

## Biotechnological Approaches to Evolve Sorghum (*Sorghum bicolor* (L.) Moench) for Drought Stress Tolerance and Shoot fly Resistance

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### Abstract

Sorghum is a model tropical grass that uses C<sub>4</sub> photosynthetic activity. But its yield is affected by many abiotic stresses like heat, drought, cold, salt and also biotic stresses such as shoot fly, midges, and stem borer from seedling stages to maturity. This article summarizes the terminal drought stress tolerance mechanism with stay-green phenotype expression during post-flowering and also mechanisms of early shoot fly resistance during seedling stages of crop growth. The trait stay-green is extensively studied and its correlation to yield makes the stay-green trait more special for research and in marker assisted back cross programs. Under terminal drought stress conditions, stay-green trait is expressed with a complex mechanism involving many transcription factors, chlorophyll retention and nitrogen remobilization from leaves to maintain longer photosynthetic activity. Shoot fly resistance on the other hand, involves many physico-chemical, biological and morphological traits. Out of the many morphological traits, seedling leaf blade glossiness and trichome density are well characterized at genetic level and can assist as shoot fly resistance sources in marker-assisted breeding programs as they are highly negatively correlated with shoot fly dead heart formation. However, quantitative trait loci (QTL) mapping studies and candidate genes identified for the

stay-green and shoot fly component traits need to be further validated with fine mapping, gene cloning and expression level studies. Pyramiding these two traits into a high yielding sorghum variety may lead to multiple stress resistance which could ultimately benefit the marginal farmers in India.

**Keywords:** Sorghum, shoot fly resistance, stay-green, drought tolerance, QTL, marker-assisted selection

### Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a cultivated tropical crop plant that belongs to the family Poaceae, tribe Adropoganeae and genus *Sorghum*. Sorghum is largely self-pollinated diploid crop (2n=2ö=20) with fully sequenced genome length of ~730Mb (1). It is the fifth most important cereal crop globally (2) providing food, feed, fiber, fuel, and chemical/biofuels feed-stocks across a range of environments and production systems. USA, India, México, Nigeria, Sudan and Ethiopia are the major producers of sorghum. Other sorghum producing countries include Australia, Brazil, Argentina, China, Burkina Faso, Mali, Egypt, Niger, Tanzania, Chad and Cameroon. Grain is mostly used as food (55%), in the form of breads and porridges in Asia and Africa, and as feed (33%) in the Americas. Its stover is an increasingly important source of dry season

fodder for livestock, especially in Asia (<http://www.icrisat.org/crop-sorghum.htm>). Its remarkable ability to produce yields under adverse conditions like arid and semi-arid regions, where water limited conditions exists alongside heat stress. This makes sorghum an important 'fail-safe' source of food, feed, fiber, and fuel in the global agro-ecosystem. Sorghum is a representative of tropical grasses that use C<sub>4</sub> photosynthesis, which results from complex biochemical and morphological specializations that improve carbon assimilation at high temperatures. While the world's average annual yield for sorghum was 1.08 tonnes per hectare in the year 2012, total production from all sorghum producing countries was 57 million tonnes. FAO reported the United States of America as the top sorghum producer with a harvest of 1.22 million tonnes followed by India, Nigeria, Mexico and Sudan (3). In India, with its large population and fragile balance in the production and demand equation for food grains, sorghum plays a crucial role in national food security. Attempts to increase the production of sorghum with the introduction of new high-yielding varieties and hybrids since 1966, was largely unsuccessful because of the susceptibility of the improved cultivars to various abiotic (drought) and biotic (shoot fly) (4,5,6,7) stresses. But the rate of loss due to biotic and abiotic stresses in sorghum year by year is increasing.

**Drought stress and stay-green trait :** Abiotic stresses are the most harmful constraints concerning the growth and productivity of crops worldwide. After soil nutrient deficiency, drought stress is the most important abiotic constraint for sorghum production globally (8). Sorghum is well adapted to semi-arid environments and regarded as model crop for studying drought stress tolerance among grass species. So, breeders mostly have focused on improving drought stress tolerant varieties of sorghum (9). If plants withstand drought spell occurring at grain filling stage, it is defined as terminal drought tolerance. Drought stress during and after flowering typically causes premature leaf senescence which in turn

lead to stalk lodging, stalk rot disease, reduced grain filling, and significant grain and stover yield losses. Plant characters best associated with post-flowering drought tolerance, may be due to delay in leaf senescence or non-senescence or "stay-green" trait (9,10,11,12,13,14,15,16). Therefore, the "stay-green" trait is more than the ability of the plant to maintain functional green leaf area (GLA), to improve quality of residues (17), to support the continuation of carbon fixation and supply of starch to the grain filling site (18), to prevent premature death and stalk lodging (19) and to sustain grain-filling under water stress to improve yield (14,20). Stay-green is of three types. Type A stay-green phenotypes have a delayed onset and a normal rate of senescence following its onset. Type B stay-green phenotypes initiate leaf senescence normally but the rate of senescence is comparatively slower. Type C stay-green phenotypes retain chlorophyll despite the normal onset and progression through senescence (21). Many crop plants other than sorghum like rice, wheat, maize, barley, cotton, tobacco have been reported till date with stay-green character.

**Mechanism of drought tolerance/stay-green and factors associated with stay-green**

: Molecular mechanisms underlying delay in senescence which extend the duration of active photosynthesis in sorghum have not been elucidated completely. Rosenow et al. (20) observed positive impact of delayed leaf senescence on crop performance of plants under water limited conditions during grain filling. Presence of stay-green phenotype is a result of balance between nitrogen (N) demand by grain and nitrogen captured by vegetative parts of plants like increasing the supply of water by modified root architecture which increases water extraction from soil or reducing water demand by reducing transpiration loss. Nitrogen remobilization from leaves maintain longer photosynthetic activity and supply adequate carbohydrates to developing grains (10,22,23). It appears that carbon, nitrogen ratios and ABA levels affect senescence. Besides them,

cytokinins also play a role in leaf senescence and increased production of cytokinins lead to delayed leaf senescence (24). Stay-green was influenced by genetic factors, environmental factors like high temperature, soil-water holding capacity, soil moisture content at planting, vapor pressure deficit, rain fall during cropping and management factors like population size and planting time (14). Leaf chlorophyll content was also significantly correlated with stay-green scores under drought conditions as pointed out by Xu et al. (25).

Nodal root angle depends on vertical and horizontal distribution of roots in soil. Their profile is relevant to drought adaptation and is co-localized with stay-green genomic regions which show that roots and their growth are related to stay-green phenotype expression (12,26,27). Stay-green is highly negatively correlated with flowering time and stover yield (9). These correlation studies indicate early flowering is associated with green leaf area. But, stay-green shows positive association with grain yield (9,11,14). Stay-green is negatively correlated with flowering time, canopy size, size of upper leaf, tillering. Under drought conditions stay-green enhances grain yield, by altering the canopy development and modifying the size of the leaf (leaf anatomy), root growth (nodal root angle) and water uptake mechanisms (11,12,28). Reduction in leaf size leads to transfer of photosynthetic nutrients to grains without undergoing the drought stress.

**Identification of genetic factors involved in stay-green :** Genomic regions responsible for stay-green trait were detected with the help of molecular markers and the phenotyping data of the stay-green lines locate the variation in the genomic regions which are important for drought tolerance breeding programs. Quantitative trait loci (QTLs) for stay-green have much importance in improving the productivity under drought stress conditions (23). Many QTL mapping studies contributing to stay-green expression under drought stress conditions have been evaluated in mapping populations (8,15,29, 30,31,32,33,

34,35,36,37,38) introgression lines (9) and near isogenic lines (29,30,31,33,34,35,15). Several stay-green sources have been field evaluated and used for crosses (39,40). Best stay-green sources are B35, E36-1, and SC56 that are involved in different marker assisted breeding programs. Cross B35 (stay-green) × R16 (senescent) was developed (9) and their introgression lines were field evaluated. B35 (stay-green) × Tx7000 (senescent) was also extensively studied and their introgression lines were used for fine mapping of different stay-green QTLs (15,33,35). B35 × Tx430 (32), SC56 × Tx7000 (36), N13 × E36-1, IS9830 × E36-1 (8), M35-1 × B35 (16) crosses were made and different stay-green QTLs were identified. Stay-green was extensively studied in crops other than sorghum like in maize (41), wheat (42), barley (43), rice (44), and *Arabidopsis* (45). It appears therefore that stay-green genotypes need to be utilized in sorghum breeding programs aimed at developing drought tolerant plants.

**Marker-assisted breeding for stay-green :** Drought stress may be alleviated by developing crops that are well adapted to dry-land environments with marker assisted breeding crop improvement programs. Increasing marker density and identifying QTLs and narrow down the QTLs to smaller regions will improve marker assisted breeding. Different types of stay-green QTLs are influenced by different backgrounds (28) and many crossing programs introgressed stay-green into senescent breeding lines. Therefore, marker assisted breeding programs help us develop drought tolerant lines in sorghum.

**Stay-green candidate genes :** An alteration in the chlorophyll break down mechanism influenced by many key factors like plant hormones, transcriptional factors and genes lead to delayed degradation of chlorophyll. Cytokinins are plant hormones involved in regulating senescence process, and the cytokinin receptor (AHK3), the type-B response regulator (ARR2) and the recently identified cytokinin response factor (CRF6) are involved in senescence signal responses (46). No apical meristem (NAC/NAM)

transcriptional factor is a developmental regulator and accelerates senescence and increases nutrient remobilization from leaves to developing grains (47). In *Arabidopsis*, AtNAP encodes NAC transcription factor which is closely associated with senescence (48). OsNAP is a NAC transcriptional activator identified in rice involved in senescence pathway. Reduced OsNAP expression lead to improved grain filling and seed setting and subsequently increased grain yield (49). Senescence associated genes (SAGs) were up- and downregulated under stress conditions (50). Chlorophyll catabolic enzymes and STAYGREEN1 (SGR1), STAYGREEN2 are regulators of chlorophyll degradation and their mutants (sgr) exhibit stay-green phenotype which is a desired phenotype for drought tolerance (45). WRKY family transcriptional factors are also involved in senescence pathway and over expression of WRKY transcriptional factors lead to improved drought tolerance (51). Thus, the above candidate genes appear to be crucial for imparting drought stress tolerance. Their overexpression in sorghum can certainly lead to transgenic sorghum lines that can withstand water limited conditions.

**Shoot fly resistance** : Apart from abiotic stresses, many biotic stresses are caused by plant pathogens and insect pests. Nearly, 150 species of insect pests damage sorghum, of which sorghum shoot fly *Antherigonia soccata* (Rondani), is the major insect pest in Africa, Asia and Mediterranean Europe (6). Shoot fly belongs to the family Muscidae and is a devastating pest in sorghum. It mostly attacks tropical grass species like wheat, barley and sorghum. Female shoot fly lays white, elongated, cigar shaped eggs singly on abaxial (lower) surface of leaf, parallel to mid-rib. Eggs hatch in 1-2 days of incubation and larvae crawl into central leaf whorl and cuts the growing tip resulting in typical wilting and drying of the central whorl leaf known as 'dead heart'. As a result of dead heart formation, the young seedlings may be killed outright or they may produce axial tillers, which are rarely productive. The axial tillers serve as a mechanism of

recovery resistance if they remain undamaged, but if shoot fly infestation continues, the seedling may die or present a rosette appearance and fail to produce any grain (52). Larvae feed on the decaying tissue which may lead to seedling mortality and the crop gets damaged within 1-4 weeks after seedling emergence.

**Mechanisms of shoot fly resistance :**

Agronomic practices (timely sowing), natural and synthetic insecticides, natural enemies and host plant resistance (HPR), are all components of integrated pest management practices used to minimize sorghum losses due to shoot fly infestation. Early sowing during rainy season can also be one of the resistance mechanisms (53); but HPR and timely sowing remains most preferred as they are cost-effective, eco-friendly and easily adapted by farmers. Mechanism of resistance to shoot fly is complex and depends on interplay of many component characters of plant, insect and environmental factors (54). Improvement in resistance will increase ecological fitness, reduces pesticide use, and facilitates creation of a sustainable production system with increased efficiency, profitability and enhances grain quality traits. Antixenosis for oviposition is the primary mechanism of resistance for shoot fly resistance in sorghum (55,56). Antibiosis and tolerance also plays important shoot fly resistance mechanism (52,57). Of many important morphological components of sorghum HPR identified, seedling leaf blade glossiness (58), seedling leaf blade trichome density (59), seedling vigor, and leaf sheath pigmentation are all positively associated with Shoot Fly Resistance (SFR). Leaf glossiness reflects the flies from the host and increased trichome density inhibits the larval movement on leaf surface and acts as barrier between the leaf and fly to prevent egg laying (antixenosis)(60). Rapid growth of seedling due to seedling vigor inhibits the larvae movement to reach the central leaf whorl and this reduces the frequency of dead hearts(60). Cytoplasmic male sterility also influences the expression of shoot fly resistance mechanism (61,62). Chlorophyll content and leaf

surface wetness, and waxy bloom have been reported to be associated with shoot fly susceptibility (63). Increased secondary metabolites also take path in shoot fly resistance mechanism (64). Shoot fly resistant genotypes were used in the breeding programs as a source for resistance. Genotypes such as IS2122, IS18551, IS2146, IS1054, IS2312, SFCR151, ICSV705, SFCR125 were used in many crossing programs as resistant donors for shoot fly resistance (65,66,67). However, many of these resistance mechanisms still need to be evaluated clearly at the molecular level. Genes associated with these mechanisms and their cloning and overexpression studies are also needed for validation.

**Factors associated with shoot fly resistance**

: Resistance to shoot fly is mediated by many physico-chemical, morphological, biological, environmental, biochemical, cytoplasmic and genetic factors. Chemicals and pesticides were used to control shoot flies in the field. Fipronil and imidacloprid were successfully evaluated for shoot fly control (68). As the chemicals and pesticides are not affordable by poor farmers and can cause serious environmental hazards, it is necessary to develop cultivars with shoot fly resistance with the help of marker assisted back cross (MABC) methods (64). Morphological traits like seedling leaf blade glossiness, trichome density in lower and upper leaf portions, leaf sheath pigmentation, seedling vigor are negatively correlated with percent shoot fly 'dead heart' and positively associated with shoot fly resistance. Significant correlation was observed between shoot fly dead hearts and yield (53). Morphological components like glossiness and trichome density are negatively correlated to shoot fly dead heart percentage and are significantly associated to shoot fly resistance. Combined effects of trichome density on abaxial (lower), adaxial (upper) and leaf glossiness have been shown to reduce dead heart percentage and high shoot fly resistance (66). These observations point out that glossiness and trichome density are vital for shoot fly resistance

in sorghum. Environment is a major factor associated with shoot fly resistance as the rainy (Rabi) season is most suitable for shoot fly infestation when compared to the post rainy (Kharif) season. Biochemical factors like p-hydroxy benzaldehyde, cinnamic acid, luteolin, apigenin, and some unidentified compounds from damaged and undamaged seedlings of sorghum were associated with expression of resistance for shoot fly as pointed out by Chamarthi et al. (69). QTLs associated with shoot fly resistance have been identified in many populations and different crosses responsible. However, candidate genes need to be identified and validated in sorghum. SFR component traits have been mapped and the putative QTLs identified for individual traits and subsequently validated by marker-assisted backcross (MABC)-introgression into genetic backgrounds highly susceptible to shoot fly. The cross BTx623 × IS18551 (70, 71, 72, 73) mapped the shoot fly resistance (SFR) QTLs on SBI-01, SBI-05, SBI-07, and SBI-10. Similarly, using crosses 296B (susceptible) × IS18551 (resistant) (60,74) and cross 27B (susceptible) × IS2122 (resistant) (75) mapped the SFR. In a reciprocal cross IS18551 × 296B, Apotikar et al. (76) found SFR QTLs on SBI-01 and SBI-03. Five putative QTLs for SFR component traits from IS18551 were then validated by MABC-introgression into the genetic backgrounds of elite shoot fly-susceptible hybrid seed parent maintainer lines 296B and BTx623 (77). Thus, these studies point out that it is possible to transfer shoot fly resistance through classical breeding programs.

**MABC for shoot fly resistance** : Many crossing programs at the National and International Research Centers like Directorate of Sorghum Research and ICRISAT, Patancheru, India, resulted in the development of introgression lines for shoot fly resistance which can be used in further breeding programs. Jyothi (77) introgressed SFR QTLs into BTx623 (fully sequenced) (1) and into 296B backgrounds. 296B × IS18551 and BTx623 × IS18551 (60,70,71,72,73,74,77) (crosses were

extensively studied and their introgression lines were field evaluated for the introgressed trait validation. Utilizing these introgression lines in future molecular breeding programs may help in increasing the shoot fly resistance in different genetic backgrounds and can be pyramided along with other preferred traits to attain multiple resistances to the sorghum plants. Gene pyramiding is a breeding strategy that serves to combine favorable alleles at multiple genetic loci into a single plant genotype. This process of stacking of genes/QTL into a single elite cultivar background can now be efficiently performed by marker-assisted selection (MAS), using backcrossing or pedigree approaches. This approach expedites the varietal development process by providing the opportunity to select for all desirable genes/QTLs simultaneously, as well as eliminating the time-consuming process of inoculation for different races or isolates at different time intervals (78). Pyramiding of multiple genes or common major QTLs for biotic and abiotic stresses are important approaches for genetical improvement of any sorghum genotype. Fine mapping can be achieved by large scale population with more markers showing more recombination events. In early generation populations like  $F_2$ ,  $F_3$  populations many recombination events can be utilized but, heterozygosity segregation distortion, dominance and epistasis need to be overcome to fine map the interested regions. Advance molecular tools increase the precision of crop improvement. A genome-wide association study (GWAS) is a further advanced method to understand the marker trait associations based on linkage disequilibrium and can identify the SNP associated with the candidate genes (79).

**Candidate genes responsible for shoot fly resistance** : Candidate genes underlying the target QTLs like seedling leaf blade glossiness and trichome density have been reported by Satish et al. (60,74) and Aruna et al. (75). Data derived from sorghum genome database and studies on trichome density and glossiness in different crops are consistent with the identified

QTLs. Identification of genes, pathways and mechanism involved in sorghum seedling leaf blade glossiness and trichome density have not yet been clearly studied nor cloned in sorghum. Majority of the studies were carried out in model crop plants like *Arabidopsis* and maize. But studies on sorghum are very few. Wax deficient mutant loci in *Zea mays* (maize), *Brassica napus* and sorghum are defined as 'glossy' loci whereas in *Arabidopsis thaliana* and *Hordeum vulgare* (barley), they were named as 'ceriferum' (cer) mutant loci (80). In *Arabidopsis*, shine (*shn*) mutants were reported. It has been found that the *shn* gene encodes for APETALA (AP2)/ethylene response element binding protein (EREBP) transcriptional factors that act in up- and downregulation of lipid biosynthesis (81). More than 30 'glossy' loci have been identified and a few were cloned (*gl1*, *gl2*, *gl3*, *gl4*, *gl8*, *gl13* and *gl15*) in maize (82) and their functional role in glossiness has been reported. Similarly, many studies reported that WRKY, MYB transcription factors play important roles (83,84,85,86) for developmental regulation of trichomes and trichome morphology can also play important roles in SFR (60). Further, *Mir1* gene encodes for cysteine protease which can reduce the growth of larvae as reported by (60). Transparent Testa Glabra1 (TTG1), Glabrous 2 (Gl2) and Glabrous 3 (Gl3) are involved in trichome initiation and TTG2 is also involved in trichomes throughout their development (83,87). Thus, these data appear that genes associated with both glossiness and trichome density have been identified and can be used in genetic engineering techniques for generating transgenics with better resistance.

### Conclusions

Recent advances in genomics, molecular breeding and next generation sequencing and re-sequencing methodologies can be utilized in future to decipher stay-green and morphological traits of shoot fly resistance in sorghum. We need to further fine map the mapped QTL genomic regions and look for the marker trait associations with the help of genome wide association studies

(GWAS) in sorghum. Genes responsible for stay-green, leaf blade glossiness and trichome density need to be cloned and their introgression and expression level studies should be made in sorghum in order to enhance the genetic architecture. In future, both these studies need to be targeted with MABC and it could be possible to pyramid the stay-green trait alongside shoot fly component traits in order to achieve a multiple resistant variety for improved sorghum productivity.

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