# Selecting Drought-Tolerance Markers: An Exploratory Analysis in Contrasting Soybeans

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## Abstract

Identifying high-yield genotypes under low water availability is an important goal for soybean breeding. However, a major bottleneck lies in phenotyping, particularly in selecting cost-efficient markers associated with stress tolerance and yield stabilization. Here, we conducted in-depth phenotyping experiments using two soybean genotypes with contrasting drought tolerance, MUNASQA (tolerant) and TJ2049 (susceptible), aiming to identify and statistically validate stress tolerance and yield stabilization markers. Firstly, at the R5 critical reproductive stage, the molecular differences between the genotypes responses to mild water deficit were explored through massive analysis of cDNA ends (MACE)-transcriptomic and gene ontology. MUNASQA transcriptional profile, compared to TJ2049, revealed significant differences when responding to drought. Next, both genotypes were phenotyped under mild water deficit, imposed in vegetative (V3) and R5 stages, by evaluating 22 stress-response, growth and water-use markers, which were subsequently correlated between phenological stages and with yield. Several markers showed high consistency, independent of the phenological stage, demonstrating the effectiveness of the phenotyping methodology and its possible use for early selection. Finally, these markers were classified and selected according to their cost-feasibility, statistical weight and correlation with yield. Here, pubescence, stomatal density and canopy temperature depression emerged as promising markers for early selection of drought-tolerant soybeans.

## Introduction

Soybean [*Glycine max* (L.) Merrill] represents one of the most important sources of vegetable oil and protein in the world [1]. Calculation models based on the growing global population and current agricultural production suggest that crop yields, including soybean, must be doubled to provide enough food in 2050[2]. Yield, the principal breeding target for most crop plants, is massively affected by suboptimal growth conditions primarily due to climate factors such as drought and extreme temperatures. In addition, the progressive climate change will reduce water availability for many rainfed crops like soybean, affecting their growth and productivity[3]. Hence, breeding for yield stabilization and drought tolerance in soybean and other crops is essential for sustainable agriculture and food production [4].

The development of cultivars with improved yield under water deficit has had relatively limited success for several reasons. First, the direct selection for yield improvement under drought is expensive, time-consuming, laborious and complex due to intrinsic genotype by environmental interactions[5]. Moreover, inherent stress susceptibility is often masked by spillover effects of high yield potential when determining plant performance under drought conditions. Consequently, a high-yield variety will often give significant yields during drought, even though the relative yield reduction can be substantial[6]. Instead, analytical approaches that emphasise breeding for yield stabilization through an indirect selection strategy, using morphophysiological or biochemical markers, have gained increasing attention[7,8]. However, the challenge for effectively using targeted breeding approaches lies in developing reliable and reproducible markers. These markers should be (i) strongly related with yield and stress-tolerance traits, where possible, (ii) be non-destructive, (iii) be easily measurable in early phenological stages and (iv) have a high narrow-sense heritability to facilitate the selection in breeding populations[9]. Therefore, identifying and validating drought-tolerance traits are essential steps to obtaining valuable markers for breeding programs and selecting superior genotypes. Soybean drought tolerance has been evaluated through markers such as water use efficiency (WUE), root morphology and penetrability of hardpan, leaf wilting, excised leaf water loss and relative water content (RWC) with varying degrees of success[10].

Advances in next-generation sequencing (NGS) and subsequent evolution in multi-omics technologies have contributed to understanding some underlying mechanisms of response to water deficit in soybean and other crops[11]. Omics studies, however, must be complemented with morphophysiological and biochemical approaches to ensure an integrative perspective of plant adaptation to water scarcity and accurately assess the role of individual traits regarding stress tolerance and yield. Usually, the main issue in such studies is the lack of a well-defined and reliable phenotyping methodology that validate the traits accuracy[12]. It is safe to say that, currently, phenotyping systems are the major operational bottleneck in plants breeding, limiting the translation of genetic and genomic analysis into stress-tolerant phenotypes.

In a previous evaluation of drought tolerance under greenhouse and field conditions, two contrasting soybean cultivars were identified from our germplasm: MUNASQA (drought-tolerant) and TJ2049 (drought-susceptible)[13]. In this study, in-depth phenotyping was conducted for both genotypes to identify and statistically validate markers associated with drought-tolerance and yield-stabilization traits, aiming for their future use in soybean breeding programs, including ours, to select genotypes better adapted to low water availability.

## Results And Discussion

Here, we show differences in the molecular, morphophysiological and biochemical responses of two contrasting soybean genotypes, MUNASQA (drought tolerant) and TJ2049 (drought susceptible), subjected to mild water deficit treatments in V3 and R5 phenological stages.

# 1. Molecular insights

Transcriptional changes were assessed in MUNASQA and TJ2049 after 72 h of exposure to mild water deficit. From 38.658 transcripts analysed, drought-stressed MUNASQA and TJ2049 plants exhibited 2952 and 1126 transcripts with significant changes ($P < 0.05$) in their expression levels (Suppl. Data 1), respectively. After an FDR=0.1, 399 and 15 transcripts were assigned as MUNASQA and TJ2049 DEGs (Suppl. Data 2). The transcript loss detected in TJ2049 might be explained by the larger variation among replicates observed in the water deficit samples and measured as SD/mean ratio for each gene (Suppl. Table 1, Fig. S2). However, in a previous exploratory 454 sequencing experiment, TJ2049 showed significantly fewer DEGs than MUNASQA (data not shown).

Large-scale transcriptional reprogramming has long been recognised as the first response to drought, initiating stress mitigation pathways and metabolic changes[14]. Moreover, the quickness to sense and respond to stresses could be an essential factor to differentiate tolerant and susceptible genotypes. Here, after a mild drought, far from normal field levels, the genotypes exhibited dramatic changes on their transcriptional profiles and no observable phenotypic alteration. In the heat map (Fig.1A), DEGs expression profiles were classified in 8 clusters (Suppl. Data 3), and almost 50% of MUNASQA DEGs showed repression under stress conditions, a difference reinforced by the Venn diagram (Fig.1C).

Generally, in response to drought, plants initially trigger transcriptional control and hormone signalling, leading to metabolic adjustment for coping with low water availability. Cellular mechanisms such as water/ion uptake and transport, redox homeostasis, ROS scavenging, osmoregulation and membrane protection are accompanied by physiological responses such as stomata regulation, root development and protection of the photosynthesis machinery[15]. Here, MUNASQA up-regulated several DEGs involved in these physiological responses (Suppl. Table 2). DEGs from "chlorophyll-binding" and "antioxidant activity" (MF category), "thylakoid", "thylakoid membranes" and "chloroplast" (CC category), and "photosynthesis", "response to water" and "response to desiccation" (BP category) (Fig.1B), were identified. Overall, these results indicate a tightly regulation of stress-response, growth and water use mechanisms in drought-stressed MUNASQAs.

The expression of ten randomly selected DEGs by qRT-PCR (Fig. 1D) agreed with the MACE profile data for MUNASQA and TJ2049, showing a high similarity between both methodologies. Moreover, genes encoding detoxifying proteins like SOD, CAT and APX were differentially regulated in both genotypes (Suppl. Table 3). Enzymes like these are stress-response indicators, frequently regulated at both transcriptional and post-transcriptional levels under drought[16]. Here, we detected two up-regulated SOD genes under stress conditions in MUNASQA and, contrary to TJ2049, three down-regulated genes for APX and CAT. In accordance, SOD activity measurements in R5 corroborated these differences in transcriptional activity between the two genotypes (Suppl. Table 4).

## 2. Morphophysiological and biochemical phenotyping

MACE assay, by itself, does not fully explain the extent of MUNASQA and TJ2049 responses to the applied stress. Thus, extensive morphophysiological and biochemical measurements were performed to minimise the gap between the transcriptional regulation and phenotypical alterations observed. Here, 22 markers associated with stress-response, growth and water-use were evaluated in both genotypes exposed to mild water deficit in V3 and R5 stages.

Stress-response markers

An efficient antioxidant activity is crucial for withstanding low cellular water content, and its importance in plant drought tolerance has been extensively reported[17]. Here, substantial differences were found in MUNASQA and TJ2049 SOD, APX, POX, and CAT enzymes regulation over time, under water deficit and independently of the phenological stage (Table 2). MUNASQA reached maximum activity for all enzymes after 4 d of water deficit, while the highest activity for TJ2049 occurred to 8 d after the stress onset, except for CAT. Noticeably, under non-stressed conditions, all enzymatic activities, excluding CAT, showed higher activity in the tolerant genotype than in the susceptible one, strengthening the hypothesis that TJ2049, compared to MUNASQA, presents delayed stress perception and response mechanisms. In fact, and according to Carvalho *et al.* (2019)[16], a successful response to water deficit may depend not only on which enzymes are activated but also on the activation timing. Moreover, the general gene expression profile regarding these enzymes regulation was highly consistent with the biochemical results.

Regarding PRO, one of the most common osmoprotectants in plants[18], MUNASQA and TJ2049 accumulated the osmolyte in response to water deficit over time and in both V3 and R5 stages (Table 2). Several studies have demonstrated a direct correlation between high osmoprotectant accumulation and drought tolerance in many crops[19]. Here, the tolerant genotype MUNASQA exhibited a higher and more rapid accumulation of PRO after 4 d of water deficit at both phenological stages. In agreement with our results, a recent study in soybean reported higher PRO accumulation in the drought-tolerant genotype A5009 RG, compared with the drought-susceptible ADM50048[20].

When analysing MDA production, an indicator of lipid peroxidation and stress severity[21], a higher and more rapid accumulation was detected in TJ2049 plants in response to water deficit, compared to MUNASQA (Table 2).

Drought also affects leaf pigments content[21]. Changes in photosynthetic pigments can alter various light-harvesting processes, while the accumulation of photoprotective compounds plays an essential role in preventing photo-oxidative damage[22]. Here, MUNASQA and TJ2049 showed alterations in pigment content under water deficit (Table 2). The CHL was significantly reduced over time due to stress in both genotypes, but this reduction was significantly lower in the tolerant one. Similar results were found in drought-tolerant maise that showed lower CHL reductions under stress than susceptible genotypes[23]. Regarding CAR levels, TJ2049 and MUNASQA showed an increased synthesis in response to drought, although the last one exhibited greater and faster accumulation.

According to our results, MUNASQA is more efficient at removing reactive oxygen species (ROS) under drought stress conditions, an essential mechanism for stress tolerance[24].

Growth and yield markers

The ability to produce high seed yield or biomass under limited water access is considered the optimal indicator of drought tolerance in crops[25–27]. We evaluated the effect of mild water deficit in MUNASQA and TJ2049 growth and yield by monitoring changes in various markers related to biomass and seed production, including LAI, LAR and NAR, RGR, CGR, relative yield and yield-DSI (Table 3).

Total leaf surface area and LAI are strongly associated with canopy interception, evapotranspiration and photosynthesis[28]. Here, independently of the phenological stage or water availability, MUNASQA plants exhibited a higher LAI compared to TJ2049, indicating a larger assimilatory capacity and, as a consequence, photosynthetic potential. In vegetative stages, a higher LAI denotes a more rapid canopy development, favouring greater and faster soil coverage and thus less water loss from direct evaporation. In general, drought suppresses leaf initiation and growth and consequently affects LAI[29]. Therefore, a decrease in LAI is expected in plants exposed to water scarcity. As expected, MUNASQA and TJ2049 plants showed a reduction in LAI in response to drought, more noticeable after 8 d of stress and in the R5 stage.

Drought also alters the LAR: the leaf area development in relation to the total biomass produced[30]. Here, water deficit only affected LAR during the vegetative stage, displaying a significant reduction in both genotypes exposed to stress. The highest ratio between plant leaf area and biomass is reached at the beginning of the plant life cycle[31], which explains the highest LAR values at the first sampling day during the vegetative stage.

In MUNASQA and TJ2049, the relationship between leaf area expansion and the biomass produced over time (NAR) was also reduced due to water deficit at both phenological stages. Changes in photosynthetic efficiency were more significant at V3, where both genotypes exhibited greater NAR values. Noticeably, well-watered MUNASQA plants showed a significantly higher NAR than TJ2049 ones, while, in response to stress, MUNASQA's NAR increased in contrast to TJ2049 values, which were reduced. Although not as accentuated, a similar pattern was observed in plants exposed to water deficit in R5. Here, LAI, LAR and NAR results indicated that, in response to drought, MUNASQA plants regulated photosynthates allocation to leaves and the maintenance of photosynthetic efficiency more efficiently than TJ2049 ones.

In addition, the relative growth rate (RGR) or biomass produced over time was significantly reduced by water deficit in the vegetative stage (Table 3). Interestingly, the reduction in RGR was more pronounced in TJ2049 plants. Regarding CGR, significant differences were observed in the V3 stage after 8 d of drought, while in R5 were detected after 4 d.

These results agree with the differences in yield and yield-DSI exhibited by MUNASQA and TJ2049 after water-deficit treatments in V3 and R5 (Fig. 2). According to these findings, when drought was applied in the V3 stage, TJ2049 showed a distinct but not significant yield penalty and a yield-DSI considerably higher than MUNASQA. Moreover, after a mild water deficit in R5, a highly moisture-sensitive phenological stage, TJ2049 exhibited the largest yield loss and a significantly higher yield-DSI than MUNASQA. Results that also agree with the ones reported by Pardo *et al.* (2015)[13].

Water use markers

Maintaining tissue/cellular water content and/or metabolic activity at low water potentials are physiological strategies to survive drought[32]. Traits like pubescence, changes in stomatal density, slow wilting, leaf thickening, or tight control of stomatal closure, canopy temperature, RWC, and WUE are essential to determining drought tolerance in plants. Thus, all these water-use parameters were assessed in MUNASQA and TJ2049 plants in response to drought.

Morphological drought-adaptation traits, such as stomatal, trichrome density and leaf thickness[33], showed significant differences after prolonged exposure to mild water deficit (21 d) (Table 4). The stomatal and trichrome densities were substantially altered in response to drought in both genotypes. Interestingly, in MUNASQA, stomatal density increased in response to stress on both leaf sides; in contrast, TJ2049 plants exhibited a significant decrease. Regarding pubescence, no significant alterations were observed on the adaxial surface in response to drought; however, on the abaxial side, an increase of trichrome density was quantified in MUNASQA plants, which was higher than in TJ2049. Generally, plants that grow under water deficit develop denser stomata and trichomes[34], mainly on the abaxial surface[35], which helps prevent excessive water loss through transpiration. Our results suggest that MUNASQA morphoanatomic adaptations favour the genotype ability to cope with water deficit.

When evaluating the genotypes leaf thickness, no significant differences were detected under well-irrigated conditions. However, and unexpectedly, after 21 d of water deficit, MUNASQA leaves were significantly thinner, while TJ2049 ones were thicker. The knowledge about the links between leaf morpho-anatomy and its function under non-stressed/stressed conditions is relatively poor, especially for traits like leaf thickness[36] that is strongly related to transpiration[37] and reported by some authors as a drought-tolerance trait that maintains turgor pressure and enhances photosynthesis[38]. Yet, this feature, only apparent in TJ2049 plants under water deficit, could not be associated with adjustments in transpiration, nor with an increase in photosynthesis, or any other drought-tolerant feature.

On the other hand, the regulation of stomatal aperture reinforces the drought-tolerant character of MUNASQA. Under non-stressed conditions, the susceptible TJ2049 showed more opened stomata (≅22% more than MUNASQA). Moreover, after 3 d of water deficit in V3, this difference was increased to almost ≅50% of stomatal aperture (Table 4). Although stomata represent a small percentage of the leaf lamina, large amounts of water evaporate through them[33]. Thus, the lack of stomatal control in TJ2049 might explain the fast-wilting phenotype displayed in response to air desiccation (Fig. 3). Measuring the water loss of detached leaves is a selection method for drought tolerance[39]. Here, R5 leaves of both genotypes were removed and air-dried. After 48 h, MUNASQA exhibited a greener, healthier and slow-wilting phenotype, previously linked to drought tolerance in studies with soybean cultivars[17].

MUNASQA water-saving behaviour was also confirmed through parameters such as RWC and WUE, strongly regulated under drought (Table 5). The RWC, a time-specific measurement of the hydric status of a plant, is considered a physiological character recommended for drought-tolerance selection[40]. In V3 and R5 stages, well-irrigated MUNASQA plants showed higher RWC than TJ2049, even with a smaller canopy. As expected, under water deficit, the RWC values were reduced by 14% in MUNASQA and 20% in TJ2049, indirectly confirming the effectiveness of the stress imposed.

Water use efficiency (WUE), referring to the biomass produced *per* water unit, has been widely used as a breeding target in many rainfed crops, including soybean. Conservative water-use strategies are associated with high leaf WUE[34]. In agreement, and contrarily to TJ2049, V3 and R5 MUNASQA plants showed a gradual increase of WUE in response to water deficit that agrees with the tighter regulation of stomatal movements and the reduced water loss observed in

the genotype (Table 5). Moreover, considering the discrete NAR reduction and the maintenance of a ~70% RWC under water deficit, we hypothesise that MUNASQA may display a stomatal control based on partial or total/partial closure intervals, therefore reducing transpiration and saving water through a smaller gas exchange (potential photosynthesis) penalty.

As a surrogate trait for stomatal conductance, the CTD, regarding the canopy temperature difference with the surrounding air, is a good indication of plant transpiration rate[41]. As expected, in response to drought, MUNASQA plants evidenced lower CTD values, a finding that supports the stomatal aperture results and strongly suggest the genotype water-saving behaviour. Plants with higher stomatal conductance transpire more and thus maintain a cooler canopy[42]. Thus, in TJ2049 stressed-plants, the high and positive CTD confirmed a higher stomatal aperture and transpiration rate that agrees with a water-spender behaviour. Moreover, TJ2049 also presented higher transpiration rates in unstressed conditions. Finding that could be evidence of a natural and predisposing difference between tolerant and susceptible genotypes.

## 3. Markers selection

Identifying and exploiting phenotyping markers will improve selection strategies for drought tolerance in legumes crops[17]. However, to successfully implement markers in a breeding program, it is imperative to validate their (i) accuracy, (ii) feasibility and (iii) strength of association with the desired trait. To further understand the markers contribution to drought tolerance and yield stabilization in MUNASQA and TJ2049, a Principal Component Analysis (PCA) was performed for each set of the parameters, previously grouped in (i) “stress response”, (ii) “growth” and iii) “water use” categories. In addition, markers evaluated at V3 and R5 stages were measured by Pearson's correlation to determine their strength of association between the phenological stages (accuracy) and yield stabilization (desired trait) (Tables 2, 3 and 5).

Biochemical parameters such as enzymatic and non-enzymatic ROS scavengers, leaf pigments and MDA have been confirmed as adaptive responses to desiccation stress, frequently used for selecting plant genotypes under drought[43]. Clear discrimination of MUNASQA and TJ2049 drought responses were observed in PCA results (Fig. 4A). Here, the first two principal components (PC) explained 96.6% of total variation (PC1 = 75.0% and PC2 = 21.6%). Data were clustered by irrigation treatment in PC1, suggesting that these markers are indicators of phenotypic plasticity. The CHL was associated with well-irrigated plants, while all enzymes, MDA, CAR and PRO, were related to drought stress (Fig.4A).

All these markers showed high accuracy between phenological stages due to significant ($P < 0.001$), strong ($r^2$ over 0.88) and positive correlations (Table 2). However, the correlation with yield showed inconsistent outcomes. PRO, MDA and CAR were significant and positive correlated with yield. Meanwhile, except SOD, all enzymes exhibited significant and positive correlations that were too variable in the strength of association and could not be linked with a specific genotype or treatment. Thus, we considered these “stress-response” markers suitable for discriminating susceptible/resistant responses during early drought-tolerance screenings in soybean. Still, due to the high environmental effect, their use as breeding traits is limited. Interestingly, a report[20] found that PRO and CHL were suitable markers for ranking soybean genotypes in response to drought in vegetative stages (5 days after emergence), while MDA could be useful during R5 as a sensitivity trait.

In legumes, features like LAI, smaller leaf area, leaf area maintenance and dry matter partitioning have been used to screen for drought tolerance[17]. Here, in the “growth” PCA (Fig. 4B), the first two PC explained 93.0% of total variation (PC1 = 73.0% and PC2 = 20.0%). In PC1, data were clustered by genotype, suggesting that these markers explained differences between MUNASQA and TJ2049 growth responses on their intrinsic genetic variability. Moreover, the markers analysed were only associated with MUNASQA, LAR and RGR associated with stressed plants. However, only LAI and LAR showed a weak correlation between phenological stage and yield (Table 3).

Water-saving features like denser leaf pubescence, a higher number of stomata, warmer canopies, RWC maintenance and increased WUE have been associated with drought tolerance in legumes and applied in drought-resistance breeding[17]. Here, “water-use” markers contributed the most to discriminating drought-tolerant and susceptible responses. In the two PCA performed, one for physiological markers and the other for morphological ones, data were clustered by genotype in PC1; thus, these markers are indicators of genetic variability.

In CTD, RWC and WUE PCA (Fig. 4C), the first two PC explained of total variation 87.7% (PC1 = 54.6% and PC2 = 33.1%). Markers RWC and WUE (associated with MUNASQA) and CTD (associated with TJ2049) showed significant ($P < 0.001$), strong ($r^2$ over 0.87) and positive correlations between phenological stages (Table 5). Moreover, WUE and CTD were significantly associated with yield, showing positive and negative correlations depending on the water treatments applied (Table 5).

In the PCA made with morphological “water-use” markers, the first two PC explained 96.6% of total variation (PC1 = 73.8% and PC2 = 22.8%) (Fig. 4D). Here, pubescence and stomata abaxial density were strongly related to MUNASQA. Although the data were insufficient to execute good correlation analysis, these morphological markers have been demonstrated as clear indicators of water-saving strategies in legumes[34].

A good drought-resistance marker linked to yield stabilization must also be accurate, cost-effective, if possible non-destructive and easily measurable. Hence, after evaluating marker accuracy (amid phenological stages) and assessing which ones better explained the phenotypic variability between genotypes and treatments, a final selection was performed by cost-feasibility (CF) and statistical weight (SW) rankings. Based on the CF values, the degree of complexity and cost to assess each indicator, 9 markers were further selected encompassing the categories 1 (easy and cheap) and 2 (easy and expensive) (Table 6). Subsequently, after SW re-selection, 4 markers remained with “High” SW in both PC. The selected markers were (i) stomatal density on the adaxial and (ii) abaxial leaf surface, (iii) trichrome density on the abaxial side and (iv) CTD. These four traits were categorised as the most efficient phenotyping markers considering their high accuracy, strong association to water-saving strategies, feasible and non-destructive measurement and amenability to high throughput screening using high-resolution imaging.

Taken together these results: (i) confirmed the effectiveness of the phenotyping methodology and (ii) its successful application in early phenological stages, (iii) the usefulness of MUNASQA and TJ2049 as model genotypes for screening potential drought-tolerance markers and, (iv) suggested that pubescence, stomatal density and CTD are informative markers to discriminate soybean adaptations to withstand drought via dehydration avoidance.

Traditional crops phenotyping is labour-intensive, time-consuming and often destructive. Hence, a well-characterized drought-tolerant phenotype could be useful for developing new commercial genotypes or identifying drought-tolerance markers. In this context, our findings confirm that MUNASQA and TJ2049 constitute suitable models for drought-tolerance screening in soybean. The deep-phenotyping results allowed us to ensure the methodology effectiveness and its possible implementation in early vegetative stages like V3, reducing time and money costs. Finally, identifying stomatal densities, abaxial pubescence and CTD as profitable and non-destructive markers, strongly associated with water-saving adaptation strategies, provides us with promising tools for early selection and prediction of drought tolerance in soybean.

## Methods

# 1. Experimental approach

The response of MUNASQA and TJ2049 to mild water deficit applied in the R5 stage was assessed through transcriptional and leaf morphology analysis. Subsequently, comparative studies were performed to determine the genotypes response to water deficit imposed in V3 and R5 stages. Next, all markers assessed were analysed according to their strength of association between phenological stages and yield, then were ranked by statistical weight and cost-feasibility (Fig. S1).

# 2. Plant material and growth conditions

All experiments were conducted in greenhouse conditions at the Estación Experimental Agroindustrial Obispo Colombres (EEAOC), Las Talitas, Tucumán, Argentina (S26°50', W65°12'). Seeds of MUNASQA and TJ2049 were inoculated with *Bradyrhizobium japonicum* E109 strain ($9 \times 109$ viable cells $kg^{-1}$ of seeds) and sown in 4 L plastic pots (diameter: 18 cm, height: 21 cm) filled with GrowMix® Multipro commercial substrate (Terrafertil S.A., Argentina). Topsoil was covered with perlite to minimise water evaporation. Pots were weekly rearranged to minimise environmental effects. At the V1 stage[44], two homogeneous plants *per* pot were left. Comparative trials were performed at two phenological stages according to Fehr *et al.* (1971)[44]: V3 (second open trefoil) and R5 (beans beginning to develop at one of the four uppermost nodes with a wholly unrolled leaf). During all the experiments, environmental variables were assessed with sensors every 15 min, then recorded and averaged in data loggers (Cavadevices.com, Buenos Aires, Argentina) (Suppl. Table 5).

# 3. Irrigation treatments

The volumetric water content (VWC) of each pot was estimated according to Pereyra-Irujo *et al.* (2012)[45], and the relationship between VWC and water potential (Ψs) was determined[46]. Pots were maintained at 22% of VWC (Ψs=-0.05 MPa) through daily watering until stress onset. According to Pardo *et al.* (2015)[13], the water deficit was applied by maintaining the pots at 14% of VWC (Ψs =-0.65 MPa) for ten days. The desired Ψs was reached in 2-3 days. At the end of stress, plants were fully watered until harvest. The Ψs was daily monitored and recorded. Corrections for soil water status were made by weighing two plants *per* genotype and treatment every 3 days. The plant water status was monitored through the RWC[47] to ensure stress occurrence.

All drought experiments were carried out for three consecutive years, always applying the previously described irrigation treatments. Water deficit in V3 and R5 phenological stages was imposed in independent plant sets; the sections below detailed the sampling process.

# 4. Experiments

MACE-transcriptomic analysis and validation

Three biological replicates *per* treatment (Control and Stress) and genotype (MUNASQA and TJ2049) were collected from R5 plants after 72 h of water deficit (n=12), and RNA from fully mature expanded leaf between nodes 5 to 7 node was isolated for transcriptional analysis.

MACE-Seq libraries and sequencing were performed on an Illumina NextSeq500 machine (1x75 bp reads). The conversion was made with bcl2fastq2 software (version 2.19.1), and the cleaning of duplicate sequences was performed with "TrueQuant". In MACE-seq, the TrueQuant barcodes each DNA molecule before PCR amplification. As each barcode-template combination is statistically unique, PCR-duplicates can be identified and eliminated from the dataset to prevent PCR bias. Bases with low sequencing quality were clipped. Next, reads were mapped into genome version “Gmax_275_v2.0.fa” of soybean downloaded with standard parameters from Phytozome.net and Bowtie2 (version 2.2.4). Then, expression analysis was performed by in-house scripts and DESeq2 (R-package).

DEGs were defined at an FDR of 0.1 and listed as either up or down-regulated. A heat map plot was generated using R software (version 3.4.1). Then, hierarchical clustering was applied by considering a cut-off threshold of 8 expression profiles (clusters). Venn diagrams were depicted using the VennDiagram R package.

A GO enrichment analysis was performed using the topGO R package[48], while the GO annotation file was extracted from agriGO website[49]. Each GO term, containing at least two DEGs, was analysed by Fisher's exact test. The resulting P values were corrected by FDR multiple testing approach. GO terms with an FDR lower than 0.1 were considered for further analysis.

Ten DEGs were randomly selected and measured by qRT-PCR assays (Applied Biosystems) to validate MACE results. F-BOX gene was used as an internal reference to standarise the expression of target genes, and the ratio between treatments was calculated according to[50]. All primers used are listed in Suppl. Table 6. Data analysis and primer efficiencies were obtained using LinReg PCR software[51]. Relative expression ratios and statistical analysis were performed using fgStatistics software interface[52]. The cut-off for statistically significant differences was set as $P < 0.05$, indicated as *.

Antioxidant measurements

Additionally, antioxidant proteins encoded by DEGs detected in MACE were analysed. Five biological samples were collected *per* treatment and genotype (n=20). The enzymatic extraction was performed according to Singh *et al.* (2015)[53]. The activities of superoxide dismutase (SOD, EC 1.15.1.1)[54], ascorbate peroxidase (APX, EC 1.11.1.11)[55], phenol peroxidase (POX, EC 1.11.1.7)[56] and catalase (CAT, EC 1.11.1.6)[57] were measured, as well as the total soluble protein content[58].

Leaf morphology measurements

Changes in leaf thickness (LT), adaxial and abaxial stomatal and trichrome densities (SD_AD, SD_AB, TD_AD and TD_AB) were assessed in leaves between nodes 4 to 7 of MUNASQA and TJ2049 R5 plants after 3, 10 and 21 d of water deficit. Five samples *per* genotype and treatment (n=20) were taken and fixed in FAA (10% formalin, 5% acetic acid, 50% ethyl alcohol). Diaphanised sections of the central leaflet were used for superficial observations. Different standard colourations were applied according to D'Ambrogio de Argüeso (1986)[59]. Staining samples were visualised in a Leica DM500 optical microscope and photographed with an Arcano (5 Mpx) camera (10 measurements *per* sample, n=200).

Stomatal aperture measurements

According to Gudesblat *et al.* (2009)[60], stomatal apertures were measured in three independent assays, using MUNASQA and TJ2049 plants submitted to 72 h of water deficit in the V3 stage. The aperture of 40 stomata *per* treatment and genotype (n=120) was measured in each experiment.

Wilting air desiccation assay

Response to air desiccation was evaluated in the R5 stage. Three whole leaves *per* genotype (n=6) were collected and exposed to air desiccation at 32°C. After 0, 6, 24, 36 and 48 h of air exposure, plants were photographed with a Canon Power Shot SX520 HS (14 Mpx), and the wilting rate was assessed.

Comparative analysis of genotypes responses to water deficit in V3 and R5

The responses of MUNASQA and TJ2049 to water deficit, applied in V3 and R5 stages, were compared by measuring morphophysiological and biochemical parameters grouped by biological processes (BP) (Table 1). Four treatments were defined: (i) Control-V3, (ii) Control-R5, (iii) Stress-V3 and (iv) Stress-R5, and three sampling times were performed (0, 4 and 8 d of water deficit). Ten plants *per* genotype, treatment and time were collected and used for markers evaluation (n=240).

Additionally, for treatments (i), (iii) and (iv), 50 plants *per* genotype were harvested at physiological maturity (n=300) to quantify relative yield and calculate the relative yield DSI (Drought Susceptibility Index) according to Fischer and Maurer (1978)[61].

The markers evaluated and their methodologies are detailed below.

Markers

As stress response markers, the activities of SOD, APX, POX and CAT proteins were assessed, together with the accumulation of free proline (PRO)[62], malondialdehyde (MDA)[63], total chlorophylls (CHL)[64], and carotenoids (CAR)[65].

As growth indicators, the plant total leaf area (TLA) and biomass (plant total dry weight) were determined. Then leaf area index (LAI) and leaf area ratio (LAR)[66], the net assimilation rate (NAR)[67], relative growth rate (RGR)[68] and crop growth rate (CGR)[69] were calculated.

Finally, as water-use parameters, plant RWC and WUE[70] were calculated. Moreover, the canopy temperature was monitored and recorded using a FLIR ONE-3 thermal camera (0.3456 Mpx) to calculate canopy temperature depression (CTD)[71].

# 5. Univariate analysis

Data from stomatal apertures were subjected to a two-way ANOVA (Factor 1: genotype, Factor 2: treatment). The remaining data were analysed through ANOVA with post hoc contrast by Tukey's HSD test. Data were analysed with InfoStat statistical package[52] and presented as the arithmetic mean ±SE. Means were considered significantly different at $P < 0.05$.

# 6. Correlations, multivariate analysis and markers selection

The 22 markers strength of association between phenological stages, V3 and R5, was measured by Pearson's correlation analysis adjusted by Bonferroni ($P < 0.05$ indicated as *; $P < 0.01$ ** and $P < 0.001$ ***). Then, the markers correlation with relative yield was assessed. Correlation coefficients ($r^2$) were classified

as “Strong” (> ±0.60) and “Weak” (below ±0.59).

All markers, grouped in (i) “stress response”, (ii) “growth”, and iii) “water use” sets, were subjected to PCA to determine which markers best explained the phenotypic variability between genotypes and treatments.

Additionally, the markers were ranked by CF and SW. The markers CF, in terms of their complexity and evaluation cost, was assigned according to 4 categories: easy and cheap (1), easy and expensive (2), complicated and cheap (3) or complicated and expensive (4). Meanwhile, the SW was obtained from PCA variables coefficients (autovectors e1 and e2) that were ranked and classified in “Low” (Low = [-2, 2]) and High (High = $\mathbb{R}$ – [-2, 2]), according to their weigh on PC1 and PC2. Markers strongly correlated between phenological stages, if possible with yield, together with CF values of 1 or 2 and “High” SW in both PC, were selected as the most efficient phenotyping markers.

7. General guidelines statement: The authors declare that there is no conflict for the use of commercial soybean varieties for scientific research purposes cited in this article in accordance with Argentine law (Law of Seeds and Phytogenetic Creations No. 20,247 / 73).

## Declarations


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### Author contributions

L.T. and L.S.P.B. designed and performed the experiments, compiled the data and wrote the article; A.P.M. carried out the statistical analysis and art graph; C.L. performed the morphophysiological analysis; E.M.P. performed the RNA-seq sample preparation; A.B. and D.F.D.P. designed and performed the RNA-seq analysis; B.W. and A.V. supervised and designed experiments; A.P.C., B.W. and E.M.P. conceived the project; all authors contributed to the writing.


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### Competing interests

The authors declare no competing interests.

### Additional information


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## Tables

**Table 1** Markers evaluated in MUNASQA and TJ2049, clustered by biological processes (BP).

| Set | Marker | |
|---|---|---|
| **I. Stress response** | **1** | Superoxide dismutase (SOD) |
| | **2** | Ascorbate peroxidase (APX) |
| | **3** | Phenol peroxidase (POX) |
| | **4** | Catalase (CAT) |
| | **5** | Free proline (PRO) |
| | **6** | Malondialdehyde (MDA) |
| | **7** | Total chlorophyll (CHL) |
| | **8** | Total carotenoid (CAR) |
| **II. Growth** | **9** | Leaf area index (LAI) |
| | **10** | Leaf area ratio (LAR) |
| | **11** | Net assimilation rate (NAR) |
| | **12** | Relative growth rate (RGR) |
| | **13** | Crop growth rate (CGR) |
| **III. Water use** | **14** | Relative water content (RWC) |
| | **15** | Water use efficiency (WUE) |
| | **16** | Canopy temperature depression (CTD) |
| | **17** | Leaf thickness (LT) |
| | **18** | Trichome density in abaxial surface (TD_AB) |
| | **19** | Trichome density in adaxial surface (TD_AD) |
| | **20** | Stomatal density in abaxial surface (SD_AB) |
| | **21** | Stomatal density in adaxial surface (SD_AD) |
| | **22** | Stomatal aperture |

**Table 2** Effect of mild water deficit on stress-response enzymatic markers measured in MUNASQA and TJ2049. SOD, APX, POX and CAT activities, together with PRO, MDA, CHL and CAR contents, were obtained from plants submitted to water deficit (Ψs =-0.65 MPa) and well-watered treatments (Ψs =-0.05 MPa) applied in V3 and R5 stages. Two independent experiments (n= 10 *per* genotype/treatment) were conducted, assessing parameters at 0, 4, and 8 d after stress (DAS) imposition. Additionally, 50 plants *per* genotype and the following treatments: 1: Control, 2: V3-Stress and 3: R5-Stress, were harvested at physiological maturity to obtain relative yield. Average values followed by the same uppercase letter in the column and the same lowercase letter in the row do not differ statistically among them within each phenological stage, according to Tukey's HSD test at 5%. The strength of association between markers evaluated in V3 and R5 stages (n=240) and between markers and yield (n=300) was measured by Pearson's correlation analysis adjusted by Bonferroni (P > 0.05 indicated as **ns**; P < 0.05 indicated as *****; P < 0.01 ****** and P < 0.001 *******). Correlation coefficients ($r^2$) were classified as "**S**: Strong" (> ±0.60) and "**W**: weak" (below ±0.59).

| SOD (µmol $O_2^-$ $gDW^{-1}$ $min^{-1}$) | Correlations | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | | | *vs* Yield | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | | |
| TJ2049 Control | 60.98 | Aa | 58.02 | Aa | 61.25 | Ba | 75.64 | A | 78.89 | A | 65.07 | B | 0.62 (S) | *** | - |
| TJ2049 Stress | 56.38 | Aa | 54.84 | Aa | 194.4 | Db | 73.82 | Aa | 91.78 | Bb | 153.1 | Dc | 0.93 (S) | *** | ( |
| MUNASQA Control | 85.25 | Ba | 90.05 | Ba | 83.56 | Ca | 94.58 | Ba | 100.73 | Ba | 88.55 | C | 0.97 (S) | *** | - |
| MUNASQA Stress | 76.36 | Bb | 183.55 | Cc | 31.55 | Aa | 97.51 | Bb | 299.59 | Cc | 47.33 | Aa | 0.96 (S) | *** | ( |
| Standard Error | 4.38 | | 4.99 | 5.1 | 3.41 | 6.08 | 4.46 | | | | | | | | |
| APX (µmol Asa $gDW^{-1}$ $min^{-1}$) | Correlations | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | | | *vs* Yield | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | | |
| TJ2049 Control | 99.69 | Aa | 96.07 | Aa | 99.87 | Aa | 103.9 | Aa | 100.13 | Aa | 104.09 | Aa | 0.90 (S) | *** | ( ( |
| TJ2049 Stress | 96.17 | Aa | 108.51 | ABa | 158.22 | Bb | 98.46 | Aa | 111.09 | Aa | 161.99 | Cb | 0.94 (S) | *** | ( |
| MUNASQA Control | 120.9 | Ba | 126.42 | Ba | 117.69 | Aa | 133.01 | Ba | 139.08 | Ba | 129.48 | Ba | 0.97 (S) | *** | ( |
| MUNASQA Stress | 121.84 | Ba | 205.1 | Cc | 174.46 | Bb | 134.87 | Ba | 227.02 | Cc | 193.11 | Db | 0.95 (S) | *** | ( ( |
| Standard Error | 4.21 | | 4.89 | 5.47 | 6.54 | 5.17 | 5.77 | | | | | | | | |
| POX (µmol Purpurogalline $gDW^{-1}$ $min^{-1}$) | Correlations | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | | | *vs* Yield | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | | |
| TJ2049 Control | 59.4 | Aa | 66.03 | Aa | 64.92 | Aa | 72.12 | Aa | 80.18 | Aa | 78.83 | Aa | 0.93 (S) | *** | ( ( |
| TJ2049 Stress | 62.85 | Aa | 103.78 | BCb | 143.33 | Cc | 74.24 | Aa | 122.60 | BCb | 169.32 | Cc | 0.97 (S) | *** | ( ( |
| MUNASQA Control | 91.97 | Ba | 90.02 | ABa | 87.15 | ABa | 111.39 | Ba | 109.03 | ABa | 105.55 | ABa | 0.96 (S) | *** | ( |
| MUNASQA Stress | 98.87 | Ba | 121.89 | Cb | 91.09 | Ba | 119.39 | Ba | 147.18 | Cb | 109.99 | Ba | 0.94 (S) | *** | ( ( |
| Standard Error | 3.98 | | 6.99 | 6.08 | 4.75 | 8.42 | 7.33 | | | | | | | | |
| CAT (µmol $H_2O_2$ $gDW^{-1}$ $min^{-1}$) | Correlations | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | | | *vs* Yield | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | | |
| TJ2049 Control | 101.2 | Aa | 94.32 | Aa | 95.69 | Aa | 122.88 | Aa | 114.53 | Aa | 113.75 | Aa | 0.95 (S) | *** | ( ( |
| TJ2049 Stress | 100.27 | Aa | 140.09 | Cb | 155.66 | Cc | 118.45 | Aa | 165.50 | Cb | 183.89 | Cc | 0.93(S) | *** | ( |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MUNASQA Control | 99.58 | Aa | 97.83 | Aa | 96.8 | Aa | 124.62 | Aa | 120.86 | Aa | 117.25 | Aa | 0.88(S) | *** |
| MUNASQA Stress | 97.42 | Aa | 120.93 | Bb | 127.54 | Bb | 117.63 | Aa | 146.02 | Bb | 154.01 | Bb | 0.90(S) | *** |
| Standard Error | 3.65 | 3.43 | 3.15 | 4.39 | 4.12 | 3.78 | | | | | | | | |
| PRO (µg gFW$^{-1}$) | Correlations | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | *vs* Yield | | | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | |
| TJ2049 Control | 34.43 | Aa | 35.81 | Aa | 37.42 | Aa | 58.75 | Aa | 51.68 | Aa | 44.91 | Ab | 0.12 | ns |
| TJ2049 Stress | 35.18 | Aa | 76.48 | Cb | 132.74 | Cc | 52.77 | Aa | 114.72 | Cb | 199.12 | Cc | 0.96 (S) | *** |
| MUNASQA Control | 49.43 | Ba | 51.46 | Ba | 53.85 | Ba | 73.00 | Ba | 75.29 | Ba | 78.11 | Ba | 0.92 (S) | *** |
| MUNASQA Stress | 47.86 | Ba | 158.13 | Dc | 139.68 | Cb | 74.14 | Ba | 237.19 | Dc | 209.51 | Cb | 0.94 (S) | *** |
| Standard Error | 1.41 | 2.32 | 2.85 | 2.56 | 3.51 | 4.30 | | | | | | | | |
| MDA (µmol gFW$^{-1}$) | Correlations | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | *vs* Yield | | | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | |
| TJ2049 Control | 6.09 | Ca | 6.89 | Ba | 6.05 | Ba | 9.75 | Ba | 9.42 | Ba | 9.67 | Ba | 0.90 (S) | *** |
| TJ2049 Stress | 5.39 | Ba | 10.4 | Cb | 18.54 | Dc | 9.03 | Ba | 19.84 | Db | 29.67 | Dc | 0.91 (S) | *** |
| MUNASQA Control | 3.03 | Aa | 2.73 | Aab | 2.02 | Ab | 4.85 | Aa | 4.37 | Aa | 4.22 | Aa | 0.94 (S) | *** |
| MUNASQA Stress | 3.12 | Aa | 6.43 | Bb | 10.95 | Cc | 4.99 | Aa | 10.32 | Cb | 17.51 | Cc | 0.90 (S) | *** |
| Standard Error | 0.27 | 0.35 | 0.4 | 0.43 | 0.56 | 0.64 | | | | | | | | |
| CHL (µg gFW$^{-1}$) | Correlations | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | *vs* Yield | | | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | |
| TJ2049 Control | 368.74 | Aa | 497.61 | Bb | 591.12 | Cc | 442.48 | Aa | 578.53 | Bb | 699.35 | Cc | 0.96 (S) | *** |
| TJ2049 Stress | 376.60 | Ab | 418.14 | Ac | 216.02 | Aa | 451.92 | Ab | 489.77 | Ac | 259.22 | Aa | 0.92 (S) | *** |
| MUNASQA Control | 371.00 | Aa | 490.32 | Bb | 589.85 | Cc | 445.20 | Aa | 594.18 | Bb | 707.82 | Cc | 0.85 (S) | *** |
| MUNASQA Stress | 374.50 | Ab | 377.12 | Ab | 311.25 | Ba | 449.40 | Ab | 452.54 | Ab | 373.5 | Ba | 0.88 (S) | *** |
| Standard Error | 15.11 | 13.82 | 13.07 | 18.14 | 16.58 | 15.68 | | | | | | | | |
| CAR (µg gFW$^{-1}$) | Correlations | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | *vs* Yield | | | | | | | | | |
| | 0 DAS | | 4 | 8 DAS | 0 DAS | 4 DAS | 8 | $r^2$ | P- | $r^2$ | P-value | | | |

| | | DAS | | | | DAS | | value | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TJ2049 Control** | 138.00 | **Aa** | 151.76 | **Ab** | 141.39 | **Aab** | 179.40 | **Aa** | 190.29 | **Ab** | 183.81 | **Aa** | 0.86 (S) | *** |
| **TJ2049 Stress** | 147.40 | **Aab** | 160.95 | **Bb** | 202.25 | **Cc** | 181.55 | **Aa** | 199.95 | **Ab** | 234.40 | **Bc** | 0.92 (S) | *** |
| **MUNASQA Control** | 139.65 | **Aa** | 153.81 | **Ab** | 180.31 | **Bc** | 256.62 | **Bab** | 248.24 | **Ba** | 262.93 | **Cb** | 0.89 (S) | *** |
| **MUNASQA Stress** | 132.74 | **Aa** | 256.94 | **Cb** | 279.94 | **Dc** | 258.36 | **Ba** | 334.02 | **Cb** | 363.92 | **Dc** | 0.90 (S) | *** |
| **Standard Error** | 3.16 | 3.03 | 3.47 | 4.10 | 3.93 | 4.51 | | | | | | | | |

**Table 3** Effect of mild water deficit on growth markers measured in MUNASQA and TJ2049. LAI, LAR, NAR, RGR and CGR were assessed in plants submitted to water deficit (Ψs=-0.65 MPa) and well-watered treatments (Ψs= -0.05 MPa) in V3 and R5 stages. Two independent experiments (n= 10 *per* genotype/treatment) were conducted, assessing parameters at 0, 4, and 8 d after stress (DAS) imposition. Additionally, 50 plants *per* genotype and the following treatments: 1: Control, 2: V3-Stress and 3: R5-Stress, were harvested at physiological maturity to obtain relative yield. Average values followed by the same uppercase letter in the column and the same lowercase letter in the row do not differ statistically among them within each phenological stage, according to Tukey's HSD test at 5%. The strength of association between markers evaluated in V3 and R5 stages (n=240) and between markers and yield (n=300) was measured by Pearson's correlation analysis adjusted by Bonferroni (P > 0.05 indicated as **ns**; P < 0.05 indicated as *****; P < 0.01 ****** and P < 0.001 *******). Correlation coefficients ($r^2$) were classified as "**S**: Strong" (> ±0.60) and "**W**: weak" (below ±0.59).

| LAI | Correlations | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | | | *vs* Yield | | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | | |
| TJ2049 Control | 18.88 | Aa | 23.76 | Ab | 29.00 | Ac | 121.12 | Ba | 151.24 | Bb | 187.29 | BCc | 0.94 (S) | *** | 0.01 | ns |
| TJ2049 Stress | 16.94 | Aa | 19.65 | Ab | 26.76 | Ac | 97.03 | Aa | 112.29 | Ab | 151.94 | Ac | 0.96 (S) | *** | 0.69 (S) | ** |
| MUNASQA Control | 30.18 | Ba | 32.90 | Ba | 39.71 | Bb | 177.59 | Ca | 187.94 | Ca | 194.47 | Ca | 0.79 (S) | *** | 0.04 | ns |
| MUNASQA Stress | 31.76 | Bab | 33.88 | Bb | 29.00 | Aa | 172.29 | Ca | 162.47 | BCa | 164.94 | ABa | 0.39 (W) | *** | 0.42 (W) | ** |
| Standard Error | 0.96 | | 1.43 | 1.12 | 3.8 | | 7.72 | 6.34 | | | | | | | | |
| LAR ($cm^{-2}$ $g^{-1}$) | Correlations | | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | | | *vs* Yield | | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | | |
| TJ2049 Control | 54.30 | Cb | 42.41 | Ba | 50.49 | Bab | 11.17 | Aa | 14.40 | Ab | 16.45 | Ab | 0.44 (W) | *** | -0.03 | ns |
| TJ2049 Stress | 50.07 | Cb | 31.62 | Aa | 35.41 | Aa | 8.50 | Aa | 11.20 | Aa | 17.76 | Ab | -0.04 | ns | -0.66 (S) | ** |
| MUNASQA Control | 83.73 | Bb | 58.07 | Ca | 49.40 | Ba | 18.60 | Ba | 18.38 | Ba | 16.58 | Aa | 0.36 (W) | *** | -0.04 | ns |
| MUNASQA Stress | 96.37 | Bb | 47.58 | Ba | 35.93 | Aa | 22.06 | Ba | 20.57 | Ba | 21.11 | Ba | 0.40 (W) | *** | -0.27 (W) | ** |
| Standard Error | 5.67 | | 2.45 | 2.25 | 2.62 | | 1.89 | 0.81 | | | | | | | | |
| NAR ($g^{-1}$ $cm^{-2}$ $day^{-1}$) | Correlations | | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | | | *vs* Yield | | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | | |
| TJ2049 Control | - | | | 3.93 | Aa | 3.67 | Ba | - | | 0.60 | Ba | 0.70 | Cb | 0.22 | ns | -0.03 | ns |
| TJ2049 Stress | - | | | 3.51 | ABa | 2.72 | Ab | - | | 0.44 | Aa | 0.48 | Aa | -0.04 | ns | 0.01 | ns |
| MUNASQA Control | - | | | 5.41 | Ca | 5.43 | Da | - | | 0.64 | Ba | 0.72 | Cb | 0.10 | ns | -0.01 | ns |
| MUNASQA Stress | - | | | 4.51 | Ba | 5.04 | CDb | - | | 0.63 | Bb | 0.54 | Ba | 0.17 | ns | -0.03 | ns |
| Standard Error | - | | 0.13 | 0.12 | - | | 0.04 | 0.09 | | | | | | | | | |
| RGR ($g^{-1}$ $g^{-1}$ $day^{-1}$) | Correlations | | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | | R5 Stage | V3 *vs* R5 | | | *vs* Yield | | | | | | | | |
| | 0 DAS | | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | | |
| TJ2049 Control | - | | | 1.56 | Ca | 1.34 | Bb | - | | 0.33 | Aa | 0.36 | Aa | -0.08 | ns | -0.11 | ns |
| TJ2049 Stress | - | | | 1.52 | Ca | 0.38 | Cb | - | | 0.28 | Aa | 0.32 | Aa | 0.33 | *** | -0.07 | ns |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MUNASQA Control | - | | 2.69 | Ab | 3.24 | Aa | - | | 0.30 | Aa | 0.34 | Aa | -0.45 (W) | ns | -0.05 | n |
| MUNASQA Stress | - | | 1.91 | Bb | 1.36 | Ba | - | | 0.32 | Aa | 0.33 | Aa | 0.22 | ns | -0.13 | n |
| Standard Error | - | 0.13 | 0.10 | - | 0.09 | 0.05 | | | | | | | | | | |
| CGR ($g^{-1}$ $cm^{-2}$ $day^{-1}$) | Correlations | | | | | | | | | | | | | | | |
| Genotype and Treatment | V3 Stage | R5 Stage | V3 *vs* R5 | *vs* Yield | | | | | | | | | | | | |
| | 0 DAS | 4 DAS | 8 DAS | 0 DAS | 4 DAS | 8 DAS | $r^2$ | P-value | $r^2$ | P-value | | | | | | |
| TJ2049 Control | - | | 0.96 | Aa | 0.62 | Ab | - | | 0.57 | ABb | 0.63 | Ca | -0.46 (W) | *** | -0.13 | n |
| TJ2049 Stress | - | | 0.90 | Aa | 0.72 | Bb | - | | 0.52 | Aa | 0.51 | Aa | 0.17 | ns | 0.02 | n |
| MUNASQA Control | - | | 1.37 | Ba | 0.59 | Ab | - | | 0.64 | Ca | 0.67 | Ca | -0.20 | ns | -0.05 | n |
| MUNASQA Stress | - | | 1.27 | Ba | 0.67 | ABb | - | | 0.60 | BCa | 0.58 | Ba | 0.33 | ns | 0.03 | n |
| Standard Error | - | 0.60 | 0.09 | - | 0.12 | 0.11 | | | | | | | | | | |

**Table 4** Effect of mild water deficit on water-use physiological markers measured in MUNASQA and TJ2049. LT, TD_AB, TD_AD, ST_AB, SD_AD and stomatal aperture were assessed in plants submitted to water deficit (Ψs =-0.65 MPa) and well-watered treatments (Ψs =-0.05 MPa) in R5 stage (except for stomatal aperture applied in V3). For LT, SD_AB, SD_AD, TD_AB and TD_AD, an independent experiment (n= 5 *per* genotype/treatment) was conducted, assessing parameters at 3, 10 and 21 days after stress (DAS) imposition. Here we showed the data corresponding to 21 DAS (n= 10 measured *per* sample). For stomatal aperture, three independent experiments (n= 40 stomatal measurements *per* genotype/treatment) were conducted, and the stomata evaluation was performed 72 hs after stress imposition. Average values followed by the same uppercase letter do not differ statistically according to Tukey's HSD test at 5%.

| Genotype and Treatment | SD_AB ($mm^2$) | | SD_AD ($mm^2$) | | TD_AB ($mm^2$) | | TD_AD ($mm^2$) | | LT (µm) | | Stomatal aperture (µm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TJ2049 Control | 219.14 | C | 173.05 | C | 0.82 | A | 0.49 | A | 158.06 | B | 3.48 | D |
| TJ2049 Stress | 200.05 | B | 87.21 | B | 1.07 | A | 0.30 | A | 165.29 | C | 0.97 | B |
| MUNASQA Control | 186.32 | A | 52.05 | A | 2.29 | B | 0.81 | B | 158.12 | B | 2.84 | C |
| MUNASQA Stress | 351.66 | D | 79.56 | B | 3.22 | C | 1.51 | C | 149.59 | A | 0.45 | A |
| Standard Error | 2.62 | | 2.38 | | 0.11 | | 0.07 | | 1.71 | | 0.07 | |

**Table 5** Effect of mild water deficit on water-use physiological markers measured in MUNASQA and TJ2049. RWC, WUE and CTD were assessed in plants submitted to water deficit (Ψs=-0.65 MPa) and well-watered treatments (Ψs=-0.05 MPa) in V3 and R5 stages. Two independent experiments (n= 10 *per* genotype/treatment) were conducted, assessing parameters at 0, 4, and 8 d after stress (DAS) imposition. Additionally, 50 plants *per* genotype and the following treatments: 1: Control, 2: V3-Stress and 3: R5-Stress, were harvested at physiological maturity to obtain relative yield. Average values followed by the same uppercase letter in the column and the same lowercase letter in the row do not differ statistically among them within each phenological stage, according to Tukey's HSD test at 5%. The strength of association between markers evaluated in V3 and R5 stages (n=240) and between markers and yield (n=300) was measured by Pearson's correlation analysis adjusted by Bonferroni (P > 0.05 indicated as **ns**; P < 0.05 indicated as *; P < 0.01 ** and P < 0.001 ***). Correlation coefficients ($r^2$) were classified as "**S**: Strong" (> ±0.60) and "**W**: weak" (below ±0.59).

| RWC (%) | | | | | | | | | | | | | Correlations | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype and Treatment | V3 Stage | | | | | | R5 Stage | | | | | | V3 *vs* R5 | | *vs* Yield | |
| | 0 DAS | | 4 DAS | | 8 DAS | | 0 DAS | | 4 DAS | | 8 DAS | | $r^2$ | P-value | $r^2$ | P-value |
| TJ2049 Control | 74.54 | Aa | 78.24 | Ca | 80.33 | Ca | 71.89 | Aa | 81.46 | Cb | 78.94 | Cab | 0.87 (S) | *** | - | - |
| TJ2049 Stress | 72.87 | Ab | 58.41 | Aa | 59.01 | Aa | 75.22 | Ab | 53.82 | Aa | 56.40 | Aa | 0.96 (S) | *** | - | - |
| MUNASQA Control | 83.42 | Ba | 80.07 | Ca | 85.37 | Ca | 82.89 | Ba | 85.40 | Ca | 88.65 | Ca | 0.90 (S) | *** | - | - |
| MUNASQA Stress | 83.08 | Bb | 68.16 | Ba | 65.76 | Ba | 80.55 | Bb | 64.36 | Ba | 60.30 | Ba | 0.94 (S) | *** | - | - |
| Standard Error | 1.90 | | 1.85 | | 1.88 | | 2.20 | | 2.04 | | 3.07 | | | | | |
| WUE (g $kg^{-1}$) | | | | | | | | | | | | | Correlations | | | |
| Genotype and Treatment | V3 Stage | | | | | | R5 Stage | | | | | | V3 *vs* R5 | | *vs* Yield | |
| | 0 DAS | | 4 DAS | | 8 DAS | | 0 DAS | | 4 DAS | | 8 DAS | | $r^2$ | P-value | $r^2$ | P-value |
| TJ2049 Control | 6.54 | Ca | 5.66 | Bb | 7.02 | Bc | 7.89 | Cb | 6.60 | Bc | 8.09 | Bb | 0.89 (S) | *** | -0.44 (W) | ** |
| TJ2049 Stress | 5.87 | Bb | 4.59 | Aa | 4.76 | Aa | 7.47 | Cb | 5.43 | Aa | 5.48 | Aa | 0.95 (S) | *** | 0.82 (S) | *** |
| MUNASQA Control | 5.16 | Aa | 5.72 | Bb | 6.52 | Bc | 6.23 | Aa | 6.77 | Bb | 7.51 | Bc | 0.91 (S) | *** | -0.78 (S) | *** |
| MUNASQA Stress | 5.42 | Aa | 7.68 | Cb | 8.58 | Cc | 7.03 | Ba | 8.24 | Cb | 7.91 | Bc | 0.96 (S) | *** | 0.84 (S) | *** |
| Standard Error | 0.48 | | 0.24 | | 0.54 | | 0.38 | | 0.18 | | 0.49 | | | | | |
| CTD (ºC) | | | | | | | | | | | | | Correlations | | | |
| Genotype and Treatment | V3 Stage | | | | | | R5 Stage | | | | | | V3 *vs* R5 | | *vs* Yield | |
| | 0 DAS | | 4 DAS | | 8 DAS | | 0 DAS | | 4 DAS | | 8 DAS | | $r^2$ | P-value | $r^2$ | P-value |
| TJ2049 Control | 3.11 | Ba | 2.97 | Ca | 3.21 | Ca | 3.37 | Ba | 3.50 | Ca | 3.25 | Da | 0.93 (S) | *** | 0.70 (S) | *** |
| TJ2049 Stress | 2.86 | Bb | 1.58 | Ba | 1.87 | Ba | 3.52 | Bc | 1.54 | Bb | 1.02 | Ca | 0.91 (S) | *** | -0.79 (S) | *** |
| MUNASQA Control | 1.27 | Aa | 1.19 | Ba | 1.33 | Ba | 1.77 | Aa | 1.46 | Ba | 1.61 | Ba | 0.95 (S) | *** | 0.76 (S) | *** |
| MUNASQA Stress | 1.43 | Ab | -1.32 | Aa | -1.39 | Aa | 1.60 | Ab | -0.94 | Aa | -1.12 | Aa | 0.97 (S) | *** | -0.85 (S) | *** |
| Standard Error | 0.28 | | 0.30 | | 0.37 | | 0.17 | | 0.22 | | 0.40 | | | | | |

**Table 6** Selection of phenotyping markers according to their CF and SW.

| CF | Marker selected by CF | SW | | Marker reselected by SW |
|---|---|---|---|---|
| | | PC 1 | PC 2 | |
| 1 | LAI | High | Low | - |
| 2 | SD_AB | **High** | **High** | **Sel** |
| 2 | SD_AD | **High** | **High** | **Sel** |
| 2 | TD_AB | **High** | **High** | **Sel** |
| 2 | TD_AD | High | Low | - |
| 1 | WUE | High | Low | - |
| 2 | CTD | **High** | **High** | **Sel** |
| 2 | MDA | Low | High | - |
| 2 | CAR | High | Low | - |

Markers with **CF** of **1** or **2** and High SW in both autovectors were selected.

## Figures

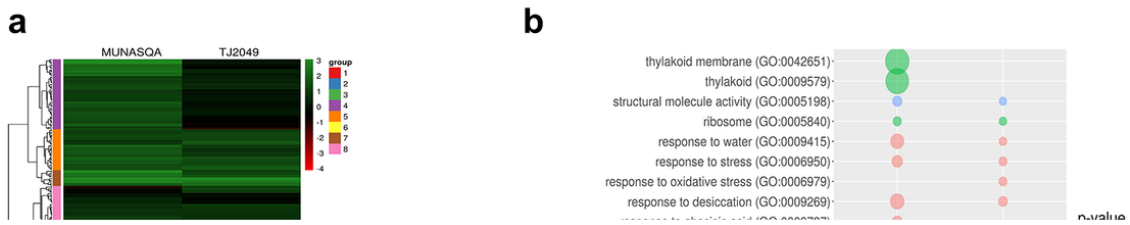


**Figure 1**

Transcriptomic analysis of MUNASQA and TJ2049 genotypes under drought. Heat-map of all DEGs for MUNASQA and TJ2049 in drought conditions. Scale colour indicates green for up-regulation and red for downregulation (a). GO enrichment in MUNASQA and TJ2049 comprises biological processes (BP, in red), molecular function (MF, in blue), and cellular component (CC in green). Relevant categories showing enrichment of DEGs for both genotypes are depicted. GO terms were plotted after applying an FDR=0.1 Bubble size correlates with enrichment factor values; for each bubble size, the P-value is indicated (b). Venn diagram for all DEGs in MUNASQA and TJ2049 under drought conditions. DEGs were plotted after applying an FDR=0.1 (c). Validation by qRTPCR of ten

genes selected from RNA-Seq. Log2 fold change (log2FC) was calculated based on the comparison drought vs control for each genotype (d). Three biological replicates were used, and the experiment was performed twice with similar results.

**Figure 2**

Effects of mild water deficit in MUNASQA and TJ2049 yield and yield-DSI. Yield in well-irrigated (Ψs= -0.05 MPa) and drought-stressed (Ψs=-0.65 MPa) V3 and R5 (a). Yield-DSI for each genotype phenotyped in V3 and R5 (b). Different letters indicate significant differences at $P < 0.05$ (two-way ANOVA). Error bars represent SE from independent experiments, n=300 per trial.

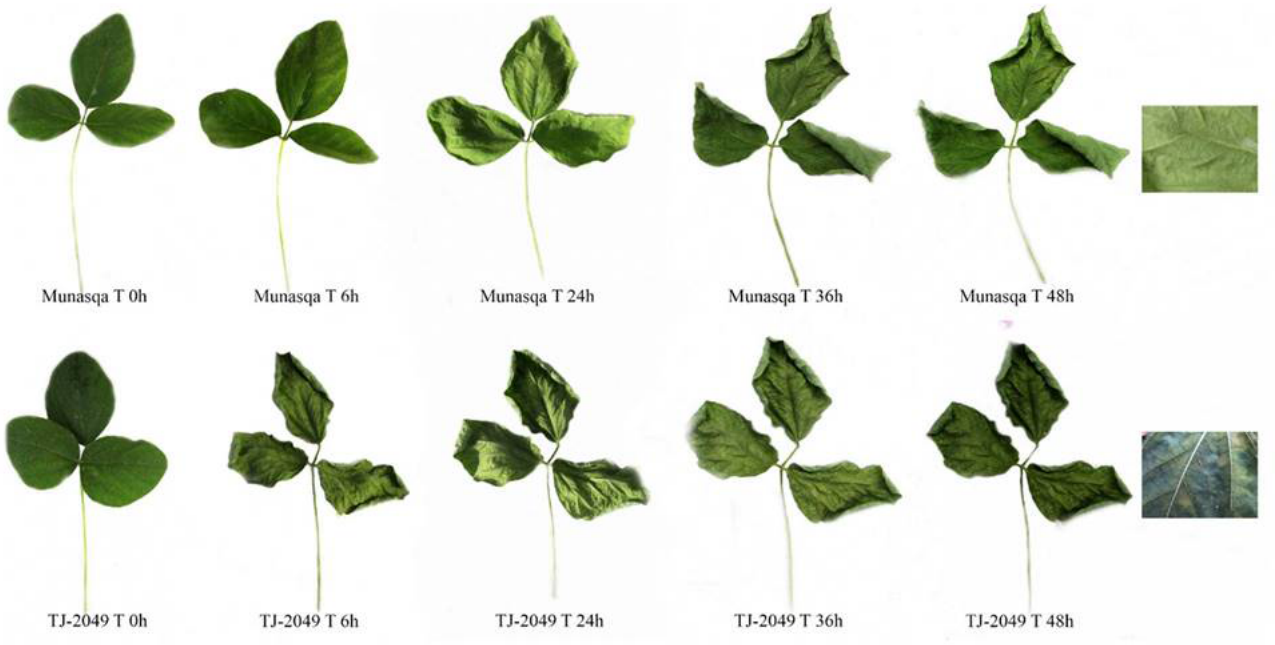


**Figure 3**

MUNASQA and TJ2049 response to wilting air desiccation. Whole leaves (n=6), collected from R5 plants, were exposed to air desiccation at 32°C and photographed after 0, 6, 24, 36 and 48 h to evaluate the appearance of wilting symptoms.

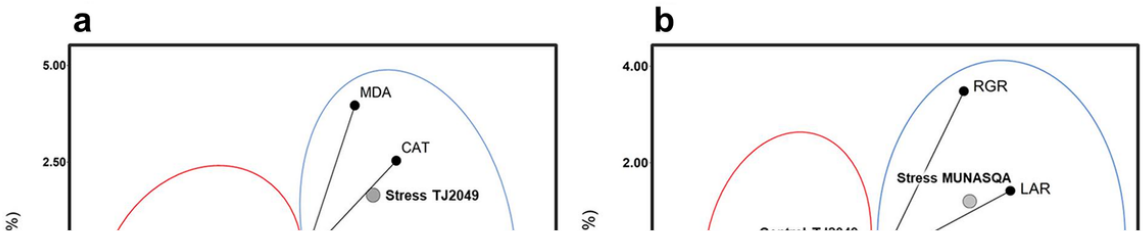


**Figure 4**

PCA for determining markers interaction with genotypes and treatments. Markers Set I: stress-response. PCA was performed using the SOD, APX, POX, CAT, MDA, PRO, CHL and CAR data (a). Markers Set II: growth. PCA was performed using LAI, LAR, NAR, RGR and CGR data (b). Physiological markers of Set III: water use. PCA was performed using the RWC, WUE and CTD data (c). Morphological markers of Set III: water use. PCA was performed using the LT, TD_AB, TD_AD, ST_AB and SD_AD data (d). For (a to c), MUNASQA and TJ2049 were exposed to water deficit (Ψs=-0.65 MPa) and well-watered (Ψs=-0.05 MPa) treatments applied in V3 and R5. Markers were determined at 0, 4 and 8 d after stress treatment was initiated. For (d), both genotypes were exposed to the same water regimen in R5. Parameters were determined 21 d after stress imposition.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1ExperimentalApproach.docx
- FigureS2SDmeanratioamongreplicates.docx
- SupplementaryData1TotalDEGswithoutFDR.xlsx
- SupplementaryData2DEGswithFDR0.1.xlsx
- SupplementaryData3ClusteredDEGs.xlsx
- SupplementaryData4GOenrichment.xlsx
- SupplTable1Totalreadsandvariabilityanalysisamongreplicates.docx
- SupplTable2MUNASQADEGs.docx
- SupplTable3Antioxidantgenes.docx
- SupplTable4AntioxidantenzymesMACEvalidation.docx
- SupplTable5Meteorologicalvariables.docx
- SupplTable6Primers.docx