Chapter 8

Molecular Breeding for Stay-Green: Progress and Challenges in Sorghum

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Abstract

The stay-green trait is regarded as the best characterized characteristic conferring drought adaptation in several crops including sorghum. Quantitative trait loci (QTLs) for stay-green have been identified using several bi-parental populations. Several of these QTLs are currently being used for introgression in a number of genetic backgrounds. Part of the challenge in the introgression of these QTLs lays in the limited polymorphism between donor and recurrent parents. As a consequence, certain QTL can’t always be distinguished, such as Stg3 and StgB which are on the same chromosome, SBI-02. Current progress in marker technology is contributing to enhancing the marker coverage of QTL intervals and this would improve breeding efficiency. Despite the knowledge of genomic regions conferring the stay-green trait, it is surprising that knowledge of the physiological mechanisms explaining stay-green are still relatively unknown. Early explanations focused on a role of stay-green as maintaining photosynthetic activity. It has also been hypothesized that the stay-green trait relates to the plant nitrogen balance and in particular to the capacity to absorb nitrogen during the post-anthesis period. It is only relatively recently that water availability during the post-anthesis period, that is, when the stay-green phenotype expresses itself, has been proposed as a possible cause for the stay-green phenotype. However, the reasons that water is left for absorption are still unexplained and could be accounted for by either a deeper soil extraction depth or water saving traits operating at early stages. As the mechanisms responsible for stay-green become more evident and as DNA-sequencing technologies offer denser genome coverage, the likelihood is that the future of manipulating the stay-green trait will be about manipulating its physiological components.

Introduction

The capacity of certain genotypes in several annual crop species to maintain green leaves during the grain-filling period (the “stay-green” phenotype, SG) is an intriguing crop feature that has long been studied and included in breeding programs in several crops, especially under water-limited conditions. Indeed, the maintenance of green leaf area has been reported
to improve the quality of residues (van Oosterom et al. 1996), support the continuation of carbon fixation and supply of starch (McBee et al. 1983), prevent premature death and lodging (Rosenow and Clark 1981), sustain grain-filling under water stress (Rosenow et al. 1983; Rajcan and Tollenaar 1999a, 199b), and improve grain yield under stress (Borrell and Douglas 1996). Here we focus on using stay-green as a breeding target under water-limited conditions and review recent progress in different areas of stay-green research, with a particular focus on sorghum, where this trait has been most studied.

Given the potential benefit of stay-green, genotypes displaying this trait have been used to identify the genomic regions responsible for this phenotype. Several QTLs have been identified, using different breeding populations and stay-green QTL donors, and different types of drought stress. This information is reviewed and the most important QTLs are identified. We also review the experimental conditions in which phenotyping for stay-green has taken place and the different ways of assessing this phenotype, either from leaf senescence curves or leaf greenness assessments. A following section then summarizes current work being done at ICRISAT (International Crops Research Institute for the Semi-Arid Tropics, in Andhra Pradesh, India) to introgress several known QTLs for stay-green into various agronomically elite genetic backgrounds.

Our understanding of the stay-green trait and of the genetic regulation of mechanisms that lead to the expression of a stay-green phenotype in sorghum is still very incomplete. Early works considered the benefit of stay-green in terms of extending the period during which a leaf could actively fix carbon (McBee et al. 1983). Subsequent work also related to the carbon economy of the plant addressed the nitrogen status of the plant and in particular the balance between nitrogen demand and nitrogen capture (Borrell et al. 2001). A large amount of work, mostly involving transgenics, has addressed the question of maintaining the production of cytokinins to prevent leaf senescence (Gan and Amasino 1995). These views, which try to address the “symptoms” of stay-green, see in the degradation of the photosynthetic pigments the key entry point for manipulating the stay-green trait. These approaches are probably the complete opposite to more recent work that addresses the “cause” of stay-green and looks at stay-green from the angle of water supply, taking the view that stay-green expression is a consequence of having water available in the soil profile during grain-filling, when stay-green is actually measured (Vadez et al. under review). Therefore, two sections of this review will deal with these “early” and more recent considerations related to the stay-green trait.

As we progress in our understanding of the physiological mechanisms and genetic regulation of the stay-green phenotype in sorghum, manipulation of the trait is likely to evolve from the current introgression of genomic regions involved in expression of the stay-green phenotype, which we now know are likely explained by mechanisms of varied nature, to the introgression of these mechanistic components individually. For instance, it was found recently that B35 (= BTx642) donor parent alleles at stay-green QTL Stg1 contributed to increased water extraction by moderately senescent caudatum variety S 35 (Vadez et al. 2011), but did not do this in the genetic background of highly-senescent durra variety R 16. Therefore, work will be needed to identify the best germplasm options as donors for each of the components of the stay-green phenotype, and these may vary with the genetic backgrounds and specific soil, water, and temperature regimes in which improved drought tolerance is desired. Work will also be needed to measure the “baseline” component trait value of potential recipient genotypes. This would involve both the development of high through-put phenotyping methods for assessing these traits, and the refinement of molecular tools for deciphering the genetic basis of these key traits. Current efforts in sorghum are exploiting
genotyping-by-sequencing data to provide full-genome scans across >100,000 SNP (single-nucleotide polymorphism) loci in each member of a portion of a global reference collection of sorghum germplasm that has been phenotyped with a lysimetric system to explore the allelic variation available for key components of the stay-green phenotype. Similar phenotype and genotype data for available sets of stay-green QTL introgression lines in several genetic backgrounds are also being used to better characterize the genomic regions associated with each of six stay-green QTLs having favorable alleles from donor B35 (= BTx642).

Last but not least, the phenotypic evaluation of traits involved in drought adaptation is limited by the number of years and sites in which these experimentations can be carried out. This is so because many of the productive processes eventually generating yield are influenced substantially by environmental cues (e.g., Reymond et al. 2003). Given the large variability of agroclimatic (including weather and soil) conditions, and the difficulty of acquiring reliable yield estimates across environments, it is risky to rely on only experimental data to assess the value of a given trait, such as stay-green and its components, for yield improvement under water-limited conditions. The literature is riddled with reports of genotype-by-environment interactions, in one recent study, in the yield performance of a reference collection of groundnut (Hamidou et al. 2012): other studies simply address the environment-specific identification of stay-green QTLs across locations and years (Tuinstra et al. 1997; Crasta et al. 1999; Subhudi et al. 2000; Tao et al. 2000; Xu et al. 2000; Kebede et al. 2001; Haussmann et al. 2002; Sanchez et al. 2002). Accordingly, here we review how the use of crop-simulation modeling can contribute to an ex-ante assessment of the likely impact of traits shown to be associated with stay-green, in term of the probability of success, the possible range of yield increase, and geographic determination of where gains can be made.

### QTL Identification

The late 1990s and early 2000s have seen a plethora of studies aimed at identifying stay-green QTLs. Several studies used as the stay-green donor parent B35 (= BTx642), a BC1 derivative of IS12555, a durra sorghum from Ethiopia (Rosenow et al. 1983). Six QTLs for pre-flowering stress tolerance were mapped (Tuinstra et al. 1996), with B35 as a source of stay-green, in a cross with pre-flowering drought tolerant line Tx7078. The stress was imposed by withholding irrigation for several weeks during the vegetative period, until flowering of about 80% of the lines. Drought tolerance was assessed either by the absolute yield under stress, or by the ratio of yield, seed number, or plant height under stress to the same parameters measured under fully-irrigated conditions. In a later study, using the same mapping population, three trials were conducted. Although two of these trials did not have any drought effect and the water stress was applied by withholding irrigation at flowering, a 40% grain yield reduction was achieved under imposed drought treatment. Stay-green was evaluated by scoring each plot for this trait, using a 1-to-5 scale, weekly from flowering until maturity. Several QTLs for stay-green were mapped, with two of these, on linkage group F (SBI-10) and I (SBI-10), also co-mapped with yield under either only drought or under both drought and fully-irrigated conditions (Tuinstra et al. 1997). Crasta and colleagues (1999) also used B35 as a stay-green donor parent and Tx430 as a senescent parent to produce a recombinant inbred line (RIL) population of 96 individuals to map seven different QTLs (StgA, StgD, StgG, as major QTLs, and StgB, StgI.1, StgI.2 and StgJ, as minor QTLs). The scoring of stay-green was done by visual assessment, with the experiment carried out in the field, with plants exposed to post-flowering stress. Tao and colleagues (2000) identified five QTLs for stay-green in trials that were conducted in five locations over three years. They used QL41 as a donor source, and QL39 as a drought-sensitive elite parent. QL41 is a derivative from a
cross between QL33 and B35. Their work clearly showed that QTLs varied across environments and years, and that three stay-green QTLs were each detected in more than two environments.

Xu and colleagues (2000) identified four stay-green QTLs (Stg1, Stg2, Stg3, and Stg4) in a mapping population based on a cross between B35 and Tx7000. Two trials were conducted in two locations and two years, and stress was imposed by stopping irrigation at anthesis. Stay-green was assessed with a plot score of 1 to 5 at physiological maturity. Stg1 and Stg2, found on linkage group A (SBI-03), were consistently identified across locations and in both years, whereas Stg3 and Stg4 were on linkage group D (SBI-02) and J (SBI-05) and were found in specific seasons only. Using the same mapping population in other field trials, Subhudi and colleagues (2000) compared their QTL results to those of Crasta and colleagues (1999) and Tuinstra and colleagues (1997), and showed consistency of QTLs in different genetic backgrounds. Subhudi and colleagues (2000) showed that Stg2, Stg3 and Stg4 of the current populations corresponded to StgA, StgD, and StgJ of Crasta and colleagues (1999) and asserted that Stg2 was likely the most important for the expression of the stay-green phenotype. Although Stg1 of Subhudi and colleagues (2000) found no equivalent in Crasta and colleagues (1999), it was likely very closely related to StgB and Stg I.1 of Crasta and colleagues (1999). Sanchez and colleagues (2002) reported on the development of near-isogenic introgression lines for four stay-green QTLs (Stg1, Stg2, Stg3 and Stg4 of Subhudi et al., 2000) in a marker-assisted backcrossing program involving B35 as donor and Tx7000 as recurrent parent.

Kebede and colleagues (2001) have mapped QTLs for stay-green with another donor parent, SC56, a conversion line (3-dwarf plant height and reduced photoperiod sensitivity) derived from a Sudanese caudatum-nigricans landrace. Another donor parent for stay-green, E 36-1, a cultivar of Ethiopian origin, has also been used to map QTLs for the stay-green trait in two RIL mapping populations from which a total of seven QTLs were identified (Haussmann et al., 2002), with three of them being common to both populations. Kassahun and colleagues (2010) reported results validating StgB, which appears to be identical to a stay-green QTL on the long arm of SBI-02 that has been incorporated into elite breeding material in Australia by conventional pedigree selection (D. Jordan and A. Borrell, pers. comm.). More recently, a third stay-green QTL on SBI-02, in addition to the previously reported Stg3 and StgB, was reported by Haryarimana and colleagues (2010) as mapping to the interval flanked by SSR markers Xtxp19 and Xtxp298 on the short arm of SBI-02. Finally, Sabadin and colleagues (2012) have mapped two stay-green QTLs on SBI-02 (St2-1 and St2-2) as well as single stay-green QTLs on SBI-03 (St3), SBI-04 (St4), SBI-05 (St5), SBI-06 (St6), SBI-08 (St8), and SBI-09 (St9), together explaining 65 to 69% of the genetic variance for stay-green in two water-stressed environments, using an SSR-anchored, DArT-saturated (Diversity Arrays Technology) linkage map for a modest-sized RIL population based on the cross of BR007, a breeding line from the Embrapa Maize and Sorghum program in Brazil, and SC283, a USDA sorghum conversion line based on guinea landrace IS7173C, from Tanzania. When flowering time and plant height were used as cofactors in the QTL analysis, many of the originally detected stay-green QTLs in this population were no longer statistically significant; on the other hand, St3, St4, St8 and newly detected St10 were statistically significant and together explained 30 to 35% of genetic variation for stay-green in the water-stressed environments in the two years of testing.

Overall, six sources of the stay-green trait (B35 = BTx642, E 36-1, QL41, SC56, SC283, and SDS 1948-3) have so far been used for the identification of QTLs for this phenotype in sorghum. Several of the stay-green QTLs identified have been validated in different backgrounds. However, there is to date only scant understanding and knowledge of the
physiological mechanisms underlying each of these stay-green QTLs and their interactions – at least little that has been published towards trait expression.

QTL Introggression – Current Progress at ICRISAT

The initial stay-green QTL mapping studies used RFLPs (restriction fragment length polymorphisms) and AFLPs (amplified fragment length polymorphisms; Tuinstra et al. 1997), but later studies extensively used SSRs (simple sequence repeats; Subudhi et al. 2000, Haussmann et al. 2002, Harris et al. 2007) and DArTs (Sabadin et al. 2012). Most of the stay-green QTL introgression and QTL validation studies reported to date (Tuinstra et al. 1996, Harris et al. 2007) have used RFLPs and/or SSRs as flanking markers for foreground selection – along with RAPD (random amplified polymorphic DNA) and/or AFLP markers.

Researchers at ICRISAT-Patancheru selected six candidate QTLs for the stay-green trait from donor B35, including $\textit{Stg1}$, $\textit{Stg2}$, $\textit{Stg3}$, and $\textit{Stg4}$ reported by Subudhi and colleagues (2000), Sanchez and colleagues (2002), and Harris and colleagues (2007), as well as additional QTLs on SBI-01 ($\textit{StgA}$) and SBI-02 ($\textit{StgB}$), and initiated marker-assisted backcross (MABC) transfer of these into a number of genetically diverse, tropically-adapted elite sorghum varieties having a range of drought tolerance (Hash et al. 2003). Recurrent parents included highly senescent post-rainy season-adapted durra variety R 16, short 2-dwarf tan white-grained caudatum variety SIAP Dorado, and tall 2-dwarf tan, white-grained, sweet-stemmed caudatum sisterline varieties S 35 and ICSV 111. The positions of flanking SSRs for these six target QTLs were putatively inferred from stay-green QTL mapping studies published in the late 1990s and early 2000s (Table 8.1). The unavailability of alternate SSRs and lack of polymorphism was a major constraint for this activity in the early 2000s, when this work was initiated. As a result, most of the stay-green QTLs targeted for this introgression work were characterized by large confidence intervals between flanking markers and scant availability of flanking SSR polymorphisms between the donor and recurrent parents. These limitations on readily available flanking marker polymorphism had many implications for this attempt to introgress stay-green QTLs into several target genetic backgrounds. The lack of enough polymorphic SSRs spread across each QTL region resulted in a high probability of losing the QTL even after flanking marker confirmation because of the possibility of recombination occurring within one or more of the putative QTL target regions, linkage drag with unfavourable traits, and ultimately a lower level of recurrent parent genome recovery. Similarly lack of polymorphic SSRs between B35 (donor) and recurrent parents (especially Indian $\textit{durras}$) meant that no progress in stay-green QTL introgression work was possible in many target backgrounds until more markers were available. Accordingly, the MABC project was focused on two genetic backgrounds, R 16 and S 35=ICSV 111.

For the R 16-background, BC$_2$-progenies were developed with foreground selection each generation to confirm the presence of alleles from the donor parent at the SSR loci flanking each of the six putative stay-green QTLs, combined with limited background selection, in order to hasten the recovery of recurrent parent alleles in genomic regions distant from one or more of the target QTLs. These progenies were evaluated for stay-green expression (Kassahun et al. 2010), and one of the backcross progenies, RSG 04005, was confirmed as carrying three QTLs ($\textit{Stg3}$, $\textit{Stg4}$ and $\textit{StgB}$). This entry was used as the stay-green donor in another round of backcrosses to R 16 to derive single-QTL introgression lines. Two of the stay-green QTLs, $\textit{StgB}$ and $\textit{Stg3}$, are mapped on sorghum chromosome SBI-02. These two QTLs are linked to the morphological marker gene $\textit{Z}/\textit{z}$ controlling the important grain quality trait of mesocarp thickness, with the stay-green alleles from B35 linked with
Table 8.1. Details of sorghum SSRs used for foreground selection for marker-assisted backcrossing of stay-green QTLs at ICRISAT.

<table>
<thead>
<tr>
<th>Staygreen QTLs</th>
<th>Sorghum chromosome&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Physical distance (Mbp)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Linkage distance (cM)&lt;sup&gt;3&lt;/sup&gt;</th>
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<td>57.1</td>
<td>92.9</td>
</tr>
<tr>
<td>XSbAGB03</td>
<td>2</td>
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<td><strong>Mesocarp thickness</strong></td>
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<td>89.9-104.4&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>Xtxp001</td>
<td>2</td>
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<td>2</td>
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<td>Xgap084</td>
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<td>55.4</td>
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<td>87.9&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>58.5</td>
<td>103.2</td>
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<td><strong>Between Stg1 and Stg2</strong></td>
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<td>Xtxp218</td>
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<td>157.9-161.6&lt;sup&gt;4&lt;/sup&gt;</td>
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<sup>1</sup>Sorghum chromosome nomenclature as per Kim et al. (2005).

<sup>2</sup>Physical map distance (in MbP) as estimated by BLAST search of primer pair sequence of individual SSR with sorghum genome sequence as described in Ramu and Deshpande et al. (2010).

<sup>3</sup>Linkage distance (in cM) of SSRs as estimated in consensus map developed by Mace et al. (2009).

<sup>4</sup>Predicted linkage map position (putative interval in cM) of morphological markers as per Mace et al. (2010).
alleles for recessively inherited thick mesocarp. Similarly Stg1 and Stg2 had overlapping confidence intervals on SBI-03, and exhibited linkage with one of two epistatically interacting genes controlling pericarp pigmentation (Y/y on SBI-01 and R/r on SBI-03) and a gene controlling the absence/presence of awns (A/a). The Indian post-rainy season (rabi) sorghum has a very specific grain quality requirement of thin mesocarp with creamy/lustrous bold grain. Utilizing the intermediate BC2-progeny for further backcrossing helped to generate segregation for desirable phenotypes at the mesocarp and pericarp loci, recombined with B35 alleles at stay-green QTL-flanking SSR markers. This permitted isolation of white-grained, thin mesocarp progenies such as ‘K369white’ in later backcross generations. Such “clean” single-QTL introgression lines can now be used individually as donor parents of specific stay-green QTLs shown to have the largest favourable phenotypic effects across several genetic backgrounds.

Similarly, for stay-green QTL introgression in S 35 = ICSV 111-background, backcrossing was continued up to BC4 and BC5 with complete foreground selection and incomplete background selection during each backcross generation. This has finally produced sets of BCnF4/BCn+1F3 progenies. These progenies have been evaluated across locations and years for their stay-green expression in field, pot, and lysimeter studies under fully-irrigated non-stress conditions and rain-fed or supplemen tally-irrigated terminal drought-stress conditions during the cool, dry post-rainy (rabi) season in peninsular India. Several progenies still segregated for plant height and flowering time, and so were advanced by selfing for several additional generations following pedigree selection. Even though it took four to five seasons more to isolate clean versions of stay-green QTL introgression lines in this genetic background, the fact that it was achieved with limited SSR availability in the early days of marker-assisted backcrossing must be appreciated. These recently developed and validated “clean” stay-green QTL introgression lines are now available in several genetic backgrounds and can be used as parents directly in breeding programs to feed the current and future product pipeline.

We also tried utilizing another well characterized stay-green source, namely Ethiopian landrace germplasm accession E 36-1 (Haussmann et al. 2002). The major problem for utilizing this source was lack of marker (SSR) polymorphism, as E 36-1 belongs to same set of of zera-zero landraces that have contributed extensively in development of the agronomically elite caudatum and guinea-caudatum derivatives across global sorghum breeding programs over the past fifty years. Further, the major stay-green QTL from E 36-1 mapping to SBI-10 had a very large confidence interval and is linked with unfavourable alleles at neighboring shoot fly resistance component QTLs associated with seedling leaf blade trichome density and seedling glossines score. We are currently developing a fine-mapping population to break this unfavorable linkage by crossing an intermediate derivative of E 36-1 with E 36-1 alleles at the stay-green QTL on SBI-10 chromosome and a QTL introgression line with shoot fly resistance QTLs, utilizing newer marker techniques such as DArT, and single-nucleotide polymorphisms (SNPs) identified with a genotyping-by-sequencing (GbS) pipeline exploiting one of the new generation sequencing (NGS)-platforms (Elshire et al. 2011).

Fine-mapping populations for the best of stay-green QTLs validated in R 16 and S 35 backgrounds are being developed. These populations will be screened for several physiological parameters. This, along with newer marker systems such as DArTs and GbS-SNPs, will help to achieve deeper genome coverage for tracking important recombinants across large genomic region(s) present between the QTL-flanking SSR markers. These recombinants will help to identify near-isogenic lines for each of the stay-green QTLs, with no or minimal negative linkage drag, for direct utilization by breeding programs and as well for use in pyramiding multiple QTLs
to confirm whether or not their epistatic interactions are of economic importance. Currently a few selected best-performing stay-green QTL introgression lines are being used in microarray assay and proteomics studies seeking up- and/or down-regulated genes and to identify gene products specific to these gene combinations.

**Mechanisms Explaining Stay-Green**

**The Nitrogen and Carbohydrate Route**

The potential benefit of stay-green was initially viewed from the angle of the maintenance of photosynthetic activity (Rosenow et al. 1983; Thomas and Smart 1993; Borrell et al. 2000). Results showed that indeed delayed senescence of fully-irrigated *Lolium temulentum* leaves would increase the carbon fixation by 11% over the entire life of the leaf, simply by delaying senescence by two days (Thomas and Howarth 2000). Other results also showed that levels of basal stem sugars (Duncan 1984) or carbohydrate contents (McBee et al. 1983) were higher in stay-green sorghum genotypes. Sanchez and colleagues (2002) also made the assumption that the delayed leaf senescence from stay-green would sustain photosynthetic activity. Hence, a number of studies have documented the worthiness of maintaining photosynthetic activity of the leaf for more time. While this may be true in situations where there is no water limitation, and where there is a light-capture interest of delaying leaf senescence, this may be less of a value in situations where water is limited and photosynthetic activity is bound to be regulated by stomata opening. Therefore, we would argue that the contribution of stay-green in terms of carbon fixation under water-stress conditions may likely be very limited.

Another approach to explaining stay-green differences has been to assess their role in the nitrogen balance of the plant. In crops producing grain, the most important nutrient required to fill up grain is nitrogen and it is remobilized from the N-rich leaf tissues (Sinclair and Vadez 2002). As rubisco, a central enzyme for the conversion of CO₂ into carbohydrates, accounts for about half the nitrogen in leaves of C3 plants and about 25% of the leaves of C4 plants, remobilizing N from rubisco and photosynthetic pigments implies that the photosynthetic rate is bound to decrease during grain filling. For instance, Borrell and Hammer (2000) showed that senescent and stay-green sorghum hybrids differed in the supply-demand balance for N, with stay-green having a shortfall in N that is about 25% lower than that in senescent hybrids, and explaining a slower rate of leaf senescence in the stay-green genotypes. A similar case was reported in maize, where a stay-green hybrid acquired up to 60% of its N supply during the grain-filling period, whereas a senescent hybrid acquired only 40% of its total N during the same period (Rajcan and Tollenaar 1999a). This showed the importance of maintaining N uptake during grain filling in staygreen lines across different species. Subedi and Ma (2005) also clearly showed that in both stay-green and senescent maize hybrids, stopping the supply of N from V8 to maturity dramatically accelerated the decrease in leaf greenness, measured by SPAD readings, compared to a treatment in which N supply was maintained. Another study also showed that under low-N conditions, there were genotypic differences in sorghum in the capacity to extract N from the soil profile (Nakamura et al. 2002). For these reasons, the N status of a plant is still considered an important factor in the expression of stay-green. Among the five cases of stay-green reviewed by Thomas and Howarth (2000), the type E stay-green is a case where senescence initiates at a similar date and follows a similar rate to a senescent type, but the higher initial N content in the leaves buffers the grain-filling-induced decline in leaf-N. That is, the current view is that an increased N uptake by roots during grain-filling leads to longer duration of leaves, and the higher specific leaf N (SLN) levels maintains the photosynthetic activity of these leaves at high levels for a longer period.
Given the tight N supply-demand balance, it has been shown that de-topping plant panicles can indeed delay leaf senescence. For example, Rajcan and Tollenaar (1999b) showed that green leaf area maintenance was higher in situations of high source:sink ratios, achieved by partial or full prevention of maize cob fertilization. A similar phenomenon occurs in the case of genotypes having a poor grain yield potential and therefore a poor sink for N, leading to the expression of a “yield-resistant” stay-green phenotype. This association of stay-green expression with a low grain-yield trend has been one of the main criticisms of the use of the stay-green trait in crop improvement (Ludlow and Muchow 1990). However, Borrell and colleagues (2000) and Haussmann and colleagues (2002) showed a positive correlation between stay-green expression and grain yield under terminal drought-stress conditions; although in the latter study, one of the two RIL populations that was used showed no correlation between grain yield and stay-green expression. Tuinstra and colleagues (1997) also identified two QTLs for sorghum stay-green that co-mapped with grain-yield QTLs. So it appears that the relationship between stay-green expression and grain yield under terminal drought-stress conditions depends both on the environment and on the background that are considered. One of the future challenges will surely be to identify the genotypes and the environments in which stay-green expression is not at the expense of grain and/or stover yield potential. The potential of stay-green genotypes to accumulate N during the grain-filling period, provided that grain yield potential is not compromised, is the most promising hypothesis to explain an N effect on the expression of stay-green that could have agronomic relevance. Further work is needed to understand the processes that allow N absorption to be sustained during grain-filling, especially under water-limited conditions. As discussed in the next section, the capacity to absorb N during grain-filling under such conditions is bound to be closely related to water status issues (having water left in the profile to allow N absorption).

Addressing the Symptoms or Addressing the Causes?

Thomas and Howarth (2000) reviewed different modalities resulting in the display of a stay-green phenotype. They have suggested five ways to stay green. Of these, four were concerned with the rate and onset of pigment decline. Pigment degradation is a self-programmed process in plants during maturation and there are a number of factors affecting N remobilization that alter that natural process. For example, de-topping the panicle delays leaf senescence. However, even after removing the effect of N-status-altering processes, pigment degradation continues its natural way. Research initiated to counter that process found that enhancing cytokinin production delayed pigment degradation in tobacco leaves (Gan and Amasino 1995; Roitsch and Ehneβ 2000). A number of studies have followed in which transgenics were developed, which contained a gene contributing to enhanced cytokinin production to retard pigment degradation (Rivero et al. 2007; Peleg et al. 2011), and which were reported to be drought tolerant. While the maintenance of green leaf area may be beneficial in situations when water is not limiting, as earlier shown in *Lolium temulentum* (Thomas and Howarth 2000), the overall approach and hypothesis of its value under water-limited situation remains questionable. Plants exchange carbon dioxide for water through the stomata and under water-limited situations the degree of stomatal opening is what sets the photosynthetic rate, because of the absolute necessity to match stomatal opening to the limited water available. Therefore, the cytokinin-related maintenance of green pigmentation under water-limited conditions may be assigned to a “type-C” stay-green as defined by Thomas and Howarth (2000), that is, a type that stays green but in which the photosynthetic functionality is equivalent to a senescent line because of the effects of water limitation on stomatal opening. Although the work on the overexpression of cytokinins to retard leaf senescence is intriguing, we would argue that it may fit
situations of mild water stress or no water stress, where indeed maintaining longer leaf life could be beneficial.

Surprisingly, the past 25 years of research on the stay-green phenotype have only lately led to examination of the possible association between stay-green expression and plant water status, although some relation between stay-green expression and plant water status had been hypothesized early (Tuinstra et al. 1998). More surprising is the fact that several reports had shown that stay-green was likely associated with maintenance of root growth (Hatlitligil et al. 1984; MacKay and Barber 1986), with the hypothesis that enhanced root growth would contribute to enhanced N absorption. As we saw in the previous example, the maintenance of a functional stay-green under water-limited conditions, that is, a plant type having both green leaf area remaining and active photosynthetic activity, depends on having water available in the soil profile at the time of leaf senescence. The difficulty in testing this hypothesis is concerned with methods that can be precise enough to assess plant water extraction at a fairly late stage of plant growth when stay-green expression is at its maximum. Recently, a lysimetric system has been developed at ICRISAT-Patancheru (Vadez et al. 2008), which consists of long and large plastic tubes in which plants are grown with the spacing and soil exploration volume they would have in a natural field conditions. This system has allowed the measurement of the pattern of water uptake to support transpiration in several crops, including sorghum (Vadez et al. 2011), chickpea (Zaman-Allah et al. 2011), and peanut (Ratnakumar and Vadez 2011). Using this system, a set of pearl millet topcross hybrids contrasting for their level of terminal-drought tolerance were assessed under conditions of terminal-drought stress, imposed by stopping irrigation at flowering time. The results clearly showed that hybrids differed in their stay-green expression as the stress developed and showed highly significant correlation ($R^2 = 0.76-0.79$) with the water extracted three weeks after panicle emergence (Vadez et al. unpublished). These results have been confirmed in several experiments of pearl millet and offer an outstanding demonstration that stay-green directly relates to the water availability during the grain-filling period. One of the exciting challenges of the coming year, using that system, is to test the hypothesis, which could not be tested before, that maintaining water uptake during the grain-filling period would also indirectly drive N uptake during the same period. As seen above, several stay-green genotypes in different species have been shown to enhance N uptake during the post-anthesis period. Since N uptake requires that this nutrient be dissolved in water to be taken up, what remains to be establish is whether the higher N uptake could be a consequence of a higher water uptake.

Water in the soil profile can become available during the grain-filling period through several possible mechanisms. The most immediate one is the capacity to extract water. This has been shown in wheat (Manshadi et al. 2006), where a stay-green wheat genotype extracted more water from deeper layers of the soil profile than did a senescent line. Recently, the stay-green QTL $Stg1$ in sorghum has also shown its capacity to enhance water uptake in senescent S 35 background (Vadez et al. 2011b). However, the effect of $Stg1$ was not visible in the R 16 background. The likely explanation for this is the higher “baseline” capacity for extracting water in R 16 than in S 35. This highlights the importance for future research on stay-green to precisely decipher the mechanisms involved, and to determine whether any of these mechanisms are already available in intended target recurrent parent genotypes.

The case of pearl millet described above is interesting because the materials that differed in stay-green (Vadez et al. in preparation) did not differ in the total water extracted from the soil profile. In other words, stay-green in this case was not related to an effect on rooting. By contrast, other studies have showed that these materials vary in constitutive water-saving traits, that is, through a lower leaf conductance (Kholova
et al. 2010a) and a further restriction of leaf conductance under high vapor-pressure deficit conditions (Kholova et al. 2010b). The expression of these traits, at the vegetative stage in the absence of water stress, leaves water available in the soil profile eventually leading to stay-green expression differences (Vadez et al. in preparation). Similar findings have been reported in stay-green Miscanthus genotypes, where the stay-green genotype Sin-H6 appeared to have a lower leaf conductance (Clifton-Brown et al. 2002). In the case of pearl millet, several QTLs have been identified for these water-saving traits (Kholova et al. 2012). Interestingly, water-saving traits, measured in pots, and stay-green expression and yield measured under field conditions co-map to the same genomic regions (Sehgal et al. in preparation).

Other possibilities for saving water during the vegetative growth stage, before any stress occurs, involve the development of smaller leaf area. One recent report shows that having a faster leaf-appearance rate reduced tillering and then decreased the overall plant leaf area at anthesis. The effect was to decrease water use prior to anthesis, leading to higher grain yield under terminal drought conditions (van Oosterom et al. 2011). In our current work at ICRISAT-Patancheru, we have also demonstrated the capacity of certain stay-green QTLs from donor parent B35 to reduce the leaf size in S 35 background (Kholova et al. unpublished). However, it was also shown that faster leaf-appearance rate was sensitive to temperature and that the beneficial effects were reduced in higher temperature environments (van Oosterom et al. 2011). Similarly, leaf expansion is highly dependent on both the evaporative demand and soil moisture in maize (Reymond et al. 2003). Therefore, future challenges with the use of stay-green expression will also be to better understand how some of the explanatory mechanisms of stay-green, like the leaf-area development addressed here, respond to the environment. Unless this is precisely known, the prediction of the effect of stay-green mechanisms will be inaccurate and the use of stay-green in breeding will be a blind exercise at best. Therefore, the use of stay-green in the future will very likely evolve to introgressing genomic elements involved in its key mechanisms rather than introgressing QTLs for stay-green per se. This implies that a more thorough understanding is needed that can decipher the mechanisms underlying stay-green expression in sorghum (and other crops in which this trait might be found useful), and the interaction of these mechanisms with the environment. This work is on-going at ICRISAT in India and in Niger.

**Advances in Sorghum Genomics and Applications for Stay-Green Research**

Among the available marker systems, simple sequence repeat (SSR) markers gained breeders’ interest for mapping and introgression of different traits in crop species because these markers are amenable to simple assays, multiplexing, and reproducibility, and more importantly are co-dominantly inherited. SSR markers have been greatly exploited for the mapping of different traits in sorghum, and the stay-green trait is no exception (Haussmann et al. 2002, Harris et al. 2007, Habyarimana et al. 2010, and Sabadin et al. 2012). The major limiting factor for utilization of SSR markers is their resolution power. Recent advances in sorghum genomics, including availability of an aligned sorghum genome sequence (Paterson et al. 2009), access to larger numbers of markers including both SSRs (e.g., Ramu et al. 2010) and DArTs (Mace et al. 2010), with very large numbers of GbS-SNPs on the way (Elshire et al. 2011, Nelson et al. 2011). Alignment of major trait genes and QTLs to integrated linkage and physical maps (Mace et al. 2011) has strengthened the foundation for better integration of molecular marker technologies in applied sorghum breeding programs.

With the invention of next generation sequencing (NGS) technologies, identification of a large number of markers, especially single
nucleotide polymorphism (SNPs), has become very inexpensive compared to other marker systems. Utilizing an Illumina NGS platform, Ed Buckler’s lab at Cornell University has developed a technically very simple and highly multiplexed (96-plex/384-plex) method for rapidly and inexpensively sequencing large numbers of DNA samples, and subsequently analysing the sequencer output with an associated bioinformatics pipeline for genotyping germplasm of any species. This protocol is referred as genotyping-by-sequencing (GbS) (Elshire et al. 2011). For this procedure, genome complexity is reduced by digestion of each DNA sample with restriction enzymes, and the resulting restricted fragments are then ligated with sample-specific “barcodes,” called “restriction site-associated DNA tags” (RAD tags), and the restricted, barcoded DNA samples are then multiplexed (at 48-, 96- or 384-plex) and subjected to “skim” sequencing to a depth of 0.1X. The resulting 66-base pair sequence reads (after sorting by barcode) are aligned to the reference genome sequence of BTx623 (Paterson et al. 2009) to identify SNPs with the help of customized bioinformatics pipelines. This analytical pipeline can readily be adapted for species lacking a reference-aligned genome sequence.

By employing GbS, ~265,000 SNPs have been identified for stay-green donor parents E 36-1 and B35 by aligning their skim sequence reads against the sorghum reference-genome sequence. The primary challenges involved in handling these large data sets are the need for substantial computational power. Analysis of combined field, pot, and lysimeter phenotype data sets for QTL introgression line sets and RIL populations is underway at present, and in the near future we expect to be able to identify genomic regions (major and minor effect QTLs) associated with putative components of the stay-green phenotype, with or without terminal-drought tolerance, in sorghum. The high marker-density and genome-wide coverage that is possible with this GbS-SNP platform will help us to identify SNPs closest to or inside the individual genes and/or regulatory elements associated with variation in stay-green phenotype. Once genomic regions associated with underlying mechanisms of the stay-green trait are identified, tagged SNPs (reduced representations of SNPs based on their linkage) can be identified and converted to a customized SNP assay using the BeadXpress platform (currently available in ICRISAT’s Genomics Service Laboratory at Patancheru) or CAPS (cleaved amplified polymorphic sequences) markers. Such SNP markers can be used individually or in small multiplexes at a much lower cost than that required for genome-wide genotyping with the GbS platform and will be appropriate for use in foreground genotyping and identification of recombination events occurring in QTL-flanking regions during the transfer of this trait to desired sorghum recurrent-parent backgrounds. This will greatly improve the efficiency of introgression of the stay-green trait and its components, by reducing the number of breeding cycles required (for recurrent-parent background genotype recovery) and facilitating stacking of complementary stay-green alleles at various loci as may be needed for improved variety development.

Application of NGS tools such as GbS for dissecting complex traits such as stay-green at the DNA-sequence level will capture most of the functional factors of the genome related to trait expression. However, another application of NGS tools in RNA-sequencing (commonly referred as RNA-seq) will help to capture the regulatory elements (Ozsolak and Milos 2011). For a complex development trait such as stay-green, many plant growth and development pathways are involved, probably throughout the life cycle of the plant, for trait expression. Application of RNA-seq can help us understand the role of regulatory and transcription factors (including small RNA, micro RNA) and their interactions with other pathways. We hope to utilize recent advances in RNA-seq technologies with the recombinants identified from the ongoing fine-mapping exercise to move toward a better understanding of stay-green expression.
associated with one or more stay-green QTLs in sorghum.

**Use of Modeling to Manipulate Mechanisms Associated with Stay-Green**

Among research areas attempting to address the food and feed demand of growing human and livestock populations living under conditions of harsh climate and erratic rainfalls across the semi-arid tropics, crop improvement efforts are not only particularly challenging but are also particularly promising. Despite the progress made in the field of crop breeding strategies, for example quantitative genetics, marker-assisted selection processes, improvement of trait-screening techniques, investigation of stress tolerance differences, and so forth, the progress made in the development of improved cultivars has been slow because of complex interactions of genotype and environmental factors, including management practices (i.e., the G × E × M problem). This slowed progress is partially because investigating these interactions in vivo requires years of precisely managed multi-locational field trials, which are extremely time- and cost-intensive, and often simply impossible to do properly covering all possible relevant environments.

This as yet unresolved G × E × M problem suggests that the existence of crop genotypes that can adapt to a broad range of stress environments is very unlikely and that breeding strategies for stressful environments should probably instead focus on the development of crop genotypes suited to particular environments. In recent years a pragmatic way appeared for at least beginning to decipher the complexity of G × E × M interactions by crop simulation modeling. This approach interlinks mechanistic knowledge of crop growth characteristics and allows estimates of crop productivity across the region(s) of interest. The crop model sensibility is highly dependent on knowledge of a given production environment (weather, soil) as well as knowledge of the crop, making it extremely useful as a guideline for “precision breeding,” that is, analysis of the environments from the crops’ perspective and development of genotypes possessing specific features that permit maximum utilization of environment potential. In other words, modeling allows reasonable diagnostics of environment-restricting factors, such as type of drought stress and probability that a crop will face a particular stress type at a specific location (Chenu et al. 2011, Hammer and Jordan 2007, Chapman et al. 2008).

Such knowledge can be used further for (1) in vivo selection and screening for crop traits providing putative adaptation in the well-defined target environmental conditions and management practices (2) in silico designing of virtual genotypes possessing hypothetical/existing traits and estimation of their benefits across time in the location of interest with a given suite of management practices. This approach has already been used for the characterization of environments for wheat and sorghum in Australia (Chenu et al. 2011, Hammer and Jordan 2007, Chapman et al. 2008) and there is an on-going effort to diagnose the sorghum production constraints using this methodology for winter cropping seasons (post-rainy season) of the semi-arid tropics in peninsular India. Here the modeling tool allowed, for the first time, the differentiation between various water-stress types, quantification of stress types’ frequencies, and their effects on sorghum production across heterogeneous parts of major production regions (Kholova et al. in preparation). At the same time, substantial progress has been made in understanding the mechanisms contributing to drought adaptation (e.g., water utilization dynamics and efficiency, plant developmental dynamics, and N utilization – many of which may result in so called “stay-green” phenotypes; see section above on “Mechanisms Explaining Stay-Green”). The well-defined physiological basis of any genotypes’ specific machinery can be simulated using such models and tested across a range of specific environments. In this way, modeling can help approximate the
benefits or possible negative trade-offs of any given trait/mechanism and thereby estimate its potential value for region-specific breeding programs.

As an example, the genotype-specific stomatal closure at high evaporative demand, one of the “water-saving” traits reviewed above, is a feasible proposition. The idea of intra-specific variability in VPD(vapor pressure deficit)-driven stomatal closure was proposed long ago (e.g., Squire 1979), although it was not until recently that this mechanism was explored by modeling (Sinclair et al. 2005; 2010). In the meantime, variability for VPD response was found across other crop species (e.g., groundnut: Devi et al. 2010; pearl millet: Kholova et al. 2010; and chickpea: Zamman-Allah et al. 2011). There is evidence that similar variability exists in sorghum (Gholipoor et al. 2010), and, indeed the model suggests that this mechanism will lead to desired improvement of the post-rainy season sorghum cultivation in terms of absolute grain yield as well as yield stability (our work in progress).

**Conclusions**

Much progress has been achieved in the deciphering of genomic regions responsible for the expression of the stay-green phenotype in sorghum. This information has been used in a number of breeding programs worldwide, mostly through marker-assisted backcrossing to move donor parent alleles for this trait into otherwise locally-adapted agronomically elite open-pollinated varieties and/or hybrid parental lines. However, the physiological mechanisms underlying the expression of stay-green are less clear. It appears now that the availability of water during the grain-filling period, when stay-green is scored, is the most likely candidate, and may likely be additive to N absorption after anthesis. The sources of water availability could be several, including water-saving traits but also possibly deeper rooting capacity. Clearly, the more recent progress pointing at clear mechanisms explaining the stay-green phenotype will likely reorient the breeding of stay-green towards the breeding for its most important components. This will require a “re-mapping” of these explanatory traits and possibly the identification of new/better donor sources for these traits than the donors for stay-green that are currently in use. The recent dramatic progress achieved in terms of density of marker coverage across the full nuclear genome will be extremely useful for precisely mapping these explanatory traits. Several of the putative traits leading to stay-green expression closely interact with the environment. Therefore their manipulation will also require a thorough understanding of these interaction effects, and the use of crop simulation modeling will then become increasingly important to help the breeding program navigate the complexity of plant-trait interactions with the environment (including crop management practices), in order to better target the type of trait combinations needed for each specific target environment.

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**References**


Mace ES and Jordan DR. 2010. Location of major effect genes in sorghum (Sorghum bicolor (L.) Moench). Theoretical and Applied Genetics, 121: 1339–1356.


