UNIVERSIDADE FEDERAL DE VIÇOSA

FLAVIANE SILVA COUTINHO

MECANISMOS MOLECULARES DE TOLERÂNCIA AO DÉFICIT HÍDRICO EM SOJA: CARACTERIZAÇÃO DE VIAS METABÓLICAS E REDES REGULATÓRIAS

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Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada, para obtenção do título de *Doctor Scientiae.*

Orientador: Humberto Josué de Oliveira Ramos
Coorientadores: Camilo Elber Vital
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Assentimento:

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RESUMO

COUTINHO, Flaviane Silva, D.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Mecanismos moleculares de tolerância ao déficit hídrico em soja: caracterização de vias metabólicas e redes regulatórias**. Orientador: Humberto Josué de Oliveira Ramos. Coorientadores: Camilo Elber Vital, Elizabeth Pacheco Batista Fontes, Pedro Marcus Pereira Vidigal e Pedro Augusto Braga dos Reis.

A soja [*Glycine max* (L.) Merr.] é uma das mais importantes culturas em todo o mundo, sendo fonte de alimento, energia e recursos industriais. Diante dessa versatilidade, grandes esforços têm sido realizados para aumentar sua produção, bem como tolerância às variações nas condições ambientais. No âmbito mundial, a seca se posiciona no primeiro lugar como desastre natural, restringindo a produção e expansão agrícola. Portanto, o desenvolvimento de genótipos tolerantes visando reduzir o impacto do déficit hídrico na produção de soja é fundamental. No entanto, a realização de tal objetivo é altamente dependente da elucidação dos mecanismos fisiológicos, bioquímicos e moleculares de tolerância à seca e sua interação com alterações ambientais. Mesmo com o advento das tecnologias de alto rendimento, ainda é um desafio abordar de modo integrativo todos os níveis da expressão gênica até o fenótipo. Portanto, a perspectiva da biologia de sistemas é necessária para entender as redes em escala de genoma necessárias para obter cultivares tolerantes à seca. Neste trabalho, apresentamos dados fisiológicos e genômicos funcionais obtidos a partir de análises comparativas de transcriptoma e proteoma entre dois genótipos de soja, BR16 e Embrapa 48, contrastantes para tolerância à seca. As plantas foram avaliadas em condições de plena irrigação (controle) e sob déficit hídrico. As análises fisiológicas mostraram que esses genótipos exibem comportamentos diferenciais em resposta ao déficit hídrico. De fato, o genótipo Embrapa 48 retardou a desidratação foliar, apresentado maiores taxa fotossintética, eficiência no uso da água, carboxilação, taxa aparente de transporte de elétrons e eficiência fotoquímica efetiva do fotossistema II em comparação ao genótipo BR16 sob mesmas condições. Apesar de compartilharem as mesmas categorias funcionais de genes responsivos ao estresse, as análises dos transcriptomas das folhas demonstraram uma menor reprogramação gênica para o genótipo Embrapa 48, sugerindo que a tolerância à seca resulta de um certo nível de transcritos que predispõem a planta mesmo antes do início da seca. A tolerância também pode ser atribuída a genes envolvidos no processo de biossíntese da parede celular em

resposta à seca. Assim, propomos uma hipótese de que as plantas do genótipo tolerante expostas ao déficit hídrico exibem mudanças transcricionais que são resultantes de modificações da parede celular pelo aumento de cadeias laterais dos polímeros pécticos ramnogalacturonano II, formando géis hidratados capazes de limitar o dano às células nas folhas. Houve uma manutenção do crescimento radicular e redução do crescimento relativo da parte aérea no estresse severo, além de maiores níveis radiculares de ácido abscísico. A análise diferencial das proteínas de raízes apresentaram maior abundância de proteínas relacionado a respiração, metabolismo antioxidativo e metabolismo de aminoácidos, sugerindo que o estado da água é mantido nas raízes do genótipo Embrapa 48 por meio do acúmulo de solutos orgânicos, como aminoácidos, que ajudam a manter o fluxo de água do solo para a planta, aumentando o teor relativo de água nas raízes do genótipo tolerante. Concluímos que a triagem de características radiculares e foliares, bem como a identificação de genes, proteínas e metabólitos envolvidos, foram essenciais para se obter uma melhor compreensão do mecanismo de tolerância à seca do genótipo Embrapa 48.

Palavras-chave: Fisiologia vegetal. Transcriptoma. Tolerância à seca.

ABSTRACT

COUTINHO, Flaviane Silva, D.Sc., Universidade Ferderal de Viçosa, February, 2020. **Molecular mechanisms of tolerance to water deficit in soybeans: characterization of metabolic pathways and regulatory networks**. Adviser: Humberto Josué de Oliveira Ramos. Co-advisers: Camilo Elber Vital, Elizabeth Pacheco Batista Fontes, Pedro Marcus Pereira Vidigal and Pedro Augusto Braga dos Reis.

Soybean [*Glycine max* (L.) Merr.] Is one of the most important crops in the world, being a source of food, energy and industrial resources. In view of this versatility, great efforts have been made to increase its production, as well as tolerance to variations in environmental planting conditions. Worldwide, drought ranks first as a natural disaster, restricting agricultural production and expansion. Therefore, the development of tolerant genotypes to reduce the impact of water deficit on soybean production is essential. However, the achievement of this objective is highly dependent on elucidating the physiological, biochemical and molecular mechanisms of drought tolerance and their interaction with environmental changes. Even with the advent of high-performance technologies, it is still a challenge to approach all levels of gene expression up to the phenotype in an integrative way. Therefore, the perspective of systems biology is necessary to understand the genome-scale networks necessary to obtain drought-tolerant cultivars. In this work, we present physiological and functional genomic data obtained from comparative analyzes of transcriptome and proteome between two soybean genotypes, BR16 and Embrapa 48, contrast for drought tolerance. The plants were evaluated under conditions of full irrigation (control) and under water deficit imposed by the suspension of irrigation. Physiological analyzes showed that these genotypes exhibit different behaviors in response to water deficit. The Embrapa 48 genotype delayed leaf dehydration, maintaining photosynthetic rate, apparent electron transport rate and effective photochemical efficiency of photosystem II, even under deficit. In addition, no changes in stomatal conductance or isotopic composition of 13C were observed, suggesting the importance of hydraulic conductivity for tolerance in this genotype. Analysis of leaf transcriptomes reported that genotypes, despite sharing the same functional categories of stress-responsive genes, gene reprogramming occurred to a lesser extent for Embrapa 48. Our analysis suggests that drought tolerance results from a certain level of gene transcription that predisposes the plant even before the drought begins. Tolerance can also be attributed

to genes involved in the cell wall regulation and biosynthesis process in response to drought. Thus, we propose a hypothesis that plants of the tolerant genotype exposed to water deficit exhibit morphological changes that are the result of modifications of the cell wall by increasing side chains of the phamic polymers rhamnogalacturonan II, forming hydrated gels capable of limiting damage to cells in the leaves. There was a maintenance of root growth and a reduction in the relative growth of the aerial part, in addition to higher root ABA levels. Differential analysis of root proteins revealed mechanisms of tolerance to water deficit related to respiration, antioxidative metabolism and metabolism of amino acids. Suggesting that the state of the water is maintained in the roots of the Embrapa 48 genotype through the accumulation of soluble organic solutes, such as amino acids, which help to maintain the flow of water from the soil to the plant, increase the RWC in the roots of the tolerant genotype. We concluded that the screening of root and leaf characteristics, as well as the identification of genes, proteins and metabolites involved, were essential to obtain a better understanding of the drought tolerance mechanism of the Embrapa 48 genotype.

Keywords: Plant physiology. Transcriptome. Drought tolerance.

SUMÁRIO

1.INTRODUÇÃO GERAL

A produção de soja está entre as atividades econômicas que, nas últimas décadas, cresceram mais significativamente devido ao seu múltiplo uso em alimentos e aplicações industriais (HIRAKURI E LAZZAROTTO, 2014; BATTISTI *et al*., 2017). Apesar desse *status* e fato da soja ser uma das leguminosas mais estudadas, sua produção tem sofrido perdas significativas diante da imprevisibilidade das variações climáticas que levam o surgimento de estresses abióticos, com ênfase na seca, que reduz o rendimento médio em mais de 50% (BRAY, 2004; LISAR *et al*., 2012; FITA *et al.,* 2015).

Os eventos iniciais das respostas das plantas ao déficit hídrico se dão pela percepção do sinal de estresse e a subsequente transdução do sinal, que levam à ativação de várias respostas moleculares, bioquímicas e fisiológicas (HADIARTO E TRAN, 2011; MA *et al.,* 2012). As respostas de curto prazo estão relacionadas ao ajuste da condutância estomática, diferenças de potencial hídrico entre os tecidos, condutância hidráulica, conteúdo osmolítico, pressão do turgor e crescimento de órgãos. Respostas a longo prazo estão associadas à duração do ciclo da colheita, aborto de grãos, arquitetura radicular, alocação de nutrientes, ciclos fenológicos de folhas/raiz, mecanismos de tolerância à desidratação celular e senescência tardia. Parte dessas respostas visam economizar água, como fechamento estomático, enquanto outras visam lidar com o baixo nível de água, como osmorregulação (HU E XIONG, 2014; DEMIDCHIK, 2018).

Uma vez que a parte aérea das plantas está exposta ao ambiente atmosférico, perdendo água constantemente para o meio, essa água deve ser reposta com novos suprimentos hídricos, provenientes principalmente do solo. Assim, a absorção, o transporte de água das raízes para a parte aérea, e a transpiração são processos acoplados e inseparáveis do balanço hídrico da planta. O movimento de água das raízes para a folha, via xilema, pode ocorrer devido a uma pressão positiva na base (raiz) ou a uma pressão negativa (tensão) no topo (folha). Tanto a resposta a curto prazo quanto as mudanças de longo prazo requerem uma dinâmica do metabolismo da planta, frequentemente envolvendo mudanças na expressão de genes (CHEN *et al.,* 2016; JANIAK *et al.,* 2018). Espécies reativas de oxigênio (ROS) e sinalização de

Ca^{+2} entram em cena muito cedo e permitem moldar e propagar o sinal em resposta ao déficit hídrico (NIEVES-CORDONES *et al.,* 2019).

Durante exposição à seca, as plantas apresentam lesão celular por meio da geração de ROS, além de alterações em interações proteína-proteína e desnaturação proteica (FAROOQ *et al.,* 2008). O declínio acentuado no volume celular torna-se evidente como um sintoma instantâneo causado pela desidratação, levando a diminuição da área e do peso foliar e da taxa fotossintética, o que limita alguns processos metabólicos (QUAN *et al*., 2016; CHEN *et al.,* 2016). Além disso, sob desidratação prolongada, as plantas podem apresentar murchamento e branqueamento foliar, que eventualmente resultam na sua morte (SAHOO *et al.,* 2013).

O ajustamento osmótico tem sido considerado um dos processos cruciais na adaptação das plantas à seca, por sustentar a atividade metabólica do tecido e permitir a retomada do crescimento quando houver disponibilidade hídrica (CHAVES *et al.,* 2003; BLUM, 2017). Todos estes ajustes metabólicos visam manter um potencial de turgescência alto nas células vegetais, o que pode trazer como consequências a reabertura dos estômatos, a expansão foliar, a transpiração e a fotossíntese por mais tempo. Adicionalmente, estudos evidenciam o envolvimento de metabólitos primários (açúcares, aminoácidos e intermediários do ciclo de Krebs) como marcadores diretos da disfunção fotossintética, bem como efetores de ajustamento osmótico. No entanto, os tipos de metabólitos osmoprotetores e suas relativas contribuições na redução do potencial osmótico diferem muito entre as espécies de plantas (SILVENTE *et al*., 2012).

Nossa equipe de pesquisa vem estudando características de dois genótipos brasileiros de soja contrastantes quanto a tolerância ao déficit hídrico. Os genótipos BR 16 e Embrapa 48, sensível e tolerante, respectivamente, tem sido também estudada por outros pesquisadores. Eles descobriram que, no estádio vegetativo, sob condições de seca no campo, o genótipo tolerante apresenta maior número de vagem (OYA *et al.,* 2004). Ainda, Embrapa 48, apresenta maior conteúdo de clorofila, taxa de crescimento relativo (CARVALHO *et al.,* 2015) e biomassa, assimilação de CO_2, densidade e condutância estomática (SOUSA *et al.,* 2019), em comparação com plantas sensíveis.

Uma caracterização da resposta dos perfis de expressão de proteínas, fosfoproteínas e metabólitos em folhas dos genótipos BR16 e Embrapa 48 foram realizadas por Lima *et al.* (2019). Análise do proteoma indicou que o metabolismo do genótipo tolerante não está comprometido em relação ao sensível, e envolveu ativação de cascatas reguladoras da atividade metabólica e expressão proteica para melhorar os mecanismos de manutenção de água nas folhas. Além disso, o genótipo Embrapa 48 apresentou maior potencial hídrico, no mesmo tempo de progressão do estresse. Dados de metabolômica sugerem que a tolerância parece não ser conferida por osmoproteção proveniente de acúmulo de aminoácidos ou por cascatas dependentes de ácido abscísico (ABA) em folhas (LIMA *et al.* 2019). As plantas do genótipo Embrapa 48 estão mais bem adaptadas à seca, mas relativamente pouco se sabe sobre as bases moleculares para tolerância nesse genótipo.

A análise do transcriptoma oferece uma oportunidade para ter uma visão mais ampla da complexidade da resposta das plantas ao déficit hídrico (NETO *et al.,* 2013). Diante disso, investigar a dinâmica do transcriptoma é fundamental para desvendar os elementos funcionais do genoma, principalmente regulatórios e de resposta funcional, e compreender as variações fenotípicas produzidas por combinações de fatores genotípicos e ambientais.

As novas tecnologias de sequenciamento começaram a ser comercializadas em 2005 e estão evoluindo rapidamente desde então. Esses sequenciadores de nova geração e suas plataformas possibilitaram a utilização de um novo método de sequenciamento de bibliotecas de cDNA, o RNA-seq (HAAS, 2012). Esse método, amplamente utilizado, permite uma avaliação quantitativa e precisa do transcriptoma de maneira eficiente. As grandes vantagens deste método são a ausência quase total de ruídos, o fornecimento de alta cobertura capaz de detectar numerosas cópias de RNA por célula (PINTO *et al.,* 2011; TRIPATHI *et al.,* 2016), aredução dos erros experimentais e a simplicidade no preparo das amostras. Aplica-se para detectar novas transcrições, variações de um único nucleotídeo (SNPs), "*indels*" (pequenas inserções e deleções) e outras mudanças previamente desconhecidas (MOROZOVA E MARRA, 2008).

Diversos trabalhos utilizando análise de transcriptoma têm sido realizados com a finalidade de tentar esclarecer os mecanismos utilizados pelas plantas para se defenderem do déficit hídrico, como os desenvolvidos por Severin *et al.* (2010), que

investigaram a expressão de genes de soja em vários tecidos. Outros estudos investigaram a variabilidade na expressão de genes nos genótipos BR16 e William 82 (GOMES *et al.,* 2015; CHEN *et al.,* 2016). Esses estudos, juntamente com outros envolvendo várias espécies de plantas expostas ao déficit hídrico, apontam para a importância de grupos de genes modulados pelo estresse. Esses genes codificam principalmente proteínas que têm função metabólica ou reguladora, como aquelas envolvidos na desintoxicação, biossíntese de osmólitos, transportadores de íons, proteína HSP (*Heat shock protein*) e proteínas LEA (*Lateembryo genesisabundant*) (VARSHNEY *et al.,* 2009; JANIAK *et al.,* 2018). Várias proteínas cinases e fosfatases (MOLINA *et al.,* 2008; RANJAN E SAWANT, 2015), juntamente com fatores de transcrição (TFs) de várias famílias, incluindo DREB, AP2/ERF, NAC, bZIP, MYB/MYC (SAHOO *et al.,* 2013; REJEB *et al*., 2014) são reguadas com a percepção do estresse.

Genes envolvidos na biossíntese e nas vias de sinalização de hormônios vegetais, como ABA, auxina, etileno, ácido jasmônico ou salicílico, também foram identificados como diferencialmente expressos sob a seca (AIMAR *et al.,* 2011; SAH *et al.,* 2016). Esses hormônios agem sobrepondo vias de transdução de sinal ou alterando perfis de expressão gênica pela rápida indução ou prevenindo a degradação de reguladores transcricionais (ARRAES *et al.,* 2015). O ABA foi identificado como um dos principais sinais químicos envolvidos no funcionamento estomático pela regulação do transporte de longa distância e modulação da concentração de ABA nas células de guarda (CHAVES *et al.,* 2003). No entanto, o aumento da concentração de citocinina na seiva do xilema diminui a sensibilidade estomática ao ABA e promove a abertura estomática, podendo ser um mecanismo de tolerância para manutenção da fotossíntese durante estresse hídrico. Nas pontas das raízes, o acúmulo de ABA aumenta em direção ao ápice da raiz (SAAB *et al*., 1992) e é necessário para a manutenção do alongamento da raiz primária em baixo potencial hídrico (SHARP *et al.,* 2004; YAMAGUCHI E SHARP 2010, YANG *et al.,* 2011). Muitos genes expressos durante as condições de estresse hídrico são dependentes da presença de ABA, outros, porém, não respondem à aplicação de ABA exógeno. Isso sugere a existência de vias de transdução de sinal ABA-dependente e ABA-independente que convertem o sinal inicial de estresse em respostas celulares, podendo ocorrer um cruzamento entre os sinais das respostas (*cross-talk*) a diferentes tipos de estresses nestas diferentes redes sinalizadoras (XIONG *et al.,* 2002).

Embora muitos genes, proteínas e metabólitos estejam associados a resposta da planta ao estresse e utilizados para gerar plantas tolerantes, o sucesso da estratégia para entender o mecanismo de tolerância torna-se limitado devido ao conhecimento fragmentado dos processos fisiológicos, bioquímicos e moleculares de órgãos individualizados na planta (REGUERA *et al.,* 2012). Poucos estudos envolvendo a integração desses mecanismos foram publicados, de forma a confirmar o fenótipo e descrever os mecanismos envolvidos na tolerância.

Vincular sistema foliar e radicular, bem como fenótipo da planta aos padrões de expressão gênica e proteica e acúmulo de metabólitos é um dos principais desafios, mediante a grande variedade de vias ativadas no estresse hídrico (KOMATSU *et al.,* 2013; CHAUDHARY *et al.,* 2015). No entanto, o estudo das respostas de plantas de maneira sistemática possibilita a construção de redes ou modelos que fornecerão detalhes de várias respostas das plantas a um ambiente dinâmico, permitindo projetar a melhor estratégia de engenharia para o desenvolvimento de genótipos tolerantes à seca. A biologia de sistemas, que foi revolucionada pelo sequenciamento do genoma, tecnologias e conhecimentos teóricos, nos permite obter uma visão holística de todos os processos regulatórios e reações de um organismo em resposta a perturbações ambientais, estudando os papéis de diferentes componentes moleculares (genes, transcritos, proteínas e metabólitos) e suas complexas interações em resposta a estímulos ambientais (FUKUSHIMA *et al.,* 2009; WECKWERTH, 2011).

Visto isso, o presente trabalho objetivou obter dados utilizando parâmetros fisiológicos e tecnologias ômicas de folhas e raízes de soja, e integra-los funcionalmente, de modo a fornecer informações e proposições sobre os mecanismos de respostas envolvidos no déficit hídrico, e identificar determinantes moleculares que controlam a tolerância à seca no genótipo Embrapa 48. Para atingir o objetivo várias abordagens experimentais diferentes, porém complementares, foram realizadas e, portanto, esta tese está organizada como uma compilação de três capítulos, apresentados na forma de artigo científico. Cada capítulo é composto por uma introdução, resultados e discussão, bem como detalhes dos métodos utilizados. No final desta tese, as principais conclusões do trabalho e uma breve integração dos mecanismos ativados em resposta ao estresse hídrico no genótipo Embrapa 48 são apresentados.

Layout e objetivos dos capítulos

2.CAPÍTULO I – Abordagem fisiológica para decifrar a tolerância à seca de genótipo de soja da Savana Brasileira

O déficit hídrico é um dos fatores limitantes à obtenção da máxima produtividade nos cultivares de soja. Compreender como as plantas respondem ao déficit hídrico e identificar os mecanismos de tolerância à seca são fundamentais para predizer os impactos na produção, além de serem cruciais no desenvolvimento de cultivares mais tolerantes. Neste capítulo, avaliamos características fisiológicas dos genótipos BR16 e Embrapa 48, sob déficit hídrico e reidratação do solo, como: (i) parâmetros de crescimento radicular e foliar, (ii) parâmetros fotossintéticos, (iii) composição de 13C nas folhas, (iv) peroxidação lipídica de folhas e raízes, e (v) atividade enzimática antioxidante em folha. O genótipo Embrapa 48 apresentou os melhores desempenhos nos testes fotossintéticos e menores danos oxidativos nas folhas submetidas ao estresse. Além disso, apresentou maior redução no crescimento relativo da parte aérea, juntamente com uma maior indução do crescimento do sistema radicular sob seca, sem alterar a área foliar. Coletivamente, nossos resultados indicam que o genótipo tolerante possui um mecanismo diferencial de alocação de carbono para as raízes, que evita uma redução na fotossíntese, mas reduz a altura da planta.

3.CAPÍTULO II - Remodelamento da parede celular como mecanismo de tolerância à seca de um genótipo de soja revelado por análise global de expressão gênica

A parede celular é de extrema importância para o formato da célula e fornece resistência mecânica para suportar a pressão do turgor. A dinâmica da parede celular não é fácil de analisar e, como consequência, a maioria dos estudos sobre modificações da parede celular sob estresse abiótico se concentra principalmente em genes envolvidos no metabolismo da parede celular. Neste capítulo, avaliamos a expressão gênica em folhas de dois genótipos de soja, BR16 e Embrapa 48, submetidos a estresse hídrico moderado utilizando sequenciamento de última

geração, RNA-Seq. Os resultados revelaram que, em resposta ao estresse, o genótipo tolerante, Embrapa 48, apresenta menor reprogramação gênica, além de expressar proteínas cinases e fatores de transcrição que indicam serem moléculas sinal que atuam para melhorar a tolerância à seca na soja. Surpreendentemente, Embrapa 48 superexpressa genes que codificam proteínas do metabolismo carboidratos como ramnogalacturonano, xilosiltransferases e expansinas, responsáveis pelo remodelamento da parede celular. Coletivamente, nossos resultados indicam a capacidade de remodelar a parede celular, permitindo a manutenção da turgescência e a continuidade na utilização de assimilados, seja o mecanismo correlacionado com a tolerância à seca nessas plantas.

4.CAPÍTULO III - Compreendendo as respostas ao estresse hídrico em soja - Uma perspectiva de Biologia de Sistemas

O desenvolvimento de um entendimento abrangente da resposta à seca exige uma visão global dos complexos mecanismos envolvidos. Pesquisas sobre tolerância à seca geralmente são realizadas usando abordagens específicas. No entanto, a resposta ao estresse da planta é complexa e está interligada a um nível em que abordagens específicas não fornecem uma análise global completa de todos os mecanismos interligados. A perspectiva da biologia de sistemas é necessária para entender as redes comumente reguladas após a exposição a seca. Neste capítulo, adotamos análise de proteoma e fisiologia de raiz, juntamente a uma abordagem integrativa dos resultados de fisiologia, transcriptoma, proteoma e metaboloma em resposta à seca no genótipo Embrapa 48, considerando a conexão raiz e parte aérea, como meios para identificar intermediários importantes na tolerância ao estresse e como uma ferramenta para rastrear variações na plantas. Tomados em conjunto, os resultados revelaram uma maior capacidade de absorção de água pela raiz no genótipo Embrapa 48, supostamente por osmoproteção através do acúmulo de aminoácidos. Este evento aumenta a pressão de turgor, ajudando na expansão radicular e otimizando o uso e transporte de água para a parte aérea. O mecanismo de osmoproteção foliar parece estar relacionado à expansão da pectina, por biossíntese de ramnogalacturonano II. Em conclusão, observamos que as abordagens baseadas em biologia de sistemas ajudaram a entender como esses fatores e

mecanismos individuais (bioquímicos, moleculares e metabólicos) "interagem" no genótipo Embrapa 48 para garantir sua tolerância à seca.

CHAPTER I

Physiological Approach to Decipher the Drought Tolerance of a Soybean Genotype from Brazilian Savana

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Physiological Response Approaches to Decipher the Drought Tolerance of a Soybean Genotype from Brazilian Savana

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Abstract

Drought is one of the major constraints for soybean production in Brazil. In this study we investigated the physiological traits of two soybean parental genotypes under progressive soil drying and rewetting. The plants were evaluated under full irrigation (control) conditions and under water deficit imposed by suspending irrigation until the plants reached predawn leaf water potentials (Ψ_{PD}) of -1.0 MPa (moderate) and -1.5 MPa (severe). Physiological analyses showed that these genotypes exhibit different responses to water deficit. The Embrapa 48 genotype reached moderate and severe water potential two days after the BR16 genotype and was able to maintain higher levels of *A*, ETR and ΦPSII even under deficit conditions. This result was not related to changes in *g*s, ^{13}C isotopic composition and presence of a more efficient antioxidant system. In addition, *Fv/Fm* values did not decrease in Embrapa 48 genotype in relation to irrigated condition showing that stress is not causing photochemical inhibition of photosynthesis. The greater reduction in the relative growth of the shoots, with concomitant greater growth of the root system under drought, indicates that the tolerant genotype is able to preferentially allocated carbon to the roots, presenting less damage in photosynthesis. Therefore, the physiological responses revealed that the tolerant genotype postpone the leaf dehydration by a mechanism involving a more efficient use and translocation of water from root to the shoot to maintain the cell homeostasis and the photosynthetic metabolism under the stress.

Keywords: Plant breeding; plant physiology; tolerance mechanism; plant growth; water-use efficiency.

Abbreviations: Water potential **(Ψ_{PD});** assimilation rate of CO_2 **(A);** electron transport rate **(ETR),** photochemical efficiency of photosystem II **(ΦPSII);** stomatal conductance to water vapor **(gs);** potential photochemical efficiency **(Fv/Fm).**

1.Introduction

Drought impairs normal growth, disturbs water relations and affects water-use efficiency and plants respond to this stress through a variety of physiological and biochemical responses at both the cellular and organism levels. Water stress can lead to increased allocation of photosynthate to the roots, increasing the root/shoot ratio and facilitating water and nutrient absorption (Huck et al. 1983; Kunert et al. 2016; Kumar et al. 2018). In soybean, significant correlations have been found between drought tolerance and root system characteristics such as dry weight, total length, volume and number of lateral roots (Liu et al. 2005; Manavalan et al. 2009; Fried et al. 2018).

In this context, the root system is of crucial importance in the provision of water and nutrients for the shoot and, in many circumstances, reductions in shoot growth can be explained by the inability of the root system to meet these needs (Dood, 2005). For these reasons a number of studies have aimed to understand the relationship between roots and shoots in terms of signaling and responses to stress (Christmann et al. 2007; Neumann et al. 2007; Thompson et al. 2007; Vishwakarma et al. 2017; Kumar et al. 2018). Additionally, plants are able to detect soil water deficiency independent of changes in the water status of the aerial part by transferring chemical signals from root to shoot (Liu et al. 2005; Schachtman and Goodger, 2008), further reinforcing the existence of close communication between both organs.

Water stress is characterized by reductions in water content and leaf water potential and loss of cellular turgor, which decreases stomatal conductance and cellular expansion, consequently constraining plant growth (Jaleel et al. 2009). Thus, occurrence of water deficit during vegetative growth can reduce the leaf area indices and consequentially the crop growth rate and yield (Raper and Kramer, 1987). Reduction of net photosynthesis in soybean plants can be induced by both stomatal and non-stomatal factors, such as problems in electron transport and photophosphorylation (Flexas et al. 2006). Depending on the intensity and duration of drought stress, metabolic limitations can affect the electron transport and photophosphorylation (Catuchi et al. 2011). Metabolic limitations are frequently correlated with decreases in ATP, which reduces the capacity for ribulose biphosphate (RuBP) turnover (Parry et al. 2002). The mechanisms involved in drought tolerance encompass innumerable processes, such as signaling and stress perception, as well

as gene activation and metabolism alterations. Thus, understanding the mechanisms at work in drought tolerant genotypes is of great importance (Chinnusamy et al. 2005; Manavalan et al. 2009; Carvalho et al. 2015), particularly when considering genotypes that are able to maintain the growth and yield potential related to parental lines.

Considering the great economic importance of soybean and the losses caused by drought the breeding of the drought tolerant of the soybean genotypes that are able to grow in environments with limited supplied of water is an important goal. Different soybean genotypes have been reported to exhibit extensive variation in drought tolerance and these genetic resources have been used to develop new genotypes by genetic crosses that combine agronomic and tolerance characteristics (Oya et al. 2004; Ku et al. 2013; Fang and Xiong, 2015). The genotypes BR 16 and Embrapa 48 which share a common ancestor were studied by Oya et al. (2004) under drought conditions. They found that at the vegetative stage the tolerant genotype (Embrapa 48) showed higher growth rate compared to sensitive BR16 plants. Due to the superior performance of Embrapa 48 under water deficit, the drought tolerance mechanism has been evaluated at the molecular and genetic levels (Rodrigues et al. 2012; Lima et al. 2019; Coutinho et al. 2020). However, physiological traits essential for drought tolerance elucidation, such as leaf expansion rate, gas exchange, water relations, total chlorophyll content, lipid peroxidation and root growth have not been investigated for the soybean parental genotypes (Embrapa 48 and BR 16) under progressive soil drying followed by rehydration. Therefore, in this study the physiological responses indicated that the tolerant genotype postponing the leaf dehydration by a mechanism involving a more efficient use and translocation of water from root to the shoot, in accordance with gene expression and metabolomic data (Lima et al. 2019), to maintain cell homeostasis and the photosynthetic metabolism unchanged under the stress condition in contrast to the sensitive genotype.

2. Materials and methods

2.1. Plant material and drought stress treatments

Seeds of soybean genotypes BR 16 and Embrapa 48 were obtained from the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA SOJA, Londrina, Paraná). Seedlings were grown in plastic trays containing Plantmax® commercial substrate,

where they remained for 10 days. After germination, seedlings were transplanted to pots containing 10 L of a mixture of soil, sand and manure (2:1:1) each. Plants were grown under natural sunlight in a greenhouse with average daytime temperature 15–35°C and relative humidity 65–85%.

Soybean plants were watered daily with the same volume (30 mL per plant) until the fourth trifoliate leaves emerged, after which water restriction treatment was imposed. Control plants for each genotype received regular watering. Treatment plants were submitted to water deficit, imposed by the suspension of irrigation, until they reached predawn leaf water potentials (Ψ_{PD}) of –1.0 ± 0.1 MPa and -1.5 ± 0.1 MPa, considered conditions of moderate and severe stress for soybean plants, respectively. Once these conditions had been reached individual leaves were removed, frozen in liquid nitrogen and stored at -80ºC. In addition to these treatments, a further group of plants was rehydrated following severe deficit by regular watering and evaluated after four days of rehydration in order to analyse recovery responses. A Scholander pressure pump was used to measure leaf water potential and define the different treatments (Scholander et al. 1965).

2.2. Evaluating the plant growth parameters

Four growth parameters were evaluated: root length and volume (VR), leaf area per plant (LA), number of leaves per plant (NL) and relative growth (RW). A) VR: The plant roots from each genotype under drought stress conditions were collected after each treatment and washed following the moisture was removed before measurement of root length and volume. B) LA: In order to measure leaf area per plant, ten fresh leaves were taken at random from each control and treatment group, and the thickness of the middle portion of each leaf was measured with a micrometer. One square inch area was carefully marked and cut with a scalpel. This area was weighed and the total plant leaf area was calculated by multiplying the fresh weight of one square inch leaf by the total fresh weight. The leaf area per plant was calculated by dividing the total leaf area by the total number of plants. C) NL: Number of leaves per plant was counted manually. D) RW: Relative growth was calculated as a function of the height variation (the final height minus the initial height) and the time interval (days) during water deficit period.

2.3. Measurement of chloroplastic pigments, gas exchange and chlorophyll fluorescence

Pigment contents (chlorophyll a, chlorophyll b, chlorophyll total and carotenoid) were determined using extraction with dimethylsulfoxide (DMSO) saturated with calcium carbonate ($CaCO_3$) as described by Wellburn (1994).

The assimilation rate of CO_2 (*A*), stomatal conductance to water vapor (*g*s), transpiratory rate (*E*) and internal and external carbon ratio (*Ci/Ca* ratio) were determined for the fourth leaf from the apical meristem of each plant (IRGA, portable model LI-6400XT, LI-COR Biosciences Inc., Lincon, Nebraska, USA). Measurement was performed from 8h-11h a.m. with a photosynthetic flux density (PPFD) of 1000 μmol m^{-2} s^{-1}. The air humidity in the leaf chamber was around 50%, with CO_2 concentration of 350–400 μmol mol^{-1} and ambient air temperature 28 ± 2 °C. Instantaneous water use efficiency (WUEi) was calculated as *A/E* and carboxylation efficiency as *A/Ci*.

Chlorophyll fluorescence was measured using a Fluorometer coupled to the LI-6400XT (IRGA). After leaves were dark-acclimated by enclosing them a leaf clip for 30 min, the initial fluorescence (*Fo*) was estimated with weak modulated light (<0.1 μmol photons m^{-2} s^{-1}), and then leaves was immediately illuminated with an intense saturating flash (>6000 μmol photons m^{-2} s^{-1}) to obtain the maximum fluorescence (Fm). Leaves were then immediately exposed to actinic irradiation for 30 min to measure steady state Chl a fluorescence (Fs) and saturating pulses (>6000 μmol photons m^{-2} s^{-1}) were applied to determine the maximum fluorescence in the light-adapted state (*Fm*') following each actinic irradiation. Finally, leaves were illuminated with far-red radiation to determine the minimal fluorescence during the light-adapted state (*Fo'*). Other parameters were calculated as follows: PSII maximum photochemical efficiency—*Fv/Fm* = (*Fm-Fo*)/*Fm*; excitation energy capture efficiency of PSII reaction centres—*Fv'/Fm'* = (*Fm'- Fo*')/*Fm'*; the quantum yield of PSII— ΦPSII = (*Fm'- Fs)/Fm'*; photochemical quenching—*qP* = (*Fm'—Fs*) /(*Fm'- Fo'*); non-photochemical quenching—NPQ = (*Fm—Fm'*)/*Fm*'; the apparent photosynthetic electron transport rate (ETR) was calculated as PPFD×0.84×0.5×ΦPSII (Maxwell and Johnson, 2000; Baker, 2007).

2.4. Leaf carbon isotope ratio ($\delta^{13}C$)

The leaf samples were dried for 48 h at 70°C then ground with mortar and pestle to a fine powder before analysis for $\delta^{13}C$ on a mass spectrometer (ANCA GSL 20-20, Sercon, Crewe, UK) at the Stable Isotope Laboratory (LIE) of the Department of Soils (DPS) of the Federal University of Viçosa.

$\delta^{13}C$ values are expressed in parts per thousand differences from the international standard Vienna-Pee Dee belemnite using the equation (Bernoux et al. 1998):

$$\delta^{13}C=[(^{13}C/^{12}C_{sample} - {}^{13}C/^{12}C_{standard})/(\ {}^{13}C/^{12}C_{standard})].1000$$

2.5. Determination of the lipid peroxidation and enzymatic activities

Five leaf disks and 0,15g the roots were crushed in liquid nitrogen and homogenized in 2.0 mL of trichloroacetic acid (TCA) 0.1% (m/v), followed by filtering through 4 layers of gauze and centrifugation at 10,000x*g* for 15 min at 4 °C. For the reaction, 0.5 mL of the supernatant was added to 1.5 mL of thiobarbituric acid (TBA) 0.5% (w/v) in TCA 20% (w/v). The tubes were closed and incubated in a water bath at 95 °C for 30 min. The reaction was stopped on ice for 1 min and samples were centrifuged at 9.000 x*g* for 4 min at 25 °C. Absorbance was measured at 600 and 440 nm. The concentration of the malonic aldehyde-TBA complex was obtained through subtraction of the absorbance and the use of the molar absorption coefficient of 155 $mM^{-1}\cdot cm^{-1}$ (Hodges et al. 1999), and the results were expressed as $mmol\cdot g^{-1}$ fresh weight.

For the enzymatic assays, 0.3 g leaf sample was ground with a mortar and pestle, homogenized in liquid nitrogen and solubilized in 2.0 mL of a 100 mM potassium phosphate buffer (pH 6.8), containing 0.1 mM EDTA-Na_2, 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (w/v) polyvinylpyrrolidine-40 (PVP-40). The homogenate was filtered and centrifuged at 12,000x*g* for 20 min at 4 °C. The peroxidase (POX), catalase (CAT) and ascorbate peroxidase (APX) activities were determined according to the methods of Chance and Maehly (1995), Havir and Mchale (1987) and Nakano and Asada (1981), respectively.

2.6. Hydrogen peroxide production by DAB staining

Hydrogen peroxide (H_2O_2) production in plants was evaluated using 3,3-diaminobenzidine reagent (DAB-Sigma) according to Weigel and Glazebrook (2002). The leaves were collected and immediately submerged in 10 mM potassium phosphate (K_2HPO_4) solution containing 1 $mg.mL^{-1}$ DAB, pH 3.8, and kept in this solution for 8 hours under constant illumination. The leaves were then boiled in ethanol for 10 min to remove the dye. Brown precipitates were observed in the leaves, indicating the presence of an H_2O_2 burst.

2.7. Statistical analysis

The experimental design was completely randomized, in a factorial arrangement 2x4, with the first factor corresponding to the plant water potential and the second constituted by the two different genotypes, with five repetitions. Data were submitted to analysis of variance (ANOVA), and a Tukey averages test at 5% probability using the Assistat® software.

3. Results

3.1. Effect of drought stress on water relation parameters leaf and on root growth traits

For the control plants of both soybean genotypes, the predawn water potential (Ψ_{PD}) was always greater than -0.25 MPa, and the tolerant genotype had lower potentials throughout the experimental period (Fig.1). Following suspension of irrigation, from the second day onwards, we observed a progressive time-dependent reduction in Ψ_{PD} that occurred at a greater rate in sensitive genotype (BR 16) (Fig.1).

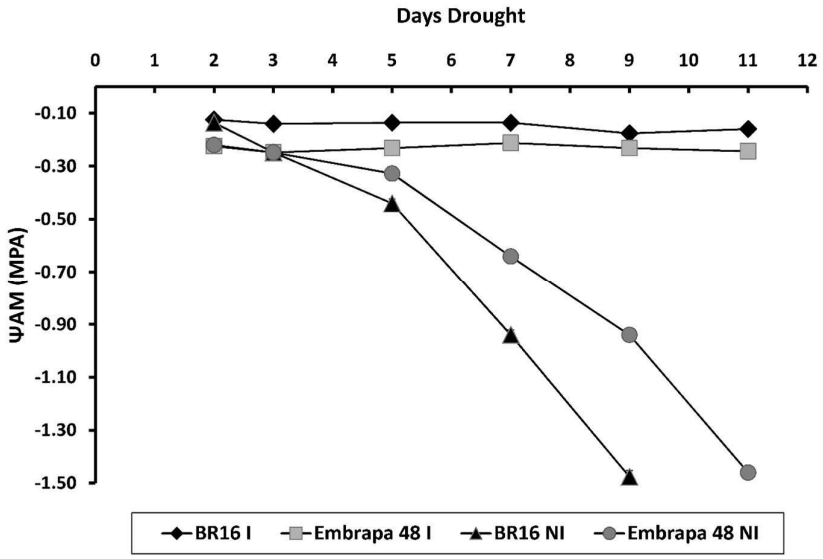

Figure 1. Temporal profile of leaf water potential in the morning (Ψ_{PD}) in two soybean genotypes, one sensitive (BR 16), and another tolerant (Embrapa 48) in relation to the water deficit. Each point represents the mean + standard error (n = 5, where n represents the number of plants), I = irrigated, NI = non-irrigated (not irrigated after imposition of stress).

BR 16 plants reached Ψ_{PD} around -1.0 and -1.5 MPa, on the seventh and ninth day after the suspension of the irrigation, respectively. In contrast, Embrapa 48 plants required more time to reach the same levels of Ψ_{PD}, around nine and eleven days, respectively (Fig.1). These results are consistent with a better economy of water in the Embrapa 48 genotype. Importantly, the plants that were reirrigated after reaching the point of severe stress (Ψ_{PD} -1.5 MPa) were able to recover their initial water status, reaching Ψ_{PD} of -0.16 MPa in the sensitive genotype and of -0.26 MPa in the tolerant genotype. Figure 2 shows plants of the two genotypes during the experiment. BR 16 plants (Fig. 2A) presented greater leaf wilt in the moderate (-1.0 MPa) and severe (-1.5 MPa) deficits when compared to the Embrapa 48 plants at the same water deficit levels (Fig. 2C). The roots, under severe water deficit, were noticeably more voluminous in the tolerant genotype (Fig. 2D) than in the sensitive genotype (Fig. 2B). Root volume analysis revealed a lower root volume in both genotypes under water deficit, with the tolerant genotype exhibiting greater root volume under both the moderate and severe treatments. Under the stress of Ψ_{PD} -1.0 plants of the BR16 genotypes presented a reduction of the root volume of approximately 17%, which increased for 22% when the plants reached Ψ_{PD} -1.5. In contrast, the plants of the Embrapa 48 genotype had a reduction of 6% and 11% under Ψ_{PD} -1.0 and -1.5, respectively (Fig. 2E).

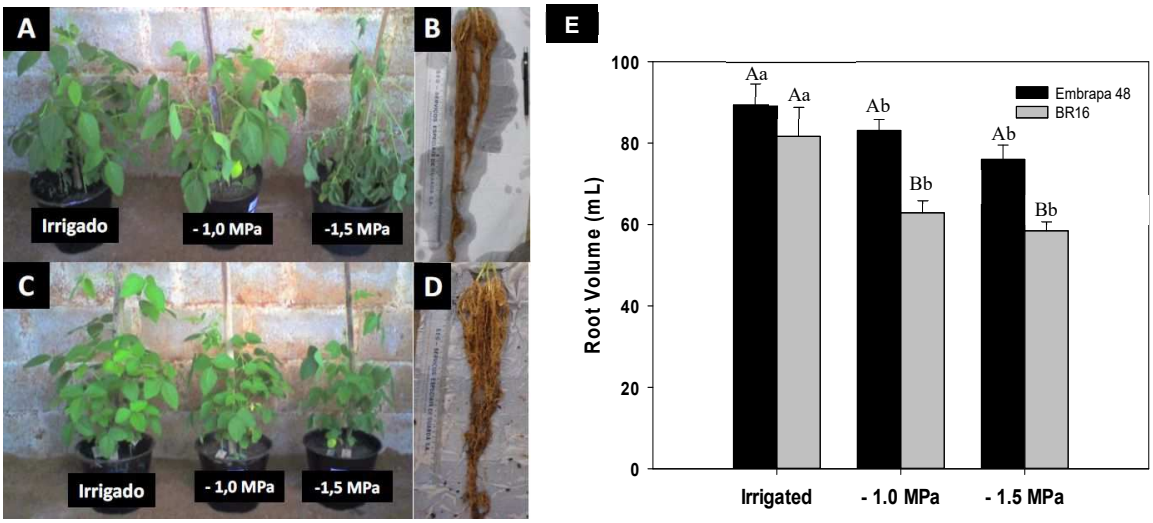

Figure 2. Overview of the plants at the end of the experiment. (A) Plants of genotype BR16 (sensitive) in the three levels of irrigation; (B) Root system of genotype BR16 under severe deficit; (C) Plants of the Embrapa 48 genotype at the three levels of irrigation; and (D) Root system of the genotype Embrapa48 (tolerant) under severe deficit. (E) Root volume in soybean genotypes. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants). The different groups correspond to the treatments. Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype, (Tukey, $p < 0.05$). The data represent the mean ± standard error (n = 5).

3.2. Effect of drought stress on the growth rates of soybean genotypes

There was no difference in leaf area between the soybean genotypes under water deficit. However, in both genotypes this parameter was lower in plants exposed to water deficit (Supplementary material Fig. S1A). The number of leaves (NF) was greater in the Embrapa 48 genotype compared to BR 16 in all treatments, and no differences in NF were detected was a result of water deficit (Supplementary material Fig. S1B). However, the leaves of the sensitive genotype were greater in size, so that, even though they had a smaller number of leaves, the two genotypes did not differ, under any treatment, with regards to their total leaf area. Water stress produced a dramatic inhibition of plant growth (height) in both genotypes, around 50%. However only under severe water stress did we measure a greater inhibition of growth in the tolerant genotype (Fig. 3). This result is due to the larger size (height) of the sensitive genotype and indicates a differential allocation of carbon between genotype under

severe stress, with the tolerant genotype exhibiting lower height and higher root volume.

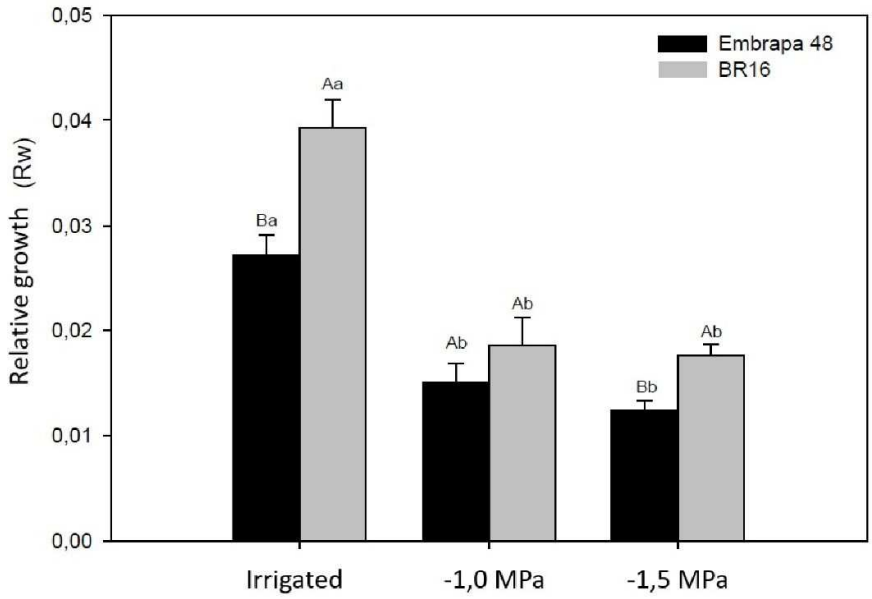

Figure 3. Relative growth (Rw) in soybean genotypes under water deficit. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants). The relative growth was calculated according to the formula Rw = (lnh1-lnh0) / t1-t0, where h1 corresponds to the final height at the time of collection and h0 corresponds to the initial height at the moment of imposition of the deficit, t1 corresponds to the age of the plant in days until the time of collection and t0 is the age in days at the time of imposition of the deficit. Each bar represents the mean ± standard error (n = 5), where n represents the number of plants). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments. The data represent the mean ± standard error (n = 5).

3.3. Effect of drought stress on levels of chlorophyll and leaf gas exchange

Regarding the pigment contents, there were lower levels of chlorophyll a in the tolerant genotype throughout treatments, showing values of approx. 15% any less of BR16 genotype in moderate stress. Furthermore, was detected a variation of approx. 30% any less of how BR16 genotype in severe deficit. For chlorophyll a, the levels increased approx. 20% with the imposition of deficit in the sensitive genotype (Fig. 4A). We also observed approx. 32% of reduction for the chlorophyll b in the tolerant genotype under severe deficit, while this decrease approx. 16% was only detected in the sensitive genotype under moderate deficit (Fig. 4B). Under severe stress, this genotype showed an increase of approx. 28% of chlorophyll b compared to the amounts during moderate stress (Fig. 4B). Total chlorophyll content followed the same

pattern as chlorophyll a (Fig. 4C). Levels of carotenoids increased 20% in the sensitive genotype with the imposition of severe stress, while this variation was observed in the tolerant genotype with the imposition only of moderate stress. In contrast, in severe stress, the Embrapa 48 genotype showed a reduction in carotenoids of approx. 30%, when compared to amounts in moderate stress (Fig. 4D).

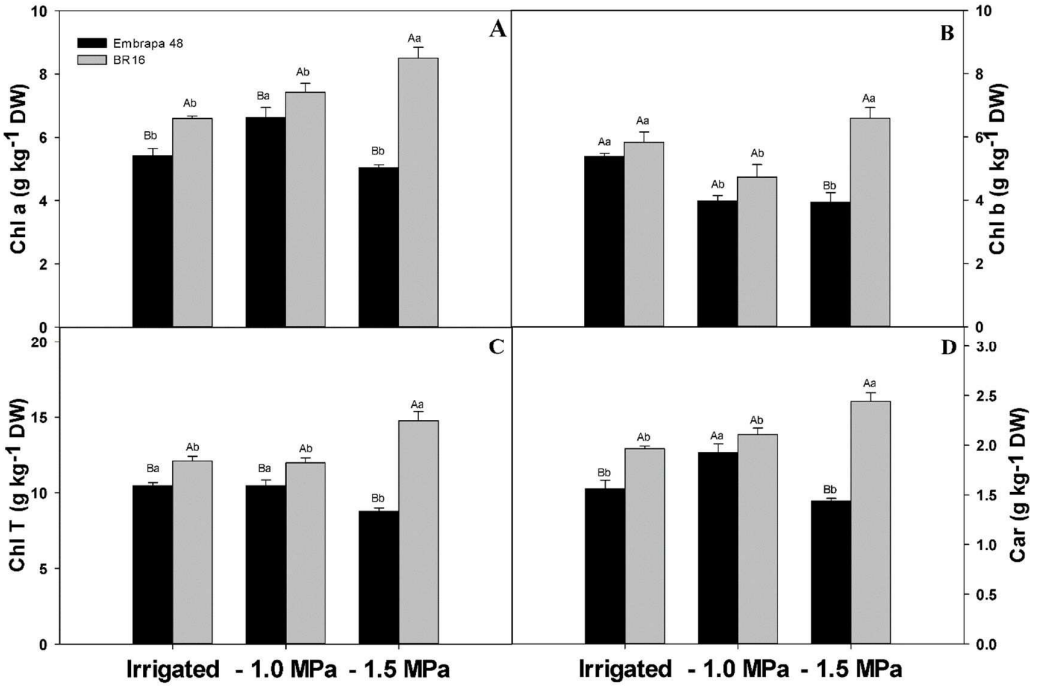

Figure 4. Pigment contents in soybean genotypes. Chlorophyll a (A), chlorophyll b (B), total chlorophyll (C) and carotenoids (D). Each bar represents the mean ± standard error (n = 5, where n represents the number of plants) (Tukey, p <0.05). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments. The data represent the mean ± standard error (n = 5).

The net photosynthetic rate (*A*) was 28% higher in the Embrapa 48 genotype than in the BR 16 genotype under control conditions (24 and 18 μmol CO_2 m^{-2} s^{-1} respectively). Values of *A* decreased 77% and 80% for BR16 and Embrapa 48 genotypes under moderate deficit. Embrapa 48 maintained net photosynthetic rate than BR 16. Interestingly, the resumption of irrigation allowed an almost total recovery in the photosynthetic rate of both genotypes, indicating that the negative effects of the severe deficit on photosynthesis were reversable (Fig. 5A). The differences between the genotypes in stomatal conductance (*g*s) were more pronounced in the irrigated and

reirrigated plants, with higher values in Embrapa 48 (Fig. 5B). Under irrigated conditions Embapa 48 presents 25% more *gs* than BR16, when reirrigated this variation increases to 31% more *gs* in the Embrapa 48 genotype compared to BR16. Under moderate stress, although photosynthesis was higher in the Embrapa 48 genotype, no difference was detected in *g*s. The ratio between internal and external CO_2 (*Ci/Ca*) concentrations was, however, lower in genotype Embrapa 48 under stress (Fig. 5D). In the severe stress treatment there was no difference in *g*s, however the tolerant genotype presented lower *Ci/Ca* (Fig.5B and 5D). The transpiration rate (*E*) was reduced approx. 80% on in both genotypes when water deficit was imposed (Fig. 5C). In the irrigated condition there was no difference between the genotypes, however, in the reirrigated plants E was 26% higher in the Embrapa 48 genotype (Fig. 5C).The proportional decrease in *E* compared to *A* under severe water stress was greater in Embrapa 48 plants, contributing to a greater instantaneous water use efficiency (*A/E*) under severe stress (Fig. 5E).

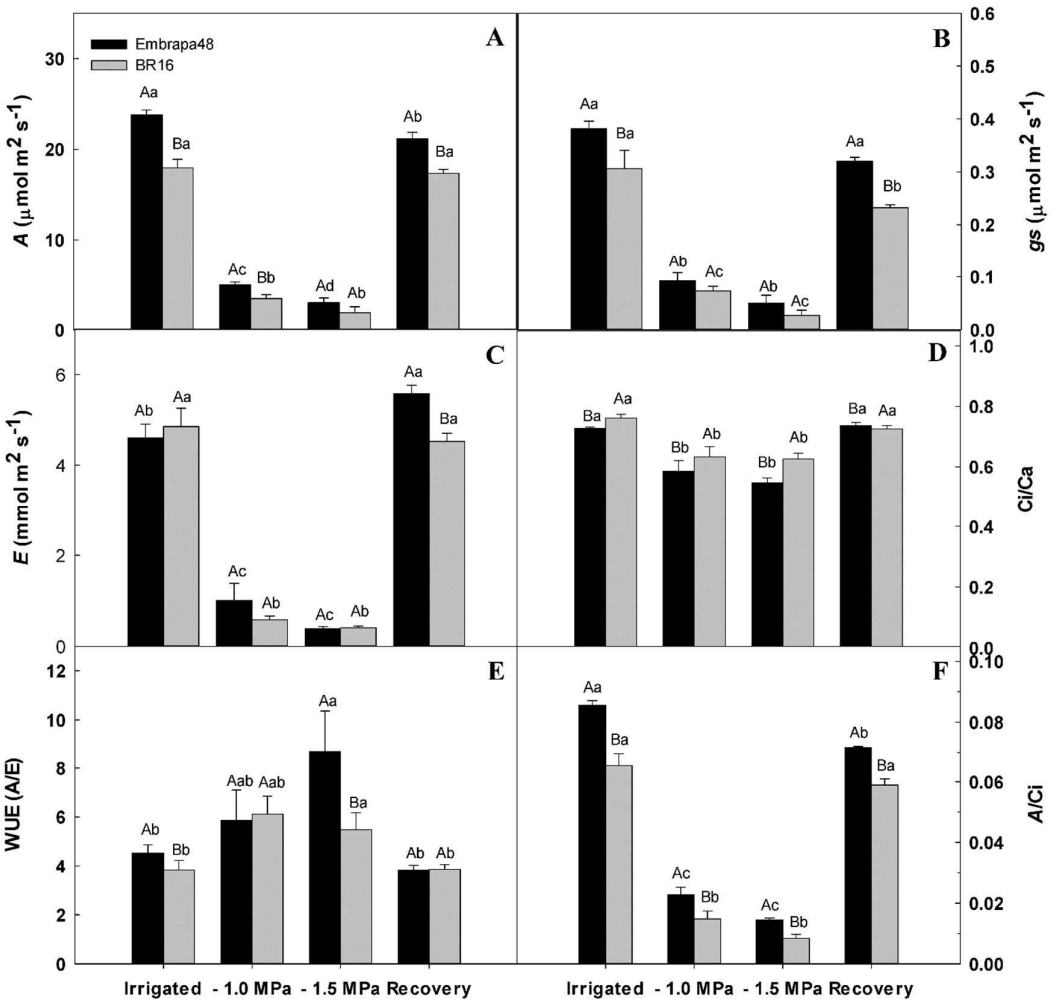

Figure 5. Effect of water deficit on the net (A) Photosynthetic rate, (B) stomatal conductance, (C) transpiratory rate, (D) ratio *Ci /Ca*, (E) water use efficiency and (F) *A/Ci* ratio in soybean genotypes. The different groups correspond to the treatments. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants) (Tukey, p <05). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments. The data represent the mean ± standard error (n = 5).

In all treatments, the *A/Ci* ratio (carboxylation efficiency) was higher in Embrapa 48 (Fig. 5F). Severe deficit led to reduction the 83% and 77% in genotypes BR16 and Embrapa 48, respectively. After rehydration, in BR 16 recovery being complete (Fig. 5F), while the Embrapa 48 genotype showed an 11% reduction when compared to control plants. This result suggests a greater carboxylation efficiency in the tolerant genotype, associated with its greater photosynthetic capacity (Fig. 5A). In the presence of water stress, the electron transport rate (ETR) reduced as stress increased in both genotypes (Fig. 6), reaching a loss of 66% in the BR16 genotype and 52% in Embrapa 48. Embrapa 48 presented a higher ETR in all treatments, including a balance greater

than BR16 of 17% following reirrigation (Fig. 6). Despite the decrease in *A* due resulting from water deficit, there was no photoinhibition under moderate stress for either genotype, as the potential photochemical efficiency (*Fv/Fm* ratio) remained unchanged (Fig. 7). Under severe stress, a reduction the 12,5% in *Fv/Fm* in BR 16 genotype was observed, indicating possible photoinhibitory damage in this cultivar (Fig. 7).

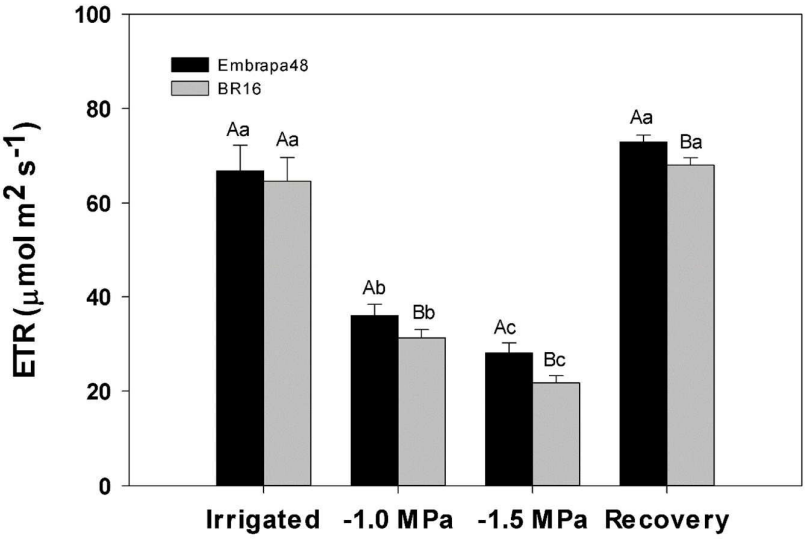

Figure 6. Effect of water deficit on the rate of electron transport in soybean genotypes. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants) (Tukey, p <0.05). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments. The data represent the mean ± standard error (n = 5).

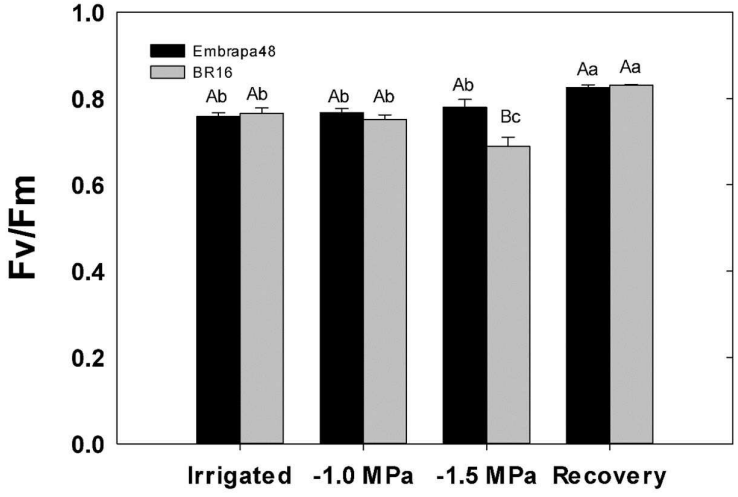

Figure 7. Effect of water deficit on the Fv/Fm ratio in soybean genotypes. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants) (Tukey, p <0.05). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments. The data represent the mean ± standard error (n = 5).

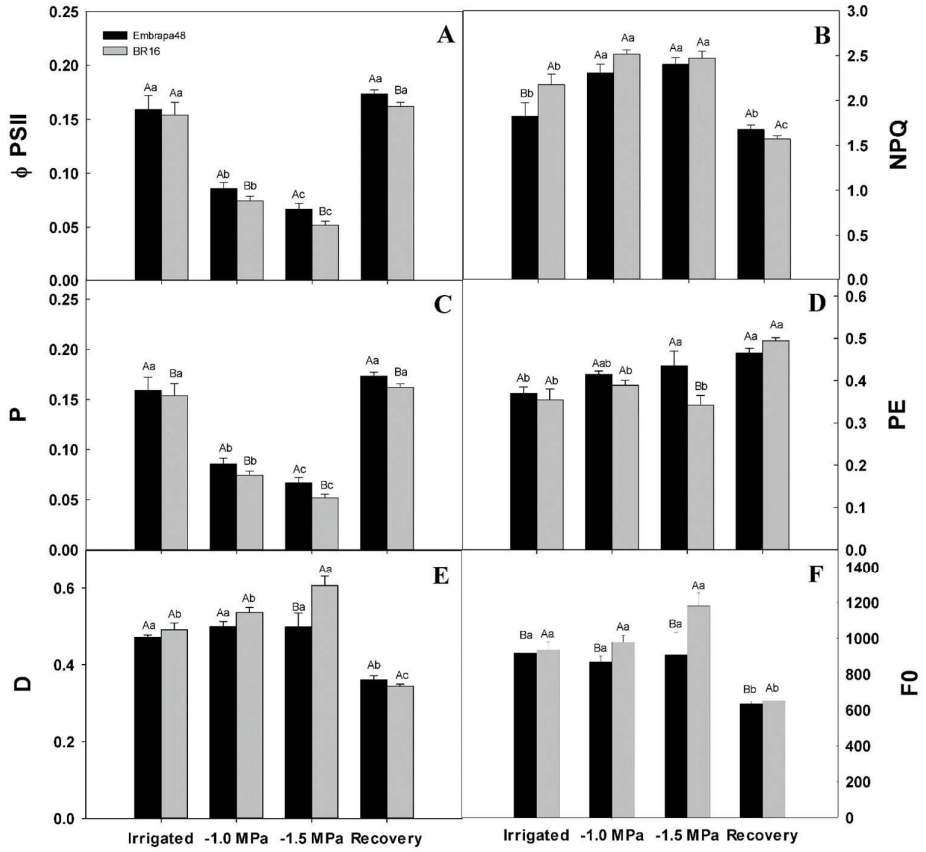

Figure 8. Effect of water deficit on ΦPSII- effective quantum yield of PSII (A), NPQ- non-photochemical extinction coefficient (B), P- absorbed light fraction that is used in the photochemical phase of PSII (C), PE- fraction absorbed light that is not dissipated thermally or used in the photochemical phase of PSII (D), D- absorbed light fraction that is thermally dissipated (E) and F_0-initial fluorescence (F) in soybean genotypes. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants) (Tukey, p <0.05). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments. The data represent the mean ± standard error (n = 5).

Embrapa 48 showed higher ΦPSII in relation to genotype BR 16 under all treatments. On severe stress, the BR16 genotype reduced ΦPSII by 66% while Embrapa 48 reduced 46% (Fig. 8A). The greater reduction in ΦPSII in the sensitive genotype under water stress is associated with the reduction in *A*, probably due to low availability of CO_2 as consequence of the decrease in gs (Fig. 5B). BR 16 presented approx. 18% a higher non-photochemical extinction coefficient (NPQ) in the absence of stress. On the other hand, whilst under stress, an increase in NPQ was observed in both genotypes, there were no differences between the genotypes (Fig. 8B). Regarding the absorbed light fraction, which is used in the photochemical phase of FSII (P), the genotype Embrapa 48 showed, aprox.33%, values higher than BR16 in severe stress (Fig. 8C).

Under moderate stress, no significant differences were observed in the fraction of absorbed light that is neither thermally dissipated nor used in the photochemical phase of photosystem II (PE). However, there was an increase aprox.33% in PE under severe water stress in the tolerant genotype compared to BR16 under the same stress (Fig. 8D). Following reirrigation PE was greater in both genotypes than under control conditions. Analyzing the fraction of absorbed light that is dissipated thermally (D), there was an increase in genotypes BR 16 only under severe water stress (Fig. 8E). It is interesting to note that reirrigation resulted in a strong reduction in aprox. 20% the D in both genotypes, with values lower than those observed in the control treatment (Fig. 8E).

The minimum fluorescence in dark-adapted leaves (F_0) was higher in BR16 genotype for all treatments (Fig. 8F). The reirrigation resulted in a strong reduction in aprox. 40% in F_0 for both genotypes, which values lower than observed in the control treatment (Fig. 8F).

3.4. Isotopic composition of $\delta^{13}C$

The isotopic composition of ^{13}C was reduced (more negative value) only in the Embrapa 48 genotype in the reirrigated treatment, indicating that the stomata of this genotype under this condition were more open.

On the other hand, in the moderate and severe deficit treatments, Embrapa 48 presented higher $\delta^{13}C$ (less negative) in comparison with irrigated controls and with the reirrigated treatment, indicating that the tolerant genotype closes its stomata more under water deficit and reopens them after reirrigation (Fig. 9). This behavior was not observed in the genotype BR 16 for which no differences were detected for any treatment.

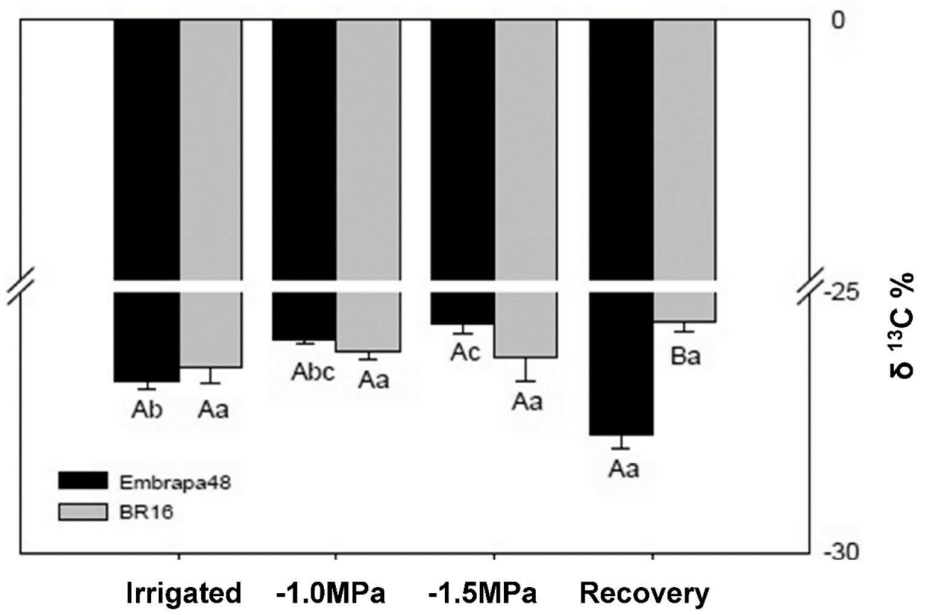

Figure 9. Isotopic composition of carbon in leaves of soybean genotypes. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants) (Tukey, p <0.05).

3.5. Cell damage and antioxidant enzyme activity

In the leaves, lipid peroxidation increased in both genotypes under conditions of moderate and severe water deficit compared to the control groups (Fig. 10A), the BR16 genotype showed an increase of 50% while Embrapa 48 increased 55% under severe stress compared to control plants (Fig. 10A). However, mention that the sensitive genotype (BR 16) presented higher absolute values than the tolerant genotype (Embrapa 48) under all treatments. Although for both genotypes there was a reduction in oxidative damage in the reirrigated treatment, the values were still higher than for the control treatment, indicating that a complete recovery from oxidative damage was not achieved after 4 days of reirrigation (Fig. 10A). When analyzing lipid peroxidation in roots, gradual increases with increasing stress were also observed, reaching 40% in severe stress, but there were no differences between the genotypes (Fig. 10B). However, unlike leaves, for roots oxidative damage after reirrigation was similar to that in the control treatment. These data suggest that there may be greater antioxidative capacity, or lower levels of oxidizing agents specifically in leaves of the tolerant genotype. Enzyme activity results show that the second option is the most likely. Lower activity values of CAT, POX and APX (Fig. 11) were found in genotype Embrapa 48 under water deficit when compared to BR 16. The imposition of severe

stress increased the activity of the POX enzyme in the BR16 genotype by 55% compared to control plants, whereas for Embrapa 48 this increase was 50% (Fig. 11A). For CAT enzymatic activity, an increase of 81% was observed in the BR16 genotype compared to control plants, whereas for Embrapa, this increase was 73% (Fig. 11B). The enzymatic activity of APX followed the same patterns, increased 66% in the BR16 genotype compared to control plants, whereas for Embrapa this increase was 46% (Fig. 11C).

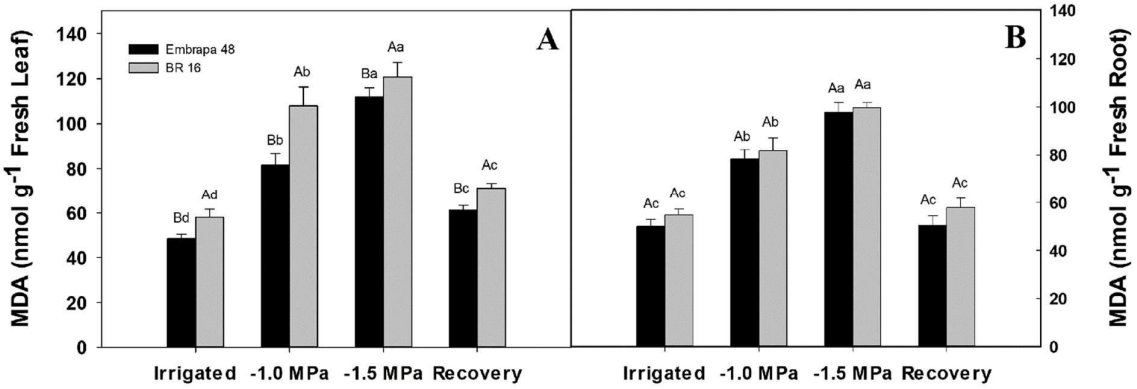

Figure 10. Effect of water deficit on leaf (A) and root (B) lipid peroxidation expressed in concentrations of malonic aldehyde (MDA) in soybean genotypes. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants) (Tukey, $p < 0.05$). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments (Tukey, $p < 0.05$). The data represent the mean ± standard error (n = 5).

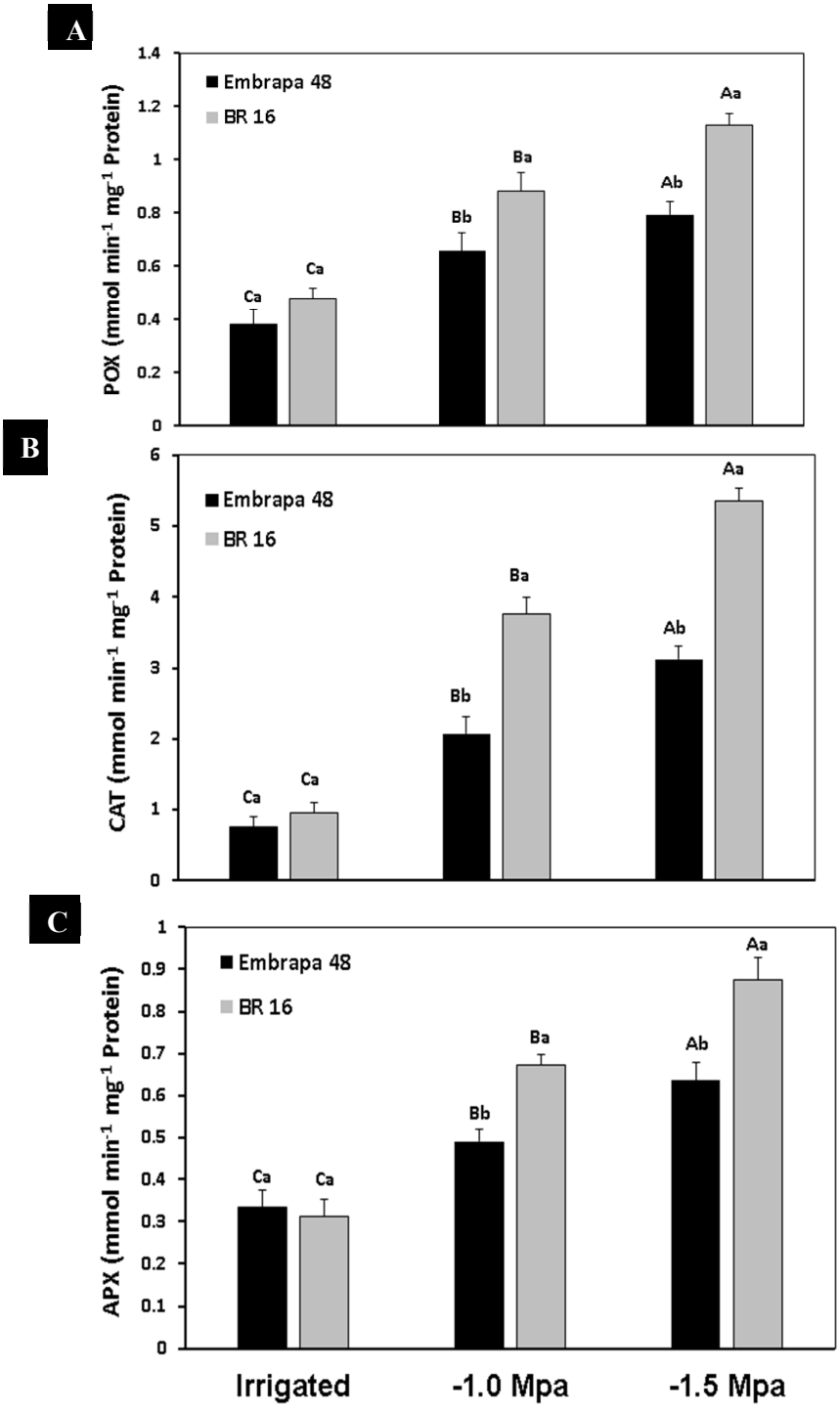

Figure 11. Antioxidant activity enzyme assays for (A) peroxidase (POX), (B) catalase (CAT) and (C) ascorbate peroxidase (APX). Each bar represents the mean ± standard error (n = 5, where n represents the number of plants) (Tukey, p <0.05). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments. The data represent the mean ± standard error (n = 5).

3.6. Reactive Oxygen Species in leaf During Stress

There are different kinds of reactive oxygen species (ROS) in plants, among them H_2O_2 is thought be the signaling ROS to regulate stress responses, and thus was evaluated using the DAB assay. Leaves which were collected from plant grown under -0,5 and -1,0 MPa showed more distribution of the brown pigments comparing to the control (Irrigated). A stronger DAB staining in the leaves BR16 compared with Embrapa48 plants was readily observed. The BR16 genotype stressed plants showed higher the distribution of brown pigments when compared with Embrapa 48 genotype under the same stress conditions (Fig. 12).

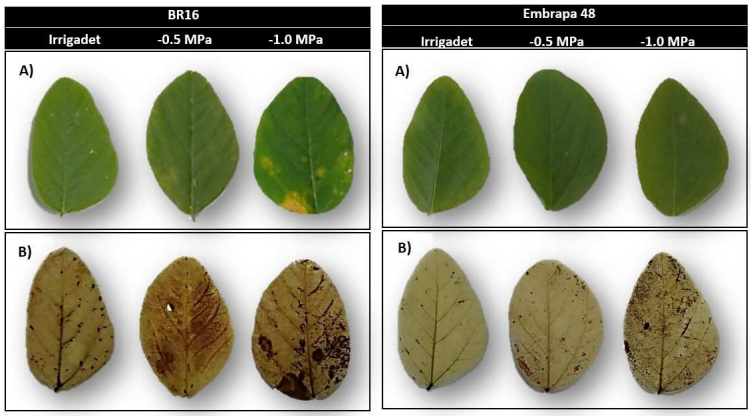

Figure 12. Hydrogen peroxide detection by DAB staining in soybean leaves. (A) Leaves at the three levels of stress (irrigated, -0,5MPa and -1,0MPa), (B) Reactive oxygen species (ROS) accumulation visualized by staining hydrogen peroxide using DAB. For DAB staining, leaves were detached from plants. The entire leaf was floated in DAB staining solution for 8h and destained to visualize the brown DAB precipitate.

4. Discussion

In plants, tolerance or sensitivity to water stress depends on the species and genotype under study, on the duration and severity of the deficit, as well as on the stage of development of the crop (Doss and Thurlow, 1974; Kron et al. 2008). Productivity (Oya et al. 2004; Giordani et al. 2019), proteomic and metabolomic analyzes (Lima et al., 2019) indicates that there are differences in tolerance between the soybean genotypes Embrapa 48 and BR 16 (Oya et al. 2004; Giordani et al. 2019), however do not investigated the physiological behavior under drought stress. We therefore carried out physiological analyses of leaves and roots of both genotypes in order to identify the mechanisms responsible for differential tolerance to water deficit,

subjecting plants to progressive water restriction and rehydration, and analyzing its effects on leaf gas exchange, fluorescence, water relations traits, root and shoot growth.

The dehydration curve showed that Embrapa 48 took longer to reach the different levels of water deficit. However, stomatal conductance at both stress levels was the same for both genotypes, and, furthermore, there were no differences in leaf area. According to Jaleel et al. (2009), water stress is characterized by reductions in water content and leaf water potential and loss of cellular turgor, which decreases stomatal conductance and cellular expansion, consequently constraining plant growth. In the absence of stress, the tolerant genotype has a lower predawn leaf water potential, which can be explained, in this situation specifically, by greater stomatal conductance.

Our results show that the tolerant genotype when subjected to water deficit allocates differently photoassimilated carbon. Higher investment in roots occurs and being more efficient could partly explain higher water potentials, in the genotype Embrapa 48 initially adopts the drought avoidance strategy. Additionally, although not measured in the experiment, a higher hydraulic conductance could help explain the higher tolerance as suggested by gene expression study indicating a reprograming of genes involved in remodeling of the cell wall (Coutinho et al. 2020). This study indicated that the metabolism of pectin is differently modulated in response to drought in soybean and may play a role in the plants defense mechanism against desiccation, through the increase of elasticity and crosslink of the cell wall.

However, it is prudent to emphasize that the tolerant genotype was more hydrated under the same water potentials suggesting a possible osmotic adjustment. A key role in the regulation of the response to drought is related to the hormone abscisic acid (ABA) and higher levels of this phytohormone were observed in the sensitive BR 16 (Lima et al. 2019). Additionally, it was observed that this sensitive genotype had a greater number of metabolites showing significantly altered abundances in drought conditions and therefore, more noticeable perturbations in the metabolic pathways (Lima et al. 2019). Otherwise, proline is widely regarded as the main osmoprotectant in drought stress tolerance in plants. Lima et al. (2019) also observed that drought triggers an increase in the proline synthesis or accumulation on both genotypes, however higher levels were found for the sensitive BR 16. Thus, the

signal for drought by ABA and proline were more noticeable in the sensitive BR 16. In the same way, amino acids and sugar were more abundant during drought in the sensitive genotype (Lima et al. 2019), which suggests that these compounds were not involved in osmoprotection of the tolerant genotype.

The decrease in CO_2 diffusion from the atmosphere to the carboxylation site of ribulose biphosphate carboxylase/oxygenase (Rubisco) is generally considered to be the main cause of reduced photosynthesis under conditions of mild and moderate water deficits (Grassi and Magnani, 2005; Pinheiro and Chaves, 2011). In the moderate deficit net photosynthesis was reduced to a lesser extent in the tolerant genotype. Given that there were no differences in stomatal conductance between them, this results may indicate a more pronounced inhibition of the biochemical or photochemical phase of photosynthesis in the sensitive genotype (*Ci/Ca*, Fig. 5D *Fv/Fm*, Fig. 7). Indeed, carboxylation efficiency, which was strongly affected by drought, was higher in the tolerant genotype under all treatments. Other evidence suggesting the participation of a non-stomatal event in the relative drought tolerance of Embrapa 48 comes from comparison of *Ci/Ca* and *gs*. Despite the absence of differences in *gs* at both stress levels we observed a lower *Ci/Ca* in the tolerant genotype, even though under severe stress there was no difference in net photosynthesis rate. In addition, *Fv/Fm* values did not decrease in Embrapa 48 genotype in relation to irrigated condition showing that stress is not causing photochemical inhibition of photosynthesis.

When the decrease in stomatal conductance is combined with high light levels, the leaves are subjected to an excessive amount of incident energy in relation to the amount of intercellular CO_2 available for photosynthesis, and the rate of reductant energy production can therefore overlap with the rate of its consumption by the Calvin cycle. Under these circumstances, down-regulation of photosynthesis or even photoinhibition can become a powerful defense mechanism for plants (Pinheiro and Chaves, 2011; Sanda et al. 2011). The contribution of photochemical reactions to the differential tolerance of these genotypes to water deficit can be seen in the higher ETR and ΦPSII of the tolerant genotype at both stress levels, even when there is no difference in *g*s; this higher ETR and ΦPSII can be explained by the higher P. Studies have revealed that PSII of soybean plants is resistant to moderate water stress (Kirova

et al. 2008), and the potential quantum efficiency of PSII (*Fv/Fm*) and electron transport rate (ETR) are not altered by the imposition of water stress (Ohashi et al. 2006).

Under severe stress BR16 has a greater fraction of thermally dissipated light, while, the tolerant genotype presents a greater light fraction that is neither dissipated thermally nor used in the photochemical reactions (PE). Higher PE can tentatively be explained by a greater cyclic electron transport, while the higher D, even with lower electron transport rate in the sensitive genotype, indicates a defense mechanism and a reduction of the efficiency of the photochemical reactions, as suggested by the higher level of oxidative damage in this genotype. This combined analysis of fluorescence data indicates that there is a clear contribution of the photochemical reactions to the differences in drought tolerance between the genotypes.

Moreover, when the availability of CO_2 and biochemical activity are reduced due to water deficits, the excess reductants in the photochemical apparatus must be dissipated as heat or drained through alternative electron sinks (Miyake, 2010) to reduce photoinhibition and the production of ROS.The higher tolerance to drought of the Embrapa 48 genotype can also be seen in the levels of oxidative damage (lipid peroxidation) under water deficit. Although in both genotypes damage increased with increasing stress, it was always higher in leaves of BR16 in all treatments. However, this is not related to lower activity of antioxidant enzymes. In fact, metabolomic profiles showed that the tolerant plants maintain cell homeostasis under the stress condition in contrast to the sensitive genotype that showed several dysregulated pathways (Lima et al. 2019). Furthermore, just small deviations in the metabolic pathways were observed for drought-tolerant plants in comparison to the sensitive genotype.

Reirrigation may represent a moment when oxidative stress can be even greater than that occurring under severe water stress (Bartels and Sunkar, 2007), and hence the evaluation of responses to this recovery treatment can help to illustrate additional tolerance mechanisms that act specifically in this phase. The fact that both genotypes still have a higher level of lipid peroxidation than the control treatment indicates that at four days of reirrigation there was not a complete recovery from water deficit damage. The higher levels of leaf oxidative damage in the sensitive genotype may be both the cause of its lower photochemical efficiency. The reirrigation treatment also showed interesting results, since although the water potential returns to normal in both genotypes, the conductance only returns to values similar to the control in the tolerant

genotype, being greater in this. Water stress also increased lipid peroxidation in roots, but no difference was observed between the genotypes and overall oxidative damage was slightly lower than in leaves. This observation suggests that the difference in drought tolerance between the genotypes may partly result from differences in antioxidative mechanisms in the leaves, but not in the roots. Increased MDA with water deficit and recovery with reirrigation was also observed by Iftekhar et al. (2010) in a proteomic study in roots of a single soybean genotype. Growth data demonstrate that root induction is greater in the tolerant genotype, which occurs at the expense of reducing shoot relative growth but did not affect the leaf area. Although the number of leaves is higher in all treatments for the tolerant genotype, the leaves are larger in the sensitive genotype, meaning that total leaf area is not altered. This indicates a lower allocation of carbon to stem growth, reductions in growth, reduction in *A*, and causing a greater allocation of carbon to root growth.

5. Conclusion

Based on the drought responses of leaf gas exchange, water relations, and root physiological traits of soybean genotypes to progressive water restriction, we can conclude that the drought-tolerant Embrapa 48 genotype copes better with water stress than the susceptible BR16 by maintaining higher water potential in leaves, and probably by increasing the proportion of fixed carbon invested in root growth. The postponement of water dehydration, unrelated to changes in *gs* or the isotopic composition of ^{13}C, suggest that differential hydraulic conductivity may be important to this tolerance, and to a greater induction of root growth under water deficiency. Lower inhibition of photochemical reactions in the tolerant genotype could explain, at least partially, the greater photosynthetic rate of this genotype under moderate stress. The lower oxidative damage in leaves of the tolerant genotype may have reduced the generation of free radicals. The greater reduction in relative growth of the shoot, together with a greater induction of the growth of the root system under drought, without altering the leaf area, indicates that the tolerant genotype has a differential mechanism of allocation of the carbon for the roots, that avoids a reduction in photosynthesis but reduces the height of the plant.

As result, the cell homeostasis and the photosynthetic metabolism were maintained unchanged under the stress. This study reinforces the view that drought

tolerance is a quantitative trait, in which a number of different but interconnected mechanisms are involved. Therefore, the combination of physiological and molecular data is essential for understanding the drought tolerance mechanism.

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Supplementary Information

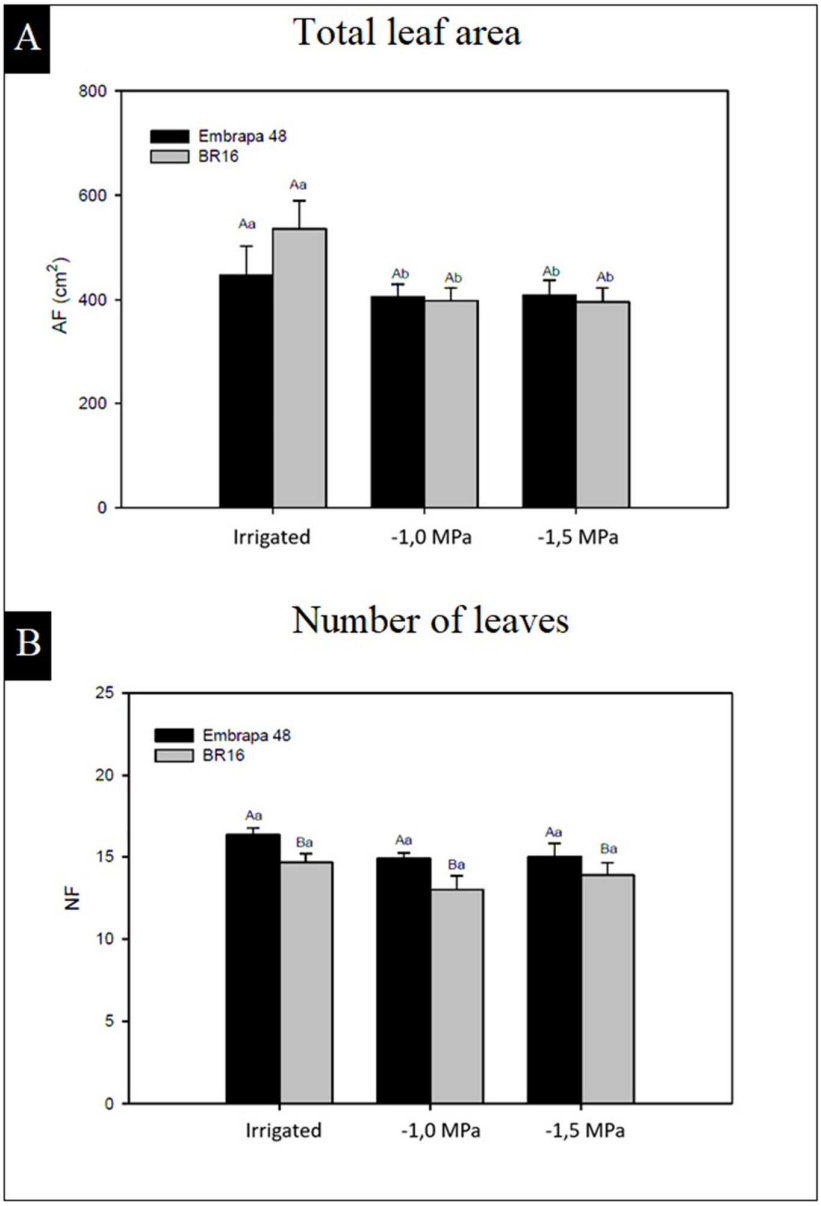

Figure S1. Effect of drought stress on leaf expansion rates. (A)Total leaf area (AF) in soybean genotypes under water deficit. (B) Number of leaves (NL) in soybean genotypes under water deficit. Each bar represents the mean ± standard error (n = 5, where n represents the number of plants) (Tukey, $p < 0.05$). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments (Tukey, $p < 0.05$). The data represent the mean ± standard error (n = 5).

CHAPTER II

Remodeling of the Cell Wall as a Drought Tolerance Mechanism of a Soybean Genotype Revealed by Global Gene Expression Analysis

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Remodeling of the cell wall as a drought tolerance mechanism of soybean genotype revealed by global gene expression analysis

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Abstract

Drought stress is a major abiotic stress that affects soybean production. Therefore, it is widely desirable that soybean becomes more tolerant to stress. However, identifying the major genetic determinants of tolerance is a challenge. As transcriptomic studies offer an insight into the regulatory mechanisms of the stress response, we compared the global gene expression profiles of leaves of two soybean genotypes that display different responses to a water deficit (BR 16 and Embrapa 48, drought-sensitive and drought-tolerant, respectively). After the RNAseq analysis, a total of 5335 down-regulated and 3170 up-regulated genes were identified in the sensitive genotype. On the other hand, the number of genes differentially expressed was markedly lower in the tolerant genotype, including 355 up-regulated and 471 down-regulated genes. The genotype Embrapa 48 has less gene reprogramming, in addition to expressing protein kinases and transcription factors that indicate they are signal molecules that act to improve drought tolerance in soybeans. These results suggest that the metabolism of pectin is differently modulated in response to drought stress and may play a role in the soybean defense mechanism against desiccation stress. This occurs via an increase of the cell wall plasticity and crosslink, which may contribute to an efficient water usage. Remodeling of the pectin component of the cell wall has the potential to be an important mechanism offering drought tolerance to the Embrapa 48 soybean genotype, contributing to the maintenance of cell turgor and growth under drought stress.

Keywords: Leaves, Molecular mechanism, Transcriptome.

1. Introduction

Drought is the main environmental factor that negatively influences both plant growth and development, thus restricting productivity and agricultural expansion. Projections of climate change indicate that drought will become more intense in some areas of the world and therefore the development of tolerant plants is necessary to maintain production (Passioura, 2007; Stanke et al. 2013; Spinoni et al. 2017). On the other hand, plants have evolved to create several strategies to cope with drought, including a short life cycle or phenotypical plasticity, enhanced water uptake and reduced water loss, as well as osmotic adjustment, antioxidant capacity, and desiccation tolerance (Fang and Xiong, 2015). The evaluation of tolerance mechanisms and drought-responsive genes from soybean is essential for genetic breeding programs (Rodrigues et al. 2012; Brown, 2017). Thus, a transcriptome analysis of contrasting genotypes may contribute to the understanding of the molecular and physiological responses.

The main difficulty in selecting genes as targets for plant breeding that's aimed at drought-tolerance is the complexity of the physiological responses to drought stress. Plant survival strategies under drought involve transient responses, such as reduced transpiration, changes in the root system, reduction of leaf area and adjusted osmotic status leading to a minimal water loss and improving water uptake (Hu and Xiong, 2014). Transient response and developmental changes require a substantial rebuilding of plant metabolism and changes in the expression of a high number of genes. Global transcriptome analysis has been used to provide a deeper insight into the complexity of plant response to drought stress on the molecular level.

External drought stimuli is perceived by sensors on the membrane, and then the signals are delivered through multiple signaling pathways, resulting in the expression of responsive genes so as to confer drought tolerance in the plants (Zhu, 2002; Hirayama and Shinozaki, 2010). In general, gene expression studies of various plant species have been performed to classify several groups of genes, which are regulated in response to drought. Among them are those encoding calcium-dependent protein kinases, calmodulin and calmodulin-related calcium sensor proteins and protein phosphatases class 2C (PP2C) (Molina et al. 2008; Guo et al. 2009; Ranjan and Sawant, 2015), along with a number of transcription factors (TFs) (Sahoo et al. 2013;

Janiak et al. 2018). These signaling proteins are usually classified as ABA-dependent and ABA-independent stress response pathways (Shinozaki and Yamaguchi-Shinozaki, 2007). Genes involved in biosynthesis and signaling pathways of other plant hormones, such as auxin, ethylene, jasmonic or salicylic acid, were also identified as differentially expressed under drought (Jakoby et al. 2002; Aimar et al. 2011). Moreover, genes related to antioxidation processes, osmoprotectant synthesis and various factors from late embryogenesis abundant (LEA) family were also reported as differentially expressed in response to drought (Shinozaki and Yamaguchi-Shinozaki, 2007; Talame et al. 2007).

The main challenge when performing gene expression studies is to identify which genes are not only responsive, but also confer a differential physiological behavior when compared to a sensitive genotype. To achieve this goal it is necessary to use parental plant genotypes contrasting in drought-tolerance. The physiological response of the genotypes BR 16 (drought-sensitive) and Embrapa 48 (drought-tolerant) under drought conditions was studied by Oya et al. (2004), Carvalho et al. (2015) and our research group. They found that, in the vegetative stage under drought conditions in the field, the tolerant genotype had the highest number of pods.The studies of the proteome, phosphoproteome and metabolomic profile were also performed by Lima et al. (2019) in order to detect the metabolic pathways which are affected by drought stress. An integrative overview showed that tolerant plants maintain cell homeostasis and photosynthetic metabolism under stress conditions, as indicated by an abundance of protein and regulation by phosphorylation. Drought stress marker in roots were also evaluated to understand the mechanism of tolerance in theses genotypes. The GmaxRD20A-like and GmaxRD22-like genes, homologs of Arabidopsis genes of the ABA-independent pathway, are highly induced by water deficit, being these potential drought marker genes in these genotypes (Neves-Borges et al. 2012).

In order to complement these studies and understand the molecular mechanisms to improve the tolerance, we conducted RNA-Seq analysis from soybean leaves to generate the expression profiles between BR16 and Embrpa 48, growing under drought stress and well-watered (unstressed) control conditions. The metabolic/regulatory pathways and biological processes were explored via Gene Ontology (GO) enrichment and indicated differences in the gene expression

reprogramming in the drought-tolerant genotype that correlated with the physiological mechanisms of drought tolerance. The results revealed that in response to stress, the tolerant genotype, Embrapa 48, has less gene reprogramming, in addition to expressing protein kinases and transcription factors that indicate are molecules that act to improve drought tolerance in soy. Expression of the genes relating to pectin remodeling, in the leaves, may be involved in a mechanism that contributes to the maintenance of leaf turgor of the Embrapa 48 genotype under drought stress.

2. Materials and methods

2.1. Plant material, growth and drought stress treatments

Seeds of soybean genotypes BR 16 and Embrapa 48 were obtained from the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA SOJA, Londrina, Paraná). Seedlings were grown in plastic trays containing Plantmax® commercial substrate, where they remained for 10 days. After germination, seedlings were transplanted to pots containing 10 L of a mixture of soil, sand and manure (2/1/1) each. Plants were grown under natural sunlight in a greenhouse with average daytime temperature 15–35°C and relative humidity 65–85%.

The plants were grown under normal water conditions until reaching the development stage V3 (fully expanded third trifoliate). The control plants were watered daily with approximately 30 mL water per plant. The plants were exposed to a slow drying soil treatment, which consisted of a reduction in irrigation to 40% of the daily normal (Valente et al. 2009). The hydric regimes were assigned as irrigated (IR) and non-irrigated (NI). The leaf water potential (ψw) was measured in the third emerging trifoliate at dawn by using a Scholander pump (Scholander et al. 1965) during the stress period. Samples were collected in liquid nitrogen when the plants had a water potential - 1 MPa and then stored at -80°C until use. As each soybean genotype had three plants per pot, nine leaves were collected. The analyses were performed using a pool of three leaves from each pot to obtain three independent biological replicates.

2.2. RNA Extraction, library preparation, and sequencing

Total RNA was extracted from leaves using a Trizol reagent (Invitrogen) according to the manufacturer's instructions. Five micrograms of total RNA were used to prepare paired-end 100 bp libraries using the BIOO NEXT flex Rapid Directional RNA-Seq Kit (Bioo-Scientific, Austin, TX). Library qualities were analyzed with the Bioanalyzer 2100 (Agilent, Santa Clara, CA) and the barcoded libraries were quantified by fluorometry using a Qubit instrument (LifeTechnologies, Carlsbad, CA). The libraries were then pooled in equimolar ratios, quantified by qPCR with a Kapa Library Quant kit (Kapa, Cape Town, South Africa). Three biological replicates of each treatment were sequenced using the Illumina Hi-Seq 2500 (Illumina, San Diego, CA) from NuBioMol (Center for Biomolecules Analysis – UFV, Brazil).

Raw reads were subsequently subjected to trimming using Trimmometic software (Bolger et al. 2014) with a Phred quality threshold of 20. Reads were aligned to the *Glycine max* genotype Williams 82 primary transcriptome (Wm82.a2.v1) (Schmutz et al. 2010) using the Kallisto aligner (Bray et al. 2016).

2.3. Analysis of differentially Expressed Genes (DEGs)

To identify differentially the expressed genes (DEGs), we used the DESeq2 software package, which performs pairwise comparisons (Anders and Huber, 2012). DESeq2 analyses were carried out using the Kallisto output. The DEGs were identified using the MA-plot-based method from the package DEGseq version 3.0 (Wang et al. 2009). An absolute fold-change threshold of 2.0 and a false discovery rate (FDR) of ≤ 0.0001 were used to select the DEGs identified by DEGseq.

2.4. RT-qPCR

RT-qPCR was performed to validate the differential gene expression data obtained RNA-Seq analysis. RNA was extracted from leaf tissues of the control and stressed plants using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was analyzed using agarose gel electrophoresis and quantification was performed on a Thermo Scientific NanoDrop 2000c. A total of 4 µg

of RNA was used for cDNA synthesis with the SuperScript III kit (Invitrogen) following the manufacturer's instructions.

The gene expression was assessed using an ABI 7500 Fast Thermocycler (Applied Biosystems, Foster City, CA, USA) and Fast SYBR Green Master Mix (Thermo Fisher Scientific). The cycling conditions were as follows: 15 s at 95 °C, 40 cycles of 95 °C for 3 s; 30 s at 60 °C, and final denaturation at 95 °C for 20 s, followed by a melting curve. Specific primers for RT-qPCR were designed using the Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with a melting temperature (T) between 59–61°C, a length of 18–23 bp, an amplicon product size of 120–150 bp, and a GC content of 40–60% (Supplementary Information Table S1). Gene expression was normalized using two soybean housekeeping genes, being them UNK2 and Actin. A total of three biological replicates and three technical replicates were performed for each gene. Relative quantification was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Functional classification of the differentially expressed genes

The DEGs were subjected to functional classification using *The Arabidopsis Information Resource* (http://www.arabidopsis.org). The gene ontology (GO) enrichment analysis of each gene set was performed using the ClueGO version 2.0.7 plugin tool (Bindea et al. 2009) in Cytoscape version 3.2.1 (Shannon et al. 2003) using the GO biological process category. Overrepresented biological process categories were identified using an (right-sided) enrichment test based on the hypergeometric distribution. In order to determine significantly the overrepresented GO terms, the terms with a *p*-value lower than 0.05 were considered as a Kappa significant value. Genes classified as significantly overrepresented were validated by the Benjamin test.

3. Results

3.1. Analysis of RNA transcripts

The response of the soybean to moderate drought was investigated at the transcriptional level by an RNA-Seq approach. Previous studies performed by our research group showed greater reduction in the growth of the aerial part,

simultaneously with a greater induction of the growth of the root system under drought, without changing the leaf area, indicating that the tolerant genotype has a differential mechanism in allocating the carbon for the roots (Mesquita et al. 2020). In the present study, gene expression in leaves of two soybean genotypes, BR 16 and Embrapa 48, drought-sensitive and drought-tolerant, were analyzed by Illumina Hi-Seq 2500 (Illumina, San Diego, CA). The initial sample collected after a water deficit was designated as "NI", and control plants "IR" (Fig. 1A). Approximately 45-50 million reads were generated from each sample. Raw reads were subjected to a pre-processing/trimming step to remove short or low-quality sequences and adaptor/primer sequences. The RNA-Seq analysis workflow is shown in Supplementary Information Figure S1 and was used for data analysis.

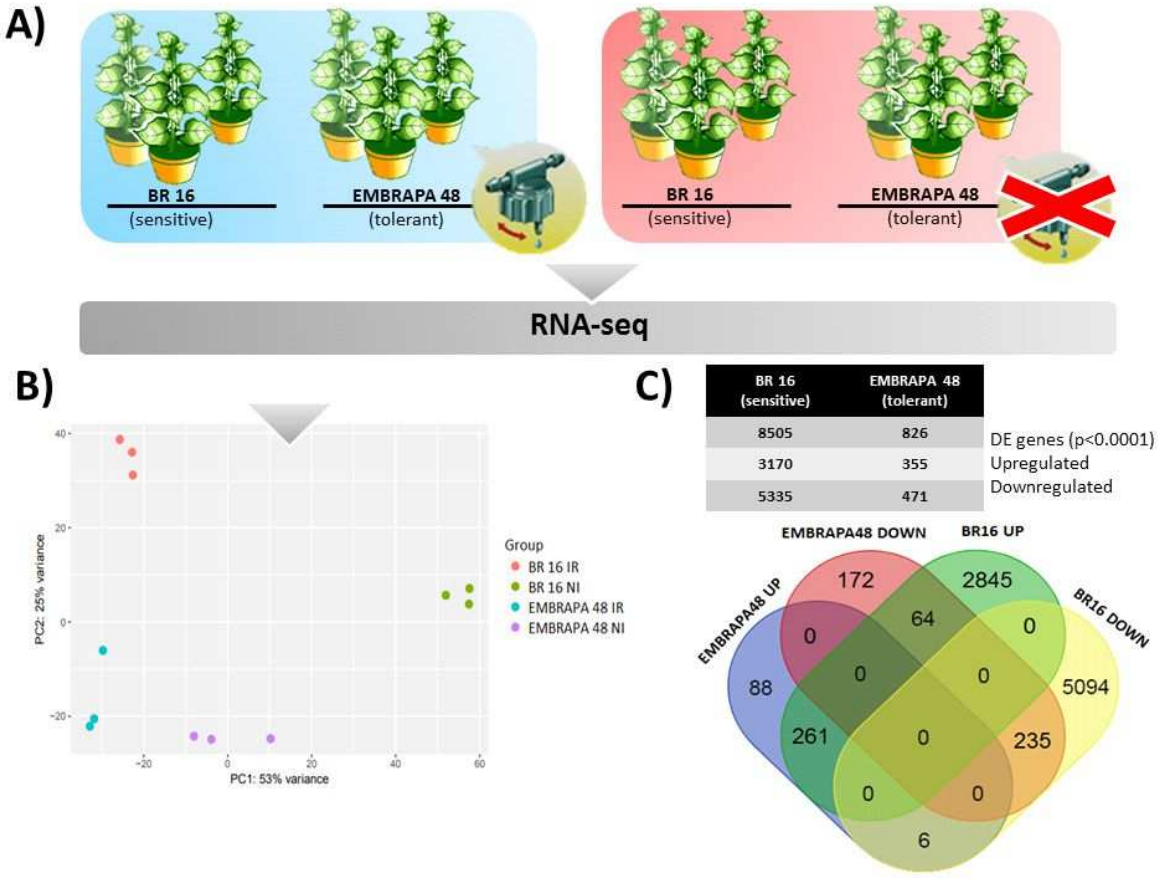

Figure 1. Overall gene expression in response to drought stress. A) Soybean plants BR 16 and Embrapa 48 exposed to a gradual drought regime to isolate total RNA for transcriptomic analysis. The water potential was measured by Scholander pump. In the blue box irrigated plants and in the red stressed plants. B) Transcriptome data used for the principal component analysis, showing distinct clusters of the different soybean genotypes in Irrigated conditions (IR) and not irrigated (NI).C) Number of differently expressed genes in drought conditions in both genotypes and Venn diagram showing the comparison of the number of genes differentially expressed. Proportion of significant results ($p \leq 0.0001$, log2 fold change ≥2 for up-regulated and ≤ −2 for down-regulated genes).

To understand further about the similarity of the genotypic responses under water deficit conditions, we used Principal Component Analysis (PCA) (Fig. 1B). The quality of the data obtained can be observed by the analysis of the sample-by-sample Euclidean distance, which is repopulated in the form of a heat map (Supplementary Information Fig. S2). High-throughput RNA-sequencing analysis was performed using a Kallisto pipeline (Bray et al. 2016) comparing the number of genes differentially regulated in response to drought combinations between controls vs. treatments, for each genotype, using DESEq2 (Anders and Huber, 2012). When comparing all genes differentially expressed, we identified for sensitive BR 16 8505 genes, including 3170 genes up-regulated and 5335 genes down-regulated under drought conditions. For the tolerant EMBRAPA 48, 826 genes were differentially expressed, including 355 up-regulated and 471 genes down-regulated (Fig. 1C). The global data shows that the transcriptional reprogramming was more pronounced in the sensitive plants, considering the highest number of DEGs in BR 16 genotype. In addition, gene-expression data obtained by RNA-seq strategy correlated with RT-qPCR measurements (Fig. 2), confirming the accuracy of our RNA-seq data.

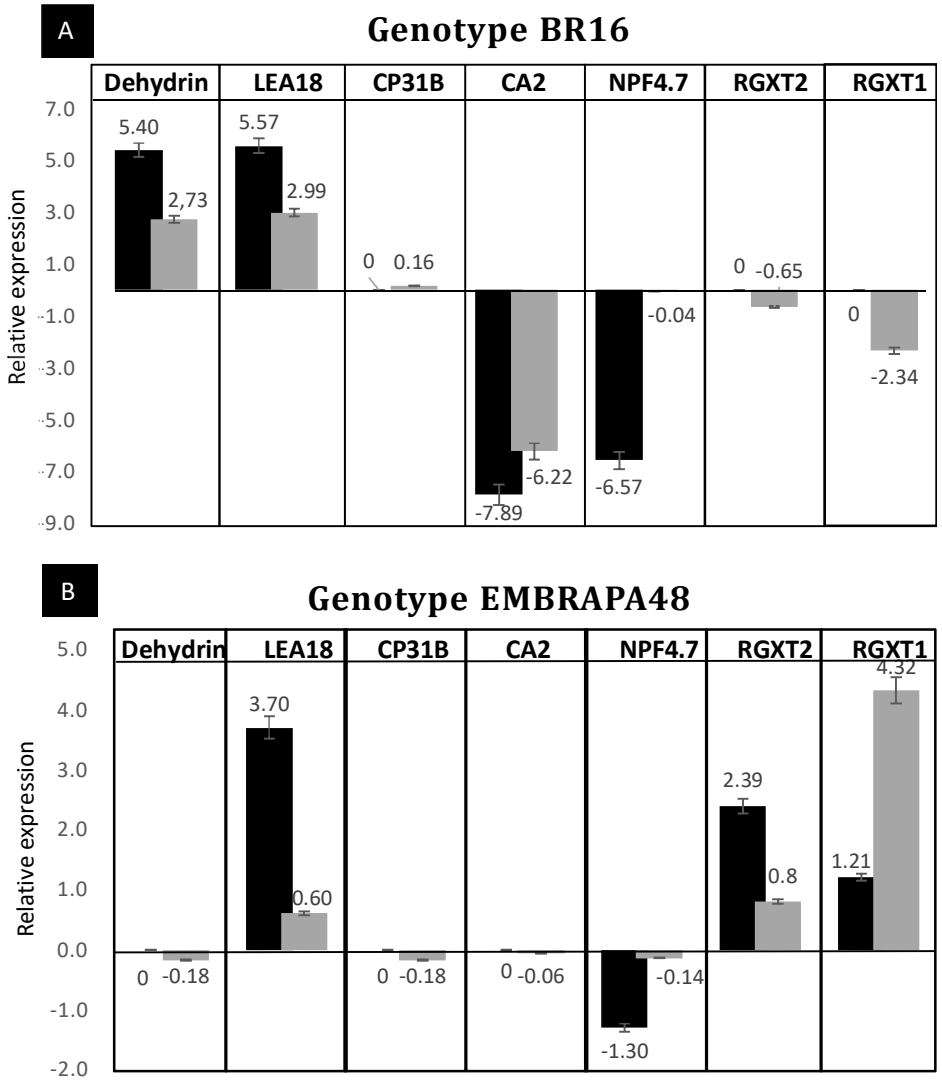

Figure 2. Validation of (■)RNA-Seq data by real-time (■)RT-qPCR. The expression variation of the RNAs analyzed in this study in plants submitted to water deficit compared with controls. The graph (A) shows the expression variation in the genotype BR16, and the graph (B) shows the expression variation in the genotype EMBRAPA48. Genes encoding Dehydryn, LEA18, CP31B (chloroplast RNA-binding protein 31B), CA2 (carbonic anhydrase), NPF4.7 (protein NRT1/PTR family 4.7), RGXT2 and RGXT1 were analyzed. The data represent the mean ± SE (n = 3).

The gene expression response to drought has been evaluated extensively in several plants including soybeans (Bhargava and Sawant, 2013). Thus, in order to select genes that may be involved not only in general stress response, but also that conferred drought tolerance in soybean, we analyze the list of genes that were differentially expressed only in EMBRAPA 48 genotype. This analysis allowed us to select candidates that could play a role in drought adaptation and tolerance, whilst also may explain the lower number of DEGs found in the tolerant genotype. Thus, 260

genes were selected as being differentially expressed exclusively in the tolerant genotype, including 88 up-regulated and 172 down-regulated (Fig. 1C). We identified 20 genes encoding protein kinases (Table 1): four genes encoding S/T PKs, two were up-regulated (Glyma20G14580.1, Glyma18G18720.1) and two were down-regulated (Glyma09G09800.1, Glyma20G17650.1) under dehydration stress; three genes encoding CDPKs all were up-regulated (Glyma16G02240.1, Glyma16G14250.1, Glyma15G14340.1); fourteen PKS genes, nine of them were down-regulated (Glyma10G19570.1, Glyma07G21590.1, Glyma13G26610.1, Glyma13G24090.1, Glyma18G12450.1, Glyma13G28310.1, Glyma04G19530.1, Glyma07G21660.1) and five were up-regulated genes (Glyma10G22220.1, Glyma13G22430.18, Glyma13G22430.22, Glyma13G22430.11, Glyma17G14990.2).

Table 1. Protein kinases responsive to dehydration only in the drought-tolerant genotype.

PROTEIN KINASE		
GENE	**Log_2 ratio**	**Annotation**
	S/T PKS	
GLYMA09G09800.1	-0,709405048	CBL-interacting serine/threonine-protein kinase
GLYMA18G18720.1	1,162165	Serine/threonine-protein kinase wnk with no lysine
GLYMA20G14580.1	1,035192	Serine/threonine-protein kinase wnk with no lysine
GLYMA20G17650.1	-0,624644997	Serine/threonine-protein kinase
	CDPKS	
GLYMA16G02240.1	1,123958	Calcium-binding protein
GLYMA16G14250.1	1,102288	Calcium-binding protein cml41-related
GLYMA15G14340.1	0,827979131	WTF9
	PKS	
GLYMA10G19570.1	-1,091893292	Leucine-rich repeat receptor-like protein kinase pepr1-related
GLYMA07G21590.1	-1,570048333	Leucine rich repeat N-terminal domain (LRRNT_2)
GLYMA13G26610.1	-1,640632312	Protein tyrosine kinase (Pkinase_Tyr)
GLYMA13G24090.1	-0,484742282	AMP-activated protein kinase, gamma regulatory subunit
GLYMA18G12450.1	-1,188983376	Protein kinase domain (Pkinase) // Leucine Rich Repeat (LRR_1)
GLYMA13G28310.1	-0,964734668	Cysteine-rich receptor-like protein kinase 27-related
GLYMA04G19530.1	-1,480822749	SNF1-related protein kinase regulatory subunit gamma-1
GLYMA10G22220.1	1,094759	Cell division protein kinase
GLYMA07G21660.1	-0,539613982	1-Phosphatidylinositol-3-phosphate 5-kinase fyab1c-related
GLYMA13G22430.18	1,81788	Protein tyrosine kinase
GLYMA13G22430.22	1,648667	Protein tyrosine kinase
GLYMA13G22430.11	1,506839	Protein tyrosine kinase
GLYMA17G14990.2	2,101687	Protein tyrosine kinase

In addition, 23 genes were identified encoding transcription factors (TFs) (Table 2). These genes were grouped into major groups. The first group contained one auxin response factor (ARF) gene down-regulated (Glyma18G18450.1) in the drought-tolerant genotype. The second group was composed of zinc-finger protein family genes, containing five members (Glyma07G12680.1, Glyma08G03140.1, Glyma05G22440.1, Glyma01G00500.1, Glyma17G15100.1) which were induced by dehydration, and three (Glyma06G19660.2, Glyma12G17890.1, Glyma04G06660.1) were suppressed. The third group was constituted by five MYB family genes down-regulated (Glyma08G02080.1, Glyma19G22220.1, Glyma11G18340.1, Glyma15G14190.1, Glyma12G18470.1). The fourth group consisted of ring-finger family genes; two members (Glyma20G07670.1, Glyma04G03980.4) were induced by dehydration in leaves, and one (Glyma10G12460.4) was suppressed. The fifth group consisted of heat shock factors (HSFs); two members were induced (Glyma01G21740.1, Glyma09G19060.1) and two suppressed (Glyma08G02590.1, Glyma08G15850.1) by dehydration stress. The remaining TF genes encoded members of families AP2/EREBP (Glyma16G01260.4) and NAC domain protein (Glyma11G18200.1).

Table 2. Transcription factors responsive to dehydration only in the drought-tolerant genotype.

TRANSCRIPTION FACTORS (TFS)		
GENE	**Log_2 ratio**	**Annotation**
	AUXIN-RELATED PROTEIN	
GLYMA18G18450.1	-0,754799159	Auxin response factor
	ZINC FINGER PROTEIN	
GLYMA07G12680.1	1,293328	CCCH Zinc finger protein
GLYMA08G03140.1	1,437808	CCCH Zinc finger protein
GLYMA06G19660.2	-0,967052969	C3HC4 Zinc finger protein
GLYMA12G17890.1	-1,279118701	C2H2 Zinc finger protein
GLYMA04G06660.1	-2,353948183	C2H2 zinc finger protein
GLYMA05G22440.1	1,214217	CCCH Zinc finger protein
GLYMA01G00500.1	1,406536	CCCH Zinc finger protein
GLYMA17G15100.1	1,06469	C2C2 Zinc-finger of the FCS-type
	MYB TRANSCRIPTION FACTOR FAMILY	
GLYMA08G02080.1	-3,100653512	Leucine Rich Repeat (LRR_1) -MYB-LIKE DNA-BINDING PROTEIN MYB // ATMYB103
GLYMA19G22220.1	-1,554766923	MYB transcription factor
GLYMA11G18340.1	-0,993510023	MYB transcription fator-MYB 2
GLYMA15G14190.1	-0,599749666	MYB
GLYMA12G18470.1	-1,655029264	MYB transcription fator
	RING-H2 PROTEIN	
GLYMA20G07670.1	2,672113	Ring finger domain
GLYMA04G03980.4	2,921892	Ring finger domain
GLYMA10G12460.4	-2,716919566	Ring finger domain

Table 2. *Cont.*

HEAT SHOCK PROTEIN		
GLYMA08G02590.1	-0,507591383	Heat shock protein 70kDa
GLYMA08G15850.1	-0,745310757	Small heat-shock protein 20KDa
GLYMA01G21740.1	1,480779	Heat stress transcription factor B-2B
GLYMA09G19060.1	1,187051	Heat stress transcription factor C-1
AP2/EREBP FAMILY		
GLYMA16G01260.4	-0,946836287	AP2 domain
NAC FAMILY		
GLYMA11G18200.1	1,480184	NAC domain protein 61

We found that some genes that code for proteins involved in cell wall dynamics were differentially expressed for both genotypes, which appear to be regulated by drought stress (Table 3). The genes related to the metabolism of Rhamnogalacturonan were only expressed in the tolerant genotype (Glyma08G09360.1, Glyma15G07400.1, Glyma05G22440.1). Two expansion protein genes (Glyma05G06580.1, Glyma20G03390.1) and two responsive xyloglucans transferase genes (Glyma10G15100.1, Glyma05G13870.1) were expressed in both genotypes. Two glycosidases responsive (Glyma10G20000.1, Glyma03G10450.1) were only expressed in the tolerant genotype. A synthase-like D3 Cellulose (Glyma01G23250.1) overexposed in the sensitive genotype and the pectinesterase inhibitor (glyma08G14790.1) down-regulated in the tolerant genotype and up-regulated in the susceptible.

Table 3. Genes coding for proteins involved in cell wall dynamics differentially expressed for both genotypes.

GENE ID	PROTEIN	BR 16-LOG^2	EMBRAPA 48-LOG^2
GLYMA10G15100.1	Xylosyltransferase MGP4	3.473208	1.975452
GLYMA08G09360.1	Rhamnogalacturonan xylosyltransferase 1 (RGXT1)	NF	2.308536
GLYMA15G07400.1	Rhamnogalacturonan xylosyltransferase 2 (RGXT2)	NF	2.397853
GLYMA05G22440.1	Rhamnogalacturonan specific Xylosyltransferase 1 (RGTX3)	NF	1.214217
GLYMA05G13870.1	Xyloglucan endotransglucosylase 27	3.006961	1.068432
GLYMA10G20000.1	UDP-Glycosyltransferase superfamily protein	NF	3.68817
GLYMA03G10450.1	Hydroquinone glucosyltransferase	NF	1.567052
GLYMA08G14790.1	Pectinesterase inhibitor 51	1.29512	-2.04434
GLYMA05G06580.1	Expansin-like B1	8.871832	3.815932
GLYMA20G03390.1	Expansin -A14	6.47856	NF
GLYMA01G23250.1	Cellulose Synthase-like D3	2.404065	NF

3.2. Functional classification of Differentially Expressed Genes

We used the enrichment analysis of DEGs based on up and down-regulated genes, performed by Cytoscape plug-in ClueGO, which identified significantly over-represented enrichment networks present in both genotypes for the drought treatment (Fig. 3 and 4). The down-regulated genes from the sensitive genotype BR16 showed clusters relating to biological processes, such as regulation of protein catabolic, gibberellin-responsive, acyl-CoA metabolic process, response to glucose, carbohydrate and lipid transport, proteolysis, response to red or far red light, stamen filament development, among others (Fig.3A). However, for Embrapa 48 (Fig. 3B), the down-regulated genes showed distinct clusters related to lipid transport, membrane fusion, toxin catabolic process, and regulation of secondary cell wall biogenesis.

The analysis applied for up-regulated genes also showed distinct results for the genotypes. Notably, we observed in the sensitive BR16 under drought the predominance of clusters related to the amino acid catabolic process, alcohol biosynthetic process, response to monosaccharide stimulus, hormone signaling pathway, monocarboxylic acid metabolic process, nucleotide salvage (Fig. 4A).For the tolerant genotype, the up-regulation of the DEGs was mainly corresponded to the pathways rhamnogalacturonan II biosynthetic process, endosperm development, serine family amino acid metabolic process as well as photosynthesis and light-harvesting (Fig. 4B).

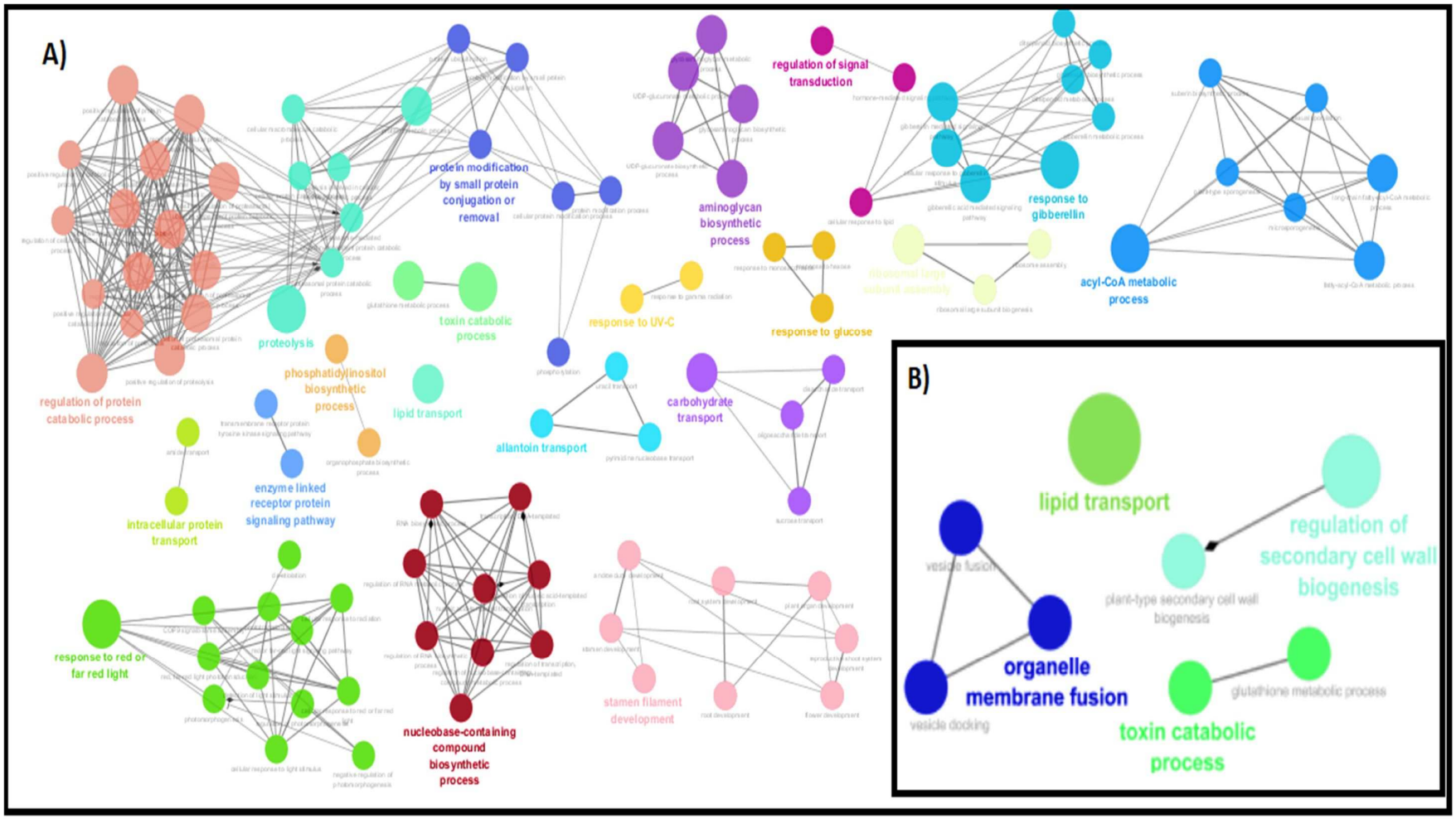

Figure 3. Over-representation analysis of down-regulated genes by using the Gene Ontology biological process database. A) Clusters containing down-regulated genes in the sensitive genotype BR16. B) Clusters containing down-regulated genes in the tolerant genotype EMBRAPA 48. The size of the node represents the integration of genes and the thickness of the edge shows a significant Kappa value.

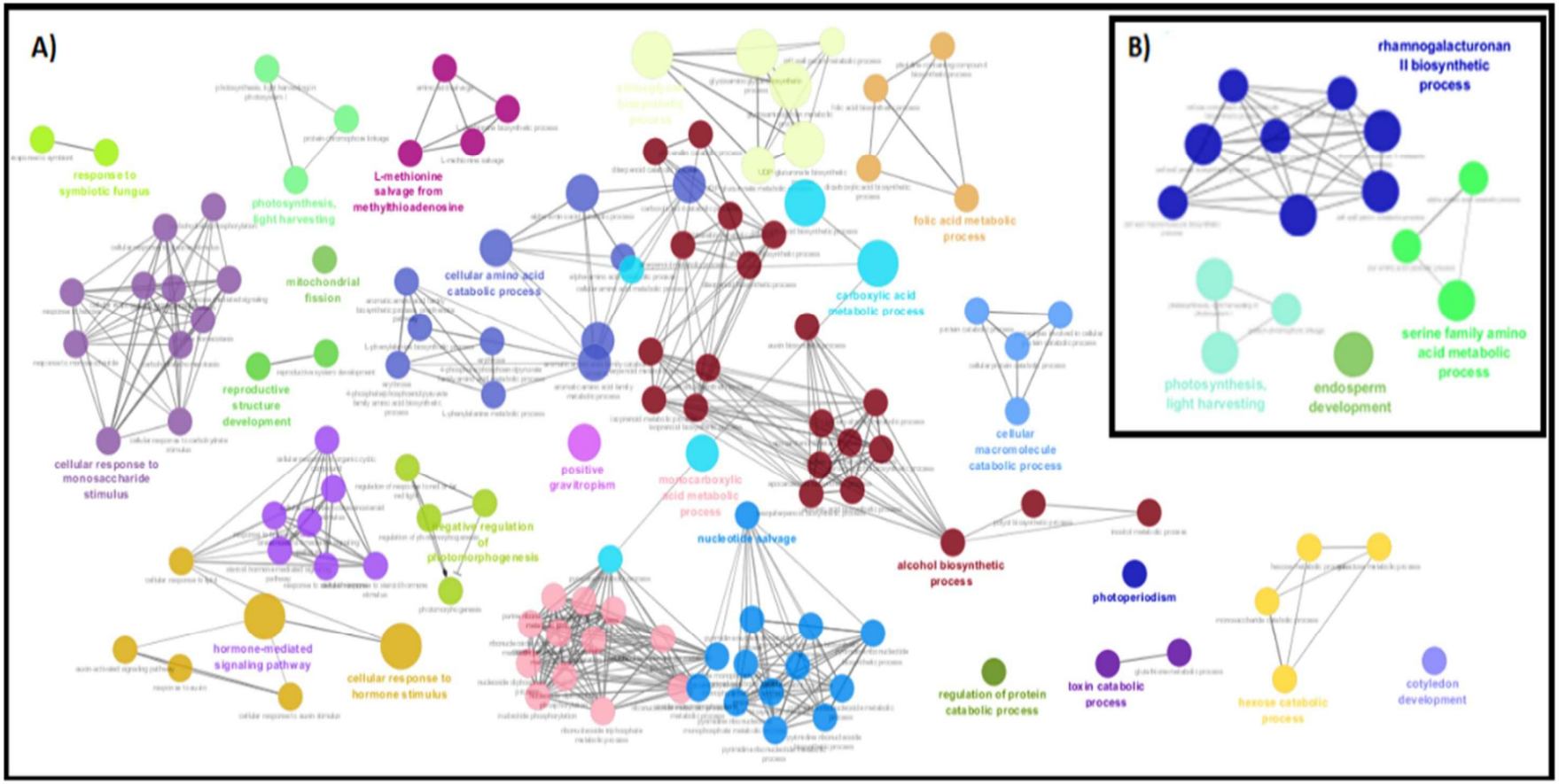

Figure 4. Over-representation analysis of up-regulated genes by using the Gene Ontology biological process database. A) Clusters containing up-regulated genes in the sensitive genotypeBR16. B) Clusters containing up-regulated genes in the tolerant genotype EMBRAPA 48. The size of the node represents the integration of genes and the thickness of the edge shows a significant Kappa value.

4. Discussion

The early events of plant responses to drought stress are signal perception and subsequent signal transduction, which lead to the activation of various molecular, biochemical and physiological changes (Rejeb et al. 2014; Joshi et al. 2016). With the availability of genomic sequences from various plant species and the recent advances in sequencing technologies, the genes involved in drought/dehydration responses have been identified in a number of plant species, such as Arabidopsis (De Oliveira et al. 2011; Borkotoky et al. 2013; Shariatipour and Heidari; 2018), and crops, such as rice and soybean (Prabha et al. 2011; Nakashima et al. 2014; Zhu et al. 2016; Sahebi et al. 2018). Thus, knowledge on gene expression reprogramming in response to drought stress has been obtained thoroughly. However, identifying the genes that contribute the most to the physiological and molecular adaptation mechanisms is a challenge.

In this study, we focused on two soybean genotypes that share a common ancestor (Davis genotype). The general response of Embrapa 48, when compared to the sensitive BR16, showed a very distinct physiological behavior (Oya et al. 2004; Carvalho et al. 2015) and a molecular response (Lima et al. 2019). The overriding feature observed in the gene expression response of BR16 genotype was the transcriptional induction of a relatively large number of genes. As this genotype is drought-sensitive, this large number of differentially expressed genes is in accordance with the other RNAseq studies in other plants (Yates et al. 2014; Fracasso et al. 2016; Yang et al. 2017), showing that sensitive plants dramatically reprogram gene expression under drought stress. This could be explained by the fact that sensitive species undergo greater changes in phenotype, physiological and biochemical properties when mitigating the effects of stress conditions. On the other hand, the drought tolerant genotype, Embrapa 48, showed a low alteration of gene expression under drought stress, as indicated by the notably lower number of identified DEGs. This general behavior was also observed in the proteomic and metabolomic data (Lima et al. 2019). Thus, the tolerance may reflect a lower level of stress when compared to BR16 and as a consequence result in a reduced reprogramming of the transcriptome (Fig. 1). These data corroborate with those obtained by Rodrigues et al. (2012), who used the Suppressive Subtractive Hybridization (SSH) technique to investigate differentially expressed genes under water deficit conditions in these genotypes. This

“more subtle” response is probably due to differently expressed genes observed in Embrapa 48, when comparing the genetic background among genotypes (Supplementary Information Fig. S3). The results suggest that mechanism of the drought tolerance involves genes that are expressed even before the onset of drought treatment, how positive regulation of cellular catabolyc process, organic cation transport, dicarboxilic acid biosynthetic process, diterpenoid biosynthetic process and responsive to far red light. Tyet these processes may be responsible for the highest photosynthetic rate, stomatal conductance and carboxylation, observed in this genotype even before stress (Mesquita et al. 2020), once an early report by Withrow et al, (1952) described red and far-red effects on pigment synthesis and Melis and Harvey (1981) in the increase the ratio of PSII/PSI. Transcriptome study performed by Janiak et. al. (2018) reveals that drought tolerance in barley may be attributed to stressed-like expression patterns that exist before the occurrence of stress. Still the results the in genetic background, revealed greater expression of genes related to cell wall biosynthesis.

The ability to tolerate a water deficit is a complex trait that could be controlled by many genes (Molina et al. 2008; Ergen and Budak, 2009). In this context, plant cells detect stress stimulus through sensors or receptors that activate second messengers and initiate the corresponding signaling pathways to transduce the signals (Bhargava and Sawant, 2013). In this study we focused on the gene expression patterns that were distinct between contrasting genotypes aiming to understand the physiological behavior and identify specific candidates that correlate with drought tolerance.

Genes that stimulate the plant to survive better in drought conditions play a role in the regulatory network of gene expression, including several kinase proteins and transcription factors. The higher levels of phytohormone ABA and proline were observed for the sensitive BR 16 in accordance with a more pronounced perturbation in the metabolic pathways under drought for this genotype (Lima et al. 2019). However, water potential the -1,0 Mpa, the genotype Embrapa 48 showed an less changed metabolism, with higher photosynthetic rate, less oxidative damage in leaves and greater root growth compared to the genotype BR 16, indicating different signaling and regulation of the metabolism of the soybean leaves (Mesquita et al. 2020). Three calcium-dependent protein kinases (CDPKs) were up-regulated, and mainly function in the abscisic acid (ABA) signaling pathway and are plant-specific calcium sensors that play important roles in various aspects of plant physiology (Yang et al. 2011;

Huang et al. 2012). Several works report the transcript levels of CDPKs are highly induced by drought, suggesting their important roles during abiotic stress responses in soybean (Hettenhausen et al. 2016), especially in the modulation of ABA signaling to reduce the reactive oxygen species (ROS) (Asano et al. 2012; Neto et al. 2013). Transgenic lines the Arabidopsis expressing WNK9 showed healthy phenotypes such as green leaves, achieved higher fresh weight and longer roots under drought stress as compared to wild-type (WT) (Manuka et al. 2018). Other kinases differentially expressed in the tolerant soybean Embrapa 48 have not been associated to drought response so far.

In recent years, a wide range of TF families holding relevance in drought stress response have been identified, such as AREB, DREB, MYB, WRKY, NAC, ZFP and bZIP (Golldack et al. 2011; Jin et al. 2014; Anbazhagan et al. 2015). Genes that encode C3HC4 and C2H2-type Zinc finger proteins were down-regulated in Embrapa 48 under drought stress (Table 2). Zhang et al. (2016) reported that the family C2H2-type Zinc finger protein negatively regulates the drought response in transgenic Arabidopsi, because the overexpressing plants these genes might lose large volumes of water by increasing the width/length and number of completely open stoma, leading to drought stress sensitivity. Most of the family CCCH-type zinc finger proteins have shown up-regulation, which has been associated with RNA metabolism by directly binding to RNA targets and have been involved in abiotic and biotic stresses. Studies have indicated that CCCH zinc finger proteins are associated with senescence delaying effect, and they can interact with ABA and drought response regulators (Jan et al. 2013; Bogamuwa and Jang, 2016; Chen et al. 2019). In summary, the higher expression of these CCCH- type zinc finger proteins and CDPKs genes in leaves of the tolerant cultivar may be responsible for the lower production of reactive oxygen species and, consequently, less cell damage, as observed in the ROS in leaf assay conducted by Mesquita et al. (2020).

Other TFs, such as the MYB and NAC, were also characterized for their role in stomatal movement controlling pore closure or in inhibiting its opening (Cominelli et al. 2010; Baldoni et al. 2015). Soybean MYB genes may contribute to the coordination of both cellulose and lignin biosynthesis in secondary wall formation. Yang et al. (2017) showed that plants engineered to accumulate less lignin or xylan are more tolerant to drought and activate drought responses faster than control plants. This is an important finding because it demonstrates that modification of cell walls must occur in the primary

wall, and the analyzes showed a low expression of secondary wall biosynthesis genes in the stress tolerant genotype. In addition, the soybean orthologue coding Cellulose Synthase-like D3 was up-regulated only in the sensitive genotype and not altered in tolerant Embrapa 48. Evidence suggests that C2H2 transcription factors are also involved in the secondary metabolism and cell wall structure (Rao and Dixon, 2018).

We found that some genes that code for proteins involved in cell wall dynamics were differentially expressed for both genotypes. Xyloglucan endotransglucosylase/ hydrolase (XTH) and Expansin are cell wall proteins involved in cell wall extension, which appear to be regulated by drought stress as observed for soybean genotypes. Some xyloglucan transferase and glycosidase were also responsive in both genotypes, however some proteins involved in pectin metabolism were up-regulated in the tolerant genotype Embrapa 48 (Table 3). The major group of polymers in primary dicot cell walls are pectins, a heterogeneous group of homogalacturonic acid, rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Mohnen, 2008). Pectins are often modified in plants exposed to drought, facilitating an increase in cell wall plasticity that can contribute to the maintenance of cell turgor or symplast volume (De Diego et al. 2013; Martínez et al. 2007). This plasticity can be correlated with drought tolerance mainly by increasing side chains of the pectic polymers rhamnogalacturonan I and II (RGI and RGII), possibly because the pectins form hydrated gels, which limit the damage to cells (Leucci et al. 2008). Despite its highly complex structure, RG-II is evolutionarily conserved in the plant kingdom as its present in the primary cell wall of all higher plants (O'neill et al. 1996; Kobayashi et al. 1996). RG-II biosynthesis is a complex process and it is involved in several glycosyltransferases (GTs). One α3-xylosyltransferase (α3-XylT), named RGXT, is able to transfer a xylose residue onto the fucose of the side chain. The Arabidopsis RGXT family has four members linked to RG-II synthesis (Egelund et al. 2006; Liu et al. 2011). Three soybean orthologous genes RGXT1, RGXT2 and RGXT3 were up-regulated only in the genotype Embrapa 48.

An increase in cell wall elasticity can contribute to the maintenance of cell turgor. These results are an indication that the cell wall molecular modifications on the tolerant genotype could contribute to a more efficient water usage observed in Embrapa 48. Only in drought-tolerant wheat genotypes, the side chains of rhamnogalacturonan I and II significantly increased in response to water stress (Leucci et al. 2008). The results confirm the role of the pectic side chains during water stress response. In

addition, in this study we also found a gene encoding for pectinesterase inhibitor differentially expressed between genotypes. Pectin is converted by the pectin methylesterase (PME) in pectate and methanol. PME activity is regulated by inhibitor proteins known as the pectin methylesterase inhibitor (PMEI), which plays a key role in wounding, osmotic stress, senescence and seed development. A gene coding for a Pectinesterase inhibitor 51 was down-regulated in tolerant Embrapa 48 and up-regulated in the sensitive genotype. These results indicate that the metabolism of pectin is differently modulated in response to drought in soybean and may play a role in the plants defense mechanism against desiccation, through the increase of elasticities and crosslink of the cell wall. Interestingly, the amount of side chains of RGI and/or RGII has been crucial to determine the hydration status of the cell wall matrix (Gall et al. 2015). The comparison with tolerant wheat genotypes indicates an increase in the amount of side chains during water stress, which consequently affects the viscosity status of the cell wall (Piro et al. 2003; Leucci et al. 2008; Gall et al. 2015).

5. Conclusion

Drought tolerance in plants is performed by different complex mechanisms, and to evaluate which genes contribute the most for this phenotype is a challenge, especially because an extensive gene reprogramming is activated. However, we have used two soybean parental genotypes to investigate the molecular responses under drought stress. Although many genes showed similar gene expression patterns in both genotypes, genes involved in the signal transduction cascades and regulation of gene expression, such as kinase of the family CDPKS and TFs of the family C2H2 and CCCH, were only differentially expressed in the drought tolerant genotype. Gene expression regulation analysis indicated that cell wall metabolism may be changed in this genotype and could be correlated with the more efficient water used. Remodeling of the pectin component of the cell wall may be an important mechanism that causes drought tolerance in the Embrapa 48 soybean genotype, contributing to the maintenance of cell turgor.

To conclude, our global transcriptomic study shows that drought tolerance result from expression profile of many drought response genes, which is operating even before the occurrence of stress and makes the plant ready to respond to adverse

environmental conditions, which reflects the lower genetic reprogramming when subjected to drought.

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Supplementary Information

Table S1. Candidate genes and characteristics of RT-qPCR primers.

GENE SYMBOL	GLYMA/GENE ID	PRIMER SEQUENCES (5′-3′)	AMPLICON LENGTH (BP)
UNK2 (Unknown 2) -endogenous	Glyma.06G03850.1	GCCTCTGGATACCTGCTCAAG ACCTCCTCCTCAAACTCCTCTG	79
ACTIN - endogenous	Glyma.18g52780.1	ATGTCGTGAGCCATCCTGTC ACACCGGATTCGTGCGGCAT	142
CP31B (Chloroplast RNA-binding protein 31B)	Glyma.05G02800.1	CCATGGGAAGTTGACGATG CATTGTGACAAAGCCAAAACC	123
Dehydrin	Glyma.09G185500.1	GGTAGACAGCATTCTAGTGG TACCATAGACACCGGTAGTT	115
LEA18	Glyma.17g164200.1	AAAGGCACAGAGTGATGAAT CTTGATGACCTTGTGTACCA	131
CA2 (Carbonic anhydrase)	Glyma.19G007700.1	GCTGTGAATGTTTCCCTTGG GATGCAAGGCCAAACTGAAG	146
RGXT1	Glyma08G09360.1	TTCACTCCCACTCCCACTTC GAGCCAGTTGTTGAGGAAGG	135
RGXT2	Glyma15G07400.1	TGGCCAAGGTGTTGATGTTA TATTGCGTAAGCCAGCTCCT	185
NPF4.7 (PROTEIN NRT1/ PTR FAMILY 4.7)	Glyma05G02990.1	AAGGTGACTGGTGCTTTTGG TTGGCCCAGAAGAGAAAATG	134

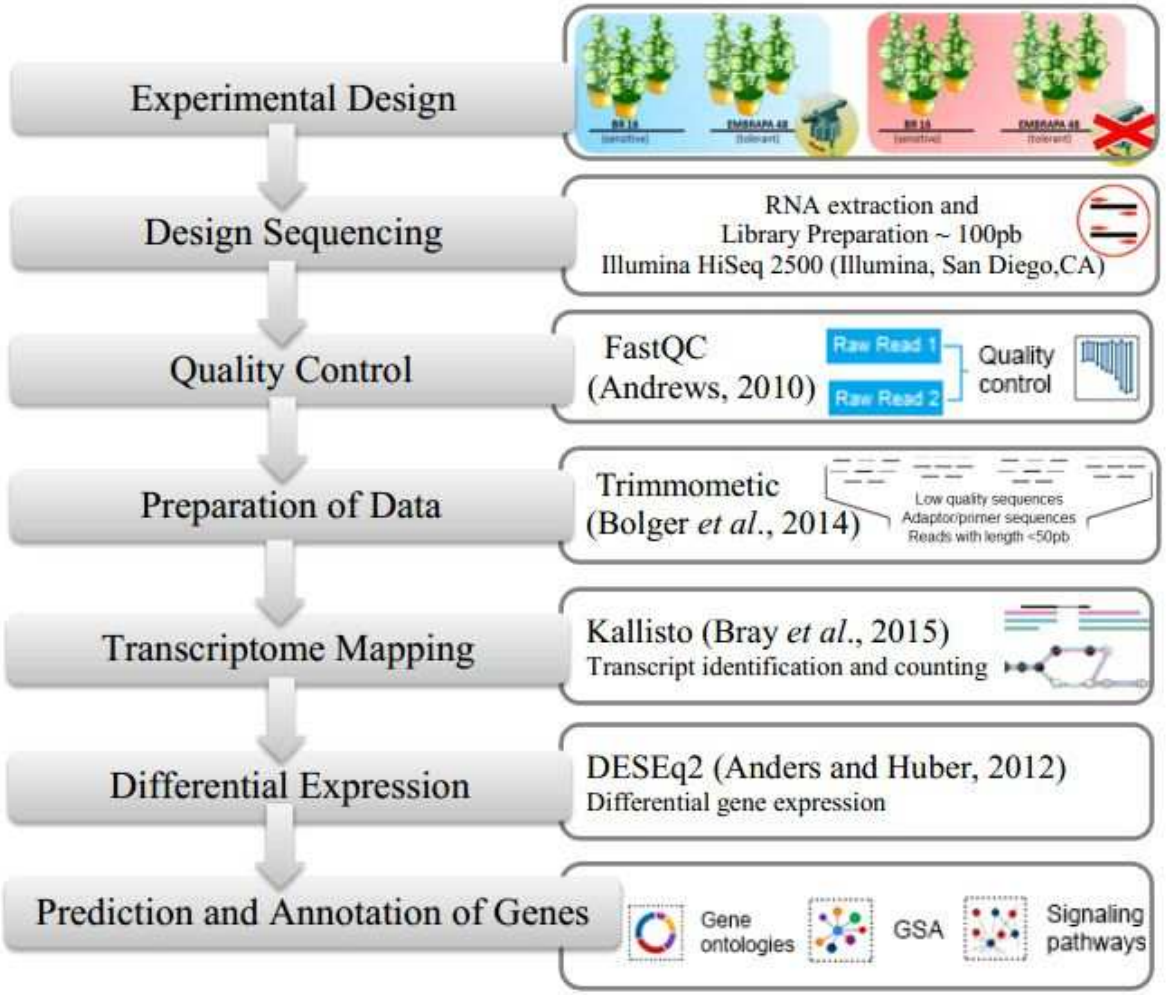

Figure S1. Overview of the workflow for analysis of RNA-Seq data. Transcriptomes of three biological replicates of two soybean genotypes, BR 16 and EMBRAPA 48, sensitive and tolerant to drought, respectively, were isolated from leaf tissue for and sequenced on the Illumina Hi-Seq 2500 (Illumina, San Diego, CA). The initial sample collected after water deficit was designated as "NI", and control plants "IR". Curated RNA sequence data was quality filtered using Trimmometic tool. High quality raw sequence reads were processed and pseudo aligned to soybean reference transcriptome using Kallisto and output collected was submitted to the DESEq2 for differential expression analysis. Finally, the differential genes expression was submitted to analyzes of prediction and annotation of genes

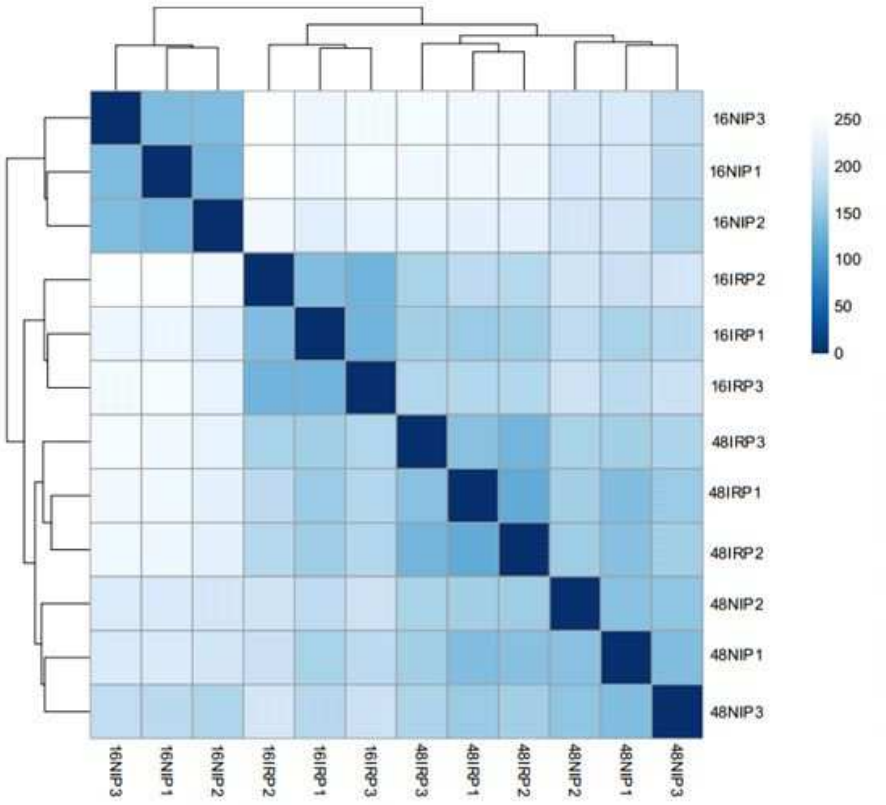

Figure S2. Heatmap generated with DeSeq2 software packages showing the Euclidean distances between the samples. 16IRP= genotype BR 16 irrigated, 16NIP= genotype BR 16 not irrigated, 48IRP= genotype Embrapa 48 irrigated, 48IRP= genotype Embrapa 48 not irrigated. The numbers represent the biological repetitions.

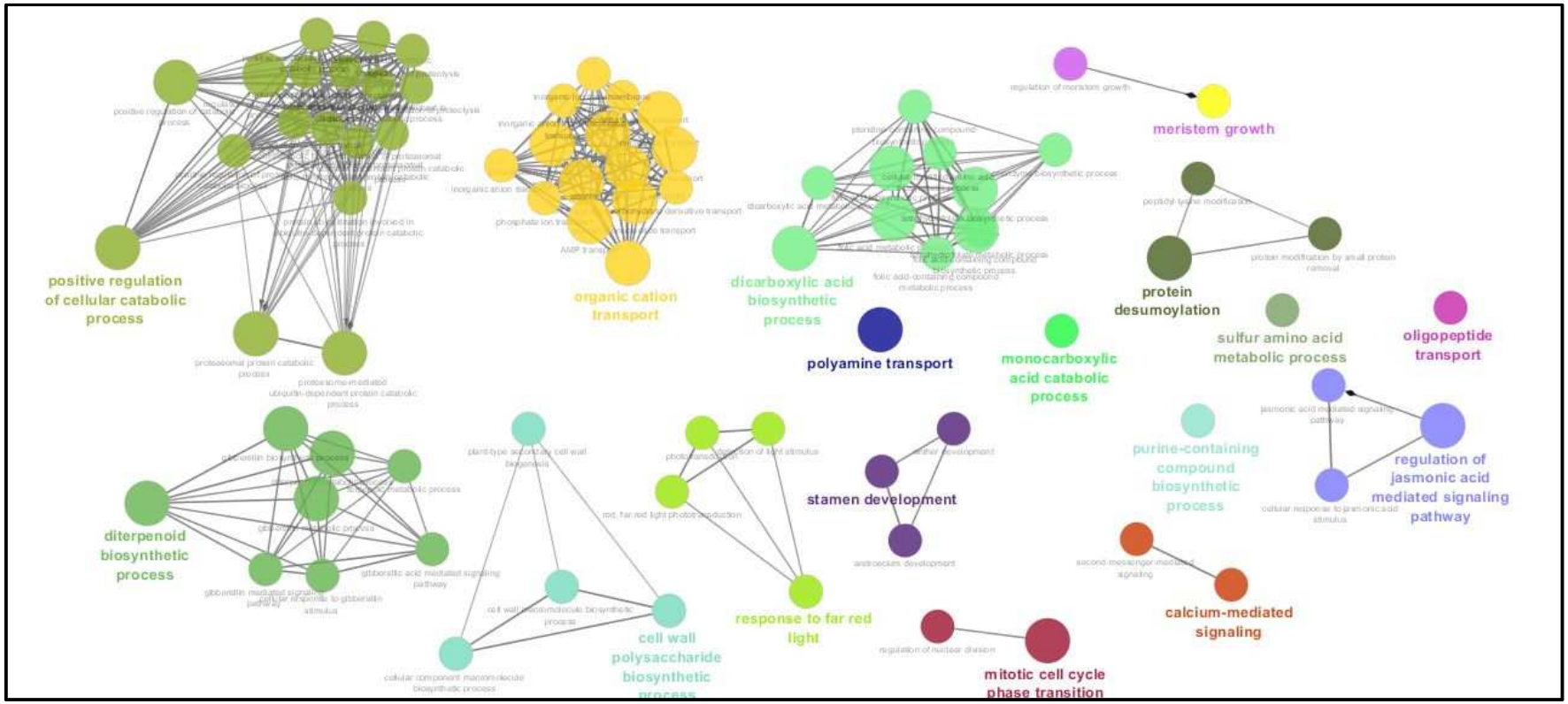

Figure S3. The over-representation analysis of up-regulated genes in EMBRAPA 48 genotype observed when studying the genetic background among genotypes by using the Gene Ontology biological process database. The size of the node represents the integration of genes and the thickness of the edge shows a significant Kappa value.

CHAPTER III

Understanding Water-Stress Responses in Soybean —A Systems Biology Perspective

Flaviane Silva Coutinho, Rosilene Oliveira Mesquita, Juliano Mendonça Rodrigues, Lucas Leal Lima, Verônica Aparecida Faustino, Camilo Elber Vital, Marcelo Ehlers Loureiro, Elizabeth Pacheco Batista Fontes, Humberto Josué de Oliveira Ramos. **Understanding Water-Stress Responses in Soybean —A Systems Biology Perspective**. Manuscript under construction.

Understanding Water-Stress Responses in Soybean —A Systems Biology Perspective

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Abstract

The variation in rainfall distribution and longer drought spells in much of the tropics during the main growing period of crops are becoming increasingly important yield-limiting factors. Even with the advent of high-throughput technologies, it is still challenging to understand all traits affecting a plant phenotype. Thus, using a strategy for reduce the complexity can certainly direct us toward the right candidates, if not completely help us to resolve the issue. The present work focuses in the physiological parameters and root proteome under drought conditions from two soybean genotypes, BR 16 and Embrapa 48. Along with an approach for integrative molecular physiology evaluating the transcriptome, proteome and metabolome of the Embrapa 48 genotype and connecting root and aerial parts, to understand networks required for building drought tolerance. The results revealed a greater capacity for water absorption by the root in the Embrapa 48 genotype, supposedly by due to root cell osmoprotection by accumulation of amino acids and its larger root volume. This event seems to increase the turgor pressure, that coupling with ABA signaling, helping in root expansion and optimizing the use and transport of water to aerial parts. In this way, the root of the Embrapa 48 genotype was less impacted by stress, showing efficient antioxidative mechanisms. The leaf protection mechanism seems to be related to cell wall remodeling and pectin expansion, thus could be the main genetic trait determinant the drought tolerance in the soybean Embrapa 48 genotype.

Keywords: Omic technologies, drought tolerance, metabolic adjustment.

1. Introduction

Climate changes foreshadow a increase in the intensity and duration of drought in major soybean production regions (Dai, 2013), which could cause crop failures and food shortages. This is because the drought stress can directly induce a wide range of injury symptoms in plants, such as the inhibition of photosynthesis, increased oxidative stress, changes in metabolism and a series of morphological adaptations (Yang et al. 2013; Du et al. 2020). Reduction of the photosynthesis also results in the shoot growth inhibition because of decreased cell proliferation (Lawlor and Tezara, 2009; Wu et al. 2018, Iqbal et. al. 2019).

Since crop growth largely depends on the ability of roots to explore the soil and absorb water and nutrients, restriction of the development of the root system can reduce crop yields if water and nutrients are limiting (Goldman et al. 1989; Trachsel et al. 2010; Kell, 2011). Root-to-shoot signaling is often considered to be important in regulating shoot growth and water use when soil conditions change (Dodd, 2005; Tardieu, 2016). Chemical signals are generally accompanied by increase in the concentrations of abscisic acid (ABA) in the root that result in oxidative damage by unnecessary production of reactive oxygen species (ROS) (Beis and Patakas, 2015). Otherwise, in the plasma membrane, the induction of hydrogen peroxide (H_2O_2) by ABA is an essential signaling event in modulating stomatal closure to decrease water loss through the activation of calcium-permeable channels (Pei et al. 2000). However, studies show that in the root tips, the accumulation of ABA increases towards the root apex (Saab et al. 1992) and is required for the maintenance primary-root elongation at low water potentials (Sharp et al. 2004; Yamaguchi and Sharp, 2010).

Several studies have provided strong evidence that root types either penetrating deep into the soil and attaining greater “root mass at depth” (Lopes et al. 2011; Wijewardana et al. 2019) or roots with large xylem diameters and/or larger lateral root systems with more root hairs are advantageous under drought conditions (Tanaka et al. 2014; Vadez, 2014, Du et al. 2020). In addition, Du et al. (2020) observed that increment of sucrose and starch in soybean roots contributed to the increased root/shoot ratio in plants that were under drought stress, seeming to be this the mechanism for maintaining root growth and metabolism in response to drought stress (Baldwin et al. 2013; Thitisaksakul et al. 2017).

Although the roots are important sensors of drought, the responses of this organ and its role played in the tolerance in Embrapa 48 genotype remain poorly characterized. Therefore, research on the changes in the leaf and root ratio in soybean under drought stress is important to further understand adaptability of genotype to drought. Once tolerance not depend only on assimilating source, but also on the pathways established by structural growth (Reddy et al. 2017), a comprehensive study of root morphological parameters and physiological and gas exchange traits for to understand the overall performance of the plant is important. Systems biology, which has been revolutionized by genome sequencing and omic technologies, combined with physiological traits, allows us to obtain a holistic overview of processes and reactions of an organism in response to environmental perturbations (Fukushima et al. 2009; Weckwerth, 2011).

Thus, the objective of this study was in the analyse physiological parameters and root proteome in dry conditions in two soybean genotypes, BR16 and Embrapa 48, sensitive and tolerant to stress, respectively, along with an data integration of physiology, transcriptome, proteome and metabolome of Embrapa 48 genotype considering connection in between root and aerial parts. The results suggest that the stress tolerance through may be associated with increased osmoprotectors accumulation in roots, allowing to obtain a higher relative water content in this organ, which can increases turgor pressure, efficiency in the use of water and keeps the leaves in better photosynthetic functioning.

2. Materials and Methods

2.1. Experimental Condition and Plant Culture

Seeds of soybean genotypes BR 16 and Embrapa 48 were obtained from the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA SOJA, Londrina, Paraná). Seedlings were grown in plastic trays containing Plantmax® commercial substrate, where they remained for 10 days. After germination, seedlings were transplanted to pots containing 10 L of a mixture of soil, sand and manure (2:1:1) each. Plants were grown under natural sunlight in a greenhouse with average daytime temperature 15–35°C and relative humidity 65–85%.

The plants were grown under normal water conditions until reaching the development stage V3 (fully expanded third trifoliate). The control plants were watered

daily with approximately 30 mL water per plant. The plants were exposed to a slow drying soil treatment, which consisted of a reduction in irrigation to 40% of the daily normal (Valente et al. 2009). The hydric regimes were assigned as irrigated (IR) and non-irrigated (NI), respectively. The leaf water potential (ψw) was measured in the third emerging trifoliate at dawn by using a Scholander pump (Scholander et al. 1965) during the stress period. Samples were collected in liquid nitrogen when the plants had a water potential - 1 MPa and then stored at -80°C until use.

2.2. Measurement of the relative water content (RWC) of leaves and roots

The fresh weight (FW) of leaf discs or roots was measured immediately after they were removed from the stem. Then, tissues were incubated in distilled water for at least 4 h in the dark, and the turgid weight (TW) was measured. Finally, dry weight (DW) was measured after incubation at 85°C until the sample reached a constant weight in the oven. The relative water content (RWC) was calculated using the equation: RWC (%) = [(FW-DW)/(TW-DW)] *100.

2.3. Determination of soil moisture

Moisture content determination in soil was performed by the method oven-drying method is based on removing soil moisture by oven-drying a soil sample until the weight remains constant. The moisture content (%) were calculated from the sample weight before (Pt) and after drying (Ps), using the equation: Pa= Pt-Ps and h(%)= (Pa/Ps)*100.

2.4. Analysis of abscisic acid (ABA)

Root samples were lyophilized and subsequently ~10 mg fresh weight was mixed with methanol: isopropyl alcohol: acetic acid (20: 79: 1).The hormones were extracted according to the methodology described by Mu¨ller and Munne´-Bosch (2011) with modifications. The samples were mixed in vortex 4 times for 20 s (on ice), sonicated for 5 min, placed on ice for 30 min and then centrifuged at 13,0009 g for 10 min at 4°C. After centrifugation, 350 µL of supernatant were removed and transferred to a new tube. The process was repeated with the resulting pellet and then the

supernatants were pooled. Approximately 300 µL of the extracts were placed in vials and 5.0 µL was injected into the LC/MS system at NuBioMol (Center for Biomolecules Analysis – UFV, Brazil). We used a chromatography column (Agilent Eclipse Plus, RRHD, 1.8 um, 2.1x50 mm) with a flow rate of 0.3 mL/min, coupled online to a mass spectrometer QQQ triple quadrupole Agilent 6430. Three biological replications were performed for LC/MS analyzes. The data were processed using the MassHunter platform (Agilent). A standard curve of each hormones, in a concentration range of 0.1 to 300 ng/mL, was used to convert the area values from XIC in ng/g of plant tissue (Supplementary Table S1).

2.5. Extraction of total protein

The samples were prepared by pooling roots of three plants. The roots was powdered in liquid nitrogen and approximately 1,5 g were suspended in 5 mL of ice-cold buffer [2% (v/v) b-mercaptoethanol, 40mM Tris-HCl pH7,5, 10mM EDTA, 250 mM sucrose, 1% (v/v) Triton-X,1 mM phenylmethylsulfonylfluoride, 50µL protease and phosphatase inhibitor (Sigma P9599 and P9455) and 1% (w/v) polyvinylpolypyrrolidonein]. Protein extration were performed as described by Mesquita et al. (2012).

Protein quantification was performed by the Bradford method with a bovine serum albumin standard to ensure a protein concentration between 1 and 15 mg/mL (Bradford 1976).

2.6. Separation of proteins by two-dimensional gel electrophoresis

The isoelectric focusing (IEF) was performed in 24 cm IPG strip pH 3–10 (GE Healthcare). Initially, the strips were rehydrated for 16 h in 450 µL of rehydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.002% (w/v) bromophenol blue, 2% IPG-buffer, 0.2% (w/v) DTT] containing 1.0 mg protein for each sample. The protein extracts were separated in the first dimension at 20 °C using an IPGphor3 isoelectric focusing system (GE Healthcare). The voltage settings for IEF was 200 V for 2 h, 500 V for 2 h, 800 V gradient to 1.000 V, 16.500 V gradient to 10.000 V, and 22.000 V in a single step of 10.000V. Following electrophoresis, the protein in the strips was reduced with equilibration buffer [50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 4% (w/v)

SDS, 0.002% (w/v) bromophenol blue, 1% (w/v) DTT] and then alkylated by incubation with the same buffer containing 2.5% (w/v) iodoacetamide instead of DTT for 20 min at room temperature. The second-dimension electrophoresis was performed on a 12,5% gel using a DaltSix electrophoresis unit (GE Healthcare). Gels were stained with Coomassie Blue G-250 solution [8% (w/v) ammonium sulfate, 0.8% (v/v) phosphoric acid, 0.08% (w/v) Coomassie Blue G-250 and 20% (v/v) methanol.

The 2DE gels were scanned using ImageScanner III scanner and LabScan 6.0 software (GE Healthcare). The protein profiles of the irrigated and non-irrigated treatments for both genotypes were compared using ImageMaster2D Platinum 7 software (GE Healthcare) for the detection of differentially abundant protein spots. As threshold, the proteins were considered as differentially expressed, if they fitted the criteria: (i) a measured overlay variation above 1.5 (ratio normalization), (ii) ANOVA with p value less than 0.05 and (iii) presence in the three gels (biological replicates).

2.7. In-gel digestion and mass spectrometry for the protein identification

Protein spots were manually excised from the gel and subjected to in-gel trypsin digestion according to Shevchenko et al. (2007). The peptides were analyzed using the AB SCIEX TOF/TOF 5800 (Applied Biosystem). An aliquot of 0.3 µL of the solution with the sample (resuspension in 10 mL of acetonitrile 50% in trifluoroacetic acid 0.1%) was applied to a polished steel dish at a ratio of 1:1 in relation to the α-cyano-4-hydroxycinnamic acid matrix. The MS1 data were acquired through the reflective method in the positive mode, with a detection range of 500 to 5000 Da. After obtaining the masses of the triptych fragments, the fifteen most intense peptides were automatically selected and submitted to analysis by MS/MS. Fragmentation was performed using the post-source decay method.

Protein identification was performed using MS/MS ion search method by Mascot server version 2.2.07 (Matrix Science, 2012). The searches were carried out against NCBI (National Center for Biotechnology Information) Viridiplantae and Phytozome *Glycine max* protein databases. The parameters used: methionine oxidation as a variable modification, cysteine carbamidomethylation as a fixed modification, one missed cleavage, trypsin-like cleavage enzyme and a mass error of 0.1 Da. The Scaffold software, version 3.6.4 (Proteome Software INc., Portland, OR) was used to validate Mascot results by applying the Peptide Prophet (Keller et al. 2002) and the

Protein Prophet (Nesvizhskii et al. 2003) algorithms. The proteins were statistically validated when the probability of identity was equal or above 95 %.

2.8. Starch and sugars content

Leaf and root samples were lyophilized and subsequently 10 mg of powder was used. Then, 1.5 mL of extraction solution (1:2.5:1 water, methanol, chloroform) were added. The samples were shaken in Thermomix for 30 min at 4 °C, centrifuged at 14,000 x *g* for 5 min and the supernatant was collected. Then, 750 µL of water were added and the samples were vortexed, followed by centrifugation at 14,000 x *g* for 5 min. An aliquot of 200 µL of the supernatant was transferred to a new tube and dried by vacuum centrifugation and eluted in ultra pure water. From this extract the sucrose, glucose and fructose contents were determined enzymatically in an ELISA plate reader. In the insoluble fraction, derived from precipitated material in the extraction of metabolic profile, starch contents were analyzed (Praxedes et al. 2006).

2.9. Statistical analysis

The experimental design was completely randomized, in a factorial arrangement 2 x2, with the first factor corresponding to the plant water potential and the second constituted by the two different genotypes, with five repetitions. Data were submitted to analysis of variance (ANOVA), and a Tukey averages test at 5 % probability using the Assistat® software.

3. Results

3.1. Effect of water stress on plant and soil

The water deficit regime resulted in a progressive decline in the leaf water potential (ψw), leading to changes in the leaf turgor from both genotypes; as for the irrigated plants, the water potential was higher than -0.2 MPa throughout the experiment. While the BR 16 genotype reached ψw= -1.0 MPa at 14-day, the Embrapa 48 genotype displayed values close to ψw= -0.5 MPa during the same period of water deficit, reaching the potential of ψw= -1.0 MPa only in the 16-day after stress (Supplementary Fig. S1).

Fourteen days after of reduction in the water supply, visual differences in the leaf turgor between in genotypes BR 16 and Embrapa 48 were registered (Fig. 1A). The leaf relative water contents (RWC) decreased with the progression of the stress in both genotypes (Fig. 1B). In the leaves ψw = -1 MPa, the RWC in BR16 was 60.61% while of the Embrapa genotype, the in RWC was 68.05% (Fig. 1B).

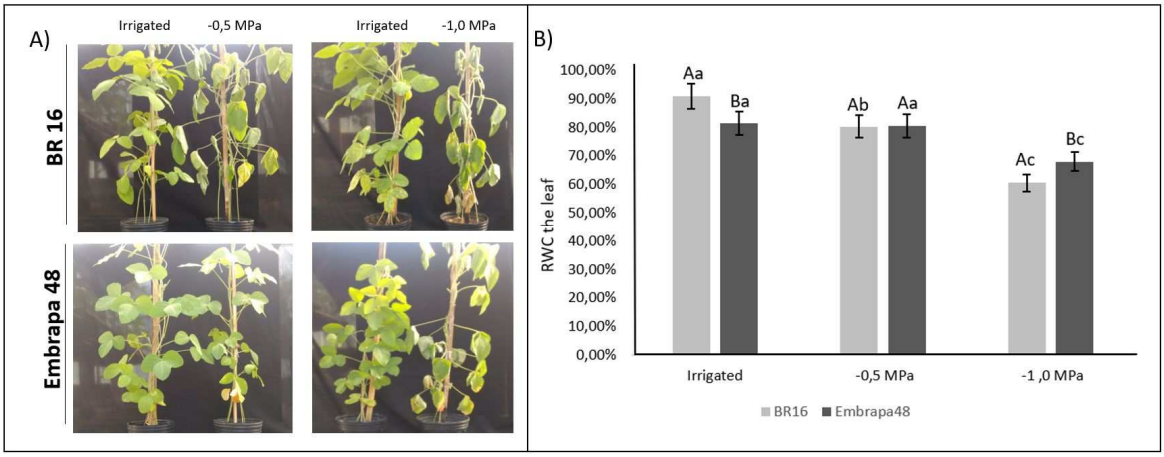

Figure 1. Overview of the plants at the end of the experiment. (A) Plants in the three levels of irrigation; (B) Relative water content RWC (%) of the soybean leaves from the BR 16 and Embrapa 48 genotypes under different water potential. Bars represent mean ± SE. Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments (Tukey, $p < 0.05$).

Under drought stress there was a decrease in root growth in both genotypes, however, the tolerant genotype presented greater length (Fig. 2A). Furthermore, the RWC of roots decreased with the progression of the stress only in BR16 genotype. The RWC in BR 16 roots went from 85,4% in irrigated condition to 71,09% in drought condition (Fig. 2B). Interestingly, drought stress increased the RWC in the tolerant genotype, going from 84.44% under irrigated conditions and going to 91.69% under stressed conditions (Fig. 2B). These results are consistent with a better water absorption efficiency in the Embrapa 48 genotype, when evaluating the the soil moisture contents (h) in the Figure 2C. Under irrigated conditions the Embrapa 48 genotype soil presented h = 49.94% while the BR16 genotype soil h = 65.20% (Fig. 2C).

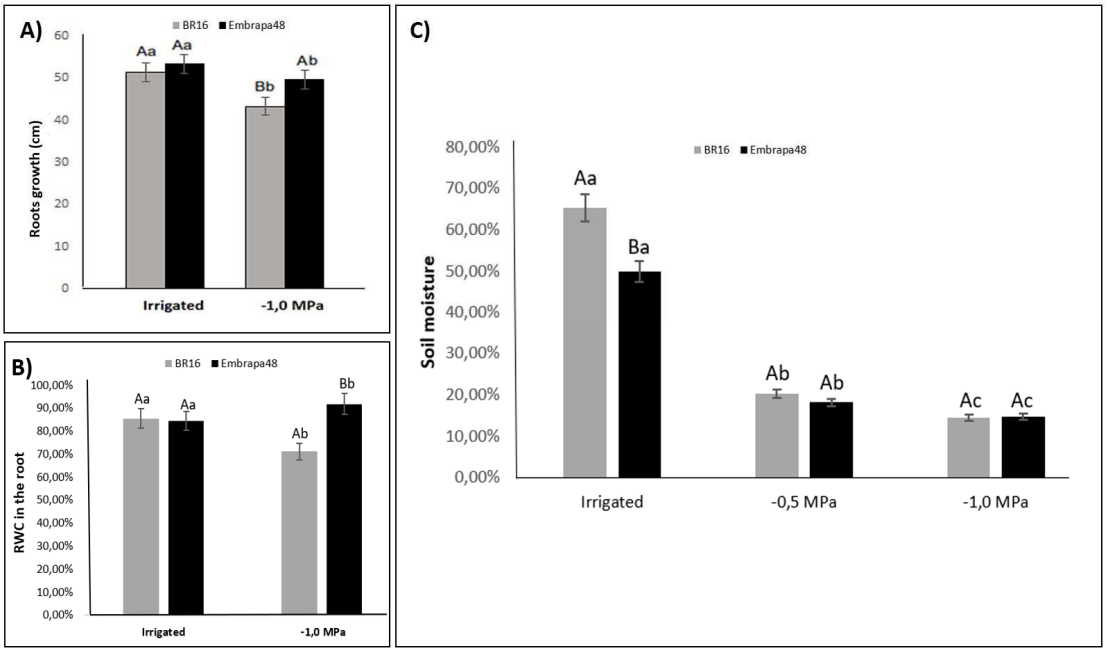

Figure 2. Overview of the roots plants at the end of the experiment. (A) Root system growth of genotypes under deficit; (B) Relative water content (RWC %) of the soybean roots from the BR 16 and Embrapa 48 genotypes. (C) Soil moisture contents (h%) of the soybean roots from the BR 16 and Embrapa 48 genotypes in different water potential. Bars represent mean ± SE. Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments (Tukey, $p < 0.05$).

3.2. Accumulation of ABA in plant roots

The concentration of the phytohormones ABA was determined by mass spectrometry coupled to liquid chromatography. The concentration of ABA in the roots of both genotypes increased in response to drought (Fig. 3).

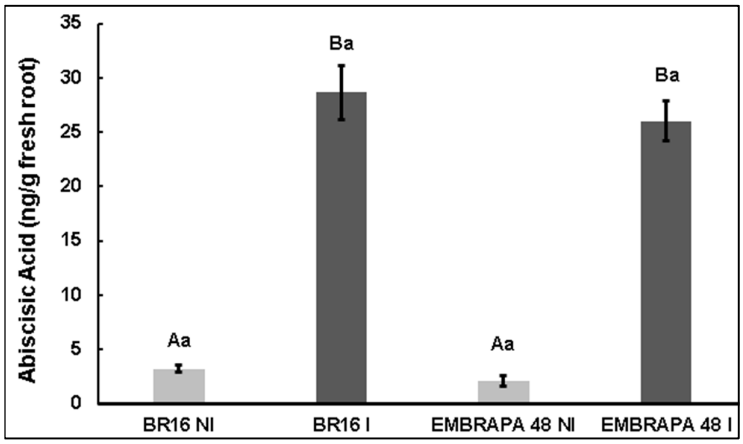

Figure 3. Absolute abundance of the phytohormone Abscisic Acid (ABA) of the soybean roots quantified by LC MS. The treatments were irrigated, under moderate drought stress (−1.0 MPa). Each bar represents the mean ± standard error (n = 3, where n represents the number of plants). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments (Tukey, $p < 0.05$).

3.3. Proteomics: identification of differentially abundance proteins by drought conditions

Protein expression profiles were generated by 2-DE from drought -sensitive and tolerant soybean plant roots. As the genotypes have different genetic backgrounds, contrasts were performed between them under irrigation conditions for diagnosis of genetic background differences. Subsequently, the tolerant genotype was compared for proteins expressed in drought conditions in order to understand the responses of the tolerant genotype and to provide information about as the roots contribute for the tolerance of this genotype.

In the absence of stress, the roots of the tolerant genotype Embrapa 48, compared to the sensitive genotype BR 16, presented a total of 32 differentially abundance spots, of which observed an increase in relative abundance in 19 spots and a reduction in 13 spots (Supplementary Table S2). Of these 32 spots identified, 25 corresponded to different proteins, as some possible isoforms or modifications of the same protein, represented by more than one spot in the same gel were found (Fig. 4). Relative abundances were normalized by the sensitive genotype, attributing the value 1.0 for abundance levels of this genotype, thus abundance above and below 1.0 were considerated up and down-regulated, respectively. The up-regulated proteins of the genotype Embrapa 48 share functional clusters related to mainly nitrogen and

antioxidant metabolism (Fig. 4). Highlighted are the leghemoglobin (LGB), peroxisomal betaine-aldehyde dehydrogenase (BADH) and nucleoside diphosphate kinase (NDPK) proteins, for antioxidant metabolism, and format dehydrogenase (FDH) for nitrogen metabolism (Fig. 4).

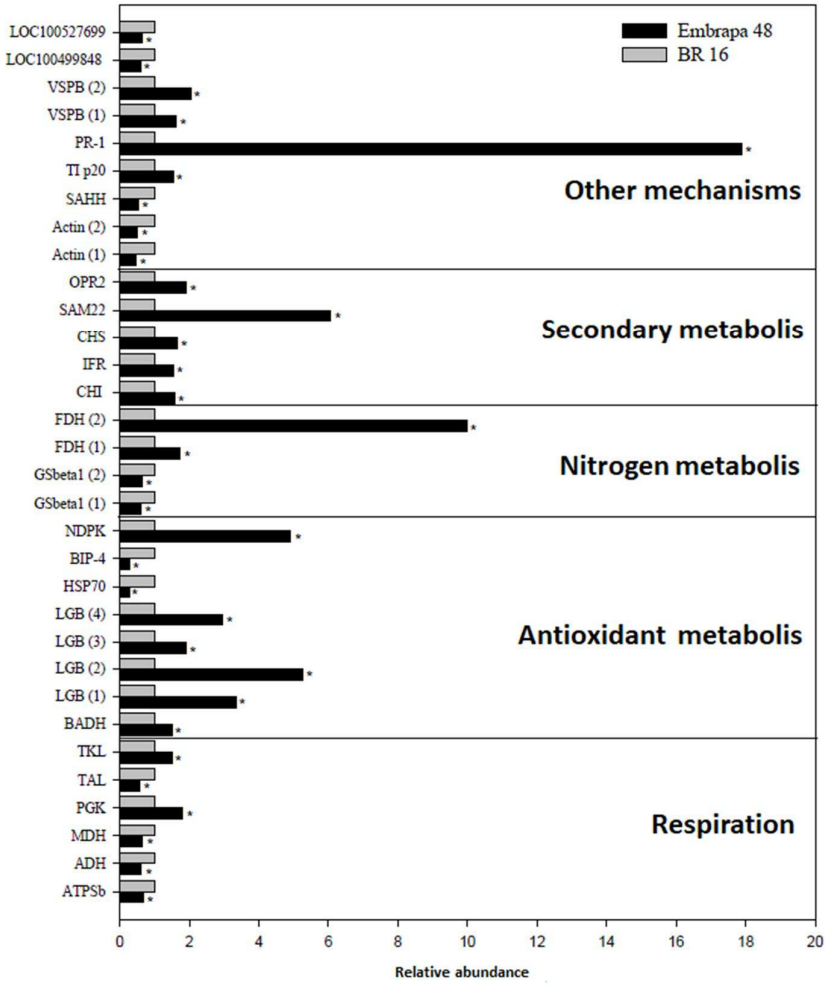

Figure 4. Relative abundance of differentially proteins in the irrigated condition (control). Relative abundance normalized by the sensitive genotype (BR16). Leghemoglobin (LGB), ATP mitochondrial synthase (ATPSb), Alcohol dehydrogenase (ADH), Malate dehydrogenase (MDH), Phosphoglycerate kinase (PGK), Transaldolase (TAL), Transketolase (TKL), peroxisomal betaine-aldehyde dehydrogenase (BADH), 70 kDa (HSP70), 4-like Luminal-binding protein (BIP-4), Nucleoside diphosphate kinase (NDPK), Glutamine synthetase (GSbeta1), Format dehydrogenase (FDH), Chalcona isomerase (CHI), Isoflavone reductase (IFR), NAD (P) H-dependent 6'-deoxychalcone synthase (CHS), S-adenosylmethionine synthase (SAM22), 2-like 12-oxophytodienoate reductase (OPR2), Actin (Actin), S-adenosyl-L-homocysteine hydrolase, partial (SAHH), Trypsin inhibitor p20 precursor (TI p20), Protein related to pathogenesis (PR-1), Precursor of the reserve glycoprotein of 31 kDa (VSPB), Not Known (LOC). (*) indicates increase or decrease in % volume (P <0.05).

The analyzes carried out for the differential proteome of proteins responsive to water deficit in the tolerant genotype showed 21 proteins with differential abundance in relation to the control. Of these, 6 were down and 15 up-regulated in response to water deficit (Fig. 5 and Supplementary Table S3). It is observed, by the functional distribution, that proteins heat shock 70kDa (HSP70), luminal-binding protein 4-like (BIP-4), peroxisomal betaine-aldehyde dehydrogenase (BADH), glutationa S transferase (GST-8), involved in the antioxidant metabolism, were up-regulated in the stress condition. Just like proteins glutamina sintetase (GSbeta1), aminopeptidase (PEPA), cobalaminindependent synthase family protein (MetE), involved in the nitrogen metabolism. The Embrapa 48 genotype also showed an increase in proteins involved in cellular respiration, such as ATP sintase mitocondrial (ATPSb), malato desidrogenase (MDH), triose fosfato isomerase (TPI), peroxisomal voltage-dependent anion-selective channel protein (VDAC), when subjected to water stress.

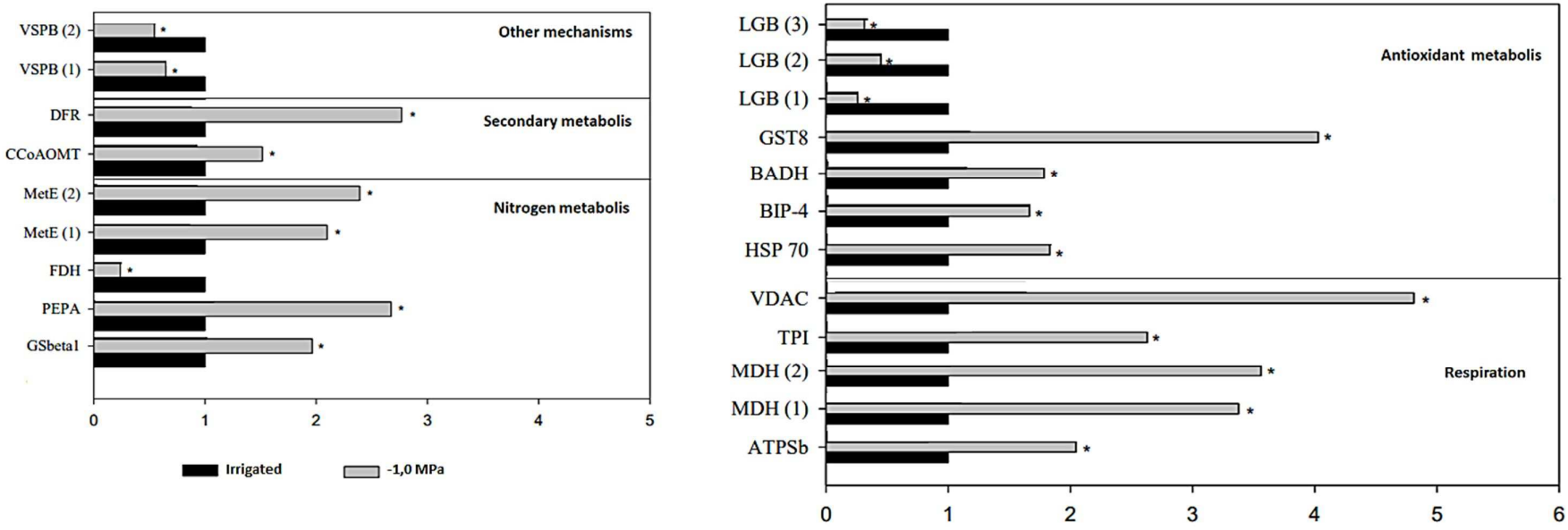

Figure 5. Relative expression of differentially expressed proteins responsive to moderate water deficits (-1.0 MPa) in Embrapa 48. Glutamina sintetase (GSbeta1), aminopeptidase (PEPA), Formato desidrogenase (FDH), Cobalaminindependent synthase family protein (MetE), Cafeoil-CoA metiltransferase (CCoOAMT), Dihydroflavonol-4- reductase-like (DFR), Precursor of the reserve glycoprotein (VSPB). ATP sintase mitocondrial (ATPSb), Malato desidrogenase (MDH), Triose fosfato isomerase (TPI), Peroxisomal voltage-dependent anion-selective channel protein (VDAC), Heat shock 70kDa (HSP70), Luminal-binding protein 4-like (BIP-4), Peroxisomal betaine-aldehyde dehydrogenase (BADH), Glutationa S transferase (GST-8), Leghemoglobina (LGB). (*) indicates increase or decrease in % volume (P <0.05).

3.4. Effect of drought stress on soluble sugar, sucrose, and starch content in leaves and roots

To investigate the effects of drought stress on the carbon distribution in leaves and roots of soybean, we measured the contents of glucose, fructose, sucrose, and starch in soybean under drought stress. The glucose, fructose and sucrose contents in leaves the Embrapa 48 genotype slightly increased with under the stress conditions (Fig. 6A, 6C and 6E). However, drought stress induce a significantly increased of these sugars in leaves the BR16 genotype compared with the control. The content of soluble sugar in the roots, also increased with stress in both varieties, however the BR16 genotype the magnitudes were lower than in the leaves. For tolerant genotype the effects of drought stress on the soluble sugar contents in roots were pretty much the same as in leaves (Fig. 6A-F). In all case the levels were higher in sensitive genotype, thus these metabolite do not act as osmoprotectors.

The starch content of soybean leaves was significantly decreased under drought stress, especially in the BR16 genotype (Fig. 7A). For starch in roots, we did not observe any alteration under stress, but it the concentrations was superior in genotype BR 16 in all treatments (Fig. 7B). Notably, the starch content in leaves was more affected than in roots under drought stress.

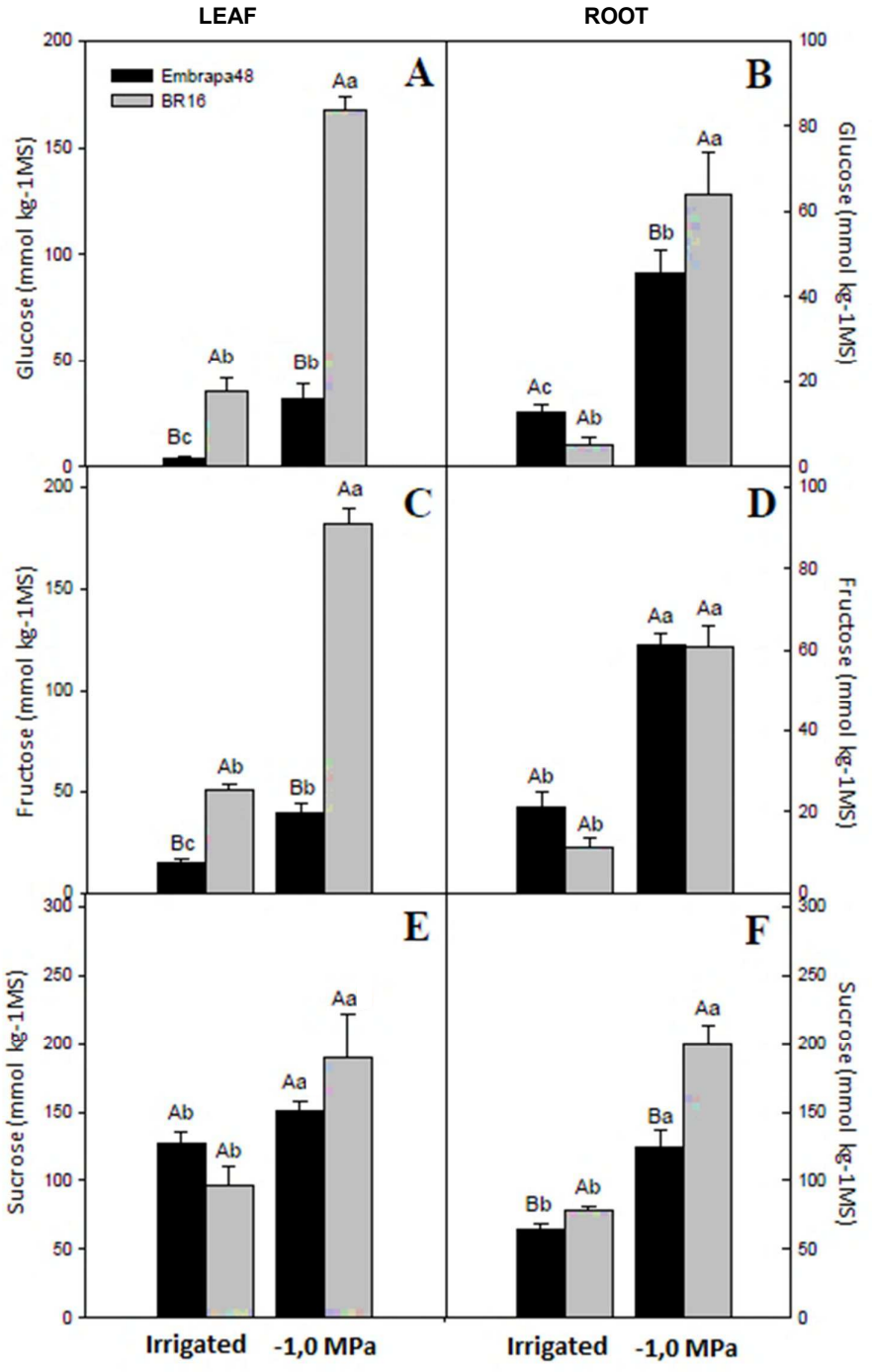

Figure 6. Effect of drought stress on soluble sugar glucose, fructose and sucrose contents. In(A), (C) and (E) for leaf content. In (B), (D) and (F) for root content.in soybean genotypes under water deficit. Each bar represents the mean ± standard error (n = 3, where n represents the number of plants). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments (Tukey, $p < 0.05$).

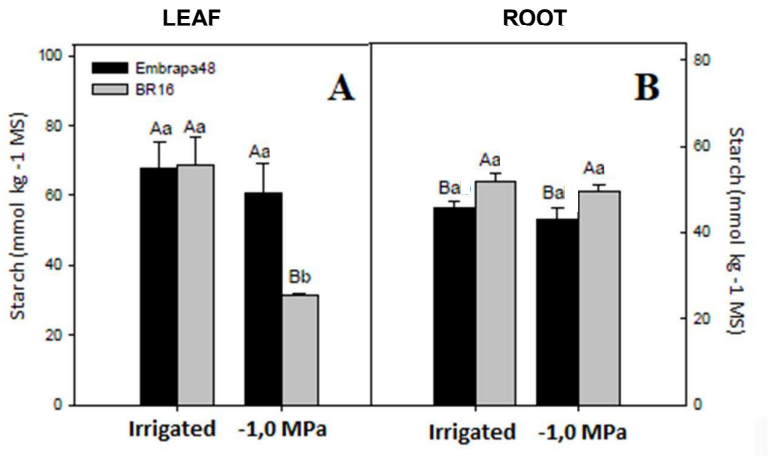

Figure 7. Effect of drought stress on starch contents**.** Leaf (A) and root (B) starch contents in soybean genotypes under water deficit. Each bar represents the mean ± standard error (n = 3, where n represents the number of plants). Different capital letters indicate significant differences between averages of the same treatment in different genotypes, and lower case letters show significant differences between averages within the same genotype under different treatments (Tukey, p <0.05).

4. Discussion

Indicate the genetic and physiological traits which are determinant for drought tolerance is a great challenge, because the plants respond by expression of several genes and showed alteration in behavior, morphology and metabolism of the leaves as well as roots. Thus, the combination of different approaches and experimental data is necessary, however when it is attained the new challenge is apply a functional integrative interpretation, providing the mechanism molecular as well as indicate the genes and pathways target for genetic breeding. To achieve these goals and overcome these challenges, we have performed several evaluations, belong the omics and the molecular physiology, of the response between two contrasting genotypes for drought tolerance. The genotypes BR 16 and Embrapa 48 share a common ancestor and were studied by Oya et. al. (2004) and Carvalho et al. (2015) under drought conditions. They found that at the vegetative stage, the tolerant genotype (Embrapa 48) showed a higher growth rate and a larger leaf area when compared to the sensitive BR 16 plants. The drought tolerance was also confirmed by desiccation curves evaluating the water potentials (Lima et al., 2019; Mesquita et al., 2020) and was confirmed in this study (Supplementary Fig. S1). We showed here that this delay in leaf dehydration in the tolerant genotype, when comparing at the same Ψw, was at least partially due to the

maintenance of the leaf turgor for a longer period and of a better water-use efficiency, because the RWC was higher than in BR16 leaves (Fig. 1).

Drought tolerance in plants is due to many complex mechanisms and to evaluate which is the most significant for this phenotype is also challenging because an extensive gene reprogramming is activated. Thus, a physiological and molecular analysis of the contrasting genotypes, showing a genetic background, could also be an efficient approach. Futhermore, the mechanisms of drought tolerance associated with major physiological characteristics not only occur in the aerial part but also in the root (Sharp et al. 2004; Manavalan et al. 2009). Root morphology and development are key traits correlating with mechanisms of drought resistance. Although the roots directly sense soil water deficit, we found that drought stress increased RWC in roots of the tolerant genotype (Fig. 2B). Soil moisture content variations between both genotypes could mark the differences in their root system architecture. The water content in the soil in the genotype Embrapa 48 was lower in relation to genotype BR16, during all irrigation conditions (Fig. 2C), indicating that plants of Embrapa 48 are able to absorb water more strongly retained in the soil, which can mean greater drought tolerance. These mechanisms of water accumulation in the roots may come from a metabolites accumulation important in osmotic adjustments and osmoprotectants in roots, widely regarded as an adaption for plants to maintain cell turgo (Li et al. 2015). Those provides activation of signal molecules that are important mediators of shoot physiological processes, and may be responsible for maintaining in leaf water potential in the tolerant genotype during stress besides allowing greater CO_2 fixation, photosynthetic rate and water-use efficiency (Mesquita et al. 2020).

In addition, studies reveal that chemical signals are produced in the root zone sensing the stress and culminating in the increased concentrations of ABA as observed for both genotypes. This signal in the shoots could induce the reduction of stomatal opening, transpiration rate and oxidative damage by unnecessary production of ROS, finally reducing shoot growth (Beis and Patakas, 2015; Tardieu et al. 2016). However, this behavior was only observed for sensitive genotype BR16.

In Embrapa 48 genotype, the results suggest that ABA in the roots may be inducing ABA dependent cascades increasing the root respiration, as suggested by greater abundance of proteins involved in this process (Fig 5), to allow relative root growth even under drought. In fact, maintenance of root elongation in the root apex is mainly achieved by three potential mechanisms: osmotic adjustment, modification of

cell-wall extension and the accumulation of ABA (Sharp et al. 2004; Yamaguchi and Sharp 2010). In the root tips, the accumulation of ABA increases towards the root apex and is required for the maintenance of primary-root elongation at low water potentials (Saab et al. 1992; Sharp et al. 2004; Yamaguchi and Sharp 2010; Rosales et al. 2019; Dowd et al. 2019).

Interestingly, the protein background among genotypes revealed the greater abundance of antioxidant proteins in the tolerant genotype, among which stand out the leghemoglobin (LGB), peroxisomal betaine-aldehyde dehydrogenase (BADH), and nucleoside diphosphate kinase (NDPK) (Fig. 4). With stress, heat shock 70kDa proteins (HSP70), luminal-binding protein 4-like (BIP-4), peroxisomal betaine-aldehyde dehydrogenase (BADH) and glutationa S transferase (GST-8) showed greater abundance. The antioxidant function of GST may be important in nitrogen-fixing root nodules due to the high risks of oxidative damage. These enzymes catalyze the conjugation of glutathione (GSH) to the electrophilic groups of a large variety of hydrophobic molecules to detoxify cells (Dixon et al. 2008). BADH catalyze the oxidation of betaine aldehyde to glycine betaine (GB), known molecule to protect organisms against abiotic stresses via osmoregulation. In addition, other roles of GB like cellular macromolecule protection and ROS detoxification have been suggested as mechanisms responsible for abiotic stress tolerance in plants (Giri, 2011).

Proteome comparisons of the contrasting genotypes (BR16 and Embrapa 48) further suggests that the tolerance the Embrapa 48 genotype can occurs by root osmoprotection by accumulation of molecules like to glutamine, gamma-aminobutyric acid, alanine and glycine betaine (GB). This is due the Embrapa 48 genotype shows an increase in relative abundance Glutamine synthetase (GSbeta1) only after the stress, this enzyme catalyzes condensation of glutamate and ammonia to form glutamine, thus playing an essential role in nitrogen metabolism and ammonia assimilation (Singh and Ghosh, 2013; Simova-Stoilova et al. 2015). Moreover, upregulation of Glutamine synthetase (GS) enhances gamma-aminobutyric acid and alanine accumulation, which have roles in maintenance of root osmotic potential (Reggiani et al. 2000), which can justify the greatest RWC in the roots of this genotype when subjected to stress. Conversely, osmoprotection not appears operate in the leaves because amino acids, such as proline, were lower in Embrapa 48 (Lima et al., 2019) and genes for their biosynthesis were not altered (Coutinho et al., 2020). Changes were also observed to greater abundance of proteins aminopeptidases in

roots in tolerant genotype just after applied stress (Fig. 5), and a decrease in the comparison between the genotype under irrigation condition (Fig. 4). Aminopeptidases liberate free amino acids from the N-terminus of the polypeptide chains, which corroborates the idea that root osmoprotection is due to the accumulation of amino acids.

Carbon/nitrogen metabolism related proteins such as triosphosphate isomerase and malate dehydrogenase were reported to be more abundant in roots of soybean (Toorchi et al. 2009; Alam et al. 2010; Mohammadi et al. 2012) after drought treatment. This reflected an increased energy demand as well as enhanced cellular activities in the root tissues at this stage of the stress, proven by the greater abundance of ATP synthase (ATPS1) protein during stress (Fig. 5). To deal with hydric deficit, roots also developed other mechanisms such as enhanced pumping of protons, as the greatest abundance of protein as voltage-dependent anion-selective channel (VDAC) (Fig. 5). The general importance of VDAC in plant physiology has only recently emerged. In Arabidopsis, the genes encoding VDAC have important functions with respect to plant such regulation of respiration (Yang et al. 2011) and in the plant stress response (Homblé et al. 2012; Takahashi and Tateda, 2013).

In an integrative way, evidence from our own studies and other published/related work provide the hypothesis that water stress promotes the accumulation of ABA in the root, however was higher in Embrapa 48, culminating in increasing in the respiration genes, besides of the expressions of antioxidant enzyme genes (Sharp et al. 2004). Overall, the accumulation of amino acids in the roots during water stress could be promoting in the highest relative water content, increasing the pressure of the turgor, helping in root expansion and optimizing the use and transport of water for aerobic parts. As the leaves of the Embrapa 48 genotype maintain the photosynthetic and metabolic status less changed during drought (Lima et al., 2019 and Coutinho et al., 2020), it could translocate photosynthates to maintain the protective processes in the root and allowed the root expansion and growth (Mesquita et al., 2020), turn possible capabilities to water uptake and higher water content relative (RWC).

The fact that the tolerant genotype leaves were more hydrated under the same water potentials suggesting a possible osmotic adjustment, however the higher levels ABA and proline were observed in the sensitive BR 16 leaves (Lima et al., 2019). Thus, the signal for drought by ABA and proline were more noticeable in the sensitive BR 16.

In the same way, amino acids and sugar were more abundant during drought in the sensitive genotype (Lima et al., 2019). Furthermore, evidence suggest the participation of a non-stomatal event in the relative drought tolerance of the Embrapa 48, because even under severe stress there was less damage to the net photosynthetic rate (Mesquita et al., 2020). Thus, the postponement of water and physiological response suggest that differential hydraulic conductivity may be important to this tolerance. Therefore, the maintenance of higher water content in the leaves and lower inhibition of photochemical reactions in the tolerant cultivar could be explain, at least partially, the greater photosynthetic rate of this cultivar. Overall, it result in a greater induction of root growth under water deficiency. Likewise, we found that some genes that code for proteins involved in cell wall dynamics, xyloglucan endotransglucosylase/ hydrolas (XTH), Expansin and glycosiltransferases (RGXT), are up-regulated by drought stress in the genotypes Embrapa 48 (Coutinho et al. 2020). This elasticity can be correlated with tolerance mainly by increasing side chains of the pectic polymers rhamnogalacturonan II, possibly because the pectins form hydrated gels, which limit the damage to cells (Leucci et al. 2008).

5. Conclusions

Despite of challenged to combined several physiological and omic data for evaluation of the drought stress response and tolerance mechanisms, it was possible to elect some traits and genes determinant in the tolerant genotype Embrapa 48. The drought tolerance mechanism proposed for Embrapa 48 is based on the increase in cell wall elasticity, which can contribute to maintenance of cell turgor, resulting in the highest leaf RWC, photosynthetic rate (*A*), transpiration (E) and carboxylation (*A/Ci*) under conditions of water stress (Mesquita et. al. 2020), as illustrated in the Figure 8. It is believed that carbon could be directed to the roots via phloem, providing root growth in this genotype. The results provide the idea that respiratory-related proteins and antioxidant pathways responsive to ABA and the accumulation of osmoprotective amino acid in roots of the genotype Embrapa 48 result in root water uptake. This event increases the pressure of the turgor, helping in root expansion and optimizing the use and transport of water for areas parts.

These mechanisms of water accumulation in the roots provide activation of signal molecules that are important mediators of shoot physiological processes, may

be responsible for maintaining in leaf water potential in the tolerant genotype during stress besides allowing greater CO_2 fixation, photosynthetic rate and water-use efficiency. In this context the, can lead to increased allocation of photosynthate to the roots, increasing the root/shoot ratio and facilitating water and nutrient absorption.

Finally, we suggest that differential hydraulic conductivity may be important to this tolerance, therefore to maintain higher water content in the leaves and lower inhibition of photochemical reactions several genes involved in the signaling and regulation and increase of the cell wall elasticities and crosslink, which may contribute to an efficient water usage. Remodeling of the pectin component of the cell wall has the potential to be an important mechanism offering drought tolerance to the Embrapa 48 soybean genotype, contributing to the maintenance of cell turgor and growth under drought stress.

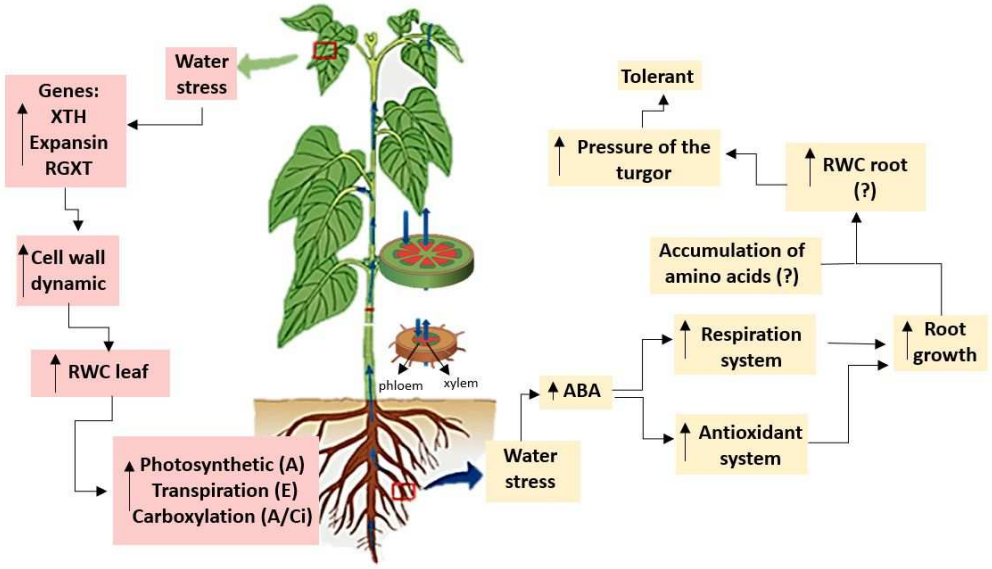

Figure 8. Overview of the possible molecular mechanisms in roots and leaves of the Embrapa 48 genotype, which ensures drought tolerance. Reactive oxygen species (ROS), abscisic acid (ABA), relative water content (RWC).

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Supplementary Information

Table S1. Transition list of the ions monitored for quantification analysi of the phytohormone from soybean root by MRM. The scan mode, the mass of the molecular ion and respective fragment ions are indicated.

PHYTOHORMNE	MS1	MS2	SCAN MODE
ABA	263	153	Negative

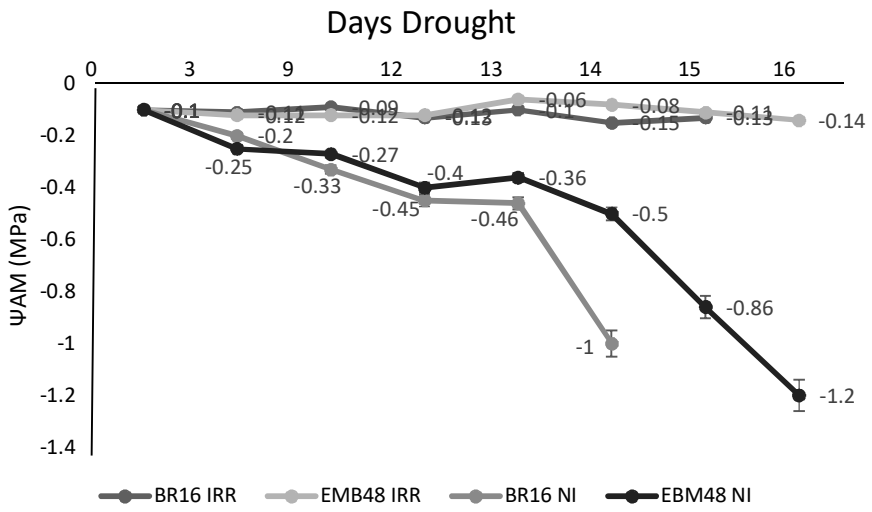

Figure S1. Temporal profile of leaf water potential in the morning (ψaam) in two soybean genotypes, one sensitive (BR 16), and another tolerant (Embrapa 48) in relation to the water deficit. Each point represents the mean + standard error (n = 3, where n represents the number of plants), IRR = irrigated, NI = non-irrigated (not irrigated after imposition of stress).

Table S2. Level of relative expression of differentially expressed proteins (P <0.05) based on the average volume% in roots of contrasting soybean plants in the absence of water deficit (control), with their identification of proteins by MS.

Spot ID	Protein	Average of % of volume ± SD (Irrigated control)		SCORE	Number Peptides	Cover (%)	Expression level
		Tolerant	Sensitive				
	RESPIRATION						
282	ATP synthase subunit beta, mitochondrial-like	0,7020 ± 0,0702	1,0496 ± 0,1157	721	8	22	-1,50
419	Alcohol dehydrogenase 1-like	0,1022 ± 0,0050	0,1633 ± 0,0099	315	2	19	-1,60
197	Malate dehydrogenase, mitochondrial-like	0,0312 ± 0,0021	0,0482 ± 0,0099	275	2	18	-1,54
202	Phosphoglycerate kinase, cytosolic-like	0,9049 ± 0,0265	0,5003 ± 0,0061	1043	9	33	+1,81
393	Transaldolase-like	0,0143 0,0040	0,0251 0,0050	208	3	11	-1,76
330	Transketolase, chloroplastic-like	0,0765 ± 0,0035	0,0509 ± 0,0061	289	3	15	+1,50
	NITROGEN METABOLISM						
388	Cytosolic glutamine synthetase GSbeta1	0,5154 ± 0,0093	0,8578 ± 0,0225	424	3	16	-1,66
389	Cytosolic glutamine synthetase GSbeta1	0,7891 ± 0,0083	1,2501 ± 0,0245	496	5	23	-1,58
212	Formate dehydrogenase 1, mitochondrial-like	0,9088 ± 0,2647	0,5234 ± 0,0610	496	5	19	+1,74
213	Formate dehydrogenase 1, mitochondrial-like	1,0098 ± 0,0462	0,1012 ± 0,0054	598	6	25	+9,98
	ANTIOXIDATIVE METABOLISM						
3	Chain A, Ferric Soybean Leghemoglobin Complexed With Nicotinate	0,3120 ± 0,0258	0,0930 ± 0,0044	604	5	12	+3,35
5	Chain A, Ferric Soybean Leghemoglobin Complexed With Nicotinate	1,8031 ± 0,2645	0,3434 ± 0,0268	420	5	13	+5,25
326	Heat shock cognate 70 kDa protein-like	0,1048 ± 0,0060	0,3921 ± 0,0061	326	2	14	-3,74
0	Leghemoglobin C3	3,6216 ± 0,2453	1,9110 ± 0,1265	743	6	13	+1,9
1	Leghemoglobin C3	1,8226 ± 0,2015	0,6145 ± 0,1210	375	4	13	+2,97
324	Luminal-binding protein 4-like	0,1085 ± 0,0013	0,3807 ± 0,0026	464	4	19	-3,51
358	Nucleoside diphosphate kinase	1,4622 ± 0,0943	0,2976 ± 0,0461	305	4	17	+4,91
400	Peroxisomal betaine-aldehyde dehydrogenase	0,2562 ± 0,0315	0,1707 ± 0,0352	188	2	15	+1,50
	SECONDARY METABOLISM						
220	12-oxophytodienoate reductase 2-like	0,0712 ± 0,0053	0,0369 ± 0,0039	486	5	23	+1,93

Table S2. *Cont.*

59	Chalcone isomerase A	0,3311 ± 0,2084	0,2083 ± 0,0639	985	11	62	+1,59
161	Isoflavone reductase homolog	1,0013 ± 0,0826	0,6529 ± 0,0576	612	6	23	+1,53
144	NAD(P)H-dependent 6'-deoxychalcone synthase	0,1064 ± 0,0200	0,0642 ± 0,0343	761	8	18	+1,66
359	Stress-induced protein SAM22	1,9451 ± 0,0995	0,3215 ± 0,0274	490	6	55	+6,05
	NOT KNOWN						
150	Uncharacterized protein LOC100499848	0,0516 ± 0,0053	0,0862 ± 0,0081	389	4	35	-1,67
20	Uncharacterized protein LOC100527699	0,0327 ± 0,0032	0,0511 ± 0,0027	458	3	31	-1,56
	RESERVATION						
90	Stem 31 kDa glycoprotein precursor	0,4883 ± 0,0265	0,3034 ± 0,0610	630	6	15	+1,61
91	Stem 31 kDa glycoprotein precursor	1,0298 ± 0,0462	0,5030 ± 0,0054	788	6	14	+2,05
	DEFENSE						
24	Pathogenesis-related protein PR-1	1,9540 ± 0,1641	0,1093 ± 0,0064	420	4	64	+17,87
	REGULATION						
214	Actin-101-like	0,1041 ± 0,0013	0,2176 ± 0,0026	576	4	15	-2,09
216	Actin-101-like	0,0552 ± 0,0060	0,1093 ± 0,0061	248	3	12	-1,98
264	S-adenosyl-L-homocysteine hydrolase, partial	0,2039 ± 0,0031	0,3677 ± 0,0279	370	2	17	-1,80
33	Trypsin inhibitor p20 precursor	0,0842 ± 0,0017	0,0543 ± 0,0061	520	5	40	+1,55

The (+) and (-) signs indicate an increase and decrease in protein abundance in tolerant Embrapa 48 compared to genotype sensitive BR 16, with reference to the tolerant Embrapa 48. SD = standard deviation. The values represent the averages of the percentage by volume (relative abundance) ± the standard deviation of three biological replicates.

Table S3. Level of relative expression of root proteins based on the mean% volume of soybean plants responsive to water deficit in the tolerant genotype.

Spot ID	Protein	Deficit (MPa)	Average of% of volume ± SD		Expression level
			Irrigated	Deficit	
	RESPIRATION				
71	ATP synthase alpha/beta family protein	-1,0	0,3606 ± 0,0111	0,7379 ± 0,0126	+2,05
11	Malate dehydrogenase, cytoplasmic-like	-1,0	0,1285 ± 0,0095	0,4341 ± 0,0054	+3,38
12	Malate dehydrogenase, cytoplasmic-like	-1,0	0,1295 ± 0,0091	0,4611 ± 0,0046	+3,56
28	Peroxisomal voltage-dependent anion-selective channel protein	-1,0	0,1781 ± 0,0137	0,8570 ± 0,0109	+4,33
33	Triosephosphate isomerase	-1,0	0,4901 ± 0,0602	1,2890 ± 0,1157	+2,63
	ANTIOXIDATIVE METABOLISM				
5	Chain A, Ferric Soybean Leghemoglobin Complexed With Nicotinate	-1,0	1,8031 ± 0,2645	0,4693 ± 0,0268	-3,84
27	Glutathione S-transferase GST 8	-1,0	0,1532 ± 0,0114	0,6173 ± 0,0633	+4,03
326	Heat shock cognate 70 kDa protein-like	-1,0	0,1048 ± 0,0060	0,1921 ± 0,0061	+1,83
0	Leghemoglobin C3	-1,0	3,6216 ± 0,2453	1,6310 ± 0,1732	-2,22
1	Leghemoglobin C3	-1,0	1,8226 ± 0,2015	0,5725 ± 0,0821	-3,18
324	Luminal-binding protein 4-like	-1,0	0,1085 ± 0,0013	0,1807 ± 0,0026	+1,67
400	Peroxisomal betaine-aldehyde dehydrogenase	-1,0	0,2562 ± 0,0315	0,4573 ± 0,0352	+1,79
	NITROGEN METABOLISM				
96	Cobalamin-independent synthase family protein	-1,0	0,2808 ± 0,0452	0,5891 ± 0,0483	+2,10
97	Cobalamin-independent synthase family protein	-1,0	0,2091 ± 0,0383	0,5001 ± 0,0245	+2,39
93	Cytosol aminopeptidase family protein	-1,0	0,2320 ± 0,0036	0,6201 ± 0,0064	+2,67
389	Cytosolic glutamine synthetase GSbeta1	-1,0	0,7891 ± 0,0297	1,5501 ± 0,0725	-1,96
213	Formate dehydrogenase 1, mitochondrial-like	-1,0	0,1781 ± 0,0527	0,2412 ± 0,0114	+4,19
	SECONDARY METABOLISM				
124	Caffeoyl-CoA O-methyltransferase-like	-1,0	0,6290 ± 0,0702	0,9537 ± 0,0812	+1,52
65	Dihydroflavonol-4-reductase-like	-1,0	0,1865 ± 0,0084	0,5162 ± 0,0242	+2,77
	RESERVATION				
90	Stem 31 kDa glycoprotein precursor	-1,0	0,4883 ± 0,0265	0,3165 ± 0,0701	-1,54
91	Stem 31 kDa glycoprotein precursor	-1,0	1,0298 ± 0,0462	0,5624 ± 0,0254	-1,83

The signs (+) and (-) indicate an increase and decrease in the expression of the protein in comparison with the deficits, having as reference the irrigated control of the tolerant cultivar (Embrapa 48). SD = standard deviation.

5.CONSIDERAÇÕES FINAIS

Em resumo, os resultados deste trabalho mostraram mecanismos das folhas e raízes envolvidas na tolerância do genótipo Embrapa 48 à seca. Embora cada capítulo apresentado tenha uma discussão independente, focando os resultados descritos, nas considerações finais dessa tese buscamos detalhar as principais descobertas em um contexto mais amplo. Diante disso, apresentamos agora uma integração das respostas de forma a possibilitar a compreensão dos mecanismos moleculares e fisiológicos desenvolvidos pelo genótipo Embrapa 48, como possíveis caminhos para pesquisas futuras.

Em hipótese, acreditamos que o aumento da sinalização por ABA nas raízes parece ter promovido um impacto significativo no seu desenvolvimento, pela ativação de vias respiratórias e antioxidantes que permitiram um maior crescimento radicular. Essa manutenção do crescimento radicular durante o déficit hídrico permite explorar melhor o ambiente sendo um benefício para manter um suprimento adequado de água na raiz (Sharp *et al.,* 2004), comprovado pelo maior teor relativo de água (TRA). Este evento supostamente está relacionado a osmoproteção por acúmulo de aminoácidos, que aumentam a pressão do turgor, otimizando o uso e transporte de água para as partes aéreas. Assim, as folhas do genótipo Embrapa 48 foram menos impactadas pelo estresse, apresentando melhores taxas fotossintéticas (*A*), carboxilação (*A/Ci*) e transpiração (*E*), quando comparadas com o genótipo BR16 sobre mesmo potencial hídrico (Ψw).

Provavelmente, a capacidade do genótipo Embrapa 48 em manter seus estômatos abertos sem que isso resulte em maior perda de água e, consequentemente, grandes reduções de Ψw, está no mecanismo de alocação de fotoassimilados para as raízes. Um estudo realizado por Du *et al.* (2020) mostrou que o estresse hídrico induziu um aumento da razão raiz/parte aérea em soja submetida ao déficit hídrico. Os autores sugerem que o metabolismo, alocação e transporte de açúcar parece ser o mecanismo preferido para manter o crescimento e o metabolismo das raízes em resposta ao estresse causado pela seca. No entanto, mais estudos sobre a alocação de carbono no genótipo Embrapa 48 devem ser realizados, bem como investigações na sua condutividade hidráulica.

Além disso, ressaltamos que nas folhas do genótipo Embrapa 48, a transcrição de genes de proteínas cinases dependentes de cálcio (CDPKs) e dedos de zinco

CCCH, são capazes de retardar a senescência e reduzir a produção de ROS, evitando a fotoinibição (Oya *et al*. 2004; Carvalho *et al*. 2015). Genes que codificam proteínas envolvidas na dinâmica da parede celular, como xiloglucanas endotransglicosilase/hidrolase (XTH), expansina e glicosiltransferases (RGXT), também foram regulados pelo estresse hídrico nos genótipos Embrapa 48. Esses resultados são uma indicação de que o aumento das cadeias laterais dos polímeros pécticos rhamnogalacturonan II, possivelmente contribuam para um uso mais eficiente da água observado na Embrapa 48 (Leucci *et al.* 2008).

Concluímos que os dados de estudos fisiológicos e das ciências “ômicas” deram uma nova direção ao avanço na elucidação dos mecanismos de tolerância à seca no genótipo Embrapa 48. Além disso, embora os resultados pareçam promissores, são necessários esforços cooperativos da comunidade de pesquisa para melhorar a cobertura de informações sobre os mecanismos de tolerância adotados pela soja em condições de seca. No entanto, este trabalho abre caminhos para futuras pesquisas sobre esse tópico.

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