Role of Genetics and Genomics in Mitigating Abiotic Stresses in Soybeans

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Introduction

The soybean is one of the most important cash crops, contributing 69.74 billion dollars to the economy worldwide, with an annual production of ~280 million metric tons in 2013. The United States is the top producer, with an annual production of 86.58 million metric tons followed by Brazil, Argentina, China, and India, where the annual production is 73.78, 47.74, 13.57, and 12.89 million metric tons, respectively (FAO, 2013). It has been grown for harvesting grains, which are rich in protein (38–40%) and oil (18–20%) content. A total of 95% of the oil has been used for edible purposes, while the remaining is consumed for industrial use, especially in the pharmacological industry. Approximately 98% of the soybean meal is used as a source of nutrition for livestock and aquaculture (Liu, 2008).

Legumes have unique ability of fixing nitrogen in soils, which make them important for multiple cropping patterns. Among these, the soybean is a major contributor (Singh, 2010). Apart from these, biodiesel from soybeans has been accepted as one of the optional alternatives to fossil fuels (Hartman et al., 2011).

Historical Perspective

Accumulating evidence indicates that soybeans originated and domesticated in China between 1100 and 1700 BC (Hymowitz, 1970). Later (from the 1st to 16th centuries), it was introduced to Korea, Japan, Indonesia, Philippines, Vietnam, Thailand, Burma, North India, and Nepal; this whole region is believed to be a second center of soybean origin (Hymowitz, 1990). In the 17th century, it was introduced to Europe and the United States. Currently, it is cultivated in ~60 different countries.

Strength of Genetic Resource

The soybean is grouped in a family *Fabaceae*, and the genus *Glycine*, which is further categorized into two subgenera: *Soja*, containing two important species *Glycine max* L. (cultivated) and *Glycine soja* (wild), and *Glycine*, comprised of 26 perennial species, which are prevalent in Australia or in its surroundings.

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In a conventional plant-breeding program, genetic resources play a key role for developing new cultivars. For the genetic improvement of soybeans, a vast collection of soybean germplasm (170,000 accessions) is present in the world, with some duplicated accessions (Nelson, 2009). China has the largest collection of germplasm (>23,000 cultivated and 7000 wild accessions), and this germplasm has been preserved and maintained at the Chinese National Soybean Gene bank (Dong et al., 2004; Wang et al., 2006; Limei et al., 2005). The entire collection was divided into three subcollections, ie, core collection, mini core collection, and integrated applied core collection (Qiu et al., 2013). The USDA-Agricultural Research Service has the second largest soybean germplasm collection, which is comprised of >20,000 accessions of the genus Glycine with a wide range of natural variations (Carter et al., 2004). The details of soybean accessions are available in the International Legume Database and Information Service, USDA-Germplasm Information Resources Network (www.ildis.org, www.ars.grin.gov; Lewis et al., 2005). The USDA has also assembled a large collection of germplasm comprised of 16 perennial species of the genus *Glycine* too. These accessions are also being maintained in Australia. This germplasm has been recognized as world base collection by the International Plant Genetic Resources Institute (Mishra and Verma, 2010). Similarly, The National Institute of Agrobiological Sciences in Japan has a collection of nearly 11,300 accessions including local landraces as well as wild soybean species. This collection contains both the improved cultivars as well as advanced breeding lines in its repository, which were either developed by the regional agriculture research institutes of Japan or introduced from the other countries. In Korea, the Rural Development Administration gene bank has almost 7000 soybean landraces (Yoon et al., 2008).

Challenges to the Sustainability of Soybean Production

Food security is at a potential risk due to fluctuations in environments worldwide and may lead to starvation in regions, which are facing or at potential risk of the changing climate. These climate changes include unusual fluctuations in temperature and rainfall pattern and its frequency, increased salinization, and frequent drought periods. These abiotic factors suppress crop productivities up to 50% (Wang et al., 2003).

Like many other crop species, soybean growth, productivity, and seed quality are severely affected by drought (Mohammadi et al., 2012), temperature (Endo et al., 2009; Sakata and Higashitani, 2008), salinity (Sobhanian et al., 2010), waterlogging (Khatoon et al., 2012; Komatsu et al., 2012), and heavy metals (Hossain et al., 2012a). The soybean has been declared the most drought-sensitive plant (Clement et al., 2008), as drought alone may reduce yield up to 40% (Specht et al., 1999). Although the plant growth is negatively affected at different stages, the seedling stage is the most sensitive to drought and flooding (Tran and Mochida, 2010; Valliyodan and Nguyen, 2006). The soybean plant requires approximately 450–700 mm water from germination to maturity (Dogan et al., 2007).

In soybeans, the potential yield parameters are number of seeds, pods, nodes, and reproductive nodes per unit area, and 100 or 1000 seed weight. These parameters are negatively affected by the onset of drought. For example, drought affects leaf area index, total dry matter (TDM), crop growth rate, and plant height (Meckel et al., 1984; Pandey et al., 1984; Ramseur et al., 1985; Hoogenboom et al., 1987; Cox and Jolliff, 1987; Muchow et al., 1986; Desclaux et al., 2000; Cox and Jolliff, 1986). In another study, a reduction in intermodal length is more obvious than that of the other vegetative growth parameters (Desclaux et al., 2000). If the plant is exposed to reproductive stage one (R1), then the predominant yield losses would be due to a decreased number of pods and seeds while the seed size will be less affected (Ramseur et al., 1984; Pandey et al., 1984; Sionit et al., 1987; Meckel et al., 1984; Constable and Hearn, 1981; Ball et al., 2000). On the contrary, a number of reports have indicated a reduction in pods/m^2 under drought stress during reproductive stages (R1–R6) (Sionit et al., 1987; Ramseur et al., 1984; Pandey et al., 1984; Snyder et al., 1982; Neyshabouri and Hatfield, 1986; Cox and Jolliff, 1986; Westgate and Peterson, 1993; Ball et al., 2000). The water deficiency at the reproductive stage inhibits early expansion of ovaries due to reduced photosynthetic rate (Westgate and Peterson, 1993; Liu et al., 2004). In the reproductive period (R1–R7), initial stages (R1–R5) are more sensitive as compared to the later stage (R6–R7). Two-fold yield reduction was observed when drought stress was imposed on R1–R5 stages than that of R6–R7 (Korte et al., 1983; Brown et al., 1985; Kadhem et al., 1985; Eck et al., 1987; Hoogenboom et al., 1987). In a few reports, it has also been shown that R3–R5 stages are more sensitive within the R1–R6 period (Korte et al., 1983; Kadhem et al., 1985). Nitrogen fixation is a very important physiological process in soybeans. This process is more sensitive to drought as compared to TDM accumulation, photosynthetic rate, and transpiration rate (Purcell and Specht, 2004).

Another important abiotic stress is excessive salt in the soil. In the world, almost one-third of agriculture land is affected by excessive salt in the soil (Zhu, 2001; Munns and Tester, 2008), which induces secondary stresses, ie, toxic metabolites, disarrangement of membranes, weakened nutrient accomplishment, accumulation of reactive oxygen species (ROS), and inhibition of photosynthetic rate. All these factors together disturb the plant growth (Hasegawa et al., 2000). Salinity also causes plant injury by the accumulation of Na^+ and by the osmotic stress (Yeo, 1998; Hasegawa et al., 2000).

Excessive heat, another abiotic factor, is increasing continuously, which impacts the normal functioning of photosynthetic apparatus (electron transport chain, ribulose bisphosphate carboxylase, oxygenase activity, and activity of ribulose bisphosphate activase) and selective phenological stages (ie, development of pollen development, anther opening, pollen germination, fertilization, and grain development) (Sakata and Higashitani, 2008; Endo et al., 2009). Losses due to excessive heat are more pronounced on the crops, which are grown in temperate and subtropical areas (Lobell and Gourdji, 2012; Teixeira et al., 2013). Various studies have shown that temperature more than 35°C affects the germination of pollen and pollen tube growth in soybeans (Koti et al., 2005; Salem et al., 2007). The optimum range of temperature is 25–40°C for the normal canopy photosynthesis (Board and Kahlon, 2011).

In contrary to high temperature environments, when temperature falls to 10–12°C or below, it changes the transition phase of cell membranes from crystalline liquid to gel form (Bramlage et al., 1978). As a result, cell metabolism is interrupted, thus reducing the soybean yield. For example, 24% yield loss has been reported when the temperature in the night drops from 16°C down to 10°C. The effects of cold stress were irreversible when imposed on the flowering to pod formation stage, resulting in huge (~70%) yield losses. However, a much lower loss in yield (25%) was observed at later stages (Board and Kahlon, 2011).

Response of the Soybean Plant to Abiotic Stress

Morphological and Physiological Responses

Under stress conditions, plants use a number of strategies for their survival, including avoidance or tolerance, usually by activating metabolic processes for carrying out normal cell functions (Bita and Gerats, 2013; Hasanuzzaman et al., 2013). For avoidance, plants adapt specialized features such as short duration life cycle, production of specialized morphological structure for the protection of stress sensitive tissue, etc. Under drought stress, the plant reduces the water content by closing the stomata and decreasing transpiration, which results in a decrease of chlorophyll amount, photosynthesis, and CO_2 assimilation. Phenotypically, plants exhibit signs of leaf rolling, wilting, and etiolating during the start of water stress. A first plant part that faces drought and submergence stresses is the root. In response to flooding, plants initially decrease the absorption of water, root permeability, and intake of minerals, which reduce the photosynthetic rate, hormonal imbalance, and development of adventitious roots and parenchyma (Vartapetian and Jackson, 1997).

Response at the Molecular Level

The response of soybeans to stress is linked with the magnitude of stress, time period, and the type of stress. These factors are responsible for a number of changes at the protein level in the plant cell. The nature and degree of response may differ depending upon stress type. However, a few similar response mechanisms have been observed in response to all types of abiotic stresses. For example, an enhanced amount of antioxidant proteins have been observed in response to all types of abiotic stresses (Komatsu et al., 2013). These proteins have been used to scavenge ROS, which ultimately protect important plant cell components from oxidative damage (Hossain et al., 2012b).

Gene expression studies in different crop plants have divided the stress responsive genes into two groups: effectors and regulatory genes (Yamaguchi-Shinozaki and Shinozaki, 2006). The first group (effector) includes the genes, which encode proteins for protecting plants directly, such as the genes responsible for the synthesis of osmolytes, molecular chaperones, membrane channel proteins, antioxidants, late embryogenesis abundant proteins, and enzymes involved in different metabolic pathways. The second group, comprised of regulatory genes, encode products such as localized receptors

in membranes, kinases, calcium receptors, and transcription factors (TF). This group of genes is further involved in signal transduction and gene expression. A number of plant drought stress-tolerant TFs, such as dehydration responsible element binding (DREB) protein, are involved in ABA-independent pathway, while TFs (involved in ABA-dependent pathways), ethylene responsive factor, WRKY, MYB, basic leucine zipper domain (bZIP), and NAC have been reported (Tran et al., 2004; Hu et al., 2006; Nakashima et al., 2007; Liao et al., 2008a,b; Zhou et al., 2008; Jeong et al., 2010; Seo et al., 2010; Hao et al., 2010; Niu et al., 2012; Lopes-Caitar et al., 2013; Song et al., 2013).

In plants, the WRKY transcription factor is the largest family. A number of *WRKY* genes have been identified in Arabidopsis (Ulker and Somssich, 2004), rice (Wu et al., 2005), barley (Mangelsen et al., 2008), and wheat (Niu et al., 2012). In soybeans, 233 WRKY members have been identified (http://planttfdb.cbi.pku.edu.cn/family.php?fam=WRKY, Schmutz et al., 2010). Two *WRKY* genes (*GmWRKY21* and *GmWRKY54*) in soybeans were identified, and their role in improving tolerance to cold, salt, and drought has been demonstrated in Arabidopsis. The overexpression of *GmWRKY13* in transgenic Arabidopsis enhanced its tolerance level to salinity and mannitol (Zhou et al., 2008). Recently, the same group characterized the involvement of GmWRKY27 in response to drought and salt stress. Overexpression of GmWRKY27 and GmWRKY27 RNAi in soybeans led to increased tolerance and severe sensitivity to salinity and water-deficit stress, respectively. In the same study, interaction of GmWRKY27 with GmMYB174 was observed, which binds directly to two neighboring *cis*-elements and suppressed GmNAC29 activation, resulting in increased tolerance to abiotic stress (Wang et al., 2015). In another study, novel candidates of *WRKY* genes were found, which elucidated the novel function of WRKY transcription factors under drought stress in soybeans (Tripathi et al., 2015).

Phosphatidylinositol phospholipase C (PI-PLC) belongs to multicellular intracellular enzymes, which is one of the signaling processes involved in plant development and activates in response to abiotic stresses (PEG, NaCl, and saline-alkali). The gene coding for PI-PLC was also identified in soybeans and has been characterized (Shi et al., 1995). Recently, the expression profiling of the *PI-PLC* gene family in response to multiple stresses has been studied in soybeans. A total of 12 putative *PLC* genes were identified. These genes were located on chromosome number Gm2, 11, 14, and 18. It has been demonstrated that PLCs have an important role in imparting the ability to adapt to adverse climatic conditions to plants (Wang et al., 2015).

Heat shock proteins (HSPs) play an important role in combating adverse environmental conditions (Cho and Choi, 2009; Zou et al., 2012; Kim et al., 2014). The role of HSPs have been described in Arabidopsis (Su and Li, 2008), rubber trees (Zhang et al., 2009), wheat (Francki et al., 2002; Duan et al., 2011), pepper (Guo et al., 2014), and cucumber (Li et al., 2014a,b,c). Recently, a total of 61 *HSP70* genes were identified, which were grouped into eight subfamilies (I-VIII). These genes were found to be unevenly distributed on 17 different chromosomes. Out of these, 53 genes differentially expressed in 14 different tissues (Zhang et al., 2015a,b).

The expression profiling studies of the Homeodomain-leucine zipper (HD-Zip) gene family (comprising of 140 *HD-Zip* genes; http://planttfdb.cbi.pku.edu.cn/family.php?fam=HD-ZIP) were carried out in soybeans under water-limited and saline environments. These proteins are homeobox TFs, which are involved in conferring tolerance to different abiotic stresses. Out of the 140, 59 HD-Zip coding genes and three paralogous pairs differentially expressed, while 20 paralogous pairs exhibited similar expression under drought and saline environments in soybeans (Jin et al., 2013).

The other important class of molecules, known as osmoprotectants or compatible solutes such as proline, glycine-betaine, etc., help the plant to counter the extreme stress conditions. In soybeans, a total of 36 differentially expressed genes involved in the synthesis of osmolytes (Proline, Trehalose, Glycine betaine, Myo-inositol) were identified. Out of these, 25 were mapped in the soybean genome (Kido et al., 2013). In another study, a total of 518 and 614 genes differentially expressed in leaves and roots, respectively, of a drought-tolerant soybean cultivar Jindou, as compared to the drought-sensitive cultivar Zhoungdou 33, were identified. While 24 were commonly expressed in the root as well as leaf tissues. A total of seven genes, *Glyma15g03920*, *Glyma05g02470*, *Glyma15g15010*, *Glyma05g09070*, *Glyma06g35630*, *Glyma08g12590*, and *Glyma11g16000*, showed significantly high expression under drought conditions, demonstrating their role in imparting drought tolerance (Chen et al., 2013).

A very comprehensive proteomic study has been conducted in soybean roots grown under submergence and water stress (Oh and Komatsu, 2015). In total, 48 and 97 proteins were induced significantly under water deficit and flooding stresses, respectively. Protein synthesis-related proteins enhanced under drought stress and reduced under flooding environments, while proteins involved in glycolysis enhanced under both stresses. The synthesis of proteins involved in the fermentation process were enhanced under flooding conditions, while the synthesis of proteins involved in cell organization and redox reaction were increased under water stress. Also, three S-adenosyl methionine synthetases, commonly reduced and enhanced under flooding and drought stresses, were identified, demonstrating their role in the regulation of both stress responses (Oh and Komatsu, 2015).

The role of cyclic electron flow (CEF) toward salt tolerance in soybeans was described. It is proposed that salinity stress accelerates the CEF and the overexpression of genes associated with Na^+ transport. In result, the increased CEF raised the ATP contents in the light. The enhanced ATP content together with the genes associated with Na^+ transport assist in Na^+ sequestering into vacuoles, thus protecting the photosynthetic machinery (He et al., 2015).

The circadian clock helps plants maintain better chlorophyll contents, carbon fixation, survival, and faster growth (Dodd et al., 2005), which is under the control of multiple genes. A number of gene paralogues of multiple clocks have been identified in soybeans when exposed to drought and submergence stresses. For example, numerous clocks and *SUB 1* genes were expressed differentially in soybeans under drought and flooding stresses. There are a number of genes (pseudo-response regulators and timing of CAB expression 1—TOC1), discovered to impart tolerance under submergence and drought stresses. These genes can be used to screen the germplasm

to genetic resources containing drought and submergence tolerance. It has also been suggested that by editing the clock gene paralogues, one can develop soybean varieties with improved tolerance to drought and flooding (Syed et al., 2015).

Melatonin (*N*-acetyl-5-methoxytryptamine) has an antioxidant role, which protects plants from biotic and abiotic stresses (Tan et al., 2012; Wang et al., 2012; Park et al., 2013; Vitalini et al., 2013; Yin et al., 2013). In soybeans, this chemical (when coated on seed) increased soybean tolerance to salt and drought by upregulating the expression of genes (involved in processes like cell division, photosynthetic rate, metabolism of carbohydrate, etc.) inhibited by the excessive salt (Wei et al., 2015).

Application of Genomic Approaches for Improving Tolerance to Abiotic Stresses

Genetic approaches have revolutionized the traditional ways of improving crops including soybeans. Traditional methods such as reverse genetics and forward genetics have been quite successful in determining gene function for simpler traits. These tools have been quite useful in model plants such as *Arabidopsis thaliana*, rice (*Orzya sativa*), and tobacco (*Nicotiana tabacum*), for which well-established transformation and regeneration protocols have been developed, and genome sequence information has been determined.

Genome Organization

The soybean genome is relatively small compared to the other crops such as maize, sugarcane, and barley (Morrell et al., 2012). Its average size is ~1200 Mb containing 46,000 genes, of these ~78% are located at the chromosomal ends (Schmutz et al., 2010) and 59% belong to the transposable elements (Du et al., 2010; Morrell et al., 2012). Like many other flowering plants, these transposable elements have a key role in plant evolution through recombination, gene expression, *cis*- and *trans*- activation/repression of transcription of other genes, and many unknown mechanisms (Du et al., 2010). The location of the majority of genes in "recombinant zones" suggests that the soybean genome has undergone substantial rearrangements due to domestication, followed by intensive selection (Lam et al., 2010; Li et al., 2014a,b,c).

The soybean genome comprises 20 relatively small and homogenous chromosomes. The information obtained from the sequence of the soybean genome suggests that the genome is paleopolyploid, with large-scale genome duplications (~60%). The hybridization-based mapping showed that 61.4%, 5.63%, or 21.53% of the homologous genes were found in two, three, or even more loci (Chan et al., 2012). One distinguishing feature of the soybean genome, revealed by the resequencing data of wild and cultivated soybean accessions, was its high linkage disequilibrium (LD). For example, the average distance for LD to decay to half of its maximum value both for wild and cultivated species was found to be ~75 and ~150 kb, respectively, much higher than those observed for maize, rice, and *Arabidopsis* (Lam et al., 2010).

This feature is quite attractive from a breeding point of view, as it allows using even a small set of molecular markers in marker-assisted breeding. However, it limits the resolution of genetic maps. That is why the conventional soybean genetic maps suffer from poor resolutions. However, the advances in genomic research are likely to refine the genetic maps.

Another distinguishing feature of the soybean genome is the occurrence of high nonsynonymous to synonymous mutation (Nonsyn/Syn) ratios, which is higher than that of rice and *Arabidopsis* (Chan et al., 2012). This high Nonsyn/Syn ratio could be attributed to high LD values that play a major role in accumulating alleles. It was also observed that the soybean genome exhibited a quite high value (~10%) for large-effect single nucleotide polymorphisms (SNP) (Li et al., 2014a,b,c). These large-effect SNPs together with a high Nonsyn/Syn mutation ratio could cause accumulation of deleterious mutations in the soybean genome. Owing to polyploidization and diploidization events, the soybean genome exhibits a mosaic genome structure. For example, a duplication of 1-Mb segment between the soybean chromosomes Gm08 and Gm15 was observed (Lin et al., 2010). Furthermore, a comparison of wild type and cultivated soybean accessions using the cultivated soybean-specific SNPs showed higher Nonsyn/Syn ratio among the cultivated species, which is believed to be due to the domestication associated with the Hill-Robertson effect (Lam et al., 2010). Further analysis showed a whole array of variations between the cultivated and wild type genomes, suggesting that wild type genomes contain diverse genes/alleles, which can be used for enhancing genetic diversity in elite cultivars (Li et al., 2014a,b,c).

TILLING in Soybeans

The Targeted Induced Local Lesion in Genome (TILLING) method has been extensively used in several crop species including soybeans. In 2008, the first soybean TILLING population was developed and established its suitability for high throughput mutation screening. A total of seven genes were screened for mutations in four mutant populations (one developed by exposing Williams-82 with NMU and three with EMS at three different levels). A total of 116 mutations were identified. It has been demonstrated that the NMU-treated population and one with EMS-mutagenized population has similar mutation density (~1/140 kb), while the other EMS-mutagenized populations had shown a mutation density of ~1/250 and ~1/550 kb each (Cooper et al., 2008).

Another TILLING population was developed by bombarding with a fast neutron, aiming to find a dwarf mutant (a desirable trait that protects soybean plants from lodging) in the soybean population. One dwarf mutant was found in ~10,000 M_4 progeny lines. After doing whole genome sequencing followed by making comparisons with the wild type, a total of 13 large deletions were identified. Most of these deletions were positioned in noncoding regions of chromosome 3. While one deletion (803-bp deletion) in a mutant allele (*Blyma15g05831*) localized on chromosome 15 was responsible for the loss of a start codon that resulted in the complete loss of gene function (*Glyma15g05831*) in the dwarf mutant (Hwang et al., 2015). Thus the aforementioned studies suggest that the TILLING approach can be used to tailor the complex traits conferring resistance to abiotic stresses.

Marker-Assisted Selection

Transferring genes from one plant to another is a useful strategy to engineer traits of interest into crop plants. Several strategies have been developed for transferring genes to a cultivar, which can be grouped into transgenic and nontransgenic approaches. The former requires identification of putative genes, transformation, and then regenerating the transgenic line carrying a gene of interest (discussed in the forthcoming section). However, transgenic approaches are often hampered by the complexity of major agronomic traits. Therefore engineering simpler traits is considered much easier. The nontransgenic requires hybridization of two compatible varieties followed by DNA marker screening, known as marker-assisted selection (MAS). This approach allows combining multiple chromosomal segments containing several QTLs into a single plant. However, it is limited to the same species, and bottlenecks include narrow genetic diversity among hybridizing varieties. Currently, MAS is being applied for QTL introgression, backcrossing, and F_2 enrichment. In soybeans, MAS has been successfully used for developing disease-resistant cultivars (Concibido et al., 2004).

The domestication of crops has resulted in the accumulation of useful alleles. Multiple domestication events yielded a number of landraces in soybeans, which are adapted to a wide range of geographical locations. For the identification of these loci (helped in adaptation) in soybeans, an association-mapping procedure was deployed on 342 landraces and 1062 putative lines. A total of 125 genomic regions harboring several genes were identified, which may have a role in future crop improvement (Wen et al., 2015). These landraces could be a valuable resource for future breeding programs for developing high-yielding soybean cultivars that are resistant to various biotic and abiotic stresses. Efforts are underway to conserve the diversity by looking at the allelic signals of selection before and after domestication (Li et al., 2014a,b,c). Next-generation genomic tools such as genotyping by sequencing (GBS) and whole-genome sequencing (WGS) have greatly accelerated efforts to identify and recover the lost traits into crop plants. For example, a novel ion transporter-coding gene, *GmCHX1*, was identified from a wild soybean using the whole-genome sequencing approach (Qi et al., 2014). The group has also constructed a recombinant inbred population of wild soybeans for the identification of novel QTLs associated with crop improvement. Similarly, a pan-genome of seven accessions of undomesticated soybeans (*Glycine soja* L.) was constructed (Li et al., 2014a,b,c) that would have numerous implications in soybean improvement. In another study, WGS, RNA transcript profiling, and comparative genomics were exploited to reconstruct the evolutionary history of the SWEET gene family in soybeans (Patil et al., 2015). The SWEET gene family plays a crucial role in the development of reproductive organs. The SWEET gene family has been extensively studied in model plants such as Arabidopsis but remain largely unstudied in soybeans. The study could help soybean improvement programs by enhancing carbohydrate delivery to the floral organs to increase yield, for example.

Whole genome sequencing is useful in developing hundreds of simple sequence repeat (SSR) markers (Song et al., 2010) and millions of SNPs (Li et al., 2014a,b,c; Sonah et al., 2013). Thousands of QTLs for different traits spanning across the whole soybean genome have been identified, which are available at www.soybase.org.

Similarly, the genome wide association (GWAS) technique was deployed on 298 soybean accessions to determine QTLs responsible for protein and seed oil content (Hwang et al., 2014).

The availability of a complete genome sequence of soybeans has made MAS a robust tool, as it helps in developing locus-specific markers (Schmutz et al., 2010). With the help of the genome sequence, it is now possible to develop high-density DNA markers with genome-wide coverage that in turn allow haplotype analysis and identification of different alleles for agronomical important traits (Tardivel et al., 2014).

MAS is particularly useful for simpler traits; however, it is not as effective for complex traits such as tolerance against various stresses. The situation becomes further complicated when even major QTLs contribute even a small fraction of a particular phenotype variation and may give an unwanted phenotype in a new genetic background due to epistatic interactions. These challenges can be circumvented using another relatively new and powerful approach known as genomic selection (GS). GS takes into account the information of all available markers (genotypic as well as phenotypic) to compute a prediction model. Thus GS allows identification of "minor" QTLs that account for most phenotypic variations. In soybeans, GS has been employed to generate different models. For example, GS was used to predict primary embryogenic capacity, a highly polygenic trait, using a blend of recombinant inbred lines and SSR markers (Hu et al., 2011). In another study, 288 soybean cultivars and 79 SSR markers were used to calculate genomic-estimated breeding value to be 0.90 (Shu et al., 2012). Although the prediction accuracy was quite high in both studies, these predictions were calculated using a small set of genotypes and markers. For values that are more accurate, GS demands diverse genotypes tested under a range of environmental conditions.

Utilization of Genetically Modified Technology for Improving Tolerance to Abiotic Stresses

Nuclear Approaches

Biotech crops offered a significant increase in yield by protecting crops from the growth-damaging factors such as biotic and abiotic stresses. These are also environment friendly, as a significant reduction of pesticide use was observed on the biotech crops. Since the introduction of genetically modified (GM) soybeans in 1996 in the United States, it is now grown in several countries, including Canada, Argentina, Mexico, Brazil, South Africa, Paraguay, Uruguay, China, and India. Each year, a substantial increase in GM soybean area has been observed. For example, GM soybeans occupied a 47% of the total global area under the GM plantation. To develop drought-tolerant soybean lines, researchers expressed the Δ1-pyrroline-5-carboxylate synthase (*P5CR*) coding gene from Arabidopsis into soybeans. Overexpression of *P5CR* in soybeans resulted in increased levels of free proline, and the transgenic lines showed greater tolerance to drought and heat stresses (De Ronde et al., 2004). Introduction of a *Brassica campestris NTR1* gene coding for jasmonic acid carboxyl methyltransferase in soybeans resulted in high tolerance to dehydration during the germination stage (Xue et al., 2007). In another

study, expression of a dehydration-responsive element binding protein, DREB1A, under the drought-inducible promoter Rd29 of Arabidopsis in soybeans was reported. Upregulation of several genes including drought-responsive genes, notably *GmPI-PLC*, *GmSTP*, *GmGRP*, and *GmLEA14*, was found in transgenic soybean lines under drought stress (Polizel et al., 2011). It was also highlighted that the downregulation of strigolactone synthesis enzymes (SSE) could lead to increased tolerance to stress (Quain et al., 2014). For example, downregulation of cysteine proteases, a member of the SSE family, by overexpressing the rice cystatin, oryzacystatin-I, improved stress tolerance in soybeans as well as *Arabidopsis* (Quain et al., 2014). In Argentina, drought tolerant transgenic soybean is very close to its commercial approval (Waltz, 2015).

Like drought, salt-induced toxicity negatively affects the all-growth stages of soybean plants and thus decreases its yield. Expression of an *Arabidopsis* vacuolar Na^+/H^+ antiporter gene (*AtNHX1*) in soybeans improved salt tolerance significantly, rather than that of the nontransformed plants (Li et al., 2010). Constitutive expression of an intrinsic plasma membrane protein 1; 6 (GmPIP1; 6) from a constitutive cauliflower mosaic virus 35S promoter (CaMV 35S) improved soybean root length and Na^+ sequestration (Zhou et al., 2014). Overexpression of a *Solanumtorvum* Δ1-pyrroline-5-carboxylate synthetase gene (*StP5CS*) in soybeans resulted in a higher level of salt tolerance by increasing proline content and reducing membrane peroxidation under salt stress (Zhang et al., 2015a,b).

Although soybean transformation was first reported in 1988 (Christou et al., 1988; Hinchee et al., 1988), it has not become a routine yet. For example, the stable transformation of soybeans is still a challenging task. The success is mainly dependent on the efficient delivery of transforming DNA and the recovery of transgenic lines from a transformed cell. The apical meristem in soybeans consists of three cellular layers, L1, L2, and L3, all of which are involved in the regeneration process of new shoots. The acquisition of stable transgenic lines therefore requires transformation of these layers. Two main methods of delivering transgene at the intended cell location have been reported for soybeans: the particle delivery system and the *Agrobacterium*-mediated method. Particle bombardment has been shown as more efficient compared to *Agrobacterium*-mediated transformation (Wiebke-Strohm et al., 2011). However, its operational cost is high. Since the initial transformation and regeneration of transgenic soybeans from cotyledonary nodes, regeneration from other explants such as hypocotyles, half-seeds, organogenic callus, and immature zygotic cotyledons have been reported as well (see Homrich et al., 2012 for review).

Nonnuclear Approaches

Traditionally, plants have been engineered by inserting transgene in the nuclear genome, known as nuclear insertions. However, nuclear transformation has met with several challenges including variable and poor gene expression, outcrossing of transgenes to weedy relatives (via pollen), and nonallelic interactions, which often results in gene silencing. For example, if the transgenes confer a fitness-enhancing trait such as salt tolerance, drought tolerance, frost tolerance, or insect resistance, transgenes spread to wild relatives could lead to the evolution of unwanted plants. These unwanted

plant species will then be able to compete with the crops for nutrients, space, and light resources and would lead to a reduction in yield. Herbicide-resistance traits, if transferred to weeds, would not be killed by the weedicide. For example, the emergence of herbicide-resistant weeds in Canada and the United States is often attributed to the cultivation of transgenic crops in which herbicide resistance genes were introduced (Gilbert, 2013). This shows that cultivation of transgenic crops have a potential threat to drive the evolution of super weeds. This perceived risk has made their cultivation in open fields highly controversial.

The controversy surrounding GM technology necessitates finding alternative means of engineering plants against biotic and abiotic stresses. Plants possess DNA into various other compartments such as mitochondria and plastids that are inherited maternally. This means transgenes from these compartments will not be escaped through natural cross-pollination, and will remain "contained" inside the plant tissue. The advancements in recombinant DNA technology have made it possible to engineer these compartmental genomes. Engineering mitochondrial genomes has not been very promising. However, the engineering of the plastid genome has been quite successful and has emerged as a serious competitor to the conventional plant engineering approaches (Ahmad and Mukhtar, 2013; Oey et al., 2009).

Plastids are cellular organelles, which have arisen through endosymbiosis by engulfing a photosynthetic bacterium. Since then, plastids have evolved to perform a variety of functions in a plant cell, ranging from photosynthesis to store different brightly colored pigments as in flowers and other colored plant parts, accumulation of starch, lipids, and to perform other specialized functions. Apart from carrying essential functions, plastids have their own genome, called plastome, which exhibits a high copy number (10,000 copies per plant cell). Other features of plastome include the arrangement of genes into clusters, higher AT content, and the presence of two inverted repeat regions (in higher plant chloroplasts only). Since plastids are inherited maternally (in most gymnosperms), therefore insertion of transgenes into plastids offer a tight natural gene containment. One of the attractive features of plastid transformation is homologous recombination-based, site-specific integration of transgenes (Fig. 9.1). Plastids do not follow Mendelian laws, and thus insertions of transgenes into plastids always result in uniform gene expression. All these features are quite convincing to make the plastids a choice of genetic transformations.

The transformation of higher plant chloroplasts, or green plastids, often results in an extraordinary expression of foreign proteins (Ahmad et al., 2012; Ahmad and Mukhtar, 2013; Michoux et al., 2011; Oey et al., 2009; Ruhlman et al., 2010). The chloroplast transformation has been deployed to engineer crops for various agronomic traits including different stresses, such as biotic and abiotic (reviewed in Ellstrand et al., 2013).

Initial attempts for establishing plastid transformation in soybeans met with limited success. The development of stable and fertile transplastomic soybean lines were reported for the first time by Dufourmantel et al. (2004). However, this report was limited to the proof-of-concept and expressed only a selection marker, the *aadA* gene coding for an aminoglycoside-3″-adenylyltransferase, which confers resistance against spectinomycin and streptomycin. Dufourmantel et al. (2005) developed transplastomic soybean lines expressing the *Bacillus thuringiensis* insecticidal protoxin Cry1Ab, which

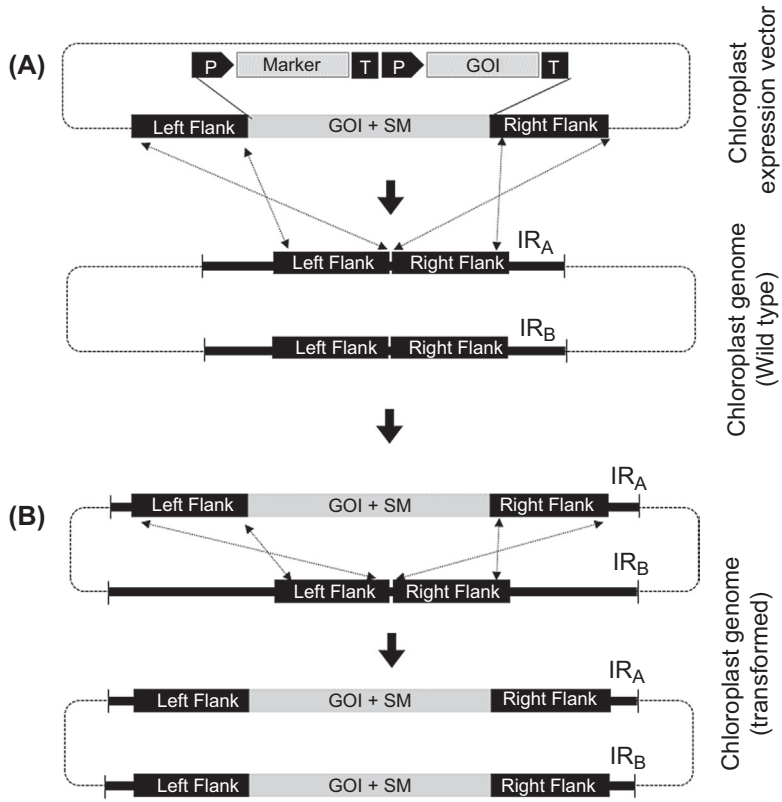

Figure 9.1 Schematic representation of site-specific genetic engineering of the plastid genome. (A) Shows integration of both the expression cassette and selection cassette into the plastid genome at a chosen site through homologous recombination, a hallmark of plastid genome engineering technology. Two flanking regions from the plastid genome at which insertion is required are therefore always incorporated into the chloroplast expression vectors to allow a homologous recombination reaction to occur for the delivery of transgenes at the intended site. The GOI and SM are placed between these flanking regions. The inclusion of these flanking regions into chloroplast expression vectors makes this technology species-specific, which means vectors constructed for one species cannot be used to transform another (when used, both the transformation efficiency as well as expression of recombinant proteins was compromised (Ruhlman et al., 2010)). In addition, the strategy could be employed to study gene functions, either reverse genetics, forward genetics, or the site-directed mutagenesis. (B) Shows the duplication of GOI and SM from IR_A into IR_B through a process known as copy-correction, also mediated by homologous recombination, which takes place between inverted repeat regions. Initially, few copies of the plastome are transformed and therefore the explant contains a mixture of both the transformed as well as untransformed copies, a state known as heteroplastomy. The wild type copies are sorted out gradually by repeating few regeneration cycles against selection to reach homoplastomy, a state when all copies of the plastome are transformed. The dotted arrows show the regions, which will undergo recombination, whereas the thick-black arrows show the progress of the reaction to purify homoplastomic plant lines (see Ahmad and Mukhtar, 2013 for further details). *IR*, inverted repeat; *GOI*, gene of interest; *SM*, selection marker.

was integrated between the *rps12/7* and *trnV* intergenic region of *Glycinemax* plastome. The transplastomic soybean plants showed a strong insecticidal activity (100% mortality in neonates after a three-day feeding) against the velvet bean caterpillar (*Anticarsia gemmatalis*), a major pest of soybeans (Dufourmantel et al., 2005). Since these reports, no other studies were undertaken for engineering the soybean plastome for other traits, in part due to difficult transformation protocols. Due to strong opposition by a large fraction of the scientific as well as public community, the use of antibiotic-based selection markers is discouraged. However, the use of heavy antibiotic-based selection markers in plastids is inevitable. Therefore protocols have been developed to remove selection markers once transplastomic lines are obtained. Excision of marker genes from the soybean plastome has been reported (Dufourmantel et al., 2007). Homology-based direct repeat (repeat length of 367 bp) excision of the *aadA* gene from transplastomic soybeans was quite efficient and was demonstrated by the restoration of a *bar* gene, resulting in a phosphinothricin-resistance phenotype (Dufourmantel et al., 2007).

Like other plant species, transforming the plastid genome of soybeans has appeared quite challenging compared to nuclear transformation. Unlike other plants, such as tobacco, where chloroplasts are targets of the gene delivery process, in soybeans, embryogenic cultures are the most preferred explants due to their regeneration potential. However, the undifferentiated cells in the embryogenic cultures contain smaller and fewer numbers of plastids compared to leaf cells. Therefore transforming soybean plastids is technically challenging, and difficult: one of the major obstacles in the application of this technology in crop plants. However, efforts are underway to develop fast and reliable protocols for the generation of fertile transplastomic soybean plants (Dubald et al., 2014).

Future Prospective

Conventional breeding will not be able to meet the food requirement in 2050, and it would also be difficult for breeders to develop varieties, which can withstand the potential hazards of climate change. Improvement in genomic tools together with the invention of new transformation procedures would be the potential area of research to address the future challenges. The WGS information of soybeans is available and assembled on chromosomes. At present, it is possible to sequence the whole genome of the soybean plant in much less time and in a more cost-effective manner than before. It is imperative to sequence the representative genotypes, which represent the whole genetic diversity available in soybean germplasm. It would help in identifying the function of different genes, and the information generated could also be used in designing new SSRs and SNPs, which would have a potential impact in initiating marker-assisted breeding for addressing the biotic and abiotic stresses. Before sequencing the representative genotypes, characterization of the available germplasm resources, preferably of both species (*Glycine max* and *Glycine soja*), would be an important step. If the resources are limited, the GBS approach can be deployed to explore variations in genomes of multiple representative genotypes, as its reference genome is available.

Studying complex traits is always a difficult task, as it hammers the progress toward initiating marker-assisted breeding (MAB). In multiple reports, a large number

of QTLs have been identified. However, it is difficult to use these QTLs in MAB, as these have been detected in different environments and genetic backgrounds. One can overcome this problem by doing a "meta-QTL" analysis for identifying the consensus QTLs associated with the trait of interest. However, there are still many gaps, which are supposed to identify the most reliable QTLs with major effects. There are a number of approaches, including association mapping and nested association mapping strategies, which can be used to develop populations. These populations can be exposed to GWAS for detecting the complex QTLs with much higher resolution. The information generated would help breeders identify DNA markers associated with the traits. Also, the information would be a useful source for geneticists for exploring the genetic mechanisms involved in conferring complex traits.

Like many other important crop species such as wheat, sorghum, pea, rapeseed, etc., it has been demonstrated that TILLING can be used as one of the reverse genetic techniques for identifying the function of different genes. Once the TILLING population has been developed, it should be characterized for variations in morphological traits. If possible, one can explore the desirable mutant plants for physiological as well as biochemical traits (studying proteins, metabolites, etc.) using various high throughput phenotyping and proteomics tools. For studying the variations in nucleotide sequences, it is important to target as many genes as possible. However, one can also sequence the whole genome of the mutants using the available second and or third generation-sequencing methods followed by the detection of putative SNPs. If resources are limited, like in many developing countries, one can carry out exome capturing followed by aligning the sequences with the wild and reference genome sequences. Once the putative SNPs associated with functional diversity are identified, these can be confirmed through crossing the mutant plant with the wild type, followed by studying the segregation of the mutant trait(s) and the associated SNP(s) in F_2 populations. The TILLING populations can also be used to identify the genes involved in different complex traits, ie, seed composition traits. There is a number of new concepts including ZFNs and CRISPR-Cas9 systems, which can precisely edit and or mutate the genes (even present in duplicate forms). This would not only help in studying the function of different genes but would also be helpful in improving the genome of the soybean plant. In the end, conventional approaches, together with marker-assisted selection and genetic engineering approaches, will be the ultimate choice for improving the genetics of soybeans for mitigating the abiotic stresses in the future.

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