starch in *tdy1* yellow mesophyll cells suggest that these tissues are subjected to a stress similar to that of failing to export fixed carbon. In addition, we observed a significant reduction in plant height, tassel and ear size, and a delay in reproductive maturity in *tdy1* mutants as compared to wild-type siblings. These phenotypes have been reported in plants with reduced levels of photoassimilates available to be transported to growing sinks (Russin et al., 1996; Burkle et al., 1998; Gottwald et al., 2000a; Niittyla et al., 2004).

*tdy1* yellow sectors have reduced levels of photosynthetic pigments and membranes. However, they remarkably accumulate 300% to 1,600% more carbohydrates than the green sectors of *tdy1* leaves or wild-type siblings. We determined that the yellow sectors have greatly reduced rates of photosynthesis and gas exchange. These data may indicate that yellow sectors are not synthesizing the fixed carbon that accumulates in these tissues, but possibly importing them from neighboring green sectors. One explanation for these data could be that a developmental block occurred in the yellow
sectors, causing them to retain sink identity and to continue to import carbon from neighboring green source tissues. This would also explain how high levels of carbohydrates accumulate in the yellow sectors against a steep concentration gradient. Alternatively, it is possible that the reduced rates of photosynthesis in the \( tdy1 \) yellow sectors are sufficient to produce photoassimilates in these tissues and the fixed carbon accumulates to high levels due to reduced capacity to export. In this case, accumulation of excess carbohydrates against the concentration gradient may be mediated by compartmentalization and storage of surplus Suc in the vacuole and starch in the chloroplasts. Future work will test these hypotheses.

Two predominant models have been proposed to explain the formation of sectors in variegated mutants (see Wetzel et al., 1994; Wu et al., 1999; Yu et al., 2004 for discussion). The first model suggests that a partially redundant function can sometimes compensate for the loss-of-function mutation and rescues the phenotype in the green sectors. The second model proposes that a threshold of activity determines sector formation: Exceeding the threshold produces one type of sector and remaining below the threshold produces the other. From our collective observations, we favor the threshold model to explain sector formation in \( tdy1 \) leaves.

Because sectoring requires high light illumination and is evident shortly after blade emergence from the whorl, \( tdy1 \) must function at or prior to this stage of development. We postulate that several nonmutually exclusive mechanisms involving sugar sensing, accumulation, or movement might explain how green and yellow sectors
form in *tdy1* mutant leaves. In yellow sectors, lack of TDY1 function results in failure to export sugar and buildup of carbohydrates in a nonclonal region of tissue. We suggest that high levels of sugars in these cells lead to feedback inhibition of photosynthetic gene expression and chlorophyll synthesis, which in turn lead to reduced chlorophyll accumulation, and ultimately in yellow coloration of the tissue. Reduced photosynthetic pigment levels could result in damage to photosynthetic membranes under high light, causing a decrease in their abundance, and thereby reducing photosynthesis and stomatal conductance rates.

To explain the formation of green tissues in *tdy1* leaves, we propose that sugar levels do not exceed the threshold necessary to trigger formation of a yellow sector. This may occur due to multiple mechanisms (e.g. variation in light levels, more efficient phloem loading, or reduction in sugar levels due to proximity to a yellow sector importing the sugar). Subsequent to the events determining sector identity, cells of both tissue types differentiate and assume their final fate. After this time, TDY1 function is no longer required and cell fate is determined. Hence, we propose that *tdy1* green tissue fails to exceed the threshold required to trigger sector formation and differentiates as normal functioning tissue escaping the consequences of the mutation. This is consistent with the fact that we observed no significant differences between *tdy1* green tissue and wild-type tissue.

A speculative function of TDY1 consistent with this model for variegation is as a sugar flux or osmotic stress sensor, which activates an export pathway under high sugar
conditions. For example, TDY1 may control induction of a high capacity sugar transporter responsible for loading Suc into the phloem (Weise et al., 2000). Differential induction of a transporter could explain both types of sector formation. In the absence of tdy1 function, under circumstances when sugar concentrations in cells are low, there is no need to activate the inducible export pathway and no consequence to the defect. However, when sugar concentrations are high, the absence of TDY1 prevents activation of the inducible export pathway. The resulting buildup of photoassimilates diffuses both laterally and longitudinally through a nonclonal region of tissue, causing formation of a tdy1 yellow sector and terminal identity as carbon-accumulating tissue. This proposed function of TDY1 does not require a redundant function to explain the formation of green tissue in tdy1 mutant leaves, simply a sugar concentration below the threshold necessary to induce the export pathway.

Our characterization of the tdy1 variegation and carbohydrate accumulation phenotypes has identified a novel pathway regulating sugar export from leaves. To our knowledge, no other mutant with this particular set of defects has been described. Other starch-accumulating mutants have been characterized, but they function in the starch catabolic pathway and affect the entire leaf (Zeeman et al., 1998; Critchley et al., 2001; Yu et al., 2001; Dinges et al., 2003; Niittyla et al., 2004). Similarly, antisense expression or mutation of Suc transporters results in starch accumulation in leaves, but likewise affects the entire organ (Riesmeier et al., 1994a; Burkle et al., 1998; Gottwald et al., 2000a). Several variegated mutants have been characterized in dicots, but none have been reported to preferentially accumulate high levels of sugars and starch. The only
characterized mutant with a resemblance to \textit{tdy1} is \textit{sucrose export defective1 (sxd1)} in maize (Russin et al., 1996; Provencher et al., 2001). \textit{sxd1} mutants develop sectors at the tip of the leaf blade that accumulate high levels of sugars, starch, and anthocyanins. However, the temporal appearance and pattern of sectors between the two mutants are quite distinct. \textit{sxd1} sectors progressively spread basipetally over the lifetime of the mature leaf. To our knowledge, these are the only two mutants that possess a nonclonal variegation pattern with differential carbohydrate accumulation, so it will be insightful to determine whether \textit{tdy1} and \textit{sxd1} function in the same genetic pathway or regulate carbohydrate accumulation in leaves via different mechanisms. Future work will elucidate how \textit{tdy1} coordinates development among nonclonally related cells and the genetic pathways regulating carbohydrate partitioning in maize leaves.
Chapter 3

*Tie-dyed*1 and *Sucrose export defective*1 independently regulate carbohydrate accumulation in maize leaves

Notes:

1. Aniline blue staining and TEM microscopy were performed by Frank Baker.

2. Chlorophyll measurement was performed by David Braun.

3. Tocopherol quantification was performed by Maria Magallanes-Lundback in Dean DellaPenna’s lab in Michigan State University.

4. Other experiments were performed by Yi Ma and this chapter has been published in Planta (see Yi Ma’s Vita attached at the end)
Chapter 3

\textit{Tie-dyed1 and Sucrose export defective1 independently regulate carbohydrate accumulation in maize leaves}

3.1 INTRODUCTION

Sugars synthesized in photosynthetic leaf cells must be transported into the veins for distribution to nonphotosynthetic tissues. Maize (\textit{Zea mays}) is a C\textsubscript{4} plant which displays Kranz anatomy in its leaves (Esau, 1977). Veins are surrounded by bundle sheath cells which in turn are surrounded by mesophyll cells. Mesophyll and bundle sheath cells cooperatively perform the reactions of photosynthesis and carbon assimilation. Sucrose is synthesized in the cytoplasm of mesophyll cells (Lunn and Furbank, 1999), diffuses through plasmodesmata into bundle sheath cells, and then into vascular parenchyma cells (Russin et al., 1996). As maize is an apoplastic phloem loading species (Evert et al., 1978), sucrose is exported from the vascular parenchyma cells to the apoplast by an unknown mechanism (see (Lalonde et al., 2004) for discussion). Sucrose is then imported into phloem companion cells and/or sieve elements by sucrose transporters located in the plasma membrane (Aoki et al., 1999; Lalonde et al., 2004; Sauer, 2007; Scofield et al., 2007). If carbon transport into the vein is blocked, carbohydrates accumulate in the photosynthetic cells, leading to down-regulation of photosynthetic gene expression and reduced chlorophyll levels (chlorosis) (Sheen, 1990; Goldschmidt and Huber, 1992; Riesmeier et al., 1994; Krapp and Stitt, 1995; Burkle et
al., 1998; Gottwald et al., 2000; Jeannette et al., 2000). Though the transport pathway for assimilated carbon has been studied in maize leaves for almost forty years (Hofstra and Nelson, 1969), the mechanisms that regulate sucrose export from leaves remain poorly understood.

To date, two recessive maize mutants have been described with defects in carbon export from leaves. *tie-dyed1* (*tdy1*) and *sucrose export defective1* (*sxd1*) mutants both develop nonclonal chlorotic leaf sectors that hyperaccumulate starch and soluble sugars (Russin et al., 1996; Provencher et al., 2001; Braun et al., 2006). They both also display growth related defects such as reduced plant height and inflorescence development due to the retention of carbohydrates in leaf tissues. However, their phenotypes also show significant differences. *sxd1* mutant leaves progressively exhibit chlorosis which initiates at the leaf tips and basipetally spreads towards the base. Interestingly, in *sxd1* mutants, only the leaf minor veins are affected in phloem loading (Russin et al., 1996). The *tdy1* mutant phenotype is distinct from *sxd1* mutants in that large chlorotic regions develop throughout leaf blade tissue. Further, *tdy1* chlorotic regions form only during a limited time as the leaf emerges from the whorl and, once formed, do not expand (Braun et al., 2006). Lastly, in *tdy1* mutants, chlorotic tissues are often bounded by lateral veins, implicating this vein class in limiting the expansion of a *tdy1* chlorotic sector (Baker and Braun, 2007).

*Sxd1* encodes tocopherol cyclase, an enzyme functioning in tocopherol (vitamin E) biosynthesis, and *sxd1* mutants lack tocopherols (Sattler et al., 2003). Absence of
tocopherols results in callose deposition over the plasmodesmata at the bundle sheath-vascular parenchyma cell interface of leaf minor veins (Russin et al., 1996; Botha et al., 2000; Provencher et al., 2001). This occludes these passageways and prevents sucrose from moving into the vein, resulting in the build-up of carbohydrates in photosynthetic cells. The function of tocopherols in preventing callose deposition in the phloem is evolutionarily conserved. RNAi mediated suppression of tocopherol cyclase activity in potato results in callose deposition in vascular associated cells and carbohydrate accumulation in the photosynthetic cells (Hofius et al., 2004). In addition, in Arabidopsis tocopherol cyclase mutants and other vitamin E deficient mutants, callose is deposited in phloem parenchyma transfer cells and the photosynthetic cells accumulate carbohydrates if the plants are subjected to nonfreezing low temperatures (Maeda et al., 2006). It is not known how lack of tocopherols causes callose deposition.

Because both mutants show defects in carbon export from leaves, we examined whether Tdy1 and Sxd1 function in the same pathway regulating leaf carbohydrate accumulation. Using aniline blue fluorescence microscopy and transmission electron microscopy (TEM) we determined that carbohydrate accumulation in tdy1 mutant leaves does not result from a similar impediment to phloem loading as in sxd1 mutants. To genetically test whether the two genes function in the same pathway, we analyzed F2 families segregating both mutations. We observed that the double mutant plants showed an additive interaction for growth related phenotypes and soluble sugar accumulation indicating that the two genes function in separate genetic pathways. To determine if Tdy1 functions in the same biochemical pathway as Sxd1, we measured tocopherol levels in
mutant plants. We found that \textit{tdy1} mutants contain wild type levels of tocopherols, indicating that \textit{Tdy1} does not function in the same biochemical process as \textit{Sxd1}. Thus, from the combination of cellular, genetic and biochemical investigations we conclude that \textit{Tdy1} and \textit{Sxd1} function independently to regulate carbon export from leaves. Additionally, genetic and cytological studies suggest that \textit{Tdy1} functions within the veins and a model of possible TDY1 functions is discussed.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

Plants were grown in the summer in the Rock Springs Agronomy Farm, Pennsylvania State University, and in the winter in Juana Diaz, Puerto Rico. The \textit{tdy1-Reference (tdy1-R)} (Braun et al., 2006) and \textit{sxd1-1} (Russin et al., 1996) mutations were backcrossed to the B73 inbred line five times prior to crossing together. F$_2$ families were generated by crossing \textit{tdy1-R} and \textit{sxd1-1} mutant plants and self-pollinating the F$_1$ individuals.

3.2.2 Morphological analyses

Plant height was measured from the soil surface to the tip of the central spike of the tassel. Tassel length was measured from the node of the flag leaf to the tip of the central spike. Anthesis was recorded as the day of first pollen shed. Plants were scored as
having produced ears if either 1) ears were clearly visible, 2) silks were visible emerging from the leaf sheath, or 3) ears developed such that they were identifiable by peeling back the ear leaf sheath. For wild type and tdy1-R single mutants n = 18 for all measurements. For sxd1-1 single mutants, 12 of 16 plants produced a tassel that shed pollen, and 13 produced an ear. For tdy1-R; sxd1-1 double mutants, seven of 15 plants shed pollen and eight made an ear. Barren tassels that did not shed pollen were not included in the tassel length or anthesis measurements.

3.2.3 Aniline blue staining

Leaf strips 1 cm in width were cut from mutant and wild type leaves. The abaxial epidermis and the mesophyll cells directly beneath it were gently scraped away with a single-edge razor to expose the inner leaf tissues. The leaf strip was placed on a slide, briefly stained with a 0.05% aniline blue and immediately viewed using a 360-370 nm excitation filter and a 420 nm long pass emission filter on a Nikon Eclipse 80i fluorescent microscope. Epifluorescent illumination was provided by a 100W mercury lamp, and images were recorded using a DXM1200F Nikon digital camera.

3.2.4 TEM analyses

For tissue fixation, leaf samples were diced into 3 × 1 mm pieces, placed in 4% glutaraldehyde, 1% paraformaldehyde, 0.3% Tween 20, 50mM sodium cacodylate, pH 7.4, and vacuum infiltrated on ice for 4-6 hours. Samples were post-fixed in 1-2%
osmium tetroxide at 4°C overnight, subsequently dehydrated through a graded acetone series and embedded in Spurr’s epoxy resin (Spurr, 1969). Thin sections (90-100 nm) were cut on a Leica Ultracut E ultramicrotome with a glass knife and lifted onto 200-mesh copper grids. The grids were stained in 2% uranyl acetate, followed by Reynold’s lead citrate and observed with a JEOL JEM 1200 EXII at an accelerating voltage of 80 kV.

3.2.5 Chlorophyll quantification

Relative levels of total chlorophylls were quantified from leaves using a SPAD 502 Data Logger Chlorophyll Meter (Spectrum Technologies). For each tissue, 30 samples were measured, and the experiment was performed three times. Representative data from one replicate are shown.

3.2.6 Sugars and starch quantification

Leaf tissues were collected at the end of the photoperiod, weighed and frozen at -80°C. Carbohydrate extraction was performed according to (Dinges et al., 2001). Sugars and starch were quantified using commercial assay kits according to the manufacturer’s instructions (R-Biopharm). Six samples from each tissue type were measured in triplicate.
For visualizing starch in leaves, samples were collected at the end of the photoperiod, decolored by boiling in 95% ethanol and stained with iodine-potassium iodide (IKI) (Ruzin, 1999).

### 3.2.7 Tocopherol quantification

11 mm leaf discs were harvested with a cork borer from leaf tissues, weighed and immediately frozen on dry ice. Total lipids were extracted from collected tissues and tocopherols measured according to (Sattler et al., 2003).

### 3.3 RESULTS

#### 3.3.1 *tdy1-R* and *sxd1-1* single mutants both display variegated chlorotic leaves

*tdy1-R* and *sxd1-1* mutants show striking parallels in their leaf phenotypes. Both develop chlorotic sectors that hyperaccumulate starch and soluble sugars in leaf blades (Figure 3.1). However, there are also some distinctions. Whereas wild type leaves have a uniform, dark green color, *sxd1-1* mutant leaves develop a chlorotic phenotype that shows a continuum of expression from strongest at the tip to mildest towards the base (Figure 3.1A, B). In addition, at the tip and leaf margins, the chlorotic tissues progressively accumulate anthocyanin and excess starch (Figure 3.1B, E). In *sxd1-1* leaves, no clear, delineated boundaries occur between green and chlorotic regions. Conversely, *tdy1-R* mutants display a variegated pattern of chlorotic and normal
Figure 3.1: *tdy1-R* and *sxd1-1* leaves display chlorotic sectors that hyperaccumulate carbohydrates. A. wild type leaf showing uniform dark green color. B. *sxd1-1* mutant leaf displaying a chlorotic gradient, strongest at the tip to mildest towards the leaf base. Anthocyanins accumulate in the chlorotic tissues at the leaf tip and margins. C. *tdy1-R* mutant leaf containing variegated chlorotic and green sectors throughout the leaf blade. D-F. cleared, IKI stained leaf tissues showing that mutant chlorotic regions hyperaccumulate starch. D wild type. E. *sxd1-1*. F. *tdy1-R*. Scale bars represent 4 cm.
appearing green regions throughout leaf blade tissue (Figure 3.1C). Additionally, the boundaries between tdy1-R chlorotic and normal appearing green tissue are sharp and distinct, and they tend to occur at lateral veins (Baker and Braun, 2007). As in sxd1-1 mutants, chlorotic regions in tdy1-R mutants hyperaccumulate starch relative to green tissues (Figure 3.1F). tdy1 and sxd1 are the only known leaf variegation mutants that hyperaccumulate carbohydrates in any plant; therefore, we investigated whether Tdy1 and Sxd1 function in a common pathway regulating carbon partitioning in leaves.

3.3.2 Carbohydrate accumulation in tdy1-R mutants occurs by a different mechanism than in sxd1-1 plants

To address whether the two genes function in the same pathway, we investigated if the mechanism for carbohydrate accumulation was similar in the two mutants. Carbohydrate accumulation in sxd1-1 mutants results from a blockage in the symplastic pathway of sucrose movement (Russin et al., 1996; Botha et al., 2000; Provencher et al., 2001). Specifically, at the bundle sheath-vascular parenchyma cell interface of leaf minor veins, callose is deposited over the plasmodesmata in bundle sheath cells. This blockage prevents sucrose from being loaded into the phloem and results in carbohydrate accumulation in the photosynthetic cells. To determine if the tdy1-R leaf phenotype is similarly caused by callose deposits plugging the plasmodesmata, we performed aniline blue staining and fluorescence microscopy. Aniline blue binds callose and fluoresces a blue-white color under UV light. In wild type plants, only the sieve plates in the end walls of phloem sieve elements stain positively for callose (Figure 3.2B). As previously
Figure 3.2: Minor veins of *tdy1-R* mutant leaves lack callose deposits. A. Cross section of a wild type minor vein shown by UV autofluorescence indicating the cell types and orientation of view (arrow) for panels B-E. Abaxial cells were removed below the black dotted line to view the bundle sheath-vascular parenchyma cell interface. X, xylem, P, phloem, VP, vascular parenchyma, BS, bundle sheath, M, mesophyll. B-E show aniline blue fluorescence images of paradermal sections along minor veins. B. wild type cells lack punctate callose deposits indicating no blockages along the symplastic pathway. The bright fluorescence observed in the phloem corresponds to sieve plates (arrowhead). C. *sxd1-1* minor veins contain many callose deposits at the bundle sheath-vascular parenchyma cell interface (arrow). D. *tdy1-R* chlorotic tissue lacks callose deposition over plasmodesmata in any cells. E. *tdy1-R; sxd1-1* double mutants contain callose deposits (arrow) at the bundle sheath-vascular parenchyma cell interface comparable to the level seen in *sxd1-1* single mutants. Scale bar in A represents 50 µm, and in B-E 10 µm.
reported, *sxd1-1* mutants display many punctate callose deposits at the vascular parenchyma-bundle sheath cell interface of minor veins (Figure 3.2C) (Botha et al., 2000). In *tdy1-R* chlorotic regions, no ectopic callose deposits were detected at this interface (Figure 3.2D). Further, no callose deposits were observed in any cells of minor or lateral veins in *tdy1-R* chlorotic tissues, nor in any cells of *tdy1-R* green regions (data not shown). These data suggest that *tdy1-R* mutants do not accumulate carbohydrates due to callose blocking symplastic sucrose movement.

Though we did not observe ectopic callose deposition in any cells in *tdy1-R* mutant leaves using aniline blue staining, it is possible that symplastic sucrose transport is precluded due to a different defect in plasmodesmal structure. To investigate this possibility, we performed TEM to inspect the plasmodesmata ultrastructure along the symplastic pathway. In minor veins from wild type leaves, the plasmodesmata between bundle sheath and vascular parenchyma cells were unobstructed, spanned the cell wall and connected the cytoplasms between the two cells (Figure 3.3A). Consistent with previous reports, minor veins from leaf tips expressing the *sxd1-1* mutant phenotype showed occlusions over the plasmodesmata on the bundle sheath cell side of the bundle sheath-vascular parenchyma cell interface (Figure 3.3B) (Russin et al., 1996; Botha et al., 2000; Provencher et al., 2001). In *tdy1-R* mutant leaves, we found no occlusions over the plasmodesmata or alterations in their structure between the bundle sheath and vascular parenchyma cells in minor veins (Figure 3.3C). In examining plasmodesmata along the symplastic pathway at all cellular interfaces in *tdy1-R* leaves, we did not observe any
Figure 3.3: Plasmodesmata in tdy1-R mutants appear normal and lack occlusions. A-D show TEM images of the bundle sheath-vascular parenchyma cell interface of minor veins. Arrows in all panels indicate the location of the plasma membrane in the bundle sheath cell. A. wild type plasmodesmata span the cell wall, lack occlusions, and connect with the plasma membrane. B. sxd1-1 chlorotic leaf tips contain occlusions over the plasmodesmata on the bundle sheath cell side of the cell wall. C. tdy1-R chlorotic sectors contain normal appearing plasmodesmata that lack occlusions. Note the plasmodesmata span the cell wall and connect with the plasma membrane. D. tdy1-R; sxd1-1 double mutant chlorotic tissues contain deposits over the plasmodesmata in the bundle sheath cell similar to sxd1-1 single mutants. Scale bars represent 200 nm.
structural perturbations or occlusions (Figure 3.4). These data indicate that carbohydrate retention in *tdy1-R* mutant leaves does not appear to result from physical blockage or plasmodesmatal structural changes. Together, the aniline blue staining and TEM studies indicate that the cellular basis for the *tdy1-R* and *sxd1-1* phenotypes is different.

### 3.3.3 Double mutant plants show more severe growth defects than either single mutant

Both *tdy1-R* and *sxd1-1* single mutant plants have growth related defects due to the retention of carbohydrates in leaves. To determine whether the two genes function in the same genetic pathway regulating carbon export from leaves, we created F₂ families segregating both mutations. Both mutations are recessive and the F₂ segregation ratio fits the expected 9:3:3:1 prediction, indicating that neither mutant shows epistasis (Table 3.1). *sxd1-1* is a molecular null allele lacking transcripts (Provencher et al., 2001), and based on dosage analysis, *tdy1-R* is a genetic null allele (Braun et al., 2006). If *Tdy1* and *Sxd1* act in a linear genetic pathway, we would expect that the phenotype of the double mutant would be similar to one or the other mutant. If the two genes function in separate pathways, then we predict that the double mutant would be more severe than either single mutant.
Figure 3.4: Plasmodesmata appear normal and unobstructed at all cellular interfaces in *tdyl1-R* chlorotic tissues. TEM images of different cellular interfaces of wild type (A, C, E) and *tdyl1-R* chlorotic tissues (B, D, F). In all panels the plasmodesmata spanned the cell wall connecting the cytoplasms of adjacent cells and lacked occlusions. BS, bundle sheath, M, mesophyll. Scale bars represent 200 nm.
Table 3.1: Genetic segregation in F2 families

<table>
<thead>
<tr>
<th>Family #</th>
<th>Total</th>
<th>wild type</th>
<th>tdy1-R</th>
<th>sxd1-l</th>
<th>tdy1-R; sxd1-l</th>
<th>$\chi^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB 5503</td>
<td>93</td>
<td>48</td>
<td>22</td>
<td>16</td>
<td>7</td>
<td>2.0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>DB 5512</td>
<td>95</td>
<td>52</td>
<td>23</td>
<td>15</td>
<td>5</td>
<td>2.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>DB 5523</td>
<td>93</td>
<td>55</td>
<td>18</td>
<td>15</td>
<td>5</td>
<td>0.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Total</td>
<td>281</td>
<td>155</td>
<td>63</td>
<td>46</td>
<td>17</td>
<td>2.9</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

$\chi^2$ analyses of tdy1-R and sxd1-l segregation in three F2 families conforms to a 9:3:3:1 expectation. Observed numbers of individuals are shown.

To examine the interactions between tdy1-R and sxd1-l, we quantified the growth parameters of plant height, tassel size, time to anthesis and ear production (Figure 3.5 and Table 3.2). At the whole plant level, the double mutants express a more severe phenotype and show a stronger reduction in growth characteristics than either single mutant (Figure 3.5A). Compared to wild type, tdy1-R single mutants have an 11% decrease, sxd1-l mutants have a 24% reduction, and the tdy1-R; sxd1-l double mutant plants show the greatest reduction in plant height of 35.5%. Similar to plant height, tassel size is reduced 13% in tdy1-R plants, 29% in sxd1-l mutants, and 50% in tdy1-R; sxd1-l double mutants (Figure 3.5C and Table 3.2). In addition, approximately half of the tdy1-R; sxd1-l double mutant plants produced very reduced tassel branches lacking spikelets (Figure 3.5D). This phenotype was observed in 25% of the sxd1-l single mutants. Correlated with the tassel height defects, both mutations retarded time to flowering. Both tdy1-R and sxd1-l single mutants first shed pollen eight days later than wild type siblings.
(Table 3.2). Double mutant plants were delayed even further by approximately another five days. Similarly, wild type and tdy1-R mutant plants invariably made ears, and sxd1-1 mutants produced ears 81% of the time. In contrast, only 53% of the double mutant plants produced an ear (Table 3.2). All of the growth defects observed in the single and double mutants are consistent with a restriction in assimilates transported to the growing portions of the plants. The double mutant plants showed the most severe phenotypes suggesting they have a greater reduction in carbohydrates exported out of leaves than in either single mutant. In support of this, the leaves of tdy1-R; sxd1-1 double mutant plants are more strongly chlorotic than either single mutant, with a corresponding reduction in the amount of green tissue (Compare Figure 3.5B with Figure 3.1B, C). In double mutant leaves, the tip to base gradient of chlorosis and anthocyanin pigmentation characteristic of the sxd1-1 phenotype is superimposed on the tdy1-R chlorotic and green sectored pattern. Double mutant leaves still contain some green tissues located at the leaf base as in sxd1-1 single mutants; however, the boundaries between the chlorotic and green regions are sharp and distinct as in tdy1-R mutants (Figure 3.5B). Thus, the double mutant leaves express the phenotypes of both single mutants, and double mutant plants display stronger growth retardation than either single mutant.
### Table 3.2: Morphometric analyses in F$_2$ families

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Plant Height</th>
<th>% of wt</th>
<th>Tassel length</th>
<th>% of wt</th>
<th>Anthesis</th>
<th>% ears</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>220.4$^y$ ± 0.39</td>
<td>100</td>
<td>55.2$^y$ ± 0.14</td>
<td>100</td>
<td>71.9$^y$ ± 0.06</td>
<td>100</td>
</tr>
<tr>
<td>$tdy1-R$</td>
<td>196.8$^x$ ± 0.92</td>
<td>89.2</td>
<td>47.9$^x$ ± 0.28</td>
<td>86.8</td>
<td>80.3$^x$ ± 0.13</td>
<td>100</td>
</tr>
<tr>
<td>$sxd1-1$</td>
<td>168.9$^y$ ± 1.24</td>
<td>76.6</td>
<td>39.3$^y$ ± 0.18</td>
<td>71.2</td>
<td>80.3$^x$ ± 0.09</td>
<td>81</td>
</tr>
<tr>
<td>$tdy1-R; sxd1-1$</td>
<td>142.2$^z$ ± 0.79</td>
<td>64.5</td>
<td>26.8$^z$ ± 0.34</td>
<td>48.6</td>
<td>84.9$^y$ ± 0.13</td>
<td>53</td>
</tr>
</tbody>
</table>

Values are the means ± SE. Plant height and tassel length are measured in cm. Anthesis is measured as the number of days after planting until first pollen shed. Percent ears indicates the frequency each phenotypic class produced an ear. Different letter superscripts within a column denote statistical significance determined using the Student’s t-test.
Since double mutants have more extensive chlorosis in their leaves, we ascertained whether the callose deposits at the vascular parenchyma-bundle sheath cell interface observed in \textit{sxd1-1} single mutants were more severe in the double mutants. Using aniline blue fluorescence, the double mutants showed no enhancement of the callose deposition phenotype and appeared similar to \textit{sxd1-1} single mutants (Figure 3.1C, E). Similarly using TEM, we found blockages over the plasmodesmata on the bundle sheath cell side of the bundle sheath-vascular parenchyma cell interface of minor veins in the double mutants comparable to what we observed in \textit{sxd1-1} single mutant leaves (Figure 3.5B, D). In neither case was there any increase in the severity of the phenotype. Hence, \textit{tdy1-R} and \textit{sxd1-1} do not display a synergistic interaction as the double mutant leaves express both single mutant phenotypes. Rather, these data suggest that the phenotypes observed in the double mutants are additive, and that the genes act in independent pathways.
Figure 3.5: *tdy1-R* and *sxd1-1* mutant phenotypes display an additive interaction. A-D show growth related phenotypes of plants from a segregating F$_2$ family. In A and C the order from left to right corresponds to wild type, *tdy1-R*, *sxd1-1*, and *tdy1-R; sxd1-1* double mutant. A. Double mutants show the greatest reduction in plant height compared to either single mutants or wild type siblings. B. Double mutant leaves are severely chlorotic with lighter green tissues restricted to the leaf base. Distinct borders between the chlorotic and green tissues characteristic of *tdy1-R* are visible at the leaf base. The strong tip to base chlorosis gradient of *sxd1-1* expression is evident. C. Tassel height is most strongly reduced in *tdy1-R; sxd1-1* double mutants compared with *sxd1-1* and *tdy1-R* single mutants or wild type siblings. D. Approximately half of the *tdy1-R; sxd1-1* double mutant plants produced barren tassels lacking spikelets. Scale bars represent 4 cm.
3.3.4 Leaf tips of double mutant plants have the greatest reduction in chlorophyll levels

*tdy1-R* and *sxd1-1* single mutants both show variegated chlorotic regions in their leaves (Figure 3.6B, C). We quantified total chlorophyll levels to determine whether the double mutants were more strongly affected than either single mutant (Figure 3.6). We observed a decreasing series in chlorophyll content with the strongest reduction in the tips of double mutant leaves < the chlorotic regions of either *tdy1-R* or *sxd1-1* single mutant leaves < the green base of *sxd1-1* or double mutant leaves < green regions of *tdy1-R* mutants or wild type leaves. The leaf bases of *sxd1-1* mutants and the double mutants displayed a mild chlorosis phenotype with approximately 70% as much chlorophyll as wild type leaves indicating that these tissues are affected by the *sxd1-1* mutation. As the tips of double mutant leaves showed a more severe chlorosis phenotype than either single mutant, it suggests that *Tdy1* and *Sxd1* function in separate pathways.

3.3.5 Sugar accumulation in double mutant leaves is greater than in either single mutant

Both single mutants accumulate excess starch in the chlorotic regions of their leaves (Figure 3.1E, F). To investigate whether *Tdy1* and *Sxd1* act in the same or separate genetic pathways regulating carbohydrate accumulation in leaves, we determined the starch and soluble sugars content in different phenotypic regions of F2 plants (Figure 3.7). For both the green regions and the chlorotic regions, we found that the double mutants
contained similar amounts of starch as the single mutants. In contrast, we determined that
the levels of soluble sugars differed with respect to genotype and phenotype. As
previously reported, we found green regions of *tdy1-R* leaves had sucrose, glucose and
fructose levels indistinguishable from wild type (Braun et al., 2006). However, green leaf
bases of the double mutants and *sxd1-1* single mutants contained approximately four-fold
higher levels of glucose and fructose as wild type or *tdy1-R* green tissues. Among the
chlorotic leaf tissues, the double mutant leaf tips had the highest concentrations of sugars,
with approximately 33% greater amounts of glucose, and a significant increase in sucrose
content compared with either single mutant. Double mutant leaf tips also contained ~30%
more fructose than *sxd1-1* leaf tips and approximately two-fold greater levels than in
*tdy1-R* chlorotic regions. Overall, sugar concentrations in green tissues of double mutant
leaves are more similar to *sxd1-1* leaf bases than to *tdy1-R* green regions, whereas in
chlorotic tissues, the double mutants contain more soluble sugars than either single
mutant. Because the double mutants show greater individual sugar accumulation than
either single mutant, these data support the hypothesis that *Tdy1* and *Sxd1* function in
independent pathways regulating carbon export from leaves.
Figure 3.6: *tdy1; sxd1* double mutant chlorotic region contain the least amount of chlorophyll. The first four bars represent green tissues from wild type, *tdy1* single mutant, *sxd1* single mutant and *tdy1; sxd1* double mutant from left to right. The last three bars represent chlorotic tissues from *tdy1* single mutant, *sxd1* single mutant and *tdy1; sxd1* double mutant from left to right. Bars in the graphs show the mean of 30 samples and error bars show the SE. dm represent *tdy1; sxd1* double mutant. The unit for chlorophyll measurement is relative amount. Different letters for each bar in all of the graphs indicate statistical analysis of significant differences using Student’s *t* test.
Figure 3.7: Sugar accumulation in double mutant leaves shows an additive interaction between *tdy1-R* and *sxd1-1*. Units for all panels are mg carbohydrate/g fresh weight. Values are the means of 18 samples ± SE. Abbreviations are wt, wild type, dm, *tdy1-R; sxd1-1* double mutant. Different letters indicate statistically significant differences between samples determined using the Students t-test.
3.3.6 *Tdy1* does not function in the same biochemical pathway as *Sxd1*

*Sxd1* encodes tocopherol cyclase, the penultimate enzyme in the vitamin E biosynthesis pathway, and *sxd1* mutants lack tocopherols (Sattler et al., 2003). To determine whether *Tdy1* functions in the same biochemical pathway as *Sxd1*, we quantified tocopherol levels in *tdy1-R* plants (Table 3.3). We found no statistically significant changes in tocopherol quantities in *tdy1-R* chlorotic or green regions in comparison to wild type tissue. *sxd1* mutants had undetectable levels of tocopherols as previously reported (Sattler et al., 2003). Hence, *tdy1-R* does not have a lesion in tocopherol synthesis or accumulation, and *Tdy1* does not function in the same biochemical pathway as *Sxd1*.

**Table 3.3: Tocopherol quantification**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>118.6 ± 3.59</td>
</tr>
<tr>
<td><em>tdy1-R</em> green</td>
<td>101.0 ± 3.17</td>
</tr>
<tr>
<td><em>tdy1-R</em> chlorotic</td>
<td>112.3 ± 6.17</td>
</tr>
<tr>
<td><em>sxd1-1</em></td>
<td>nd</td>
</tr>
</tbody>
</table>

Values are means of six samples ± SE measured in μg/ g fresh weight. nd indicates none detected. Values indicated by a were not statistically different as determined using the Student’s t-test.
3.4 DISCUSSION

3.4.1 Tdy1 and Sxd1 independently control carbon export in maize leaves

Tdy1 and Sxd1 are the only known mutations in plants that confer a variegated leaf phenotype with sectors that hyperaccumulate carbohydrates. Using a combination of biochemical, cytological and genetic analyses we investigated whether Tdy1 and Sxd1 act in a common pathway. Whereas sxd1 mutants lack tocopherol, we determined that tdy1-R mutants accumulate wild type levels of tocopherols. Therefore, Tdy1 does not function in the same biochemical process as Sxd1. sxd1 mutants accumulate carbohydrates in leaf tissues due to callose being deposited over the interface of bundle sheath-vascular parenchyma cells in leaf minor veins which blocks sucrose symplastic movement. Using aniline blue fluorescence microscopy we did not identify any ectopic callose deposition in tdy1-R mutant leaf tissues. Moreover, using TEM we did not observe any structural alterations to the plasmodesmata, indicating that the symplastic loading pathway is not impeded. This suggests that tdy1-R mutants do not accumulate carbohydrates due to a physical occlusion, but rather due to a different cellular mechanism. This was confirmed by double mutant analyses which showed that the two mutations had an additive interaction. We found that the double mutants expressed both single mutant leaf phenotypes, that the reductions in plant height, tassel size and chlorophyll levels were additive, and that the accumulation of sugars in chlorotic leaf tissues of the double mutants was greater than in either single mutant. In addition, half of the double mutants produced ears whereas the tdy1-R mutant frequency was indistinguishable from wild type.
and 81% of the sxd1-1 single mutants made ears. The increased frequencies in the failure to make ears and production of barren tassels observed in the double mutants are most likely explained by an additive retention of carbohydrates in leaf tissues and a concomitant failure to export sucrose to the developing inflorescences. Collectively, these data lead us to conclude that Tdy1 and Sxd1 function independently, and that tdy1 defines a distinct genetic pathway regulating carbon partitioning in maize leaves.

3.4.2 Chlorotic leaf tissues do not retain sink identity

We previously suggested that one possibility to explain the excess carbon accumulation in tdy1-R chlorotic leaf sectors was a block in the developmental transition from sink to source identity, such that the chlorotic sectors continued to import photoassimilates from neighboring green tissues (Braun et al., 2006). If the chlorotic tissues were to remain sinks, the large amounts of green source tissues could potentially provide the carbohydrates accumulating in the chlorotic regions. However, based on our genetic analyses, our data do not support this hypothesis. In the tdy1-R; sxd1-1 double mutants, the amount of green leaf tissue is greatly reduced and is restricted to a narrow region at the very base of the leaves. Additionally, this green tissue displays mild chlorosis and an increase in carbohydrate accumulation indicating that it is perturbed in carbon export. As this tissue is proximal to and up to a meter distant from the chlorotic tip of adult leaves, it is highly unlikely to be providing the carbohydrates that accumulate in the cells near the tip. Furthermore, half of the double mutant plants produced a tassel and an ear. These are sink tissues dependent on the photosynthetic source leaves for their
carbohydrates. Because the great majority of the area of double mutant leaves is severely chlorotic, yet still must be capable of exporting photoassimilates to produce the developing reproductive tissues, our data argues against the chlorotic tissues remaining sinks and does not support the hypothesis that Tdy1 functions to regulate the sink to source transition. Rather, the new data lead us to favor the hypothesis that the cells in the tdy1 chlorotic region are source tissues that are partly blocked in carbon export capacity.

3.4.3 A model for TDY1 function

In wild type maize leaves, sucrose is synthesized in mesophyll cells, diffuses through plasmodesmata into bundle sheath cells and then into the vascular parenchyma cells (Figure 3.8A). Sucrose is exported from the vascular parenchyma cell to the apoplast, and then loaded into the phloem by sucrose transporters in the plasma membrane of the companion cell and/or sieve element for transport through the veins (Lalonde et al., 2004; Sauer, 2007). sxd1 mutant leaf tips are blocked in this pathway at the bundle sheath-vascular parenchyma cell interface (Figure 3.8B) (Russin et al., 1996; Botha et al., 2000; Provencher et al., 2001).

tdy1 chlorotic sectors accumulate excess carbohydrates indicating they have a defect in phloem loading. In agreement with this, we previously localized the site of TDY1 function to the innermost tissue layer of leaves comprised of the veins, bundle sheath cells, and interveinal mesophyll cells (Baker and Braun, 2007). Due to the limits of the experiment it was not possible to further delineate where within this tissue layer
TDY1 acts. However, the cytological investigations presented herein resolve this ambiguity. As we observed no blockages or alterations to plasmodesmatal structure along the sucrose symplastic pathway in *tdy1-R* leaves, the defect in carbon export in *tdy1* chlorotic regions must occur at a later step in phloem loading. This suggests that TDY1 functions in the veins (Figure 3.8C). Due to the variegated phenotype of *tdy1* mutants, we hypothesize that TDY1 acts as a regulator of sucrose transport rather than as a transporter itself, as loss of transporter function would be predicted to have uniform carbohydrate accumulation and chlorosis phenotypes (see (Braun et al., 2006; Baker and Braun, 2007) for discussion). Two possibilities are envisioned for *Tdy1* function. The first possibility is that *Tdy1* may regulate the sucrose efflux step from vascular parenchyma cells. Failure to export sucrose to the apoplast would create a bottleneck restriction leading to the build-up of sucrose in the vascular parenchyma cells and, in turn, to carbohydrate accumulation in the bundle sheath and mesophyll cells. The second possibility for *Tdy1* function is controlling the activity of sucrose transporters within the companion cells and sieve elements. In this case, failure to load sucrose into the phloem is predicted to lead to excess accumulation in the apoplast. This would shift the equilibrium for sucrose export from vascular parenchyma cells thereby leading to increased concentration within these cells, and ultimately to carbon accumulation in the photosynthetic cells. In support of this, antisense expression or mutation of phloem sucrose transporters causes an accumulation of carbohydrates in photosynthetic cells and chlorosis (Riesmeier et al., 1994; Burkle et al., 1998; Gottwald et al., 2000), similar to *tdy1-R* phenotypes.
Our analyses of the *tdy1-R* mutant phenotype and genetic interactions between *tdy1-R* and *sxd1-1* have shown that *Tdy1* acts independently of *Sxd1*. Thus, the *tdy1* mutation identifies a separate genetic pathway controlling carbon accumulation in maize leaves. Future work characterizing the molecular function of *Tdy1* and its potential role in the regulation of sugar transporter activity will determine the mechanism by which *Tdy1* regulates carbon partitioning. Understanding the control of carbon export from leaves will lead to novel strategies to manipulate carbon allocation and biomass deposition which may have applications in the production of biofuels.
Figure 3.8: Models for TDY1 regulating phloem loading of sucrose. Diagrams represent the pathway of sucrose movement from photosynthetic cells into the phloem. M, mesophyll, BS, bundle sheath, VP, vascular parenchyma, CC, companion cell, SE, sieve element. Green and yellow ovals in M and BS cells represent chloroplasts. White circles within chloroplasts represent starch grains. Magenta box on VP plasma membrane represents sucrose efflux transporter, and blue boxes on CC and SE plasma membranes depict sucrose transporters. Arrows indicate direction of sucrose movement.

A. wild type tissue with normal, unimpeded sucrose movement into the phloem. B. *sxd1-1* mutant chlorotic tissue has callose deposits (grey box) over the plasmodesmata at the BS-VP cell interface blocking sucrose movement and resulting in carbohydrate accumulation in M and BS cells. C. *tdy1-R* chlorotic tissue has normal appearing plasmodesmata and lacks callose deposits. We hypothesize that either the sucrose efflux transporter (the first ×) or the CC-SE sucrose transporters (the right two ×) are defective. This would result in failure to export sucrose and lead to the carbon hyperaccumulation observed in the photosynthetic cells.
Chapter 4

*Tdy1* cloning and expression analysis

Notes:

1. The *in situ* hybridization work was performed by Frank Baker.
Chapter 4

*Ty1* cloning and expression analysis

4.1 INTRODUCTION

*Mutator* is one of the transposon families used in large-scale mutagenesis experiments in maize as it generates mutants at a 30-50 fold higher mutation frequency compared with normal lines (Robertson, 1978). *Mu* elements have conserved ~220 bp terminal inverted repeats, while the internal sequences are distinct (Walbot, 1991). Typically, *Mu1* has the highest copy number of the *Mu* elements and usually has the highest insertion frequency. Thus, most of the *Mu* induced mutations that have been characterized are due to a *Mu1* insertion (Bennetzen et al., 1993).

Transposon tagging is a technology used for both forward and reverse genetics (Brutnell, 2002; Settles et al., 2004). In forward genetics, the transposons are used as gene tags to generate mutants and isolating the corresponding genes. Many genes have been cloned using this approach (Walbot, 1991; Gao et al., 1998). Due to the high copy number of *Mu* elements in maize *Mutator* active lines, it is generally necessary to reduce the *Mu* copy number by backcrossing the mutant plant to normal non-*Mu* containing lines prior to undertaking a cosegregation analysis (Chomet, 1993). Cosegregation is a crucial step in cloning the desired gene from the mutant, and it is used to measure the linkage between the transposon and the gene of interest (Walbot, 1992).
Generally, a Southern blot is the technique used for cosegregation analysis. Ideally, the hybridized band from the restriction enzyme digested mutant genomic DNA corresponds to the transposon that is responsible for the mutation, while no band should be detected from the digested homozygous wild type genomic DNA. However, it is not always the case that the sequence flanking the transposon is inserted into the desired gene responsible for the phenotype of interest. One strategy to increase the likelihood of cloning the correct gene is to increase the sample population by testing DNA from diverse mutant sources (Walbot, 1992). Additionally, choosing proper restriction enzymes is also critical for cloning the cosegregating fragment because neither a large nor a small fragment is easy to work with for cloning the gene. If the fragment is too big, it is difficult to detect on the Southern blot, while if the fragment is too small, only a small piece of the gene can be obtained and may make future analysis difficult. Hence, multiples enzymes should be examined.

_Tdy1_ was proposed to regulate sucrose transporters based on the phenotype and from a clonal analysis indicating the gene acts in the middle tissue layer of the leaf, which consists of the bundle sheath cells and the vein tissues (Baker and Braun, 2007). Sucrose transporter genes and/or cDNAs from both dicot and monocot plants have been cloned over the past decade (Sauer, 2007). Sucrose transporters in higher plants are all members of gene families and are divided into four groups. Group 1 contains sucrose transporters from monocots, especially cereal crops, such as rice OsSUT1 (Hirose et al., 1997; Scofield et al., 2007), sugarcane ShSUT1 (Rae et al., 2005; Reinders et al., 2006), barley HvSUT1 (Weschke et al., 2000), wheat TaSUT1 (Aoki et al., 2002) and maize
ZmSUT1 (Aoki et al., 1999). Most of these SUTs have been functionally characterized and shown to transport sucrose (Hirose et al., 1997; Aoki et al., 1999; Weschke et al., 2000; Reinders et al., 2006).

Currently, three SUT genes have been identified in maize. Based on its expression pattern, ZmSUT1 is proposed to be involved in phloem loading (Aoki et al., 1999). Two other maize sucrose transporter cDNAs were isolated from immature kernels, ZmSUT2 and ZmSUT4, but the functions of these two genes are unknown. However, as functional characterizations of sucrose transporters from the same groups as ZmSUT2 and ZmSUT4 have been studied, there might be some hints for the function of these two genes. AtSUC3 (also known as AtSUT2) is in group 3 as is ZmSUT2. AtSUC3 has been localized to sieve elements in the leaf phloem of Arabidopsis, and it is expressed highly in sink tissues as well (Meyer et al., 2004). AtSUC3 has been proposed to have a sugar sensor function in addition to sugar transport activity (Barker et al., 2000), though there is debate about this interpretation (Meyer et al., 2004). ZmSUT4, AtSUC4 and HvSUT2 are all in group 4, and AtSUC4 and HvSUT2 are tonoplast localized sucrose transporters (Endler et al., 2006). However, there is some controversy in the literature because other group 4 sucrose transporter proteins, StSUT4, LeSUT4 and AtSUT4, were localized to sieve elements (Weise et al., 2000). The contradiction arises from the fact that sieve elements do not possess vacuoles. Despite this discrepancy, group 4 sucrose transporters are speculated to be vacuole-localized in non-sieve element cells and perhaps also function in sink tissues (Sauer, 2007).
Many sucrose transporters have been localized to sink tissues where phloem unloading occurs. Typically, phloem unloading uses a symplastic pathway by transporting sucrose from the sieve elements into the sink cells, such as root tips and immature leaves, through plasmodesmata although some sink cells, such as pollen grains, import sucrose apoplastically (Sauer, 2007). These sink cell localized sucrose transporters may play a role in retrieval of sucrose leaked to the apoplast, and function to transport sucrose to sink cells if an apoplastic route is used.

The previous two chapters characterized the function of \textit{Tdy1} function through genetic and physiological analyses, and we postulated that TDY1 may be involved in regulating phloem loading of sucrose. In this chapter, I will describe the cloning of \textit{Tdy1} from a \textit{Mu1}-tagged allele and present some initial expression studies. Semi-quantitative RT-PCR analyses revealed that \textit{Tdy1} is expressed in all tissues tested and is highly expressed in sink tissues except for immature leaves. As sucrose transporters function in phloem loading, the three known sucrose transporters in maize, \textit{ZmSUT1}, \textit{ZmSUT2} and \textit{ZmSUT4}, were examined for their expression pattern in the same tissues tested for \textit{Tdy1}. We detected overlapping tissue expression between \textit{Tdy1} and the three \textit{SUTs} indicating that \textit{Tdy1} may regulate multiple \textit{SUTs}. Consistent with its proposed role regulating SUT function, \textit{Tdy1} mRNA is localized in companion cells and sieve elements. Together with the high expression in sink tissues, these data suggest a possible role for TDY1 in regulating phloem loading and unloading as well as possibly interacting with sucrose transporters.
4.2 MATERIALS AND METHODS

4.2.1 Plant Materials

tdy1-R plants were crossed to Mutator (Mu) active lines to generate Mu-insertion alleles. The putative Mu insertion mutants were crossed to tdy1-R for complementation testing, and were repeatedly backcrossed to B73 and Mo17 inbred lines. In addition, Mu-inserted tdy1 mutants were crossed to Mu killer plants (Slotkin et al., 2003) to silence Mu activity and backcrossed to inbred lines to eliminate unlinked Mu elements to facilitate gene cloning.

4.2.2 Genomic DNA Southern blotting

The mutation used for the gene cloning is tdy1-D190 (hereafter D190). The inbred lines used were B73, Mo17, a1-mum2 and bz1-mum9. Genomic DNA was first extracted using “Damon’s all natural genomic DNA miniprep” protocol. 250 mg of young leaf tissue was harvested and ground in liquid nitrogen for about 1 min until the leaf tissue turned into fine powder. The power was resuspended in 1 ml of 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl and ground in the buffer. The slurry was transferred into a 1.5 ml eppendorf tube, 150 µl of 10% SDS was added and the tubes incubated at 65°C for 20 min. After incubation, 300 µl of 5 M KOAc was added, mixed and chilled on ice for 30 min. Cell debris was removed by centrifugation for 5 min at 14000 rpm. 900 µl of supernatant was transferred to a fresh microtube, 630 µl of isopropanol added,
and the mixture was spun for 5 min to pellet the DNA. The supernatant was discarded, the pellet was washed with 500 µl of 70% ethanol and spun for another 3 min. The washed pellet was dried at room temperature and was dissolved in 100µl water. 20 µl of the genomic DNA was used per lane for the Southern blots.

Genomic DNA was digested with different restriction enzymes, EcoRI, BamHI, BglII, SacI and HindIII, at 37°C for 2 hr, and the digested DNA samples were fractionated on a 0.9% agarose gel at 50 V for 20 hr. DNA was transferred to a membrane by Southern Blotting. The DNA was crosslinked to the membrane using an Ultra-LUM UVC-508 UV crosslinker (120,000 µJ of UV energy) for 1 min and hybridized with α-32P-dCTP labeled Mu1, Mu3, Mu8 and MuDR probes. The labeling procedures followed the manufacturer’s manual from Stratagene (La Jolla, CA). The blots were pre-hybridized in the hybridization buffer (6 × SSC, 2 mM EDTA, 10 mM Tris pH 7.5, 5 × Denhardts, 0.2 mg/ml sonicated salmon sperm DNA, 20 mM pH 7.4 NaPO4 buffer) for at least 15 min, the denatured probe was added and hybridized overnight for 10-12 hr. For the Mu1 probe, the temperature was 68°C and for the other probes, 65°C. The blots were washed three times for 30 min in the wash solution (0.2 × SSC and 0.2% SDS) at 68°C or 65°C and exposed to X-ray film (Kodak, BioMax, Rochester, NY) for a few days depending on the signal strength. The blots were stripped in boiling 0.1 × SSC, 0.1% SDS solution for 15 min twice before being used for hybridization with another probe.
4.2.3 Construction and screening of a \textit{tdy1-D190} sub-genomic DNA library

4.2.3.1 Preparation of genomic DNA and ligation products

Genomic DNA was isolated as described above and digested with \textit{Bam}HI for 2 hr. The digested genomic DNA was fractionated on a 0.9% agarose gel. The agarose gel slices containing the 5.3 kb \textit{Mu1} element were excised and the DNA was purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) following the manufacturer’s instruction. 2.3 µl purified DNA (0.4 µg) was ligated with 1 µg commercial \textit{Bam}HI digested and CIAP treated ZAP Express vector (Stratagene, La Jolla, CA), 0.5 µl 10× ligase buffer and 0.5 µl T4 DNA ligase at 16°C overnight.

4.2.3.2 Packaging of the DNA from the ligation

The packaging extracts were also from Stratagene (La Jolla, CA). When the packaging extract just began to thaw, 4 µl (1 µg) of the ligated DNA was immediately added to the tube and mixed well by stirring with a pipet tip. The tube was incubated at room temperature for no more than 2 hr, and 100 µl SM buffer (0.1 M NaCl, 0.01 M MgSO\textsubscript{4}, 0.05 M Tris-HCl pH 7.5, 0.01% gelatin) and 20 µl chloroform were added to the tube and the contents were mixed gently. The tube was spun briefly for a few seconds in a microcentrifuge to sediment the debris, and the supernatant which contained the phage was titered.
4.2.3.3 Titering of the packaging product

Before titering the packaging reaction product, host bacteria cells were prepared. The strain provided in the kit is XL1-Blue MRF’. The cells were first streaked onto an LB agar plate containing 50 µg/ml of tetracycline and incubated at 37°C overnight. Single colonies were inoculated in 50 ml LB broth with 500 µl 20% maltose plus 500 µl 1 M MgSO₄ and incubated with shaking at 30°C overnight. The next day, the cells were collected and resuspended in 25 ml sterile 10 mM MgSO₄. The resuspended cells were used immediately or stored at 4°C for later use. The XL1-Blue MRF’ cells were diluted in sterile 10 mM MgSO₄ to an OD₆₀₀ of 0.5. In order to test the titer of the packaged ligation product, 1µl of the final packaged reaction and a 1:10 dilution in SM buffer of the final packaged reaction were mixed with 200 µl diluted XL1-Blue MRF’ cells, and the mixture was incubated at 37°C for 15 min to allow the phage to attach to the bacteria cells. After the incubation, the cells were added to 3 ml of melted NZY top agar (~48°C) and the top agar was plated immediately onto dry, prewarmed NZY agar plates (37°C). After the top agar cooled, the plates were incubated at 37°C overnight. The next day, the plaques were visible and counted to determine the titer in plaque-forming units per milliliter (pfu/ml).
4.2.3.4 Lifting of plaques

From the titering estimates, the library was plated at ~40,000 plaques/plate and eight plates were screened. To screen the library, nitrocellulose membrane were placed onto NZY agar plates, which had been chilled at 4°C for 2 hr to prevent the top agar from sticking to the nitrocellulose, for ~2 min to allow the transfer of the phage to the membrane. A syringe needle dipped in India ink was used to mark three positions on the plate in order to orient the membrane in relation to the agar. A duplicate membrane from the same plate was also prepared and screened.

4.2.3.5 Screening of the lambda library

The membranes from the lifting step were floated on denaturation solution for 2 min, and then neutralization solution for 5 min. The membranes were then rinsed by submerging in a 2 × SSC solution for less than 30 sec and blotted on to Whatman 3MM paper. The DNA was crosslinked to the membranes and the blots were immediately hybridized with a Mu1 probe. The washing and exposure procedures are the same as for genomic DNA Southern blotting. In order to locate the hybridized plaques on the plates, the blots were exposed to X-ray films overnight. The films were then compared back to the plates to locate the plaques that correspond to the signals on the film.

To enrich and purify for the phage of interest, agar plugs of the region were removed from the primary plate using modified wide bore 1 ml pipette tips, put into
microtubes with 500 µl SM buffer and 20 µl chloroform, and incubated for 1-2 hr at room temperature. The eluted phages were titered as described above and then secondary followed by, tertiary screening was similarly performed. The purified phage stock from the final round was stored at 4°C.

4.2.3.6 In vivo excision of the pBK-CMV phagemid vector from the ZAP Express vector

XL1-Blue MRF’ and XLOLR cells were grown overnight in 50 ml LB broth (with 0.01 M MgSO₄ and 0.2% maltose) at 30°C. The next day, the bacteria solutions were transferred to 50 ml conical tubes and spun down at 1000 × g using a benchtop clinical centrifuge. The cell pellets were resuspended in 25 ml of 10 mM MgSO₄ and the OD₆₀₀ was adjusted to 1.0 (~8 × 10⁸ cells/ml) with 10 mM MgSO₄. 200 µl of the prepared XL1-Blue MRF’ cells, 250 µl of the purified phage stock and 1 µl of the ExAssist helper phage from the kit were combined together in a 15 ml Falcon tube and incubated at 37°C for 15 min for the phage to attach to the bacteria cells. 3 ml of LB broth (plus 0.01 M MgSO₄ and 0.2% maltose) was added to the tube for another 2.5-3 hr incubation at 37°C with shaking. The incubated tube was heated at 65-70°C for 20 min to lyse the lambda phage and the bacteria cells, and then spun at 1000 × g in a benchtop clinical centrifuge for 15 min to pellet cell debris. The supernatant which contained the excised pBK-CMV phagemid was transferred to a new Falcon tube and stored at 4°C.
To isolate the recombinant plasmid containing colonies, 200 µl of freshly grown XLOLR from the above step was separately combined with 100 or 10 µl of the excised pBK-CMV phagemids in two microtubes, and the tubes were incubated at 37°C for 15 min. After the incubation, 300 µl of NZY broth was added to each tube for 45 min incubation at 37°C. Then each of the incubated cell mixtures was plated on LB-kanamycin (50 µg/ml) agar plates and the plates were incubated at 37°C overnight.

4.2.3.7 Sequencing of the pBK-CMV plasmid

Single colonies obtained from the above step were incubated in 3 ml LB broth (50 µg/ml kanamycin) with shaking overnight at 37°C. The plasmids were extracted using plasmid miniprep kit (QIAGEN, Valencia, CA). The plasmids were sequenced using two primers from the plasmid sequence, a forward primer, T7, and a reverse primer, BK reverse, from each side of the vector. Additional, gene specific primers were designed from the obtained sequence for sequencing, and the procedure repeated to sequence the insert. The sequence was used to blast Genbank for related sequences.
4.2.3.8 Sequencing of different tdy1 alleles

To characterize the tdy1 alleles isolated from Mu active populations, tdy1-D24, tdy1-D46 and uniform Mu, a Mu primer (Mu34.56 5’-CGCCAACGCTCCATTTTCG TCGAATCC-3’) from the Mu TIR (Terminal Inverted Sequences) and a tdy1 specific primer (239_rev 5’-GCAGCGATGCGTCTGCGGCT -3’) were used for PCR amplification. The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and the primer used for sequencing was 239_rev. To characterize the mutation in the reference allele, tdy1-R, the coding sequence was amplified using 239_rev and YM10 (5’-ACGCCGCGCATCCAACGCTA A). The PCR product was purified and 3 μl of the purified product was mixed with 5 μl of the 2 × Rapid Ligation Buffer, 1μl of the pGEM-T Easy vector (Promega, Madison, WI), 1μl of T4 ligase in a microtube and incubated at 16°C overnight. The next day, 1μl of the ligation product was used to transform MACH1 heat-shock competent cells. For the transformation, 1 μl of the ligation was added to 50 μl of the MACH1 competent cells in a microcentrifuge tube and was mixed gently using a pipette. The tube was incubated on ice for 30 min, heat-shocked in a 42°C heat-block for 30 sec and put on ice immediately after the heat-shock. 450 μl of the LB broth was added to the tube and the tube was shaked horizontally at 37°C for 1 hr. During the shaking, 20 μl of 50 mg/ml X-Gal and 100 μl of 100mM IPTG was spread on LB plates containing 100 μg/ml ampicillin and the plates were prewarmed at 37°C for 30 min prior to use. 100 μl and 200 μl of the incubated culture were plated on two prepared LB plates and the plates were incubated
overnight at 37°C. The next day, white colonies were selected and inoculated in 3 ml LB broth (100 µg/ml ampicillin) with shaking for ~12-16 hr. Plasmids were extracted using the QIAprep Miniprep Kit (QIAGEN, Valencia, CA). The plasmids were digested with EcoRI to verify that the insert was correct, and then the plasmids were sequenced using the 239_rev and YM10 primers. The sequence of the tdy1-R mutation was compared with B73 genomic DNA sequence to identify the mutated site using the online software from http://workbench.sdsc.edu/.

4.2.4 RNA extraction and 5’-RACE

50 mg of maize mature (leaf 5, fully emerged) and immature (leaf 10, unexposed and within the whorl) leaf tissues, immature ears (1 cm long), immature tassels (1.5 cm long) and young roots (1 week old) samples were collected. Total RNA was isolated from the samples using RNAgents Total RNA Isolation System (Promega, Madison, WI) following the manufacturer’s instruction. The RNA concentration was measured using a nanodrop spectrophotometer. 5 µg RNA was used for DNase treatment and cDNA was synthesized from 1.5 µg DNase treated RNA using the iScript™cDNA Synthesis Kit (Bio-Rad, Hercules, CA) following the manufacturer’s instruction. The cDNA was stored at -20°C for later use. cDNA that was used immediately and frequently was stored at 4°C.
5’-RACE was performed using the FirstChoice RLM-RACE kit (Ambion, Austin, TX) according to manufacturer’s instruction. The primer pairs used in the first round PCR amplification were the outer primer (provided in the kit) + YM 8. In the second, nested primers used were the inner primer (provided in the kit) + 239_U1 and seq L1. The PCR products from the inner primer with the two Tdy1 primers were sequenced and the sequence was compared with the genomic sequence DNA to determine the transcription start point.

4.2.5 Gene expression analysis using semi-quantitative RT-PCR

The cDNA levels were first normalized using the maize ubiquitin gene. The primers for *Ubiquitin*, *Tdy1*, *ZmSUT1*, *ZmSUT2* and *ZmSUT4* are listed in Table 4.1. The *Ubiquitin* amplification conditions were: 94°C for 2 min; 20 cycles of 94°C for 15 sec, 65°C for 15 sec, 72°C for 15 sec; and finally 72°C for 5 min. After normalization, the same amount of cDNA was used for *Tdy1* and *SUT* PCR. The amplification conditions for *Tdy1*, *SUT1* and *SUT2* were 94°C for 2 min; 20 cycles of 94°C for 15 sec, 60°C for 15 sec, 72°C for 15 sec; and finally 72°C for 5 min. For *Tdy1* PCR, 1.5% glycerol and 5% DMSO were included in the PCR reactions. For SUT4, the reaction conditions were: 94°C for 2 min; 20 cycles of 94°C for 20 sec, 62°C for 20 sec, 72°C for 20 sec; and finally 72°C for 5 min. After the PCR reactions, the PCR products were run on agarose gels, Southern blotted to a membrane and hybridized with the corresponding probes. Signal was detected by autoradiography.
4.2.6 In situ hybridization

*In situ* hybridization was performed according to Langdale (1993). The Tdy1 probe was amplified with YM2 and YM8 as described above, and cloned into the pGEM-Teasy vector (Promega, Madison, WI). 1 µg of plasmid was linearized with Nco1 and Nde1 and the *in vitro* RNA transcription reactions carried out with T7 or SP6 RNA polymerases.

<table>
<thead>
<tr>
<th>Table 4.1: Primers used for RT-PCR</th>
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<tbody>
<tr>
<td>Gene</td>
</tr>
<tr>
<td>Outer primer</td>
</tr>
<tr>
<td>YM 8</td>
</tr>
<tr>
<td>Inner primer</td>
</tr>
<tr>
<td>239_U1</td>
</tr>
<tr>
<td>seq L1</td>
</tr>
<tr>
<td>Ubiquitin</td>
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<tr>
<td>ZmUbi4</td>
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</tr>
<tr>
<td></td>
</tr>
<tr>
<td>ZmSUT4</td>
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4.3 RESULTS

4.3.1 A *Mu* element is tightly linked with the *D190* allele

The *D190* allele is one of the *tdy1* alleles isolated from a directed *Mu* tagging experiment and the one used for cloning *Tdy1*. As *Mu* generally has the highest copy number among *Mu* elements, we first examined whether *Mu* co-segregated with *D190*. Southern blotting was used for co-segregation analysis using a *Mu* specific probe and 110 chromosomes were tested. We found out that the *Mu* element was always present in *D190* mutants but absent in wild type siblings (Figure 4.1). This indicates that the *Mu* element tightly co-segregated with *D190*, and showed that the *Mu* element is linked less than 1 cM from *Tdy1*. A *BamH*I digested genomic DNA Southern blot showed a hybridized fragment of approximately 5.3 kb. In order to clone this fragment, a lambda subgenomic DNA library containing the 5.3 kb region was constructed and screened using the *Mu* probe. By repeatedly enriching for *Mu* containing phage, I isolated a clone containing the *Mu* fragment. I sequenced the plasmid insert containing this DNA to obtain the flanking sequence and identified a *Mu* element inserted into the presumed *Tdy1* sequence.

In order to confirm the cloned sequence was the correct *Tdy1* gene, other *tdy1* alleles were sequenced to examine whether there were changes in the same sequence in different alleles. A deletion allele was previously cloned and sequenced, but since it has almost a 180 kb deletion, it was hard to determine which gene corresponded to *Tdy1*. 
However, it was very encouraging that the sequence I cloned flanking the *Mu1* element was located in the sequence encompassed by the deletion (previously determined by David Braun), and suggested that this sequence could correspond to *Tdy1*. Three other *Mu*-insertion alleles were PCR amplified and sequenced, and the results showed that one has a *Mu1* insertion, one has a *Mu3* insertion and another has a *Mu8* insertion within this same gene. In addition, the insertion sites in the *Mu1* and *Mu3* alleles are identical and are located 18 base pairs downstream of the insertion site in *D190*. The *Mu8* insertion occurs 85 bp upstream of the *D190* allele. The insertion of different *Mu* elements in the same sequence confirmed that the correct gene had been cloned. All of the insertion sites from these alleles are in the 5′-UTR of the *Tdy1* gene (Table 4.2), and suggests that *Mu* transposons may have a preference for insertion in this region. Additional alleles of *Tdy1* were molecularly characterized. The *tdy1-shadow* allele has a 453 bp deletion in the 3′ end, the *tdy1-PM* allele has a 1 base pair frameshift in the 3′ end, and the first allele isolated from an EMS mutagenized population, *tdy1-R*, has a substitution at the 112th amino acid with a proline changed to arginine (Table 4.2). The reason why this substitution eliminates *Tdy1*’s function is still unclear, but the proline residue is highly conserved and potentially important for the protein structure and/or function. The fact that the *tdy1-shadow* and PM alleles are mutated in the 3′ end of the gene indicates that the C-terminal region is likely important for *Tdy1* function.
In order to determine the transcription start site of the *Tdy1* mRNA, 5’-RACE was performed. The transcription start site is located 152 bp upstream of the start codon. Interestingly, the insertion sites of the *Mu*-insertion alleles occur close to this region.

**Table 4.2: Molecular analysis of *tdy1* mutations**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutation</th>
<th>Position</th>
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<td><em>tdy1</em>-reference</td>
<td>cct(\rightarrow)cgt</td>
<td>112nd amino acid</td>
</tr>
<tr>
<td><em>tdy1</em>-D190</td>
<td><em>Mu1</em> insertion</td>
<td>5-UTR</td>
</tr>
<tr>
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<td><em>Mu1</em> insertion</td>
<td>5-UTR</td>
</tr>
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<td>200th amoni acid</td>
</tr>
<tr>
<td><em>tdy1</em>-shadow</td>
<td>453 bp deletion</td>
<td>3’-end and 3’-UTR</td>
</tr>
<tr>
<td><em>tdy1</em>-D6</td>
<td>~180 kb deletion</td>
<td>Entire gene</td>
</tr>
</tbody>
</table>
Figure 4.1: A Mu1 fragment co-segregates with the D190 allele. Genomic DNA from both D190 mutants and wild type siblings were digested with BamHI, blotted and hybridized with a Mu1 specific probe. The Mu1 element was present only in tdy1 mutants but not in wild type plants indicating that Mu1 co-segregated with D190. The arrow points to the 5.3 kb fragment hybridized by the Mu1 probe.
4.3.2 *Tdy1* is a novel maize gene and conserved in grasses

After confirming that the correct gene was cloned, blast analysis was performed in order to determine if TDY1 contained known protein domains. Blast analysis did not find a clear homolog in Arabidopsis or other dicots, but a similar protein sequence in rice was found with 57% identity to TDY1 (Figure 4.2). Sorghum is very closely related to maize, and contains an ortholog with 75% identity (Figure 4.2). Unlike the maize and rice TDY1 proteins, the sorghum gene has an intron and does not have a conserved stop codon. But, since the sequencing of the sorghum genome is not totally completed, there could be some sequencing and assembly errors to explain these anomalies. In addition, several deduced amino acid sequences from sugarcane ESTs are also similar to TDY1 with identity around 60-70%. Though no genomic DNA sequence is available in sugarcane, we can learn from these ESTs that there must be a homologous gene in sugarcane that is similar to TDY1.

Using protein domain prediction algorithms, TDY1 is predicted to be a transmembrane protein with two transmembrane domains (Figure 4.2). The rice and sorghum genes are also predicted to have two N-terminal transmembrane domains shown by the black brackets in Figure 4.2. Additionally, both the N-terminus and C-terminus of the TDY1 protein are predicted to be inside of the cell. From the alignment we can see that the two transmembrane domains in maize, sorghum and rice TDY1 are highly conserved. The first transmembrane domain is almost completely identical except for a one amino acid substitution, and the second predicted transmembrane domain is identical
Figure 4.2: Alignment of TDY1 deduced amino acid sequences from maize, sorghum and rice. The deduced maize amino acid sequence has 75% identity to the sorghum sequence and 57% identity to the rice unknown protein. The two presumed transmembrane domains are shown by black brackets. The N-terminus and the two transmembrane domains are highly conserved among the three grasses, while the C-terminus is somewhat less conserved in rice than in maize and sorghum. The asterisk indicates the conserved proline mutated in the *tdy1-R* allele. The two boxes indicate the sequences aligned in Figure 4.3. The Genbank accession number for the rice sequence is EAY99183. No sorghum protein sequence was found in Genbank, but there are two genomic DNA sequences BZ423348.1 and CW071677.1 that have 100% identity to the sorghum sequence used from the Phytozome website (Sbi_0.37231). The alignment program used is CLUSTALW.
among the three proteins. The 110 amino acids following the second transmembrane domain in the three grass proteins are less conserved in rice than between maize and sorghum. However, the C-terminal region is conserved in the three grasses. These results show that TDY1 is highly conserved in grasses.

Among grasses, only rice and sorghum are sequenced, so we could not obtain more sequence information from other grasses, such as wheat, oat or rye. However, there are two additional regions of TDY1 protein sequence showing 50% to 80% identity, and 70% to 90% similarity respectively, to the deduced amino acid sequences from more distantly related plants (Table 4.3 and Figure 4.3). Comparing the entire amino acid sequences from the most closely related sequences in Arabidopsis, poplar, grape and medicago, which have full length sequences available, they respectively show 43%, 41%, 41% and 39% similarity to the maize TDY1 sequence. Moreover, the predicted protein topology from these four distant sequences has two predicted transmembrane domains, the intracellular small N-terminus and large C-terminus. Although all of the proteins are of unknown function, the similarities in topology and conserved amino acids among distantly related plants suggests that the conserved sequences may be important for TDY1 function in all angiosperms. No proteins with significant similarity were found in gymnosperms or more distantly related organisms, though one possible reason is the limited genome sequences currently available for these species. Additional information about the evolutionary history of TDY1 will be obtained after more plant genomes are sequenced and the related genes are characterized.
### Table 4.3: Two stretches of TDY1 protein sequence are conserved

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Region 56-65</th>
<th>Region 218-244</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Identity</td>
<td>Similarity</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>Poplar (<em>Populus trichocarpa</em>)</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>Barley (<em>Hordeum vulgare</em>)</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>Grape (<em>Vitis vinifera</em>)</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>Medicago (<em>Medicago truncatula</em>)</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>Lettuce (<em>Lactuca sativa</em>)</td>
<td>N/A</td>
<td>56</td>
</tr>
<tr>
<td>Cotton (<em>Gossypium hirsutum</em>)</td>
<td>N/A</td>
<td>56</td>
</tr>
<tr>
<td>Potato (<em>Solanum tuberosum</em>)</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Soybean (<em>Glycine max</em>)</td>
<td>N/A</td>
<td>50</td>
</tr>
<tr>
<td>Sunflower (<em>Helianthus annuus</em>)</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>Tomato (<em>Lycopersicon esculentum</em>)</td>
<td>N/A</td>
<td>53</td>
</tr>
</tbody>
</table>

Percent identity and percent similarity of conserved amino acid regions 56-65 and 218-244 of the maize TDY1 protein with either protein or translated nucleotide sequences from other plants. All of the gene functions are unknown. For lettuce, cotton, soybean and tomato, the first region was not found by tblastn, because this region was not available (N/A) from the nucleotide sequence.
Figure 4.3: TDY1 has two amino acid sequences conserved in other angiosperms. The alignments of these two amino acid sequences among these plants are shown. A is the alignment of the sequence located in the second transmembrane domain (56-65, shown by black box in Figure 4.2) and B is the alignment of the sequence in the C-terminus (218-244, shown by black box in Figure 4.2). Hv, barley; Vv, grape; Ls, lettuce; Zm, maize; St, potato; Le, tomato; Gh, cotton; Gm, soybean; Ha, sunflower; At, Arabidopsis; Mt, medicago; Pt, poplar.
4.3.3 *Tdy1* belongs to a small gene family

*Tdy1* mutant leaves show variegated chlorotic and green sectors but not uniform chlorosis. This suggests that there might be a duplicate gene functioning similar to *Tdy1* and partially compensating for the loss of *Tdy1* function in maize. To test this hypothesis, a Southern blot analysis was performed. I found that there is a duplicate gene present in the maize genome (Figure 4.4). Two hybridizing bands can most clearly be seen in the *Bam*HI and *Sac*I lanes. Because *Tdy1* was cloned using *Bam*HI, we know that in the *Bam*HI lane, the top band corresponds to the *Tdy1* gene, while the lower band indicates the duplicate gene. To determine on which chromosome the duplicate gene is located, maize BAC (Bacteria Artificial Chromosomes) filters were hybridized with a *Tdy1* probe. 12 BACs were identified based on Southern blotting (Table 4.4), among which 4 are from the same contig located on chromosome 1, and a single contig was each mapped to chromosomes 3, 4 and 9. In addition, two BACs mapped to the same contig that is not mapped to a chromosome, and three were identified that have not been mapped. Other than *Tdy1* which is located on chromosome 6, the strongest hybridizing BACs all mapped to contig 10 on chromosome 1. Hence, it most likely contains the duplicate gene identified by genomic DNA Southern blotting that is most closely related to *Tdy1*. The remaining BACs showed weaker hybridization signals, and those which were mapped, are each located on a different chromosome. The weaker hybridization signals may suggest that they are less related to *Tdy1*, and likely represent more distantly related gene family members. When the maize genome is completely sequenced (expected to be complete by the middle of 2008), it will reveal whether these sequences
Figure 4.4: A duplicate for *Tdy1* was detected in the maize genome by Southern blotting. White arrows indicate *Tdy1* and the duplicate gene. Distinct bands are seen in the *BamHI* and *SacI* lanes. In the *BamHI* lane, the top band is *Tdy1* and the lower band indicates the duplicate gene. Since we do not know the size of the *SacI* digested *Tdy1* fragment, we cannot tell which one is *Tdy1* or the duplicate, but clearly two bands are present.
encode potentially functional genes. These results suggest that there may be more than one duplicate for Tdy1 in the maize genome, and that is likely a small gene family.

**Table 4.4: BAC contigs identified by a Tdy1 probe**

<table>
<thead>
<tr>
<th>BAC</th>
<th>FPC Contig#</th>
<th>Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>b0107G13</td>
<td>290</td>
<td>6</td>
</tr>
<tr>
<td>b0121E19</td>
<td>290</td>
<td>6</td>
</tr>
<tr>
<td>b0118P13</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>b0097H07</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>b0162F02</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>b0192A10</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>b0104J21</td>
<td>120</td>
<td>3</td>
</tr>
<tr>
<td>b0130G17</td>
<td>160</td>
<td>4</td>
</tr>
<tr>
<td>b0153C21</td>
<td>390</td>
<td>9</td>
</tr>
<tr>
<td>b0192K17</td>
<td>435</td>
<td>Unknown</td>
</tr>
<tr>
<td>b0180G02</td>
<td>435</td>
<td>Unknown</td>
</tr>
<tr>
<td>b0114D20</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>b0168K01</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>b0153O13</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

The contig and chromosome mapping results were obtained from http://www.genome.arizona.edu/fpc/WebAGCoL/maize/WebFPC/. The BACs on chromosome 6 are Tdy1. Four BACs are located on the same contig on chromosome 1. An individual contig was each found on chromosomes 3, 4 and 9. Two BACs of the same contig were found without information about chromosome location. Three are not mapped to a contig or chromosome.
4.3.4 *Tdyl* is expressed in all tissues examined

Since the *tdyl* phenotype is only observed in leaf blade tissues, but not in sheath or stem (Figure 2.1), we predicted that *Tdyl* might only be expressed in leaf tissues. To test in which tissues *Tdyl* is expressed and its relative expression levels, semi-quantitative RT-PCR was performed in different maize tissues: mature leaf, immature leaf, immature ear, immature tassel and young root. RT-PCR results showed that *Tdyl* is expressed in all of these tissues (Figure 4.5). *Tdyl* was expressed similarly in mature and immature leaf tissues, but at a lower level than found in developing ear, tassel or young root. *Tdyl* was expressed at approximately three fold higher levels in immature tassels compared to leaf tissues, and developing ear tissue has approximately 2.5 fold more transcripts than tassel. *Tdyl* is most highly expressed in root tissues with about 2 fold more than ear. So, the relative expression level of *Tdyl* in different tissues is root > ear > tassel > mature leaf ≈ immature leaf. Though RT-PCR showed highest expression of *Tdyl* in root tissue, we did not notice any obvious phenotypes in young roots grown in darkness, for example, shorter root length or a change in the number of root hairs. Similarly, no phenotype was observed in ears or tassels although *Tdyl* transcripts were detected in these tissues. Since immature leaf tissue is also a carbon sink like ear, tassel and root tissues, it indicates that *Tdyl* is not always expressed at high levels in sink tissues. Further, these data suggest a broader function for *Tdyl* in other tissues besides in leaves.
Figure 4.5: Semi-quantitative RT-PCR of Tdy1 and three sucrose transporters, ZmSUT1, ZmSUT2 and ZmSUT4 in different maize tissues. The tissues tested are mature leaf (m), immature leaf (im), ear (e), tassel (t) and root (r). The images from the top to the bottom are hybridized blots of Tdy1, ZmSUT1, ZmSUT2, ZmSUT4 and the normalized cDNA expression control, ubiquitin. Genomic DNA (gDNA) was shown in ZmSUT1, ZmSUT2 and ZmSUT4 images. Because primers used for these three genes each span a small intron, it indicates that there was no genomic DNA contamination in the cDNA pools. The UBIQUITIN and Tdy1 primers do not span introns, so the gDNA lane is not shown. The water control lane indicates that there was no DNA contamination.
4.3.5 Maize sucrose transporters have overlapping expression with \textit{Tdy1}

I previously suggested that TDY1 could regulate sucrose transporters to control carbohydrate accumulation in leaf tissues. Three sucrose transporters in maize have been cloned, \textit{ZmSUT1}, \textit{ZmSUT2} and \textit{ZmSUT4}. Only \textit{ZmSUT1} has been characterized, and it is reported that \textit{ZmSUT1} is expressed in almost all maize tissues except for root and silk (Aoki et al., 1999). \textit{ZmSUT2} and \textit{ZmSUT4} cDNAs were cloned from immature kernels, but no more information about these two genes has been reported. To determine whether these sucrose transporters have overlap in their expression with \textit{Tdy1}, semi-quantitative RT-PCR was performed.

Our results showed that \textit{ZmSUT1} is expressed in mature and immature leaves and in roots, while no transcripts were detected in immature ear and tassel (Figure 4.5). Our results differ from the published data because Aoki et al. detected transcripts in glumaceous flowers by Northern blotting (Aoki et al., 1999). The differences could be due to the different developmental ages of the tissues used. The tassel I used was very young and was no more than 2 cm long, but glumaceous flowers indicate that the tissue Aoki et al. used was mature. In addition, I detected \textit{ZmSUT1} expression in roots. This discrepancy may be explained by the fact that the gene is expressed at a low level in roots and possibly below detectable limits by total RNA Northern hybridization (Aoki et al., 1999). \textit{ZmSUT2} was expressed in all tissues with higher expression in sinks, especially in immature leaf and root, while weak expression was detected in mature leaf (Figure 4.5). \textit{ZmSUT4} is expressed in all of the tissues. \textit{ZmSUT4} expression is the
strongest in root which is similar to Tdy1, while ZmSUT4 was expressed at similar levels in mature leaf, immature leaf, ear and tassel. Different expression patterns of ZmSUT1, ZmSUT2 and ZmSUT4 in maize tissues suggest that the three maize sucrose transporters likely play distinct roles in sucrose loading/unloading. Expression of Tdy1 is more similar to ZmSUT4 suggesting that there might be coordination of these two genes in regulating sugar transport.

4.3.6 Tdy1 RNA is localized to phloem cells supporting a function in phloem transport of sucrose

I have shown that Tdy1 is expressed in all tissues examined. To understand in which cells Tdy1 is expressed, RNA in situ hybridization was conducted (Langdale, 1993). Two tissues were examined: vegetative developing stem tissue directly below an immature tassel, and reproductive immature ear tissue. The results showed that Tdy1 is expressed in phloem tissues, especially in sieve elements (Figure 4.6). It also indicates that Tdy1 is expressed very early during development since the protoxylem in Figure B showed that xylem formation was not complete. The expression of Tdy1 in phloem cells suggests a function of TDY1 in phloem loading/unloading possibly by interaction with SUTs regulating carbohydrate partitioning in maize.
Figure 4.6: RNA in situ hybridization shows that Tdy1 is expressed in phloem cells in young stem tissue. A. Transverse section of stem tissue below an immature tassel with young surrounding leaves. The blue color indicates Tdy1 expression. B. Close-up view of a vein from panel A showing expression in the phloem. SE, sieve elements; CC, companion cells; PX, protoxylem. C. Transverse section of immature ear tissue showing Tdy1 RNA is only detected in phloem cells. D. Close-up of a vein from panel C.
4.4 DISCUSSION

The loss of Tdy1 function results in carbohydrate accumulation in leaf tissues leading us to hypothesize that Tdy1 regulates carbohydrate partitioning in leaves. In order to explore and understand Tdy1’s function at the cellular and molecular levels, we cloned the gene using transposon tagging. Tdy1 encodes an unknown protein and is predicted to be localized to a membrane.

Tdy1 was cloned from a Mu-insertion allele and sequence analysis showed that the insertion occurs in the 5’-UTR. Three other independent Mu-insertion alleles were also found to have insertion sites in the same region. It suggests that the 5’-UTR may be a “hot spot” for Mu transposon insertions. Presumably, the transcription of Tdy1 is decreased due to the insertions resulting in the tdy1 phenotype. 5’-RACE showed that these insertions are close to the transcription start site. Other tdy1 alleles are all affected in the C-terminus of TDY1 suggesting this part of the protein is essential for its function. All of these mutants showed phenotypes comparable in severity to the two deletion alleles suggesting they are all null alleles.

4.4.1 Tdy1 is broadly expressed

The principal visible manifestation of the tdy1 phenotype is the formation of chlorotic leaf sectors, but no phenotype was evident in other vegetative tissues, such as leaf sheaths, stems, husk sheath, glumes and roots, or reproductive tissues like ears and
tassels. We found that the chlorosis in \textit{tdy1} leaves occurs after excess carbohydrates accumulate. Presumably, the loss of \textit{Tdy1} function results in defects in sucrose loading in source leaves and the build-up of carbohydrates in the chlorotic sectors. This may partly explain why we do not see a visible phenotype in sink tissues as they may not be able to accumulate enough carbohydrates to induce the mutant phenotype.

Surprisingly, I found that \textit{Tdy1} is not only expressed in mature source leaf tissue, but is also expressed in sink tissues, such as immature leaves, ear, tassel and roots. The semi-quantitative RT-PCR results showed that \textit{Tdy1} is expressed more in ear, tassel and roots, but not as high in immature leaf, which are all sink tissues (Figure 4.5). The broad expression of \textit{Tdy1} in both source and sink tissues suggests that it has additional functions beyond potentially regulating phloem loading. The highest expression in root tissue may suggest possible functions in water uptake and response to osmotic pressure, which can also affect sucrose fluxes (Aloni et al., 1986; Buchanan et al., 2000).

\subsection*{4.4.2 \textit{Tdy1} is highly conserved in grasses}

There are putative \textit{Tdy1} orthologs in rice and sorghum with high identity to the maize TDY1. In sugarcane, there are a few deduced amino acid sequences from ESTs showing identity to TDY1. The high similarity of amino acid sequences among maize, rice, sorghum and sugarcane indicates that TDY1 function is highly conserved in grasses. No sequences with high identity throughout the predicted proteins were found in dicots, such as Arabidopsis, poplar, grape and medicago. However, two conserved stretches of
amino acid sequences were discovered in some dicot plants. Although the full length sequences of the Arabidopsis, poplar, grape and medicago homologs showed around 41% similarity to TDY1, protein topology prediction programs showed that these proteins are also predicted to be transmembrane proteins similar to TDY1. The comparisons among these genes suggest the possibility for a similar function of TDY1 in all angiosperm plants. The conserved sequence in the intracellular C-terminus suggests that this region may be important for TDY1 function. This idea is also supported by the fact that the mutations in \textit{tdy1} non-insertion alleles all occur in the C-terminus. A possible function of the TDY1 C-terminus might be as a sensor to detect certain interacting proteins. Because none of these genes have been characterized, their function is still unknown. Based on their predicted structural similarity and limited sequence conservation with \textit{Tdy1}, they may function to influence carbohydrate export from leaves. If so, it will be interesting to determine their function and evolutionary history.

\textbf{4.4.3 \textit{Tdy1} function may be compensated by other related genes}

A putative duplicate gene for TDY1 was identified by Southern blot analysis and the probe used is from the conserved 3’ end of the \textit{Tdy1} gene. There is also a second sequence from rice that was found to have similarity to TDY1 in the same region and is also predicted to have two transmembrane domains. The C-terminus of this second rice protein has high similarity to TDY1 and is probably an ortholog to the \textit{Tdy1} duplicate gene. Hence, rice has at least two members of the \textit{Tdy1} gene family. In addition, BAC Southern blotting using a probe from the conserved sequence of \textit{Tdy1} revealed several
maize BACs which mapped to different chromosomes. Since the *tdyl* mutant displays variegated leaf sectors but not uniform chlorosis, the green wild type appearing tissue might be due to the redundant function of the duplicate gene that partially compensates for the loss of *Tdy1* function (Yu et al., 2005; Aluru et al., 2006; Yu et al., 2007).

Furthermore, even though *Tdy1* is expressed in mature leaves, once the tissue passes a certain developmental point in *tdyl* mutants, the loss of TDY1 function will not cause mature green leaf tissue to undergo carbohydrate accumulation and chlorosis (Figure 2.2). This result may suggest that another related gene functions later in plant development stages and performs a similar function as *Tdy1*. More information about these duplicate genes will be obtained when they are identified and characterized.

### 4.4.4 TDY1 may regulate maize sucrose transporters controlling photoassimilates transport

Sucrose transporters have been found to be expressed and function in phloem cells in both source and sink tissues (Sauer, 2007). Transgenic or mutated plants with sucrose transporter gene silencing showed carbohydrate build up in the chlorotic tissues of source leaves and stunted growth of the plants (Riesmeier et al., 1994a; Burkle et al., 1998; Gottwald et al., 2000a). Similarly, we have found that chlorotic sectors in *tdyl* leaves overaccumulate starch and soluble sugars (see Chapter 2). *In situ* hybridization showed that *Tdy1* mRNA is exclusively localized to phloem cells in young stem below a developing tassel and in young ear tissue (Figure 4.6). The expression data are consistent with previous work that *Tdy1* functions in the inner layer of the leaf, which is composed
of interveinal mesophyll cells, bundle sheath and vein cells (Baker and Braun, 2007). These results provide support to the hypothesis that TDY1 may function in phloem loading and/or unloading by regulating sucrose transporters.

Evolutionary studies suggest that TDY1 may have additional functions other than regulating phloem loading. Two amino acid sequences were found to have high sequence similarities to TDY1 from distantly related plants (Table 4.3). This indicates that TDY1 function may be conserved in plants. Among the plants listed in Table 4.3, poplar has been characterized to be a symplastic phloem loader (Buchanan et al., 2000), which means that sucrose is loaded into phloem cells by way of plasmodesmata, not by plasma membrane sucrose transporters. Thus, in poplar, TDY1 may not function in phloem loading but possibly in regulating sucrose transporters that function to retrieve sucrose leaked from the phloem along the transport pathway (van Bel, 2003).

Sucrose transporters have been identified and characterized in many plants (Sauer, 2007). Three maize sucrose transporters have been identified, ZmSUT1, ZmSUT2 and ZmSUT4. ZmSUT1 is capable of both sucrose uptake and release (Carpaneto et al., 2005). Expression studies of the different maize sucrose transporters were performed to test for overlap with Tdy1. The expression of ZmSUT1 was not detected in reproductive tissues but in vegetative tissues (Figure 4.7). Tdy1 and ZmSUT1 are expressed in mature and immature leaf tissues and roots but only Tdy1 is expressed in ear and tassel. Additionally, ZmSUT1 transcripts are not found in silks while we detected Tdy1 in silk cDNA (data not shown). The expression of ZmSUT1 in source leaves is
approximately two-fold higher than in sink leaves and root tissues suggesting that
ZmSUT1 might mainly play a role in phloem loading rather than sucrose retrieval in the
phloem. ZmSUT2 and ZmSUT4 showed more overlapping expression patterns with Tdy1
in that the expression of these mRNAs is higher in roots (Figure 4.5).

ZmSUT2 is in the same phylogenetic group as AtSUC3 and the expression of
AtSUC3 is higher in sink tissues (Meyer et al., 2004), similar to ZmSUT2 (Figure 4.5).
ZmSUT4 belongs to group 4 sucrose transporters and it is argued that other group 4
SUTs, such as AtSUC4 and HvSUT2, are vacuolar sucrose transporters (Endler et al.,
2006). However, other group 4 SUTs, StSUT4 and LeSUT4, have been reported as
localized to SEs which lack vacuoles (Weise et al., 2000). Unlike Tdy1 expression,
ZmSUT4 in ear and tassel has similar amounts of transcripts as in mature and immature
leaf tissues (Figure 4.5). However, the expression of ZmSUT4 in roots is much higher
than other tissues. If ZmSUT4 is a tonoplast sucrose transporter, it would have no direct
role in phloem unloading, but possibly function in importing sucrose from the cytoplasm
into the vacuole for sucrose storage and future efflux from the vacuole into the cytosol
for other biochemical processes.

The detection of Tdy1 mRNA in developing ear SEs is consistent with the sink
SEs localization of some group 3 sucrose transporters and grass sucrose transporters,
such as OsSUT1 and TaSUT1 (Barth et al., 2003; Aoki et al., 2004; Hackel et al., 2006;
Scofield et al., 2007). This suggests a possible function for TDY1 in sucrose efflux and
phloem unloading. ZmSUT2 and ZmSUT4 are highly expressed in root tissue similar to
Tdy1. Roots are heterotrophic carbon sinks, and it has been shown that phloem unloading
into root occurs by a symplastic route (Wolswinkel, 1985). However, apoplastic unloading in roots was also supported by the discovery of both sucrose and hexose transporters in sugar beet roots (Patrick, 1997; Lalonde et al., 2003). The phloem unloading mechanism in maize roots has not been extensively studied. Root growth in maize is very fast, and it has been reported that root elongation is related to sugar content in the elongation zone (Muller et al., 1998). Even though sugar unloading into roots may occur mainly through the symplastic pathway (Giaquinta et al., 1983b; Warmbrodt, 1985; Oparka et al., 1994; Lalonde et al., 2003), perhaps the apoplastic pathway also contributes to import. If surplus sugars cannot diffuse through plasmodesmata, they may be pumped out of the plasma membrane into the apoplastic space by sucrose transporters, such as ZmSUT2.

Based on my work cloning and characterizing Tdy1, we determined that Tdy1 is predicted to encode a membrane protein, is potentially conserved across angiosperms, overlaps in its tissue specific expression with multiple sucrose transporters, and expressed in phloem cells. These data are in agreement with my hypothesis that TDY1 functions to regulate sugar transporters. Future experiments to test this hypothesis will be outlined in Chapter 5.
Chapter 5

Conclusions and perspectives

In this chapter, I will summarize my research results and highlight important conclusions drawn from my work. Finally, I will propose some prospects for future study to understand Tdy1’s function.

5.1 Research summary

A maize variegated leaf mutant, tdy1, with excess carbohydrate accumulation in leaf sectors was identified. Associated with the carbon accumulation defect, tdy1 plants displayed reduced growth and yield. The reduced plant growth is likely due to the fact that export of carbohydrates from leaves into developing sink tissues is hampered. tdy1 is the second maize mutant identified with carbohydrate accumulation in leaf sectors, the other being sucrose export defective1 (sxd1). Double mutant studies indicate that Tdy1 and Sxd1 have distinct roles in regulating carbon partitioning in maize and function in independent pathways. To characterize the function of Tdy1, I cloned the gene by transposon tagging. Tdy1 encodes a novel protein predicted to be membrane localized. Interestingly, BAC Southern blotting suggests that Tdy1 belongs to a small gene family. Even though we only observed a phenotype in tdy1 leaves, the expression of Tdy1 in different tissues (mature and immature leaf, ear, tassel and root) suggests a broader function of Tdy1. In situ hybridization localized Tdy1 mRNA in phloem cells suggesting
a function for \textit{Tdy1} in phloem transport. To examine whether \textit{Tdy1} has overlapping expression to sucrose transporter genes, the expression patterns in the same tissues of the three known maize sucrose transporter genes were examined. \textit{ZmSUT1} is expressed in mature and immature leaves and roots, while no transcript was detected in immature ear and tassel. \textit{ZmSUT2} and \textit{ZmSUT4} were expressed in all tissues examined. As \textit{Tdy1}, \textit{ZmSUT2} and \textit{ZmSUT4} showed higher expressions in roots, it is possible that they may interact to control sucrose flux into roots.

\textbf{5.2 Perspectives}

Based on our studies of \textit{Tdy1}, we proposed that TDY1 functions in regulating phloem loading/unloading of sucrose in maize plants. While our data is consistent with this proposal, it is not conclusive enough to prove our hypothesis. Here, I will propose some future experiments that may lead us to either support or reject the hypothesis. I will also point out potential applications of our research.

\textit{Tdy1} mRNA is detected in sieve elements and companion cells by \textit{in situ} hybridization. However, except for determining the expression of \textit{ZmSUT1} in different tissues (Aoki et al., 1999), no other expression work on maize sucrose transporters has been performed. Hence, in which cell(s) the mRNAs of these sucrose transporters are expressed could be examined using \textit{in situ} hybridization to investigate whether they are also expressed in phloem cells and if their expression overlaps \textit{Tdy1}. We might expect that they would be expressed in the phloem since most sucrose transporters are found in
phloem cells except for some in group 4 and ShSUT1, a sugarcane sucrose transporter most closely related to ZmSUT1 (Rae et al., 2005; Sauer, 2007).

If the cellular co-expression of *Tdy1* and sucrose transporters is confirmed, subcellular expression of the proteins can be investigated. Preliminary data has found the TDY1 protein to be expressed on the ER membrane (Tom Slewinski, personal communication). The membrane localization is consistent with the topology prediction of TDY1. In order to confirm this result, we could raise an antibody against TDY1 and immunolocalize TDY1. Alternatively, an ER specific dye, DiOC₆ (Martens et al., 2006a) could be used to verify TDY1’s localization. Sucrose transporters are also transmembrane proteins although they are predominantly plasma membrane proteins, and no sucrose transporters have been found on the ER membrane. Based on the expression data obtained, ZmSUT2 and ZmSUT4 should be tested first. As ZmSUT4 belongs to group 4, and proteins from the same group (AtSUT4 and HvSUT2) are tonoplast proteins (Endler et al., 2006), it is possible that ZmSUT4 is also localized on the vacuole membrane. ZmSUT2 is predicted to be localized to the plasma membrane. One possibility to reconcile the different subcellular locations would be if ER-localized TDY1 were to function as a chaperone to assist the targeting of sucrose transporters from the ER to the plasma membrane or tonoplast. To test this hypothesis, *in vitro* and *in vivo* protein-protein interaction approaches can be used, such as the modified split ubiquitin yeast two-hybrid assay (Y2H), FRET or co-immunoprecipitation.
One interesting observation is that \textit{Tdy1} is highly expressed in root tissues. Roots are the site for water absorption and transport through the stele to other parts of the plant. A type of membrane protein, aquaporins, has been identified for more than a decade in plants, and maize aquaporins have been cloned (Chrispeels and Maurel, 1994; Kammerloher et al., 1994; Chaumont et al., 1998; Chaumont et al., 2000; Chaumont et al., 2001). These proteins belong to an ancient protein family and function as a channel for water influx though some family members transport uncharged solutes (Chrispeels and Maurel, 1994). The expression and specific regulation of aquaporins can trigger changes in cell membrane permeability for water (Boursiac et al., 2005; Luu and Maurel, 2005). This can affect the osmotic pressure between the apoplast and the cytoplasm and cause sucrose uptake or release (Patrick et al., 2001). Another potential function for TDY1 is to interact with this family of proteins. To test this hypothesis, we can detect if there are changes in aquaporins expression at the transcriptional level in \textit{tdy1} mutants by quantitative RT-PCR, and at the protein level by Western blots. Additionally, similar experiments as outlined for the SUTs can be used to examine their potential interaction with TDY1.

Besides directly testing candidate proteins for interaction, other proteins that might interact with TDY1 could be detected using yeast two hybrid (Y2H) or immunoprecipitation. Because \textit{Tdy1} is highest expressed in roots and \textit{tdy1} mutants show a leaf phenotype, we can first test these two tissues. For Y2H, cDNA libraries made from leaf and young root can be tested. In addition, as preliminary data showed ER membrane localization of TDY1, microsomal membranes can be purified from maize leaves and
roots to immunoprecipitate a TDY1 protein complex. If we could co-precipitate and identify associated proteins, we can search available databases to determine their functions. Hopefully, these proteins will have characterized functions and we will obtain some hints on how TDY1 functions.

I proposed in Chapter 4 that Tdy1 likely belongs to a small gene family. Tdy1 has been shown to be expressed early in tissue development. It is possible that other genes from the family regulate sucrose transport in different developmental stages or have different subcellular locations. Hence, it would be important to clone these genes, characterize their expressions at the tissue level and determine their developmental expression patterns as well as cellular and subcellular localization. In addition, it would be informative to obtain mutants from these genes to test for genetic interaction between these genes and Tdy1. These approaches may reveal the function of additional members of the Tdy1 gene family.

The study of TDY1 function has both agricultural and industrial significances. Maize is one of the most important crops for food production for both human beings and livestock. The whole maize plant can be used as forage and ears provide much of the world’s food. Furthermore, the production of biofuels has become an active and exciting area of research. As a C4 plant, the high efficiency of photosynthesis and carbon assimilation makes maize an ideal plant for carbohydrate production. If TDY1 controls sucrose allocation, by manipulating the gene’s expression and function, we could control the amount of sucrose retained or exported from the leaves to developing sinks, such as
ears. The accumulation of higher amounts of carbohydrates will increase the sugars available for fermentation to ethanol. Hence, future studies with TDY1 may be directly applicable to the production of biofuels.
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