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Setaria viridis: A Model for C$_4$ Photosynthesis Ⓒ Ⓦ

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C$_4$ photosynthesis drives productivity in several major food crops and bioenergy grasses, including maize (*Zea mays*), sugarcane (*Saccharum officinarum*), sorghum (*Sorghum bicolor*), *Miscanthus x giganteus*, and switchgrass (*Panicum virgatum*). Gains in productivity associated with C$_4$ photosynthesis include improved water and nitrogen use efficiencies. Thus, engineering C$_4$ traits into C$_3$ crops is an attractive target for crop improvement. However, the lack of a small, rapid cycling genetic model system to study C$_4$ photosynthesis has limited progress in dissecting the regulatory networks underlying the C$_4$ syndrome. *Setaria viridis* is a member of the Panicoideae clade and is a close relative of several major feed, fuel, and bioenergy grasses. It is a true diploid with a relatively small genome of $\sim$510 Mb. Its short stature, simple growth requirements, and rapid life cycle will greatly facilitate genetic studies of the C$_4$ grasses. Importantly, *S. viridis* uses an NADP-malic enzyme subtype C$_4$ photosynthetic system to fix carbon and therefore is a potentially powerful model system for dissecting C$_4$ photosynthesis. Here, we summarize some of the recent advances that promise greatly to accelerate the use of *S. viridis* as a genetic system. These include our recent successful efforts at regenerating plants from seed callus, establishing a transient transformation system, and developing stable transformation.

Why Study C$_4$?

C$_4$ photosynthesis is the primary mode of carbon capture for some of the world's most important food, feed, and fuel crops, including maize (*Zea mays*), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*), millets (e.g. *Panicum miliaceum*, *Pennisetum glaucum*, and *Setaria italica*), *Miscanthus x giganteus*, and switchgrass (*Panicum virgatum*). In contrast with C$_3$ plants, C$_4$ plants first fix CO$_2$ into a C$_4$ acid before delivering the CO$_2$ to the Calvin cycle (Hatch and Slack, 1966; Hatch, 1971). For example, in maize and sorghum leaves, CO$_2$ entering mesophyll (M) cells is first fixed into oxaloacetate, which is then reduced to malate in the M chloroplasts. The malate then diffuses into the inner bundle sheath (BS) cells and is transported into the BS chloroplast. There, malate is decarboxylated by NADP-malic enzyme, releasing CO$_2$ close to ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). This carbon shuttle greatly lowers rates of photorespiration as Rubisco is both isolated from the site of O$_2$ evolution (oxygen evolving complex of photosystem II) and also maintained in a CO$_2$-rich environment. Indeed, in mature maize or sorghum leaves, rates of photorespiration are at the limits of detection under conditions where C$_3$ plants lose up to 30% of their photosynthetic capacity due to photorespiration (Zhu et al., 2008). Accompanying this partitioning of photosynthetic activities are several anatomical adaptations. This includes close vein spacing and large numerous plastids of the inner BS. Together, these characters enable C$_4$ plants to thrive in environments that induce high rates of photorespiration in C$_3$ plants, such as the tropics or grassland savannas (Sage et al., 1999; Sage and Pearcy, 2000). An added benefit of the C$_4$ syndrome is improved nitrogen and water use efficiencies that have likely contributed to their global distribution and high rates of productivity (Sage, 2004; Tilman et al., 2006; Edwards et al., 2010; Edwards and Smith, 2010).

What Is the Grass?

"A child said, What is the grass? fetching it to me with full hands; How could I answer the child?. . . .I do not know what it is any more than he."

Walt Whitman wrote these words over one and half centuries ago (Whitman, 1855), and in many ways they are as true today as

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they were then. Despite the agronomic and ecological importance of C_4 grasses, little is known of the molecular mechanisms that underpin C_4 differentiation. Maize is perhaps the best-studied C_4 grass, and yet not a single transcription factor, kinase, phosphatase, or receptor has been identified that can be linked directly to C_4 differentiation. Why is this? Part of the reason is that large-scale genetic screens for C_4 mutants have not been conducted. For instance, it would be informative to screen large mutagenized populations for mutants that grow well under ambient conditions, but fail to grow under low CO_2 conditions. This could potentially identify mutants with defects in CO_2 concentration pathways. Similarly, mutants that are rescued under high CO_2 but fail to grow in ambient conditions may identify mutants with leaky BS cells. However, these screens are not trivial to conduct with large plants like maize as it is difficult to scrub CO_2 from large chamber volumes or keep concentrations of CO_2 elevated to very high levels (>1000 ppm).

Instead, in the most comprehensive genetic screen for C_4 mutants, Langdale and colleagues (Hall et al., 1998b) first identified mutants with photosynthetic defects and then examined the accumulation of carbon shuttle enzymes as a secondary screen. These screens have led to the identification of a few genes that affect the accumulation of C_4 shuttle enzymes and Rubisco. Examples include *Golden 2* (Hall et al., 1998a; Fitter et al., 2002; Waters et al., 2009) and *Bundle Sheath Defective* 2 (Roth et al., 1996; Brutnell et al., 1999; Wostrikoff and Stern, 2007). These mutants display BS cell-specific defects, but this is because the process or protein that is regulated by the gene products is localized to that cell type; therefore, these genes should not be regarded as genes regulating C_4 differentiation. For instance, BSD2 is required for the assembly of Rubisco holoenzyme (Brutnell et al., 1999) and displays a BS-specific defect because Rubisco is localized to the BS chloroplast. However, the *Bsd2* gene is expressed in both BS and M cells and plays an essential role in regulating Rubisco accumulation in both C_3 and C_4 plants (Roth et al., 1996; Brutnell et al., 1999; Wostrikoff and Stern, 2007). This localization of a general C_3 function to either BS or M cells is likely to be prevalent in C_4 grasses, as over 18% of the maize transcriptome is differentially expressed between these two cell types (Sawers et al., 2007), including a sizable fraction of the plastid proteome (Majeran et al., 2005, 2008; Friso et al., 2010). Thus, screens for BS or M cell-specific defects are likely to identify many activities that are strictly compartmentalized but are not directly promoting C_4 differentiation per se.

As alluded to above, the slow progress in dissecting C_4 traits is due, at least in part, to the relative recalcitrance of the current C_4 models to high-throughput genetic screens. The most extensively characterized C_4 plants include maize, sorghum, *Flaveria* sp (Asteraceae), *Amaranthus* (Chenopodiaceae), and *Cleome* (Cleomaceae); all lack efficient transformation systems, are large in stature, and have relatively long generation times, often of several months (Brown et al., 2005). Thus, the forward genetic screens that have been the foundation of *Arabidopsis thaliana*

research have yielded little in defining the networks underlying C_4 differentiation.

The Time for a New C_4 Model Has Arrived

The critical need for major gains in crop productivity (http://www.fao.org/docrep/011/i0100e/i0100e00.htm) and the burgeoning biofuels industry have together refocused attention on understanding C_4 photosynthesis. A long-standing goal of many members of the C_4 community has been to engineer C_4 traits into rice (*Oryza sativa*) as a way potentially to increase rice yields by 50% with reduced fertilizer inputs (Mitchell and Sheehy, 2007; Sheehy et al., 2007). This ambitious project is now being driven by the generous support of the Bill and Melinda Gates Foundation and taps the expertise of an international consortium of scientists (http://beta.irri.org/projects15/c4rice). However, these engineering efforts will require a more sophisticated understanding of both a C_3 grass (rice) and its C_4 relatives to identify the genes and networks that can be manipulated to transform a C_3 into a C_4 photosynthetic system (Hibberd et al., 2008). This will include restructuring the rice leaf to place veins closer together, increasing the cross-sectional area of the BS and engineering cell walls to facilitate the metabolic flux of sugars, amino acids, and C_4 acids between the BS and M cells. Major biochemical hurdles must also be overcome, including the engineering of a C_4 shuttle to pump CO_2 into the BS cells and driving cell-specific accumulation of transporter proteins. It is accepted that this is a high-risk project that likely will not be realized within the next decade. However, if successful, C_4 rice has the potential to form the foundation of a second green revolution.

Over the past 5 years, several billion dollars have been invested in bioenergy research as a means to offset the US dependence on oil and reduce greenhouse gas emissions (Ohlrogge et al., 2009). In a highly cited report by the U.S. Department of Energy (Perlack et al., 2005), several grasses were identified as potential feedstocks for a lignocellulosic fuel industry. Interestingly, of the top seven grasses named (maize, sorghum, *M. x giganteus*, switchgrass, big bluestem, *Arundo donax*, and reed canary grass), five use C_4 photosynthesis. Thus, the primary targets of the biofuels feedstocks community (cellulose, lignin, and hemicellulose) are the products of C_4 photosynthesis.

In summary, there is a critical need to understand the networks underlying C_4 photosynthetic differentiation as a foundation for engineering these traits into rice and for manipulating existing C_4 systems to yield more.

Setaria viridis: Redefining the Model System

Recent advances in sequencing technologies are transforming the field of plant science (Wang et al., 2010). Rather than decades, it will soon be possible to obtain draft genome sequences in a matter of months or weeks (Eid et al., 2009; Pushkarev et al., 2009). Thus, the availability of genome

sequence soon will not be a limiting factor in developing a model genetic system. Instead, more mundane characteristics, such as generation time, ease of growth, crossing, size, and self-fertility, likely will dictate the choice of a model system. A case in point is *Brachypodium distachyon*. Despite the availability of the rice and sorghum genomes, *B. distachyon* is gaining momentum as a model system because it requires less space and flowers faster than sorghum or rice and, perhaps most importantly, is readily transformable (Vogel and Hill, 2008). With an extremely small and well-annotated genome, *B. distachyon* is poised to become a powerful model system for understanding grasses (Vogel et al.,

2010). However, as a C_3 grass, its utility in dissecting C_4 photosynthesis is limited.

The C_4 photosynthetic pathway has originated multiple times within the grasses (Kellogg, 1999; Christin et al., 2008; Vicentini et al., 2008), having evolved, apparently independently, in subfamilies Aristidoideae, Chloridoideae, Micrairoideae, and Panicoideae (Sinha and Kellogg, 1996; Grass Phylogeny Working Group, 2001). The number of origins has been estimated to be as high as 17, although the precise number depends on whether reversal to C_3 is considered a possibility (Vicentini et al., 2008). C_4 species are divided into subtypes, named for the primary
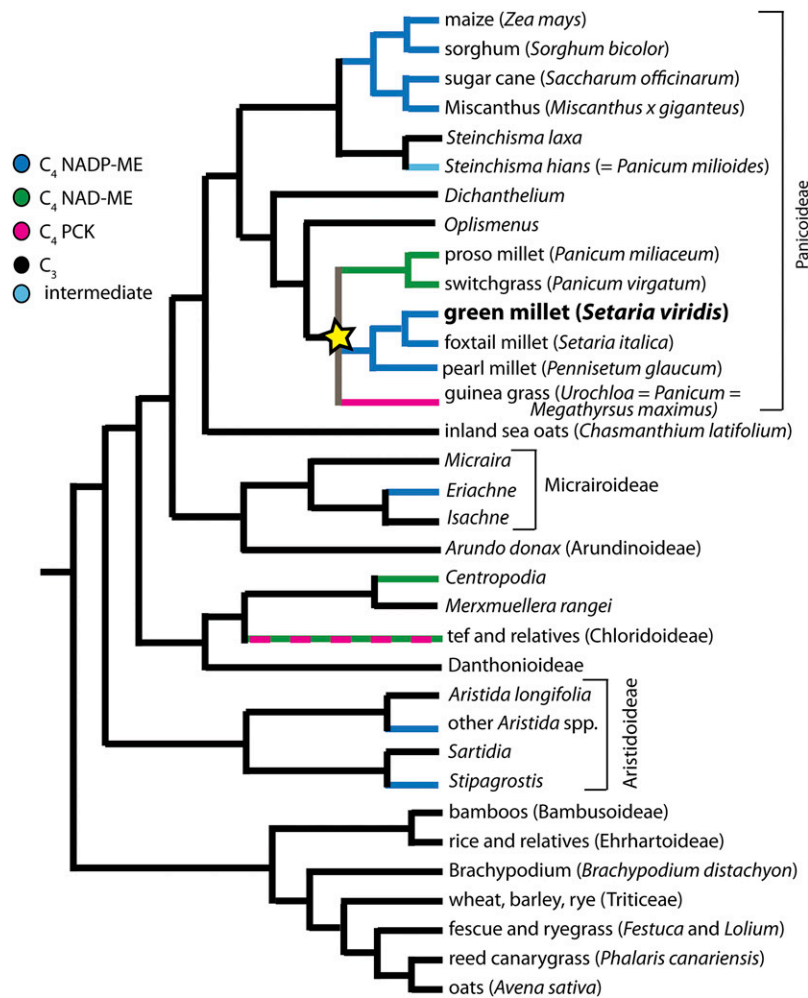

Figure 1. Phylogeny of the Grass Family.

Relationships based largely on Vicentini et al. (2008) and Christin et al. (2009), showing multiple origins of C_4 photosystems. *S. viridis* is an NADP-ME subtype C_4 grass that is closely related to the bioenergy feedstock switchgrass (NAD-ME subtype), the grain crop foxtail millet, and the agricultural weed guinea grass (PCK). The C_4 photosynthetic systems in this Setaria/Urochloea/Panicum (SUPa clade, indicated with a yellow star) arose independently from the NADP-ME family members of the Andropononeae (maize, sorghum, sugarcane, and *M. x giganteus*). Dashed lines show clades with multiple subtypes.

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decarboxylating enzyme that is localized to the BS. Maize, sorghum, and sugarcane use an NADP-dependent malic enzyme (NADP-ME subtype), whereas switchgrass and tef (*Eragrostis tef*) use an NAD-malic enzyme (NAD-ME subtype). A few taxa, such as guinea grass and some species of sand dropseed, use PEP carboxykinase (PCK) to generate a CO_2 pump in the BS cells. Most C_4 lineages are of the NADP-ME subtype, but there is a minimum of three independent origins of the NAD-ME subtype and two (and probably more) of the PCK subtype (Christin et al., 2009). Thus, C_4 lineages have likely exploited existing diversity in C_3 ancestors as a foundation for the C_4 syndrome (Hibberd and Quick, 2002; Sawers et al., 2007). Most current work has focused on maize and sorghum, which together represent only one of the C_4 origins and a single subtype. A summary of the origins and diversity of C_4 subtypes in the grasses is shown in Figure 1. This is a pruned tree based on relationships described by Vicentini et al. (2008) and Christin et al. (2008).

The clade within the Panicoideae indicated by the star in Figure 1 is of particular note as it includes representatives of all three C_4 subtypes. This clade includes a grain crop, *Setaria italica* (foxtail millet); a promising biofuel feedstock, *Panicum virgatum* (switch-grass); and a major agricultural weed, *Urochloa maxima* (guinea grass), each representing a different subtype of C_4. *S. viridis* (green millet) is closely related to pearl millet (*Pennisetum glaucum*) and foxtail millet (*Setaria italica*) and is likely the weedy relative of the domesticated foxtail millet (Dekker, 2003). As recently discussed, the crop *S. italica* offers a number of advantages as a model for bioenergy grasses (Doust et al., 2009), but as a model for forward and reverse genetics, *S. viridis* has some distinct advantages. Perhaps most importantly, *S. viridis* is significantly shorter than most *S. italica* accessions (see Supplemental Figure 1 online). We grow *S. viridis* using the same growth conditions, soil, and flats as *Arabidopsis* and typically grow 50 plants/flat to maturity in the growth chamber. Under short-day treatments, plants can be <10 cm in height at flowering. Second, *S. viridis* has a much shorter generation time. In short days, some accessions of *S. viridis* begin flowering within 2 weeks of planting, and mature seed can be harvested within 6 weeks of planting, approximately half the time of *S. italica*. One of the advantages of *S. italica* as a model system is that a draft genome assembly will soon be available from JGI-DOE as a major output of a recent DOE-USDA grant (D. Rokhsar and J. Bennetzen, personal communication; http://genomicscience.energy.gov/
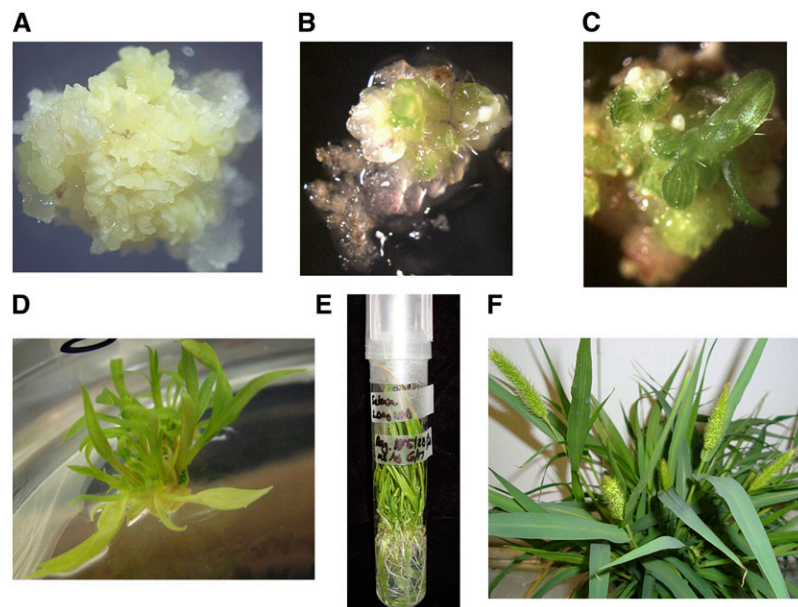

Figure 2. Regeneration of *S. viridis.*

Several steps in the regeneration process are shown, including the following.
(A) Callus formation induced from germinating *S. viridis* seeds.
(B) Initial shoot regeneration from callus.
(C) Young shoot after regeneration.
(D) Plantlets after transfer to rooting media.
(E) Rooted plant.
(F) Regenerated plants at flowering.

the inflorescence after flowering is complete. We have also found that placing the dried seed in a −80°C freezer overnight greatly improves the frequency of germination.

A recombinant inbred population derived from a cross between *S. viridis* × *S. italica* has been generated and a number of quantitative trait loci mapped using this population (Doust et al., 2005; Doust and Kellogg, 2006). In addition, some *S. viridis* accessions are available at GRIN (http://www.ars-grin.gov/),
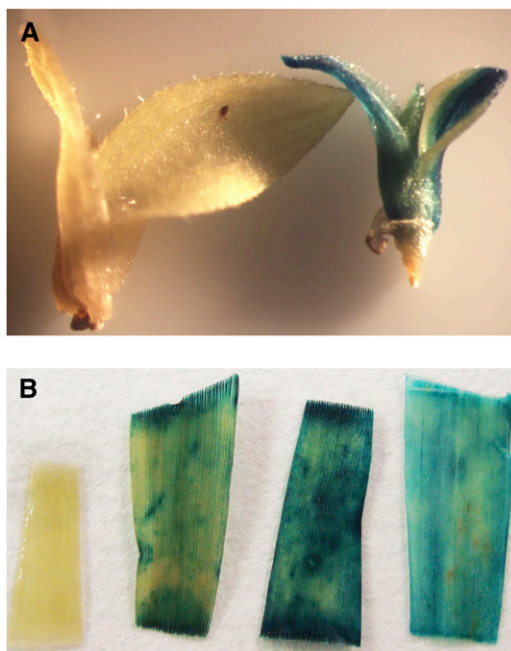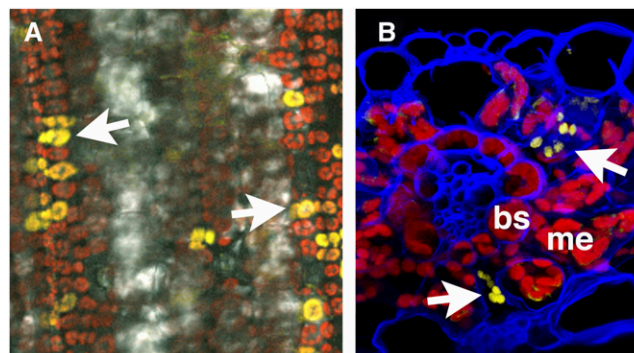

Figure 3. *A. tumefaciens*–Mediated Transformation of *S. viridis.*

(A) Developing shoots grown on selective media following cocultivation with *A. tumefaciens*. Image shows nontransformed (left) and GUS-positive (right) shoot.
(B) Mature leaf tissue from three independent GUS-positive transformants and a nontransgenic control (leftmost sample). A detailed protocol for *S. viridis* transformation is provided in Supplemental Methods online, and constructs used are shown in Supplemental Figure 2 online.

research/DOEUSDA/abstracts/2008bennetzen_abstract.shtml). However, this likely will not be limiting for long as efforts to sequence *S. viridis* are now underway as well at DOE and the Beijing Genomics Institute in China (D. Rokhsar and S. Huang, personal communication).

Although performing crosses in *Setaria* is challenging, it can be done. Because *S. viridis* flowers are bisexual, they must be emasculated or the pollen destroyed before crossing. The flowers can be emasculated by waiting until just before anthesis, cutting off the top of the anthecium with fine scissors, and then removing the anthers with forceps. This procedure is described in detail by Siles et al. (2001). Alternatively, the entire inflorescence can be dipped in boiling water for a few seconds to kill the pollen. In either case, because of the large number of flowers and the possibility of accidental self-pollination, the identity of seedlings must be verified using molecular markers. Seed propagation generally is not problematic with *S. viridis*. Even under conditions of rapid cycling (i.e., short days) a single plant can generate upwards of several hundred to several thousand seed. Although the fruits disarticulate readily, they are all caught within bags (we typically use maize ear shoot bags) that are placed over

Figure 4. Transient *A. tumefaciens*–Mediated Transformation of *S. viridis*.

S. viridis (accession A10) seedlings were grown on MetroMix 360 soil mix under a 12-h light/dark cycle with high relative humidity (75%) at a constant temperature of 23°C. The plants were watered as needed and every 3rd day with 20-10-20 fertilizer. Twelve days after germination, leaf 4 of a healthy *S. viridis* seedling was inoculated with *A. tumefaciens* strain AGL1 carrying the pPTN469 vector (Sattarzadeh et al., 2010) at a concentration of 0.05 (OD$_{600}$). The images show transient expression of a plastid-targeted YFP fusion protein. A detailed protocol for transient transformation is provided in the Supplemental Methods online, and constructs used are shown in Supplemental Figure 2 online.
(A) Low magnification fluorescence image showing abundance of transformed cells (arrows). The picture was taken using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems) using ×63 oil submerged objective lens. For the YFP signal (yellow), leaf tissue was excited with a 514-nm laser, and emitted light was collected from 525 to 575 nm. Autofluorescence (red) was captured from 650 to 789 nm. Image was compiled using Leica image software LAS-AF (version 1.8.2.) and Adobe Photoshop CS3 version 9.0.2 (Adobe Systems).
(B) Confocal reconstruction of leaf section showing transformed mesophyll cells (arrows). Chlorophyll autofluorescence is red and cell walls counterstained blue. bs, bundle sheath cell; m, mesophyll cells. Leaves were fixed in 2.5% paraformaldehyde, embedded in 7% low melting point agarose, cryosectioned, and counterstained with calcofluor white as described (Goldshmidt et al., 2008). The image was taken with a Carl Zeiss LSM 710 laser scanning microscope. A 514-nm laser excitation and 520- to 550-nm prism filter set was used to detect YFP emission (yellow), a 405-nm laser excitation and 475- to 500-nm prism filter set used for calcofluor white emission (blue), and a 488-nm laser excitation and 650- to 700-nm prism filter set used for detection of the chlorophyll emission (red). Subsequently, the confocal z-stack was reconstructed using Bitplane Imaris 7 software.

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though little molecular characterization of these materials has been performed. Thus, while *S. viridis* is suitable for classic genetic analysis, the genetics resources for this organism are clearly lagging the primary C_4 model grass, maize.

As an NADP-ME subtype C_4 plant, with a small genome of 510 Mb that will likely be available within the year, a rapid generation time, and small size, *S. viridis* is poised to be an excellent model system. The one major hurdle yet to overcome, however, is transformation. To the best of our knowledge, there are no published reports on tissue culture or transformation methods for *S. viridis*.

Transformation Methods for *S. viridis*

Often in monocot transformation, regeneration of plants from tissue culture is rate limiting. Thus, we first attempted to induce callus formation and regenerate plants from seed of *S. viridis* using published protocols for *B. distachyon* and *S. italica* (Rao et al., 1988; Rout et al., 1998; Vogel and Hill, 2008). As shown in Figure 2, we were able to successfully regenerate mature plants, which flowered and set seed, from seed callus. Although we have only propagated a small number of plants from seed callus through flowering, we have not observed any instances of somaclonal variation, and seed set in all regenerants is very high (>1000/plant). This is likely due to the relatively brief callus phase before plants are moved to regeneration media. A detailed protocol is provided in Supplemental Methods online. Most recently we have been able to transform *S. viridis* callus with a construct expressing a β-glucuronidase (GUS) transgene (the transgene pOL001 described in Vogel and Hill [2008]). We have been able to generate transformed shoots and several T0 plants (as shown in Figure 3) and are now optimizing our transformation procedure. We have also developed a transient expression system using *Agrobacterium tumefaciens*–mediated transformation (Janssen and Gardner, 1990) and have been able to introduce a plastid-localized yellow fluorescent protein (YFP) construct into leaf cells of *S. viridis* (Figure 4). We are now testing a series of constructs for subcellular localization patterns. Together, these studies are extremely encouraging and suggest that routine stable and transient transformation of *S. viridis* is now in reach.

Given the many attractive features of *S. viridis* as a genetic system, the time is right to begin detailed histological, physiological, and molecular characterizations of this plant. Systems modeling, which has shown its capacity in identifying limitations of C_3 photosynthesis (Lefebvre et al., 2005; Zhu et al., 2007), can be used to identify features critical for the high efficiency of C_4 photosynthesis. To do this, a systems model of C_4 photosynthesis must faithfully describe not only the biophysical and biochemical processes involved in C_4 photosynthesis, but also the diffusion process of major metabolites and gases through the system. Once such a model is available, combining it with measurements of the biochemical and anatomical properties of C_4 leaves will in turn allow sensitivity analysis of the CO_2 fixation efficiency to various parameters and environmental factors. Such an analysis will help identify the critical biochemical and anatomical features controlling efficiencies of C_4 photosynthesis. These features can then be focused on engineering C_4 photosynthesis into C_3 plants. Thus, the C_4 community could greatly benefit from a concerted effort to characterize the growth, development, and physiology of *S. viridis*.

Given the rapid progress that has been made in developing tools for *S. viridis*, the C_4 community may soon be able to rally around a common C_4 model. With any luck, we may be able to answer that outstanding question, "What is the [C_4] grass?", by fetching a handful of *S. viridis*.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Mature *S. viridis* and *S. italica* at Flowering.

Supplemental Figure 2. Schematic of Expression Constructs.

Supplemental Methods. Plant Regeneration and *Agrobacterium*-Mediated Transformation of *S. viridis*.

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