

# Identification and Application of Phenotypic and Molecular Markers for Abiotic Stress Tolerance in Soybean

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## 1. Introduction

Soybean is a sub-tropical crop, however, its present cultivation range extends from temperate regions to the tropics. The sustainability and predictability of soybean crop production can therefore be severely restricted by environmental stresses. Of these, drought stress is considered to be the cause of major limitations in yield, particularly for soybean crops grown in rain-fed areas (Manavalan et al., 2009; Siddique et al., 2001). The detrimental effects of drought on plant metabolism arise largely from osmotic constraints particularly to the cytoplasm (Lopes et al., 2011). Varieties that are able to grow well under stressful conditions and retain high yields have therefore great potential economic importance. Ideally, therefore, such varieties must be able to sustain growth under limited water supply, conditions that also cause nutrient deprivation and exacerbate the production of reactive oxygen species (Lopes et al., 2011; Foyer & Shigeoka, 2011).

The production of drought-tolerant soybean varieties is a major goal of many plant breeders but progress to date remains slow. Intensive research efforts have identified a variety of genes and processes that are affected by drought in soybean (see for example, Chen et al., 2007 a, b). Similarly, much is known about how drought-induced changes in plant metabolism and gene expression influence plant growth, development and yield. However, sustained increases in soybean yield under stressful conditions will require improved crop management practices as well as new soybean varieties with enhanced drought tolerance.

Many research groups world-wide are involved in the identification of phenotypic and molecular markers for application in marker-assisted breeding programs. A range of robust phenotypic and molecular markers are required to assist cultivar evaluation for stress tolerance. Ideally, any selected markers should be able to discriminate between stress-tolerant and sensitive soybean cultivars using rapid, inexpensive methods. It is an advantage to have markers that do not require destruction of the plants or plant organs, particularly as the assessment of non-destructive markers allows greater consistency in measurements over time. The routine use of molecular markers in soybean breeding

strategies is vital to the understanding of the nature of the different mechanisms that can contribute to drought tolerance and sustained crop yields under field conditions. Only then can desired traits be incorporated in an informed manner in soybean improvement programs. Drought-tolerant varieties must also yield well under both optimal and drought conditions. Markers have therefore to work well under field conditions as well as in the laboratory or in controlled environments, where there is absolute control of other parameters such as temperature, soil moisture, light, and day-length. In this regard, testing potential markers under fixed or portable rain-fed shelters is often considered to be a first step in the assessment of the potential of a marker under semi-natural conditions over the plant life cycle.

The application of a potential marker in the field must also take into account the existing infrastructure of the sites where the marker will be used and the technical expertise required for accurate assessment. While some technologies, such as the molecular markers or "omics" approaches, are excellent analytical tools in the laboratory, they are technically demanding, often costly and often require specific skills. These factors are often not compatible with the requirements of the agro-industrial environment, where any useful marker has to be cheap, simple in application and should potentially be usable in automated systems for high throughput screening. We consider here some of the achievements to date with regard to the identification of a useful marker for drought tolerance in soybean and provide insights from our own research concerning the identification and application of a phenotypic or molecular marker in soybean. We focus particularly on the identification of useful shoot and root parameters, as well as symbiotic nitrogen fixation and its relationship to photosynthesis under optimal and stress conditions.

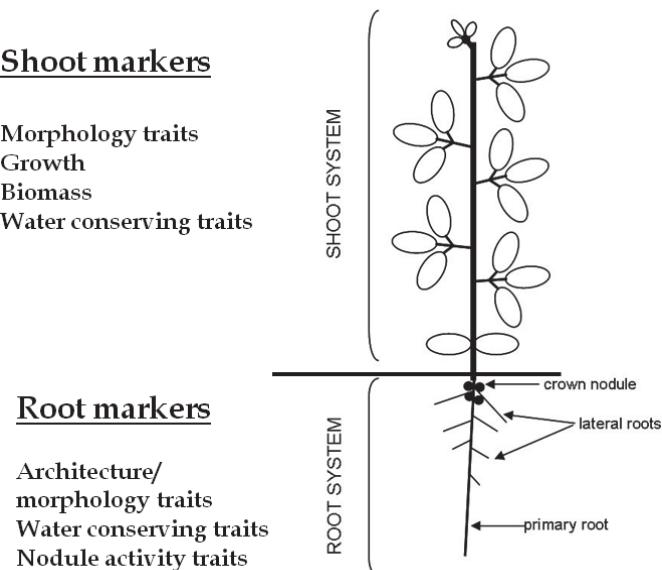


Fig. 1. Phenotypic soybean markers. Classes of phenotypic markers for evaluation of soybean plant performance under drought mentioned in the text.

## 2. Shoot markers for drought

### 2.1 Morphological and physiological markers

Of the wide range of possible morphological characteristics that can be used in the selection of soybean varieties for enhanced drought tolerance (Figures 1 and 2), shoot parameters are generally considered to be the easiest to assess under field conditions. Shoot markers remain major targets in breeding programs, particularly in developing countries, where variations in shoot morphology are often determined subjectively under field or glasshouse conditions. Often this involves visual monitoring of easily detectable plant characteristics such as the number of leaves per plant or the shoot height. These simple parameters can be measured easily in soybean at different intervals during the growing period, and they can be assessed together with a range of other less easily determined parameters such as dry matter yield per plant (Udensi et al., 2010) or photosynthetic capacity and water use efficiency (Gilbert et al., 2011). Rapid growth is often directly related to the supply of water during the growing season. Hence, early maturity, early vigour, stomatal regulation, leaf area maintenance and osmotic adjustment of roots and shoots are generally considered to be useful and effective markers of good plant performance under drought conditions.

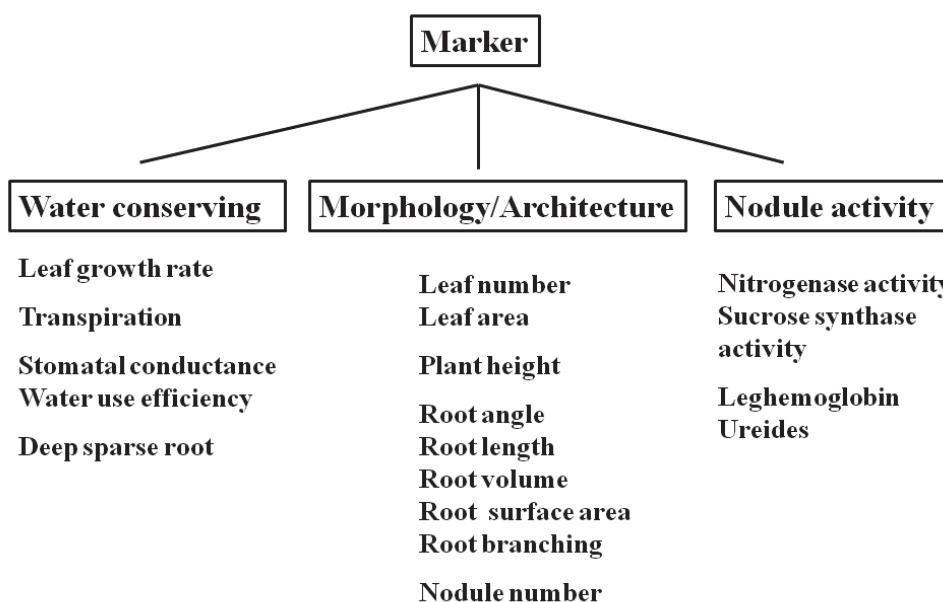


Fig. 2. Measurable phenotypic markers for drought tolerance.

A wide range of morphological, architectural, and physiological parameters are associated with water conservation and these can be used as markers for drought tolerance in soybean. Water deprivation leads to changes in turgor, osmotic pressure, leaf water potential, stomatal conductance and transpiration (Basra et al., 1999; Earl, 2002; Ribas-Carbo et al., 2005). Stomatal closure as a result of leaf water loss results in higher leaf water potentials

and allows better maintenance of the leaf water (Nakayama et al., 2007). However, stomatal closure also results in reduced CO<sub>2</sub> movement for carboxylation within the chloroplast and this can be a major cause of drought-induced decreases in CO<sub>2</sub> assimilation capacity, particularly in C3 plants (Chaves & Oliveira, 2004; Flexas et al., 2006; Warren, 2008). General plant performance parameters, such as biomass production, shoot and root length or seed yield but also leaf movements and phenological plasticity as an adaptation to drought (Acosta-Gallegos & White, 1995; Pastenes et al., 2005) might be useful as indicators for drought tolerance. Such physiological traits are therefore potential candidates for development as breeding markers for the selection of superior drought tolerant varieties. However, breeders can often be reluctant to use such traits, particularly if the associated measurements are time-consuming and technically demanding.

## 2.2 Water use efficiency

Traits that serve to conserve water (conservative traits), including low stomatal conductance, low leaf growth rate, high water use efficiency (WUE), or deep but sparse root systems, favour better water use efficiencies. Research efforts in this regard have often tended to focus on the use of instantaneous water use efficiency (IWUE) values as a physiological marker for drought tolerance. The IWUE of a plant is determined from the ratio between CO<sub>2</sub> assimilation rate and stomatal conductance values (Soares-Cordeiro et al., 2009). It is considered that higher IWUE values provide an indication of improved drought tolerance because varieties with high IWUE values are better able to assimilate carbon at low stomatal conductance and hence attain a greater yield using less water than other varieties. Genetic variation has been observed in soybean with regard to the amount of dry matter produced per given amount of water (Hufstetler et al., 2007). Moreover, soybean genotypes with a quick recovery after drought stress were more productive than genotypes with slower recovery (Hufstetler et al., 2007). Rapid recovery after drought stress is considered as an important trait in varieties grown under drier conditions with periods of drought. Water use efficiency (WUE) is increased by reduced transpiration and water use and biomass production is tightly linked to transpiration, WUE, and nitrogen accumulation. Breeding plants for high WUE under drought might actually result in low-yielding genotypes (Blum, 2011). It has been argued biomass production under most drought conditions can only be enhanced by an effective use of water which will allow maximal soil moisture capture for transpiration and minimizing water loss by soil evaporation (Blum, 2011).

Breeding efforts to improve drought tolerance in soybeans must ensure that a high level of water use efficiency is maintained in new varieties. In a study carried out in our group we compared a range of physiological shoot and root traits under optimal and drought conditions in three soybean varieties: Prima 2000, a commercial variety registered in South Africa that is suitable for cultivation in areas with longer growing seasons but suffering water stress during the growth period; A5409RG, a commercial variety that harbours a glyphosate-resistance gene and that is better suited to areas that do not experience drought or where crops are grown under irrigation; and Jackson, a drought-escaping cultivar with a shorter life cycle (Chen et al., 2007 a,b; Sall & Sinclair, 1991). Of the three genotypes studied, Prima had the highest IWUE values and shoot biomass under both well-watered and drought conditions (Fenta et al., 2011, Figure 3). Prima maintained highest photosynthetic CO<sub>2</sub> assimilation rates under drought. The IWUE values in Prima leaves experiencing long term drought stress were twice those measured under water-replete conditions. The drought-induced increase in IWUE values observed in Jackson was smaller under drought,

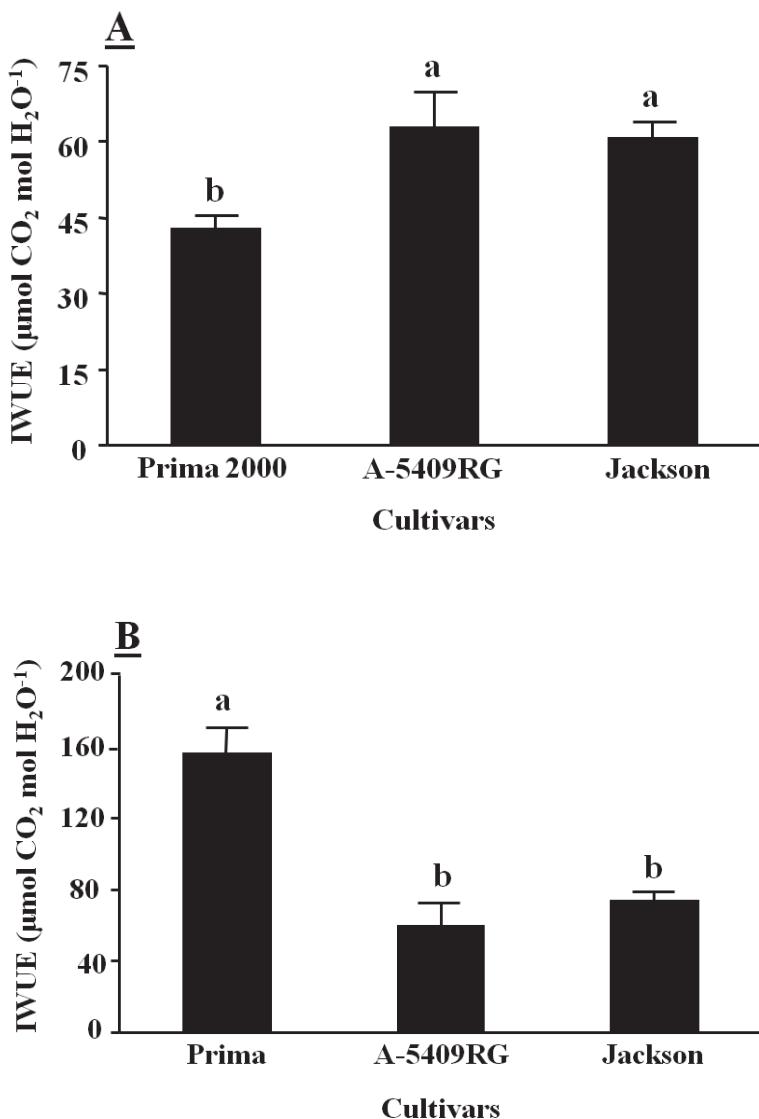


Fig. 3. Instantaneous water use efficiency of soybean. Comparison of instantaneous water use efficiency (IWUE) values measured in three soybean cultivars at day 0 (A) and after 18 days growth under drought conditions (B). Data are the means  $\pm$ SEM of four independent replicates. Means with the same letter above the bars are not significantly different.

even though Jackson had lower stomatal conductance rates than Prima. In contrast, no significant differences ( $P > 0.05$ ) in IWUE values were found in A-5409RG either under water-replete or drought conditions.

### 3. Root markers for drought

Although much information is already available on potential markers for shoot performance, there is relatively little information available on markers for root architecture or root morphology. Several of the root-related traits, such as deep penetration and dense roots, have been found to be related to improved growth under stress. For example, slow wilting in soybean may be associated to deep rooting (Hufstetler et al., 2007). The ability of the plant to extract water from deeper soil profiles is considered to be important in enabling plants to maintain optimal water relations as well as carbon assimilation under drought stress. Deep root systems often allow changes in carbon allocation patterns before water limitation adversely affects growth (Jordan et al., 1983; Jones & Zur, 1984; Blum, 1985; O'Toole & Bland, 1987; Sponchiado et al., 1989; Sinclair & Muchow, 2001; Campos et al., 2004; Manschadi et al., 2006, 2008; Reynolds et al., 2007; Lopes & Reynolds, 2010). For example, bean genotypes with deeper roots were found to have better seed yields and crop growth (Sponchiado et al., 1989).

Improving root traits could contribute to sustainable productivity under water-limited conditions. Root architecture traits, such as root angles and branching, are considered to play a basic role in water acquisition. However, measurements of such parameters under environment conditions is not always representative of the conditions experienced in the field, particularly in situations where root growth is restricted in pot experiments. Measurements of root traits under field conditions are further very difficult. Moreover, such traits, when measured, are not positively related to yield. While root traits are considered to be important in selection of improved plant performance under drought, most researchers are reluctant to use them particularly when dealing with complex root systems.

Flowering plants have two main types of a root system: an allorrhizic root system is found in dicotyledonous species, as illustrated in Figure 4 (Osmont et al., 2007) and a homorhizic root system is typical for monocotyledonous species. Adventitious roots are rare in allorrhizic root systems but they occasionally emerge from hypocotyls or stems. Soybean has a typical allorrhizic root system with two root types, a primary root (or tap root) and lateral roots (Figure 4). The first root that emerges from the hypocotyls in the soybean root system dominates the lateral roots, which can also produce branches.

Our recent research on soybean has focused on the development of a root morphology marker for drought tolerance. In the study, we applied particularly imaging analysis to determine various root parameters in field-grown plants (Figure 5). This analysis enabled the determination of a number of root traits including total length, total surface area and total volume, as well as tap root parameters and lateral root branching density. In this study, the drought tolerance of the three soybean genotypes discussed above was tested under field conditions using a randomized complete block design. Over the first four weeks of the study, all plants were watered regularly (8 mm/day) using a pivot sprinkler irrigation in addition to rain to allow optimal growth with soil moisture status at near field capacity. In some blocks (controls), plants were always grown with this adequate water supply. In other blocks, water stress was initiated one month after sowing by withholding irrigation for one month. Rain fell on only three days of this latter experimental period. This study showed

that Prima 2000 and Jackson have deeper roots with a greater branching density than A-5409RG under drought conditions (Figure 5). A-5409RG had a shallower and thicker root system under drought conditions, with lower overall numbers of roots and a lower branching density. This cultivar also had the highest shoot to root ratio under drought conditions. The production of a lower root biomass compared to the shoot would result in a lower water harvesting capacity. Prima 2000 and Jackson 2000 also had larger root systems under drought than A-5409RG with a greater root length, root surface area and a greater root volume (Figure 6). Both varieties were therefore able to extend the root system in response to drought and so withstand water stress. This study indicates the importance of root traits in the field performance of different soybean cultivars under drought conditions. The association of root traits with the higher productivity of Prima 2000 under drought conditions suggests that these parameters could be used as a marker for drought tolerance in the field.

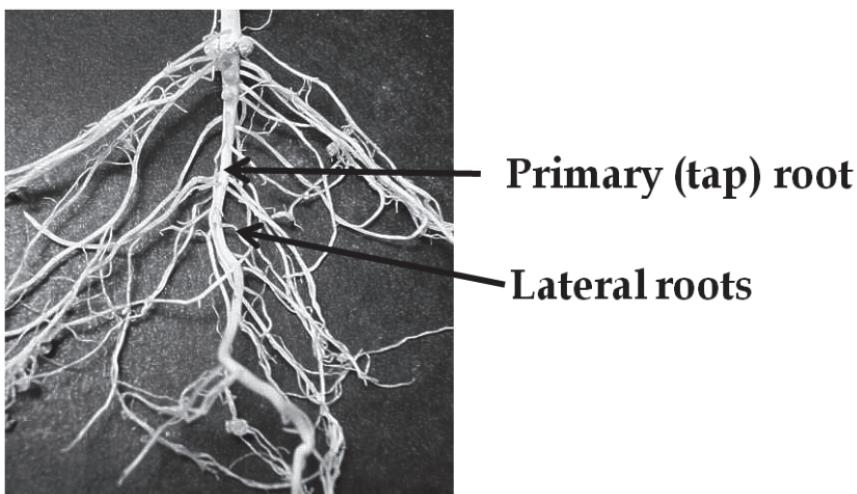


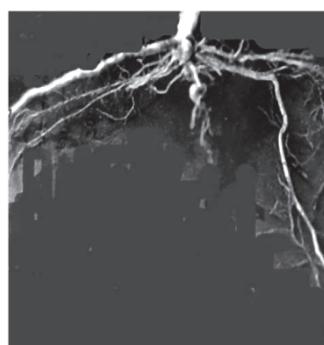
Fig. 4. Soybean root system architecture. Schematic representation of the allorhizic root system architecture of soybean.

### 3.1 Nodule markers

For soybean, the ability to maintain not only high rates of photosynthesis but also symbiotic nitrogen fixation under drought can be important "in terms of yield". Symbiotic nitrogen fixation is rapidly inhibited by drought and thus soybean plants exposed to drought are also deprived of nitrogen (Sinclair et al., 2007). Like photosynthesis, nitrogenase activity can therefore be used as a sensitive marker for drought tolerance. Metabolic markers, such as nitrogenase activity, detect the early plant responses to drought and they are more sensitive than morphology markers. Soybean genotypes can be selected for increased yield on the basis of leaf or nodule numbers (Udensi et al., 2010). Other nodule parameters such as size, leghemoglobin content, ureide accumulation can be used as markers for nitrogen fixation capacity and hence plant performance.



**Jackson**



**A-5409RG**



**Prima 2000**

Fig. 5. A comparison of the root phenotype in three soybean cultivars grown under conditions of drought. A representative shoot phenotype is shown for each of the three soybean cultivars studied: Jackson, A-5409RG and Prima 2000. Photos were taken after plants had been exposed to drought for 1 month under field conditions.

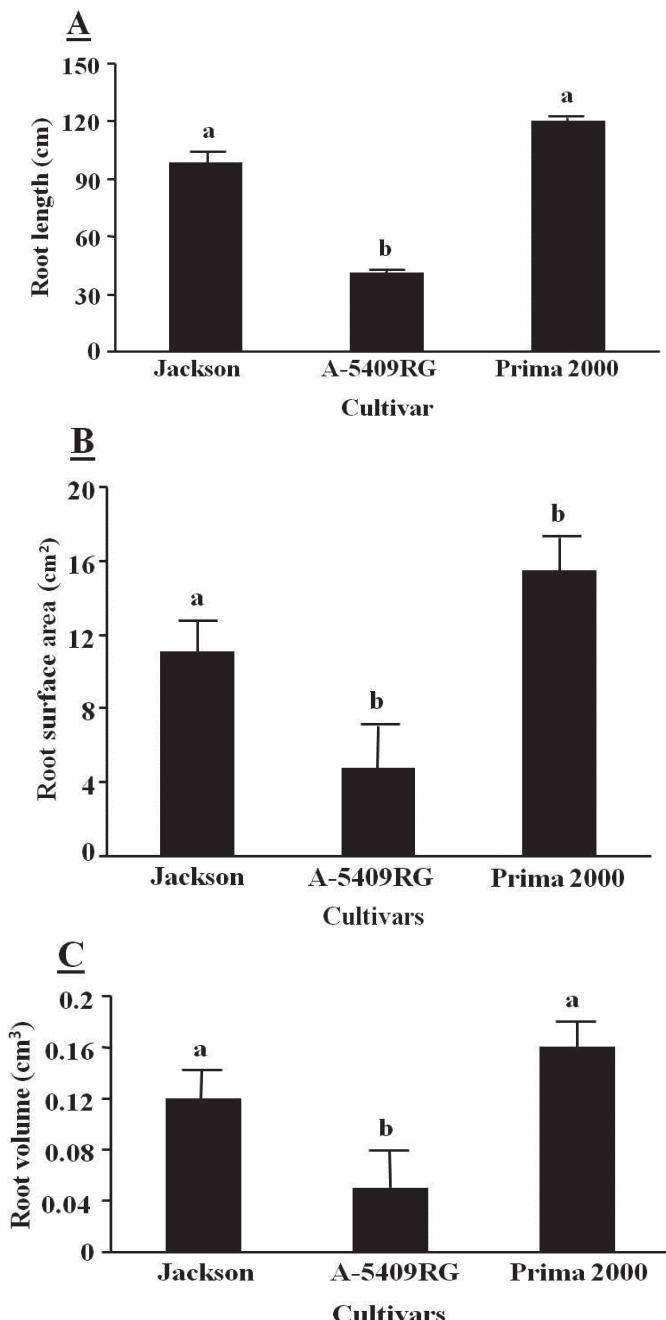


Fig. 6. A comparison of root morphology traits in three soybean cultivars after plants had been exposed to drought for 1 month under field conditions.

Root length (A), root surface area (B) and root volume (C) were measured in soybean cultivars, Jackson, A-5409RG and Prima 2000. Roots were harvested in the field to a depth of 60 cm under drought conditions. The data were obtained from the roots as illustrated in Figure 7 and are the mean  $\pm$  SE of 3 individual root systems per cultivar. Scanned root images were analyzed using the winRHIZO 2008a software which is an image analysis system specially designed for root measurement in different forms (Regent Instruments Canada Inc.). Using this software, root morphological data of root length, average diameter, total area and volume were determined. Furthermore, nodules were counted from the root images. Significance was determined using mean separation student's t-test ( $\alpha = 0.05$ ) Means with the same letter above the bars are not significantly different.

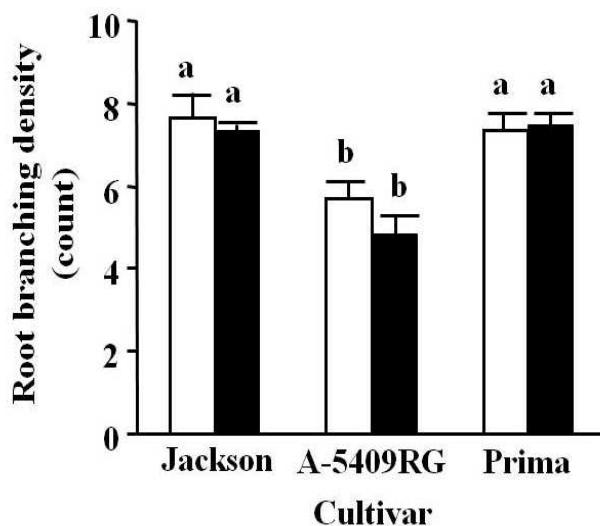


Fig. 7. A comparison of the root architecture traits in three soybean cultivars: Jackson, A-5409RG and Prima 2000 (Prima). Measurements of primary (tap) root branching (open bars) and lateral root branching density (closed bars) were performed after plants had been exposed to drought for 1 month under field conditions. Data represent the mean  $\pm$  SE for values obtained from 3 individual plants. The student's t-test ( $\alpha = 0.05$ ) was applied. The letters above the bars indicate significant differences.

When a large collection ( $> 3,000$ ) of soybean lines was screened for variations in leaf ureide contents under drought only eight were found to maintain low ureide contents under conditions of water deprivation (Sinclair et al., 2000). Thus, leaf and nodule ureide contents could be used as markers for alterations in nitrogen fixation under drought.

Of the factors that are important in preventing the inhibition of nitrogen fixation under drought perhaps the most important is the maintenance of the oxygen diffusion barrier. Other important factors include a continued supply of assimilated carbon from the leaves, together with high nodule sucrose synthase activities. In addition the export of ureides from the nodules is also important in preventing the accumulation of these metabolites and also amino acids in the nodules. Increases in nodule ureide or amino acid levels would lead to an

inhibition of nitrogenase activity. (Durand et al., 1987, Gonzalez et al., 1998, Arrese-Igor et al., 1999, King & Purcell, 2005). There is a close relationship between leaf and nodule water potentials (Durand et al., 1987). Water stress directly inhibits nitrogenase activities because of increased resistance to oxygen diffusion in the nodule. The increase in oxygen diffusion resistance is often linked to a decrease in nodule respiration as well as nitrogenase activity. In these conditions respiratory substrate accumulate as do oxidized lipids, and there is enhanced expression of antioxidant genes suggesting that impaired respiratory activity in the bacteroids under drought leads to enhanced oxidation prior to effects on sucrose synthesis or leghemoglobin (Naya et al., 2007).

In an earlier study on the stress tolerance of different soybean varieties, we investigated the responses of symbiotic nitrogen fixation to dark chilling (van Heerden et al., 2008). Nodule structure, respiration and carbon-nitrogen interactions were compared in the two soybean genotypes that differed in chilling sensitivities: PAN809 (PAN) is chilling sensitive and Highveld Top (HT) is chilling resistant. We found that nodule numbers and the abundance of nitrogenase and leghemoglobin proteins were unaffected by dark chilling. However, the chilling stress caused a large decrease in nodule respiration rates and nitrogenase activities. The large chilling-dependent decrease in ureide contents observed in the PAN nodules was linked to decreases in respiration and an inability to maintain the oxygen diffusion barrier (van Heerden et al., 2008). These data suggest that nodule respiration and mitochondrial markers could be useful tools in assisting conventional breeding efforts aimed at the development of higher yielding soybean genotypes with better chilling tolerance.

#### 4. Molecular markers for drought

Accurate phenotypic markers must ultimately be associated with molecular markers to aid and accelerate current plant breeding efforts to select improved soybean varieties with better stress tolerance. The effectiveness of morphological and physiological markers can vary greatly according to the growth stage of the plant and the many variables in the environment, particularly under field conditions. The selection of molecular markers is based either on variations in genomic DNA or on variations in gene expression (transcriptome) patterns. Comparisons of the transcriptome and also proteome signatures of organs or tissues under stress conditions can provide a direct assessment of processes and/or components that can be developed into a useful marker for stress tolerance in breeding programs. In general, a molecular marker might identify variations in plant responses to stress at the gene level, or in certain regions of DNA, the composition of DNA or in the degree of DNA methylation. While the DNA content of a cell is independent of environmental conditions, organ specificity or growth stage, DNA methylation can be regulated by these endogenous and environmental conditions and so alter also the patterns of gene expression. Variable regions of the genome (transposons), which can include single copy and repetitive genome regions, can also have considerable impact on plant stress tolerance. Such genomic regions can be functional or 'silent' without any obvious function. More recently, next generation sequencing with the identification of single nucleotide polymorphisms, is increasingly being considered as a tool to identify useful molecular markers for stress tolerance.

Further, Quantitative trait loci (QTLs) are chromosomal regions (genomic loci) that might regulate the expression levels of proteins. QTLs can be associated with DNA segments that are considered to make a significant contribution to the expression of complex phenotypic traits

such as stress tolerance. Quantitative traits are usually affected by more than one gene and by the environment. The association of morphological or physiological phenotypic markers with molecular markers in QTL analysis is considered to ultimately be the ideal approach to accelerate plant breeding programs in marker-assisted selection (MAS). MAS is the process whereby the identified markers of all types (morphological, physiological, biochemical or molecular) are used for indirect selection of required traits. MAS approaches however are far from trivial and they are often not cost effective in current breeding programs. Drought tolerance is a mutagenic trait, often influenced by large numbers of minor QTLs, rather than one or more major QTLs. This means that the development and effective large-scale application of MAS is still technically challenging, costly and time-consuming.

The identified molecular DNA markers should ideally be applicable using relatively simple methods that are amenable to automation and high throughput. However, the isolation DNA from plants is not technically demanding and can be carried out in any laboratory that has basic DNA isolation and characterisation equipment. Potential markers can be first evaluated in small sample subsets in order to confirm that the desired loci are present. Such methodologies normally require large sample collections or population sizes because the chosen markers have been applied to large segregating populations in order to determine whether the markers are linked to the required traits (Ribaut et al., 2002).

Relatively few markers for drought tolerance have been identified in soybean. However, progress in this regard will be accelerated once large collections of potential DNA sequences have been established for marker development (Shinozaki, 2007). Accurate genetic and physical maps of the soybean genome are also essential for the development of useful molecular markers for drought tolerance in soybean. Towards this goal, a total of 318 AFLP, 121 SSR, 108 RFLP, and 126 STS markers have been integrated into a linkage map composed of 509 RFLP, 318 SSR, 318 AFLP, 97 AFLP-derived STS, 29 BAC-end or EST-derived STS, 1 RAPD, and five morphological markers (Hisano et al., 2007, Xia et al., 2007). A further very useful advance has come from the sequencing of the soybean (*Glycine max* (L.) Merr. 'Williams 82') genome. Some 66,153 protein-coding loci are now available at: <http://www.phytozome.net/soybean>. Moreover, 3290 microsatellites (SSRs) identified from BAC end sequences of clones (comprising the 'Williams 82' physical map) were screened and two hundred and sixty-five SSRs were genetically mapped in at least one mapping population (Shoemaker et al., 2008).

#### 4.1 Identification of markers using microarrays

Microarrays involve the immobilisation of single-stranded DNA on a solid support that is hybridised with a single-stranded DNA or RNA population (Rockett and Dix, 1999). In microarray analysis gene expression and regulation patterns can be monitored on a large scale (Quackenbush, 2001). The technology facilitates screening for differently expressed genes in different plant varieties. Differently expressed genes can potentially be useful in MAS providing that they encode proteins involved in the traits of interest.

A spotted soybean cDNA microarray containing 36,000 elements derived from EST libraries is available that covers a wide range of tissues and organs at different developmental stages under optimal and stress conditions (Vodkin et al., 2004). Expressed sequence tags (ESTs) are coding regions within the DNA that can also be used for MAS or mapping purposes. The isolation of 6570 full-length sequences of soybean cDNAs derived from tissues exposed to different abiotic stresses will aid marker development (Umezawa et al., 2008) as will the isolation of ESTs from drought-stressed soybean root tips (Valliyodan & Nguyen, 2008).

We have analysed the crown nodule transcriptome at two stages of development (i.e. on 7 and 11 week-old plants) in order to identify possible markers for nodule development/senescence that might underpin plant performance. A comparison of the crown nodule transcriptomes was performed on two cultivars (PAN809 and Highveld Top) after 7 and 11 weeks of growth. PAN 809 is generally recommended for use under long growing seasons and this variety is often used in moderate and hot regions of Southern Africa because it is well-suited to irrigated- and rain-fed cultivation conditions. Highveld Top was developed specifically to withstand the low night temperatures that are often experienced during the growing season at high altitude in Southern Africa. The first nodule harvest point (7 weeks) was chosen because the measured capacity of symbiotic nitrogen fixation was highest at this point of nodule development. The second harvest point (11 weeks) was chosen because symbiotic nitrogen fixation had decreased by 50% at this time point relative to week 7. Figures 8 and 9 illustrate the degree of genotypic variation in the nodule transcriptome signatures observed in the nodules of 7 and 11 week-nodules under optimal growth conditions. While 702 transcripts were changed in abundance in Highveld Top nodules harvested at week 11 compared to week 7, 1737 transcripts were differentially expressed in PAN809 nodules under the same conditions (Figure 8). Of these, 226 transcripts were identical and showed similar patterns of increase or decrease at week 11 relative to week 7 in both cultivars (Figure 8). Of the transcripts that were differentially expressed in PAN809 and Highveld Top nodules at week 7, the PAN809 nodule transcriptome had a much higher number of transcripts encoding proteins involved in cell wall and stress metabolism than Highveld Top (Figure 9). For example, an extensin-like protein and proteins involved in disease resistance were much more abundant in PAN 809 nodules than Highveld Top nodules at week 7. Moreover, transcripts encoding the extensin-like protein were enhanced to a much greater extent in PAN 809 than Highveld Top. Transcripts encoding disease resistance proteins were also much higher in PAN 809 nodules than Highveld Top nodules at 11 weeks. The biological relevance of these findings is currently under investigation in relation to the different performance of these two cultivars.

## Microarray VENN graph

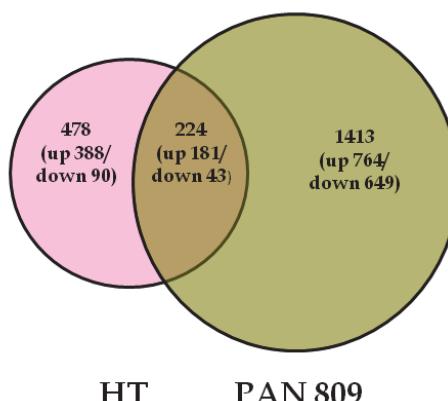


Fig. 8. A comparison of differential gene expression in Highveld Top (HT) and PAN 809 nodules harvested from 7- and 11-week old plants.

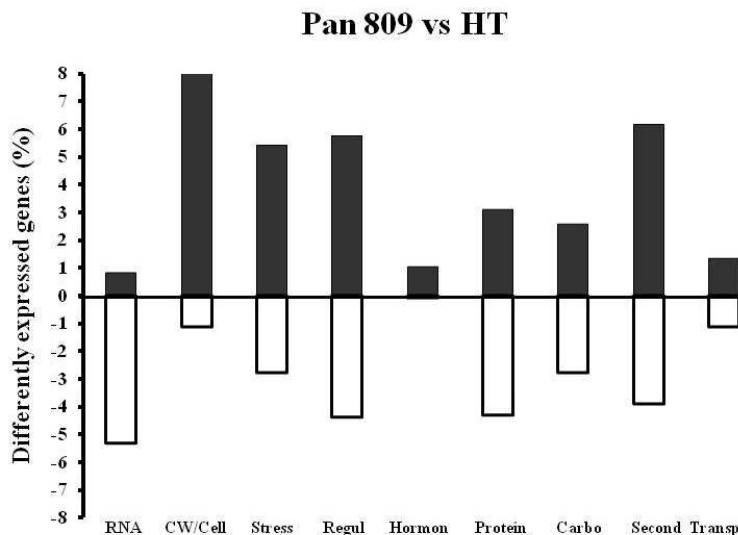


Fig. 9. A comparison of the relative expression of different transcripts in the crown nodules from Pan 809 and Highveld Top. Positive values represent relative increases in transcript abundance while negative values represent decreases in the abundance of transcripts encoding proteins involved in RNA/signaling (RNA), cell wall metabolism (CW/Cell), defense or stress responses (Stress), regulation (Regul), hormone metabolism (Hormon), protein metabolism (Protein), primary metabolism (Carbo), secondary metabolism (Second) and transport (Transp).

## 5. Conclusions

The fixation of atmospheric nitrogen in leguminous plants through the symbiotic union with soil bacteria (rhizobia) and powered ultimately by photosynthesis is an important driver of ecosystem sustainability in the face of climate change. Grain legumes such as soybean are rich in protein, starch, fibre and other essential nutrients and valuable in the production of foodstuffs and feed. They are also used as a natural nitrogen source in agriculture, particularly in Africa. Nodule development is a complex process that has been intensively studied for many years but many questions remain concerning the orchestration of bacterial infection, nodule development and nodule senescence in relation to the senescence of the whole plant. As with other major crops, grain legume production can be severely restricted by environmental stresses. Our studies, which have been summarized here, have largely focused on the effects of environmental stress on nodule senescence in soybean. Our aim has been to determine the contribution that stress-induced nodule senescence makes to the overall sensitivity of soybean plants to drought. Furthermore, we have characterised a range of physiological, metabolic and molecular parameters that could be useful in selection of genotypes for enhanced stress tolerance. The conclusions from our studies are discussed below.

### 5.1 Characterization of chilling-induced tolerance traits in soybean

We compared nodule structure, carbon/nitrogen interactions and respiration in PAN, which is chilling-sensitive and in HT, which is more chilling-resistant (van Heerden et al., 2008). Under optimal growth conditions, SNF began to decline after 9 weeks in PAN nodules and after 11 weeks in HT. A transcriptome analysis was performed on PAN and HT nodules harvested from plants at 3 until 15 weeks after germination. Of the genes that showed the same developmental pattern in both varieties transcripts encoding a cysteine proteinase gene (*Gma.8481.1.S1\_at*) that belongs to a subgroup of vacuolar processing enzymes (legumains) was up-regulated in the senescent nodules. Nodule numbers were unaffected by dark chilling in both genotypes. The abundance of the nitrogenase and leghemoglobin proteins was not changed as a result of dark chilling but nodule respiration rates, nitrogenase activity and *NifH* and *NifK* mRNAs were decreased while nodule starch, sucrose and glucose were increased. Chilling-induced decreases in nodule respiration continued in PAN nodules after return to optimal temperatures but respiration recovered in HT by the end of the chilling period. This recovery was associated with a large decrease in the area of the intercellular spaces in the nodule cortex and infected zone in HT. This acclimatory response was not seen in PAN nodules. We conclude that the ability to regulate the oxygen diffusion barrier is an important component of ability of nodules to tolerate stress (van Heerden et al., 2008). The HT nodules were able to regulate both respiration and the area of the intercellular spaces during chilling and so control the oxygen diffusion barrier. We conclude that chilling-induced inhibition of SNF in PAN nodules was caused by the inhibition of respiration coupled to the failure to regulate the oxygen diffusion barrier effectively (van Heerden et al., 2008). Furthermore, the stress-induced limitations in SNF make an important contribution to the greater chilling-induced inhibition of photosynthesis in PAN than HT.

### 5.2 Characterization of drought tolerance traits in soybean

The characterisation of simple but accurate phenotypic markers for enhanced drought tolerance is important because drought is considered to be the most important factor limiting soybean productivity in the field. A comparison of shoot, root and nodule parameters in three genotypes: Prima 2000, glyphosate-resistant A5409RG and Jackson revealed a positive correlation between SNF and photosynthesis under optimal and drought conditions (Fenta et al., 2011). Considerable genotypic variation was observed in the responses of photosynthesis to drought. While Jackson and Prima performed better than A-5409RG in short-term drought, SNF in Jackson nodules was equally inhibited in all cultivars under long term drought conditions. Drought-induced decreases in shoot to root ratios occurred in all three cultivars, together with a reduction in whole plant biomass (Fenta et al., 2011). However, the shoot to root ratios under drought were significantly higher in A-5409RG than Jackson or in Prima, showing that there is considerable genotypic variation in the control of shoot to root ratios in soybean in response to drought. We conclude that the ability to sustain shoot biomass under the nitrogen limitation caused by impaired SNF could be used as a marker for drought tolerance in soybean (Fenta et al., 2011).

### 5.3 A role for CLAVATA3/Embryo Surrounding Region (CLE) peptide signalling in soybean nodule development

Nodule development is an energy demanding process and so it might be more economical for the plant, to be able to form few larger nodules compared to many small ones. Indeed,

auto-regulation mutants that have an abundant number of nodules do not necessarily fix more nitrogen and often have a reduced shoot growth. Thus, by understanding primordium formation and meristem activity in soybean, we might use this information to alter nodulation architecture leading to less energy demanding nodule formation. CLE peptides are small secreted peptides derived from the C-terminal region of pre-proteins. They control the balance between stem cell proliferation and differentiation in plant developmental processes and fulfil as yet largely undefined roles in nodule development. A genome-wide survey of CLE peptides in soybean resulted in the identification of 39 GmCLE genes (Mortier et al., 2011). Two different CLE expression patterns were identified; one of these was linked with nodule primordium development and the other was linked with nodule maturation. We conclude that group-III CLE peptides are produced in the nodules and that they are involved in primordium homeostasis and in auto-regulation of nodulation (Mortier et al., 2011).

#### 5.4 Marker assisted selection

Phenotypic and molecular markers can be equally important in plant breeding programmes. The identification of “perfect” marker gene(s) conferring the required traits related to enhanced drought tolerance might prove to be elusive because abiotic stress tolerance is a multi-genic trait. Much current research effort in soybean breeding is focused on this goal. There is an urgent need of validated linked markers for stress tolerance. The usefulness of the different markers discussed above in MAS depends on many factors, not least the available infrastructure, technical expertise and the relevance of the technology to the traits under consideration. New and improved technologies for molecular marker selection are developing rapidly but the application of such technologies to plant breeding programmes remains slow. Unfortunately, there is still a wide gulf between advances in basic knowledge of the genes and proteins that underpin stress tolerance mechanisms and the successful application of this knowledge through MAS approaches in plant breeding programmes.

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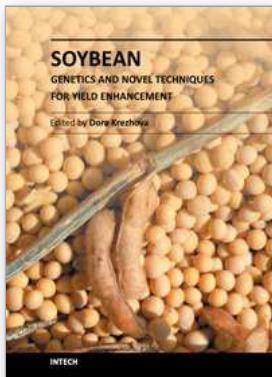
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## Soybean - Genetics and Novel Techniques for Yield Enhancement

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