UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGÍA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR MOLECULAR

O EFEITO DO ESTRESSE OSMÓTICO NO PADRÃO DE EXPRESSÃO GÊNICA E NA REGULAÇÃO POR MIRNAS EM SOJA.

Tese de Doutorado

Isabel Cristina Cadavid Sánchez

Porto Alegre, RS

Dezembro 2019

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS como requisito parcial para a obtenção do título de Doutor em Ciências

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ÍNDICE

LISTA DE ABREVIATURAS

°C	Graus Celsius
µg	Micrograma
µL	Microlitro
ABA	Ácido abscísico
ABCC10	"ATP Binding Cassette Subfamily C Member 10"
ABCG29	"ATP Binding Cassette Subfamily G Member 29"
ABFs	Fatores de ligação a elemento responsivo a ABA
ABI5	"ABA-Insensitive 5"
ABRE	"Abscisic acid (ABA) response elements"
AccA	"Acetyl-coenzyme A carboxylase carboxyl transferase "
AGO	Argonauta
AGXT	"Alanine-glyoxylate transaminase "
AKT1	"AKT Serine/Threonine Kinase 1"
AQPs	Aquaporinas
ATP	Adenosina trifosfato
ATX3	"Histone-lysine n-methyltransferase "
BAK1	"Brassinosteroid insensitive 1-associated receptor kinase 1"
BBCA	"Branched chain amino acid"
BES1	"Brassinosteroid signaling positive regulator"
BIN2	"Brassinosteroid-insensitive 2"
BKDA	"2-oxoisovalerate dehydrogenase e1 component alpha subunit "
BLAST	"Basic Local Alignment Search Tool"
BMAL	"Brain and Muscle ARNT-Like 1"
BR	Brassinosteróides
BRI1	"Brassinosteroid insensitive 1"
BSU1	"Serine/threonine protein phosphatase, positive regulator of brassinosteroid (BR) signaling"
bZIP	"Basic Leucine Zipper Domain "
BZR1	"Brassinazole-resistant 1"
CACYBP	"Calcyclin-binding protein"
CAX1	"Vacuolar cation/proton exchanger 1"
CCNA	"Cyclin A"
CDC	"Cell division control protein"
CDK	"Cyclin-dependent kinase"
cDNA	"Complement DNA"
CDS	"Coding sequence" (sequência codificadora)
CDT1	"Chromatin licensing and DNA replication factor 1"

CTF4	"Chromosome transmission fidelity protein 18 homolog"
DCLs	"DICER-LIKE "
DDM1	"ATP-dependent DNA helicase "
DEGs	"Differentially expressed genes "
DNA Mtase	"MTase Cytosine-specific methyltransferase"
DREB2	"Dehydration responsive element binding protein"
dS/m	Decisiemens por metro
dsRNA	Fita dupla de RNA
ECe	Condutividade elétrica
EF TS	"Elongation factor Ts"
EF Tu	"Elongation factor Tu"
EFF2	"Eukaryotic elongation factor 2"
EFP	"Elongation factor P"
EIF1A	"Eukaryotic translation initiation factor 1A"
EIF3I	"Translation initiation factor 3 subunit I"
ETR	"Ethylene receptors"
EUA	Estados Unidos de America
FC	"Fold change"
FERM	"FERM central domain"
FTSZ1	"Tubulin/FtsZ family, Cell division protein"
g/L	Gramas por litro
GAMT	"Guanidinoacetate N-methyltransferase"
GNB2L1	"Guanine nucleotide-binding protein subunit beta-2-like 1 protein "
h	Horas
H3	"Histone 3"
HAD6	"Histone deacetylase 6"
HATs	Histonas acetiltransferases
hcsiRNAs	siRNA heterocromático
HD2C	"Histone deacetylase 2 C
HDAC	Histona desacetilases
HDI	Inibidor de histona desacetilases
HDMs	Histona desmetilases
HDT	"Histone deacetylase "
HEC1	"Helix-loop-helix transcription factor HEC1"
HEN1	"HUA Enhancer 1 "
HESO1	"HEN1 Suppressor 1 "
HKT1	"High-affinity K+ transporter 1"
HMTs	Histona metiltransferases
HNRNPA1	"Heterogeneous nuclear ribonucleoprotein A1/A3"

HPS	"Hydrophobic protein from soybean "
HSP	"Heat shock protein"
HYL1	"Hyponastic leaves 1 "
KIFC2	"3 kinesin family member C2/C3"
Ky2	"Histone Deacetylase Inhibitor"
LCT1	"Low-affinity cation transporter"
LEA	"Late Embryogenesis Abundant"
LRRK	"Protein kinase family protein with leucine-rich repeat domain"
MAP65-3	"65-KDA Microtubule-associated protein 3-related"
MAPRE	"Microtubule-associated protein RP"
MCAT	"Acyl-carrier-protein s-malonyltransferase / MCAT "
MCM	"Minichromosome maintenance protein complex"
MCMBP	"Mini-chromosome maintenance complex-binding protein"
mg	miligramas
MIP	Proteínas intrínsecas principais
MIR	Gene que codifica para micro RNA
miRBase	Base de dados de micro RNAs
miRNA	micro RNA
mM	milimolar
mRNA	RNA mensageiro
MRPS6	"Mitochondrial 28S Ribosomal protein S6"
MSH2	"DNA mismatch repair protein "
MutS V	"DNA mismatch repair protein msh1, mitochondrial "
MYBRS	"Myeloblastosis virus transcription factor binding site"
MYBs	Proteínas intrínsecas principais
MYCRS	"Mycorrhiza transcription factor binding site"
MYCs	"Mycorrhiza transcription factor"
NAC	"NAC domain transcription factor"
NACA	"Nascent polypeptide-associated complex subunit alpha "
NHX	"Sodium/hydrogen exchanger"
NHX1	"Vacuolar Na+/H+ antiport"
NOLA4	"H/ACA ribonucleoprotein complex subunit 4"
NORC	"Nonselective outward-rectifying conductance"
NSCC	"Nonselective cation channels "
nt	Nucleotídeos
ORC1	"Origin recognition complex subunit "
PCNA	"Proliferating cell nuclear antigen "
PCR	"Polymerase chain reaction"
PCSK9	"Proprotein convertase subtilisin/kexin "

PEG	Polietilenglicol
piRNA	RNA interagindo com PIWI
PMEI	"Pectin methylesterase inhibitor "
POLD1	"DNA polymerase delta subunit 1 "
POLE4	"DNA Polymerase epsilon subunit 4"
PP2C	"Protein phosphatase 2C family protein"
pre-miRNA	micro RNA precursor
PRI	"DNA primase small subunit "
pri-miRNAs	micro RNA primário
PTGS	silenciamento de genes em nível pós-transcricional
PYL / PYR / RCAR	"pyrabactin resistance1 (PYR1)/pyr1-like (PYL)/regulatory components of aba receptors (RCAR)""
qPCR	PCR quatitativa
QTLs	"Quantitive Trait Locus"
RAN	"GTP-binding nuclear protein Ran"
RANGAP1	"Ran GTPase-activating protein 1"
RECA2	"DNA Repair protein reca homolog 2"
RECQL3	"Helicase bloom syndrome protein "
RIOK	"Serine/threonine-protein kinase RIO"
RISC	complexo de silenciamento induzido por RNA
RLP55	"Receptor like protein 55 "
RNA Pol	RNA polimerase
RNA-Seq	Sequenciamento de RNA
ROS	Especies reativas de oxigenio
RPA2	"Replication factor A2"
RPA-70	"Replication protein A 70 kDa DNA-binding subunit"
RP-L	"Large subunit Ribosomal protein "
RPO41	"DNA-directed RNA polymerase, mitochondrial"
RP-S	"Small subunit Ribosomal protein "
RRM2	"Ribonucleoside-diphosphate reductase subunit M2"
RSM-B	"16S rRNA (cytosine967-C5)-methyltransferase"
RT	Transcrição reversa
RT-qPCR	"Real-time quantitative PCR" (PCR quantitativa em tempo real) "
SAHA	"Suberoylanilide Hydroxamic Acid "
SAM2	"S-adenosylmethionine synthetase"
SDH	"Shikimate dehydrogenase "
SE	"Serrate "
siRNA	Pequeno RNA interferente
SNF2H	"Chromatin remodeling enzyme "

SNP	Polimorfismos de nucleotídeo único
SnRK2	"SNF1-related protein kinase 2"
SNU13	"U4/U6 small nuclear ribonucleoprotein "
SOS1	"SALT OVERLY SENSITIVE1"
SuSy	"Sucrose synthase "
TGS	Silenciamento de genes em nível transcricional
Thaumatin	"Pathogenesis-related thaumatin family protein"
TIFY10A	"jasmonate ZIM domain-containing protein"
TM	target mimicry
TPL	"TOPLESS"
TPS	"Alpha,alpha-trehalose-phosphate synthase "
TRI12	"Fungal trichothecene efflux pump "
tRNA	RNA transferentes
TRX	"Adenylyl-sulfate reductase (thioredoxin)"
tsRNAs	Pequenos RNA derivados RNAs transferentes
TSS	"Total suspended solids"
UHRF1	"E3 ubiquitin-protein ligase "
USP	"Universal stress protein family"
UTP15	"U3 small nucleolar RNA-associated protein 15"
VIC	"Voltage-independent calcium"
WEE1	"wee1-like protein kinase"
WTF1	"RNA recognition domain-containing protein WTF1 "
X-box TF	"X-box Transcription Factor"
YHBY	"RNA-binding protein YHBY/Poly(A)-specific exoribonuclease PARN"

RESUMO

A soja é uma cultura mundialmente importante. No entanto, sua produção é afetada pelas condições de estresse, como o osmotico. É importante entender os mecanismos moleculares de adaptação das plantas ao estresse osmotico para encontrar ferramentas para melhorar a tolerância a este. Os micro RNAs (miRNAs) são essenciais nas redes reguladoras de estresses. São pequenas moléculas de RNAs não codificantes que modulam a expressão gênica, pela clivagem do mRNA alvo ou inibição da sua tradução. No estudo, foram gerados dados de RNA-Seq (pequenos RNA e mRNA) de soja cultivar Conquista sob condição de alta salinidade. Foram feitas comparações entre Conquista (tolerante à seca) e uma cultivar sensível à seca (C08) que foi submetida a diferentes períodos de estresse salino em um estudo previo. O perfil de expressão gênica para cada cultivar foi obtido por transcriptograma. Após 4 horas de estresse salino, Conquista apresentou 647 genes induzidos e 753 reprimidos. Desses, 719 compartilham o mesmo padrão de expressão entre as duas cultivares. O estresse salino também modificou a expressão de 54 isoformas de miRNAs em Conquista, pela maturação de 39 pré-miRNAs. Os alvos previstos para 12 desses miRNAs maduros têm correspondência com 15 genes expressos diferencialmente de nossas análises. Encontramos genes das vias de sinalização de ABA e BR modulados, com possível *crosstalk* entre eles e com provável regulação pelos miRNAs. Genes relacionados à biossíntese de etileno, reparo do DNA e a tradução de plastídios podem ser regulados por miRNAs. A expressão do miR482bd-5p foi reprimida sob condições salinas em Conquista, enquanto que a expressão dos seus alvos preditos (HEC1, BAK1) aumentou. A expressão diferencial de seis miRNAs, incluindo miR482bd-5p e seus possíveis alvos, foi confirmada por RT-qPCR. Também foi avaliado o efeito de PEG na expressão de miRNAs e seus alvos preditos. miR482bd-5p, HEC1 e BAK1 tiveram o mesmo padrão de expressão sob PEG. A regulação epigenética do miR482bd-5p foi avaliada por tratamento com SAHA, um inibidor de histona deacetilases (HDAC). O miR482bd-5p foi induzido e HEC1 reprimido sob tratamento com sal e SAHA. Isso pode ser explicado por uma regulação epigenética, na qual o gene do miRNA pode ser reprimido pela HDAC sob estresse salino com um aumento associado na expressão do alvo. Assim este trabalho, com o uso de uma ferramentas nova, como o transcriptograma, permitiu entender melhor os mecanismos de resposta da soja ao estresse salino, agregando conhecimento sobre regulação genica no nível trancipcional e post trancricional.

ABSTRACT

Soybean is an important crop worldwide. However, its productivity is affected by stress conditions. It is important to understand the mechanisms of plant adaptation to abiotic stress to find tools to improve the tolerance. MicroRNAs (miRNAs) have key roles in abiotic stress regulatory networks. They are small non-coding molecules that modulate gene expression by cleavage or by translation inhibition of the target mRNA. Data from RNA-Seq libraries (small RNA and mRNA) from soybean Conquista cultivar grown under salinity were analyzed. Comparisons of drought-tolerant cultivar with a drought-sensitive (C08) subjected to different saline stress variations were made. The gene expression profile for each cultivar was produced by the transcriptogram. After 4 hours of saline stress, Conquista had 647 induced genes and 753 repressed genes. From which, 719 share the same expression pattern among cultivars, 393 being induced and 326 repressed. Saline stress also modified the expression of 54 miRNA isoforms by maturation of 39 pre-miRNAs in Conquista. The predicted targets for 12 miRNAs correspond to 15 differentially expressed genes in our analysis. Genes of ABA and BR signaling pathways were modulated, with a possible crosstalk between them and a post-transcriptional regulation by miRNAs. Genes related to ethylene biosynthesis, DNA repair, and plastid translation can also be regulated by miRNA. The miR482bd-5p expression was repressed under salinity in Conquista and its predicted targets (HEC1, BAK1) expression was increased. The differential expression of six miRNAs, including miR482bd-5p and their targets, was confirmed by RT-qPCR. The effect of PEG on miRNA and target expression was also evaluated. miR482bd-5p, HEC1 and BAK1 had the same expression pattern under PEG treatment. Epigenetic regulation of miR482bd-5p was assessed by treatment with SAHA, a histone deacetylase (HDAC) inhibitor. miR482bd-5p was up-regulated and HEC1 down-regulated under salt and SAHA treatment. This can be explained by an epigenetic regulation; which miRNA gene can be repressed by HDAC under salt stress with an associated increase in target expression. Therefore, this work, with the use of a novel tool, such as the transcriptogram, allowed a better understanding of the soybean response mechanisms to saline stress, adding knowledge about genetic regulation at the transcriptional and post-transcriptional levels.

1. INTRODUÇÃO

1.1 Estresse nas plantas

LEVITT (1980) define o estresse como uma mudança nas condições do meio em que as plantas estão submetidas e que reduz o seu crescimento. Os estresses provocam nas plantas respostas complexas que constituem um problema fundamental para agricultura, já que influenciam na sobrevivência e produtividade das culturas. Dentre os fatores que geram estresse, estão os que são provenientes da ação de organismos vivos (insetos, vírus, fungos e bactérias), da atividade antropogênica (agroquímicos, poluição do ar, ozônio, incremento da radiação UV e incremento de CO_2) e das variações nas condições ambientais, tais como temperaturas altas ou baixas; salinidade excessiva; escassez ou excesso de água; mudanças na intensidade da luz e carência ou excesso de nutrientes (LICHTENTHALER, 1996)

As respostas das plantas se manifestam no nível celular, de tecido ou de órgãos e afetam os processos bioquímicos, fisiológicos e de desenvolvimento (LARCHER, 2003). Esses processos são controlados por um importante número de genes, cuja expressão é induzida ou reprimida sob condições de estresse, os quais codificam proteínas específicas, como as proteínas de membrana, as proteinases, os fatores de proteção de macromoléculas (chaperonas e proteínas abundantes na embriogêneses tardia ou LEA), as proteínas da síntese de compostos osmoprotetores, as enzimas detoxificantes, e as proteínas reguladoras como os fatores de transcrição, proteínas quinases, fosfatases e as fosfolipases (SHINOZAKI & YAMAGUCHI-SHINOZAKI, 1997)

1.2 Estresse salino

1.2.1 Origem do estresse salino

O estresse salino é produzido por um acúmulo de sais no solo, perto da zona radicular, numa concentração que causa redução da produção das culturas. Ocorre quando a cultura não é mais capaz de extrair água suficiente da solução água/solo/sal e é agravadado pelo acumulo de íons tóxicos nos tecidos. Os sais responsáveis pela salinização são as provenientes dos íons Na^+, K^+,

Ca^{2+}, Mg^{2+} e Cl^-. Destes sais, o cloreto de sódio é o mais comum. Quando o Na^+ (sódio) predomina, os solos podem ser chamados de sódicos. Os solos altamente salinos são reconhecíveis por uma camada branca de sal seco na superfície do solo. Os solos afetados pelo sal cobrem perto de 800 milhões de hectares de terra, representando mais de 6% da área total do mundo (MUNNS & TESTER, 2008).

Existem dois tipos de salinidade do solo: a primária (natural) e a secundária (devido à atividade humana). A maioria dos solos salinos surgiram devido a causas naturais tais como acumulação de sais durante longos períodos de tempo em zonas áridas e semiáridas (MUNNS & TESTER, 2008). Isto é devido ao fato de que a rocha mãe a partir da qual se formou o solo, contém sais, principalmente cloretos de sódio, cálcio e magnésio, e em certa medida, também contém sulfatos e carbonatos. A água do mar é outra fonte de sais em áreas baixas ao longo da costa. Além da salinidade natural, uma proporção significativa de terra cultivada tornou-se salgada devido ao desmatamento, à pobre drenagem do solo, à água de irrigação com altos níveis de sais e ao uso excessivo de fertilizante (MUNNS & TESTER, 2008).

Em terras secas, o aumento da salinidade do solo pode ocorrer quando o lençol freático está entre dois e três metros da superfície do solo. Os sais das águas subterrâneas são elevados por ação capilar na superfície do solo. Isso ocorre quando a água subterrânea é salgada e é favorecida por práticas de uso da terra que permitem que mais água da chuva entre no aquífero do que ele poderia acomodar. Por exemplo, o desmatamento para a agricultura é uma das principais razões para a salinidade da terra seca em algumas áreas, uma vez que o enraizamento profundo das árvores foi substituído pelo enraizamento superficial de culturas anuais. Esses fatores elevam o lençol freático e causam o acúmulo de sais na zona radicular (MUNNS & TESTER, 2008).

A salinidade por irrigação pode ocorrer ao longo do tempo, uma vez que quase toda a água (mesmo a chuva natural) contém alguns sais dissolvidos. Quando as plantas absorvem a água ou esta evapora diretamente do solo úmido, os sais são deixados para trás no solo e, eventualmente, começam a se acumular. A água de irrigação contém cálcio (Ca^{2+}), magnésio (Mg^{2+}) e sódio (Na +). Ca^{2+} e Mg^{2+} precipitam frequentemente em carbonatos, deixando Na^+ dominante no solo. Como um resultado, as concentrações de Na^+ frequentemente excedem às da maioria dos macronutrientes em uma ou duas ordens de grandeza, e ainda mais no caso dos micronutrientes (CARILLO et al., 2011).

Existe uma variedade de procedimentos de campo e de laboratório para medir a salinidade do solo. No campo, a salinidade do solo é usualmente inferida a partir da condutividade elétrica aparente (ECe) usando uma série de dispositivos. Em laboratório, a salinidade do solo é geralmente avaliada pela determinação dos sais solúveis totais por evaporação de um extrato de água no solo (TSS), ou pela determinação da condutividade elétrica (ECe) (RHOADES & VAN SCHILFGAARDE, 1976).

1.2.2 Efeitos da salinidade nas plantas

A alta salinidade produz dois tipos de estresse nas plantas; estresse hiperiônico e hiperosmótico. Como consequência, ocorrem estresse hídrico, toxicidade iônica, distúrbios nutricionais, estresse oxidativo, alteração dos processos metabólicos como fotossíntese, desorganização da membrana, redução da divisão e expansão celular e genotoxicidade (MUNNS, 2002), levando assim à redução na produção de biomassa (ALAM et al., 2015; NEGRÃO et al., 2017).

As altas concentrações de Na^+ na solução do solo podem alterar as quantidades de nutrientes iônicos na celula e produzir proporções extremas de Na^+/Ca^{2+} ou Na^+/K^+ (GRATTAN & GRIEVE, 1998). O aumento dos cátions e seus sais, particularmente NaCl, no solo, geram potencial osmótico externo, que pode impedir ou reduzir o influxo de água pela raiz. O déficit hídrico resultante é semelhante às condições de seca e adicionalmente agravado pela presença de íons Na^+ (MUNNS, 2002). O sal é absorvido pela raiz e se difunde na corrente de transpiração à parte aérea, eventualmente se acumulando nas folhas (MUNNS & TESTER, 2008).

É bem sabido que o estresse salino produz respostas na planta semelhantes a aquelas do estresse osmótico. Em *Arabidopsis* foi demonstrado que a expressão de muitos genes responsivos a estresse foi induzida tanto em estresse salino quanto em osmótico (manitol e polietilenoglicol). Isto indica que o estresse salino implica em estresse iônico e osmótico (SEWELAM et al., 2014).

Nas leguminosas, o estresse salino limita a produtividade porque pode afetar diretamente a formação do nódulo por *Rhizobium* e o desenvolvimento, podendo influenciar na capacidade de fixação de nitrogênio (ELSHEIKH & WOOD, 1995; ZAHRAN, 1999; RAO et al., 2002). Poucas espécies de plantas se adaptaram ao estresse salino, sendo a maioria das plantas cultivadas suscetíveis (elas podem não sobreviver ou sobreviver, mas com baixo rendimento). As plantas glicófitas não crescem em altas concentrações de sal e são severamente inibidas ou não sobrevivem

em concentrações de NaCl de 100 a 200 mM. O motivo é que elas evoluíram sob condições de baixa salinidade do solo e não exibem tolerância ao sal (MUNNS & TERMAAT, 1986). Pelo contrário, as plantas halófitas podem sobreviver à salinidade de mais de 300 mM. Sabe-se que as halófitas têm capacidade de crescimento em solos salinizados de regiões costeiras e áridas devido a mecanismos específicos de tolerância ao sal desenvolvidos durante sua adaptação evolutiva (FLOWERS et al., 1977, 2010)

1.2.3   Resposta da planta ao estresse salino

Os efetores para adaptação ao estresse salino podem ser classificados como: mediadores da homeostase iônica e da biossíntese de osmólitos, aqueles que sequestram espécies reativas de oxigênio, os transportadores de água e os tradutores de sinais.

1.2.3.1 Homeostase iônica

Durante o estresse salino, existe uma redução no influxo intracelular de potássio (K^+) e um incremento do Sódio (Na^+) externo. Na^+ é tóxico para algumas reações do metabolismo, e K^+ é indispensável para vários processos fisiológicos, incluindo a manutenção do potencial de membrana e turgor, ativação enzimática, movimento estomático, regulação da pressão osmótica e tropismos (GOLLDACK et al., 2003). Para evitar que cesse o crescimento e a morte celular, o Na^+ tem que ser ou excretado ou compartimentalizado no vacúolo, ou tem que ser reduzida a sua entrada na célula (HASEGAWA et al., 2000a).

Teoricamente, o Na^+ pode ser atacado antes ou depois de entrar na célula, ou ambos. O Na^+ extracelular pode ser detectado por um receptor de membrana (Figura 1), enquanto o Na^+ intracelular pode ser detectado por proteínas de membrana ou por qualquer uma das enzimas sensíveis ao Na^+ no citoplasma. Embora, os sinais específicos de íons sejam provavelmente mais importantes que a hiperosmolaridade, na regulação do transporte de Na^+, o estresse osmótico também desempenha um papel essencial (Figura 1). Por exemplo, o estresse osmótico ativa a síntese de ácido abscísico (ABA), que pode regular positivamente a transcrição de AtNHX1, o gene que codifica o trocador vacuolar Na^+/H^+ (SHI & ZHU, 2002). O estresse osmótico pode ser sentido, em parte, por canais

mecanossensíveis, por proteínas quinases de membrana (URAO et al., 1999) e quinases associadas à parede (KOHORN, 2001). O transportador antiporte de membrana plasmática SOS1 (SALT OVERLY SENSITIVE1) que tem uma atividade trocadora de Na^+/ H^+, sendo um excretor de Na+ (Figura 1) também é responsivo a hiperosmolaridade (SHI et al., 2000)

1.2.3.1.1 *Entrada do Na^+ na planta*

Em condições fisiológicas típicas, as plantas mantêm uma alta razão K^+/Na^+ no citosol com concentrações relativamente altas de K^+ (100-200 mM) e baixas de Na^+ (1-10 mM) (NIU et al., 1995). Dada a diferença de potencial elétrico negativo na membrana plasmática (-140 mV) (HIGINBOTHAM, 1973), um aumento nas concentrações extracelulares de Na^+ estabelece um grande gradiente de potencial eletroquímico que favorece o transporte passivo de sódio desde o ambiente para o citosol. Diferentes transportadores de potássio na membrana plasmática podem mediar o influxo de Na^+ dentro das células. Como por exemplo, o AKT1 (Figura 1 e 2) que é um canal de alta afinidade por K^+ que medeia o influxo de potássio das raízes das plantas e células guarda, com uma maior seletividade por K^+ que por Na^+ (SENTENAC et al., 1992). Adicionamente, o HKT1 (Figura 1) é um simportador K^+/Na^+ de alta afinidade a K^+. Tem como função absorver Na^+ da solução do solo para reduzir os requisitos de K^+ se este é um fator limitante; e reduzir o acúmulo de Na^+ nas folhas, removendo Na^+ da seiva do xilema e carregando Na^+ na seiva do floema (SCHACHTMAN&SCHROEDER, 1994).

Outro canal, chamado de NORC (Condutância não-seletiva retificadora externa), é ativado por Ca^{2+} e não discrimina entre Na^+ e K^+ (Figura 2). Este se abre durante a despolarização da membrana plasmática (mudança da diferença do potencial elétrico para valores mais positivos), mediando a saída de K^+ e o fluxo de Na^+ (WEGNER & DE BOER, 1997). Além disso, vários estudos relataram a presença de canais de cátions independentes de voltagem (VIC) em membranas plasmáticas vegetais (ELZENGA & VAN VOLKENBURGH, 1994; DAVENPORT & TESTER, 2000). Estes tem uma mais alta seletividade por Na^+ que por K^+ (Figura 2). CLEMENS et al., (1998) identificaram um gene do trigo, LCT1, que codifica um transportador de cátions de baixa afinidade que também poderia mediar o fluxo de Na^+ em células de plantas (BLUMWALD et al., 2000).
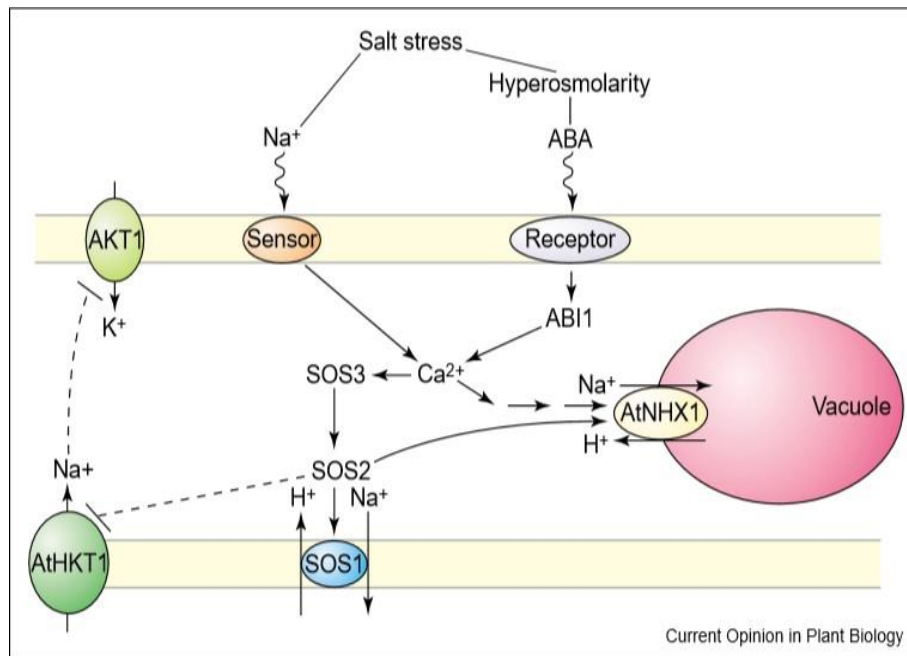
Figura 1. **Vias de sinalização que regulam a expressão e as atividades dos transportadores de íons para manter uma baixa concentração citoplasmática de Na$^+$ sob estresse salino**. O excesso de Na$^+$ e a hiperosmolaridade são percebidos por sensores desconhecidos. A via da proteína quinase SOS3 – SOS2, que é responsiva ao Ca^{2+}, medeia a expressão e atividades dos transportadores de Na$^+$. A hiperosmolaridade é proposta para induzir a síntese de ABA, que por sua vez regula positivamente a transcrição de AtNHX1 e outros genes transportadores de íons. A potencial regulação negativa de AtHKT1 por SOS3 – SOS2 e de AKT1 por Na$^+$ intracelular também é indicada por linhas discontinuas que terminam em barras T (ZHU, 2003)

1.2.3.1.2   *Extrusão de sódio*

Embora pouco se saiba sobre os mecanismos de regulação da restrição de entrada do Na$^+$ na célula, o mecanismo de efluxo é bem conhecido em *Arabidopsis*. A extrusão de Na$^+$ da célula é um processo ativo, devido ao fato de que os íons de Na$^+$ precisam ser transportados contra seu potencial eletroquímico. Nas plantas superiores, o principal mecanismo para a extrusão de Na$^+$ utiliza a H$^+$-ATPase da membrana plasmática (Figura 2). A H$^+$-ATPase usa a energia da hidrólise do ATP para

bombear H^+ para fora da célula, gerando um gradiente eletroquímico de H^+. Esta força próton-motora gerada permite a operação de antiportadores Na^+/H^+ de membrana plasmática (Figura 2), que acoplam o movimento de H^+ para dentro da célula ao longo de seu gradiente eletroquímico até a extrusão de Na^+ contra seu gradiente eletroquímico (Figura 2).

Na^+ induz uma despolarização na membrana plasmática e a ativação dos canais Ca^{2+} (Figura 1). Assim, o aumento resultante de Ca^{2+} no citosol ativará o fluxo de Na^+ através dos canais retificantes para o exterior. Além disso, o acúmulo de Ca^{2+} no citosol sinaliza a resposta ao estresse através da via do SOS (Figura 1), que é crucial para a adaptação ao sal. O Ca^{2+} se liga a SOS3 (uma proteína do tipo calcineurina) levando a associação desta com a proteína quinase SOS2 (HALFTER et al., 2000). O complexo SOS3-SOS2 estimula SOS1 (um Na^+/H^+ antiportador) regulando as concentrações citosólicas de Na^+ (QIU et al., 2002). Foi demonstrado que a SOS2 também afetou CAX1 (um antiportador vacuolar Ca^{2+}/H^+), ligando assim o Ca^{2+} celular com o transporte de Na^+ (CHENG et al., 2003)
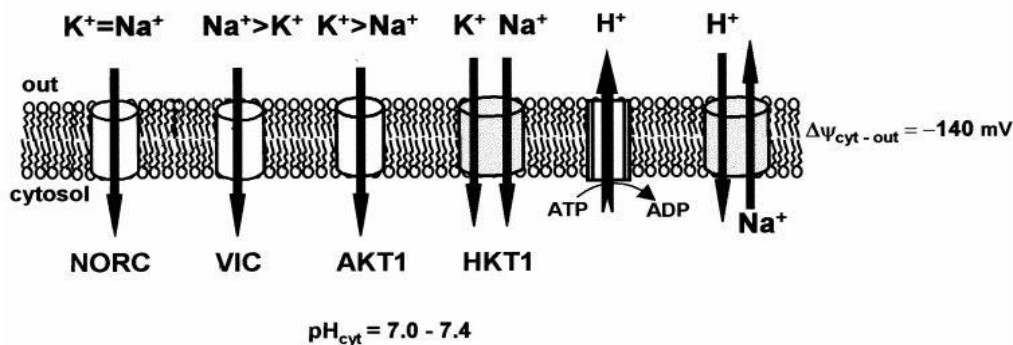
Figura 2. Entrada e extrusão do Na^+ pela membrana plasmática da celula vegetal. A entrada é proprosta que acontece através de NORC (Condutância não-seletiva retificadora externa), VIC (canais de cátions independentes de voltagem), AKT1 (Canal de potássio altamente seletivo para retificação interna) e HKT1 (transportador de potássio de alta afinidade 1) e a extrusão através dos antiportadores Na^+/H^+ com a força próton motora gerada pela H^+-ATPase (BLUMWALD et al., 2000)

A compartimentalização do Na^+ nos vacúolos é um mecanismo conservado em halófitas e glicófitas (BLUMWALD et al., 2000; FAN et al., 2015). Este processo é mediado por um trocador Na^+/H^+ (NHX, Figura 1 e 3) que é impulsionado pelo gradiente eletroquímico de prótons gerados pelas enzimas de translocação H^+ vacuolar, H^+-ATPase e a H^+-Ppiase (Figura 3). A compartimentação de Na^+ nos vacúolos permite evitar os efeitos deletérios do Na^+ no citosol. Além disso, a compartimentação de Na^+ e Cl^- permite que as plantas usem NaCl como osmólito, mantendo um potencial osmótico que conduz a água para dentro das células. Enquanto as plantas sensíveis ao sal dependem principalmente da exclusão de íons Na^+ na membrana plasmática, as espécies tolerantes ao sal acumulam grandes quantidades de Na^+ nos vacúolos. E a ausência do antiportador Na^+/H^+ parece estar relacionado com a dependência das plantas sensíveis a extrusão de Na^+ e não a acumulação deste (BLUMWALD et al., 2000). Outro mecanismo de compartimentalização de Na^+ no vacúolo é por indução de endocitose. Assim, a formação de vesículas com transportadores SOS1 permite o ingresso de Na^+ nas vesículas e posterior transporte ao vacúolo (HAMAJI et al., 2009).
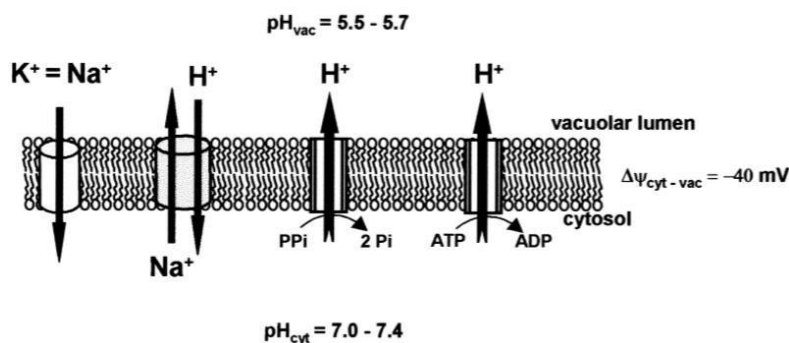
Figura 3. Transportadores usados pela celula vegetal para compartimentalizar o Na^+ nos vacúolos que incluem transportadores de K^+ e os antiportadores Na^+/H^+. O ultimo usa a força próton motora gerada pela H^+-ATPase (BLUMWALD et al., 2000).

1.2.3.2 Papel do cálcio no estresse salino

Além do papel do cálcio no efluxo do Na^+, também tem sido demonstrado que o cálcio melhora os efeitos adversos da salinidade nas plantas (KAYA & HIGGS, 2002; SHABALA et al., 2006; TUNA et al., 2007). O cálcio desempenha um papel essencial em processos que preservam a integridade estrutural e funcional das membranas vegetais (TUNA et al., 2007), estabilizam estruturas de parede celular (NEVES-PIESTUN & BERNSTEIN, 2001), regula o transporte e seletividade de íons e controla a troca iônica, também como atividades enzimáticas da parede celular (QIU et al., 2003; ASHRAF & OROOJ, 2006).

Numerosos estudos mostraram que uma variedade de condições de estresse, incluindo a seca (CHEONG et al., 2003), salinidade (TATTINI & TRAVERSI, 2008), choque térmico (GONG et al., 1998) e choque frio (CHEONG et al., 2003) induzem acumulações citosólicas de Ca^{2+}. O papel do Ca^{2+} como segundo mensageiro em muitos sistemas biológicos, juntamente com estas observações, indica que as plantas são capazes de se adaptar a ambientes com alto teor de sal, ativando um sistema de transdução de sinal envolvendo Ca^{2+} (HASEGAWA et al., 2000b; PARRE et al., 2007). A transmissão do sinal de estresse envolve aumentos transitórios no Ca^{2+} citosólico desencadeando vias a jusante pela interação com diferentes sensores, como calmodulina (domínio de proteínas quinases) e proteínas semelhantes à calcineurina B (KNIGHT et al., 1997; MANABE et al., 2008)

1.2.3.3 Biossíntese de osmólitos

Uma resposta para mudar o potencial osmótico externo é o acúmulo de metabólitos na raiz e parte aérea que atuam como solutos compatíveis. Isto significa que eles não inibem as reações metabólicas normais. São pequenas moléculas com baixo peso molecular, eletricamente neutras, altamente solúveis e não-tóxicas em concentrações molares. A função deles é proteger as estruturas, suportar o balanço osmótico evitando a perda de água a curto prazo e melhora o turgor e expansão celular em longo prazo (SLAMA et al., 2015). Os metabólitos frequentemente observados como osmólitos podem ser caracterizados em três tipos. Osmoprotetores que contêm compostos de amônio (poliaminas, glicina betaína, β-alanina, betaína, propionato de dimetilsulfônio e colina-Osulfato),

osmoprotetores contendo açúcares (sacarose e frutose), açúcar álcoois (trealose, frutano, manitol e D-ononitol e sorbitol) e osmoprotetores contendo aminoácidos ácidos (prolina e ectoína) (ABDELGAWAD et al., 2014; HUANG & JANDER, 2017). Eles são tecido-específicos e espécie-específicos (SINGH et al., 2015).

Os solutos compatíveis são tipicamente hidrofílicos, o que sugere que eles podem substituir a água na superfície das proteínas, ou membranas. Isso cria uma situação na qual as estruturas proteicas nativas são favorecidas termodinamicamente porque eles apresentam a menor área possível para a água. Pelo contrário, os sais entram na esfera de hidratação e interagem diretamente com as superfícies proteicas, favorecendo o desdobramento (CHEN et al., 2007). Assim, os osmólitos incrementam a estabilidade de enzimas, diminuindo o efeito desnaturante de altas concentrações de sais e outros solutos prejudiciais (TIMASHEFF, 1992). Em ambientes secos ou salinos, os osmoprotetores podem servir tanto para elevar a pressão osmótica no fluido celular, como para proteger os constituintes das células. Seus efeitos protetores também se estendem a temperaturas extremas (LEE et al., 2012). Nas células vegetais, os osmoprotetores são tipicamente confinados principalmente ao citosol, cloroplastos e outros compartimentos citoplasmáticos. Engenharia genética de plantas para sintetizar e acumular moderadamente osmólitos mostrou uma melhora na tolerância da planta sob estresse abiótico (RONTEIN et al., 2002).

1.2.3.4 Transporte de água através de aquaporinas

Estresses ambientais, como salinidade, seca e baixa temperatura podem reduzir rapidamente as taxas de transporte de água. Portanto, as plantas precisam ter várias respostas adaptativas para lidar com tensões ambientais e seus efeitos no balanço hídrico. As aquaporinas (AQPs) desempenham um papel fundamental na manutenção da homeostase da água e no equilíbrio sob diferentes condições de estresse ambiental (LUU & MAUREL, 2005). As AQP são proteínas hidrofóbicas de canal pertencentes à superfamília das proteínas intrínsecas principais (MIP). O principal papel das aquaporinas nas plantas é o transporte de água e outras pequenas moléculas neutras através das membranas biológicas celulares. Podem estar envolvidas na distribuição de solutos compatíveis, na transferência de gases (CO_2, NH_3), na captação de micronutrientes (ácido bórico), transporte de H_2O_2 e glicerol. As AQPs permitem o transporte de água para dentro e para fora das células ao longo do

gradiente do potencial hídrico. As AQPs estão localizadas nas membranas celulares e são encontradas em todos os organismos. No entanto, a maioria das AQPs que foram descritas em plantas estão localizados no tonoplasto e membranas plasmáticas. A regulação da atividade da AQP e da expressão gênica, também são consideradas como parte dos mecanismos de adaptação às condições de estresse e dependem de processos e vias de sinalização, bem como fatores transcricionais, traducionais e pós-transcricionais complexos. A abertura de AQPs é através de diferentes mecanismos, tais como fosforilação, tetramerização, pH, cátions, espécies reativas de oxigênio, fitohormônios e outros agentes químicos (KAPILAN et al., 2018).

1.2.3.5 *Sinalização por espécies reativas de oxigênio e produção de antioxidantes*

O estresse salino causa acúmulo de espécies reativas de oxigênio (ROS), tais como radicais hidroxila, radical superóxido, oxigênio singleto e peróxido de hidrogênio nas células vegetais. Em baixas concentrações, podem servir como sinalizadores que podem causar uma resposta ao estresse. Mas em altas quantidades podem causar danos nas estruturas celulares e macromoléculas como enzimas, DNA e lipídios (MILLER et al., 2010). Assim, as concentrações de ROS devem ser equilibradas dentro da célula. O estresse salino induz sistemas enzimáticos e não-enzimáticos para mitigar o estresse oxidativo (GILL & TUTEJA, 2010). Os métodos enzimáticos incluem a superóxido dismutase (SOD), ascorbato peroxidase (APX), catalase (CAT), glutationa peroxidase (GPX), guaiacol peroxidase (GP), dehidroascorbato redutase (DHAR), monodehidroascorbato redutase (MDHAR), glutationa redutase (GR), e glutationa S-transferase (GST) (SREENIVASULU et al., 2000; MELONI et al., 2003; DAS & ROYCHOUDHURY, 2014; BEGARA-MORALES et al., 2015; LI et al., 2015; WANG et al., 2017). Os não-enzimáticos incluem ácido ascórbico (ASH), alcalóides, carotenóides, flavonóides, glutationa, compostos fenólicos, e tocoferol. Estes mecanismos não são específicos de estresse salino, mas fazem parte da maioria das respostas à estresses abióticos.

1.2.3.6 Dinâmica do citoesqueleto

Estudos sugerem que o citoesqueleto é um participante importante nas respostas das plantas ao estresse salino. A arquitetura do citoesqueleto da célula é determinada por microtúbulos (MTs), microfilamentos de actina (MFs) e proteínas que interagem com MT/MF. O citoesqueleto vegetal participa de muitos processos celulares, tais como ancoragem da membrana, motilidade extracelular e intracelular, divisão celular e crescimento (LLOYD, 2011; BASHLINE et al., 2014). O citoesqueleto é altamente dinâmico como consequência da rápida polimerização e despolimerização dos MT / MF e pode ser reorganizado adaptativamente nas células vegetais como respostas a estresses abióticos, incluindo estresse salino (WANG et al., 2010a; WANG et al., 2011).

Foram realizados estudos em *Arabidopsis* onde se avaliou a resposta dos filamentos de actina (MF) e microtúbulos ao estresse salino (WANG et al., 2007, 2010a, 2011). Os resultados mostraram que o estresse pode induzir a montagem de MF e formação de feixes. Por outro lado, os MTs despolimerizam e depois repolimerizam quando as plântulas de *Arabidopsis* são estressadas com sal. Além disso, se observou maior sobrevivência das células ao estresse salino, quando foram primeiro submetidas a drogas desestabilizadoras de MT, do que aquelas que foram submetidas a drogas estabilizadoras de MT. Isto é devido a uma interrupção inicial do MT, levando a uma posterior reorganização. Tanto a despolimerização quanto a reorganização dos MTs são importantes para a capacidade da planta de suportar o estresse salino. Assim, a destruição da rede MT induzida por sal não é o resultado de dano celular, mas desempenha um papel vital na resposta ao estresse salino (WANG et al., 2007, 2010a, 2011).

A celulose é um componente principal da parede celular e é sintetizado pelos complexos enzimaticos de celulose sintase (CSCs) na membrana plasmática. ENDLER et al. (2015) demostrou, como as plantas mantêm sua capacidade de produção de celulose durante condições salinas. Sob condições normais, os CSCs produzem celulose na membrana plasmática e são guiados por proteínas de interação a celulose sintase (CSI1) ao longo de microtúbulos corticais. Após exposição ao estresse salino, os microtúbulos são despolimerizados e os CSCs deslocados da membrana plasmática para dentro de compartimentos pós-Golgi, ou compartimentos associados a microtúbulos. As proteínas companheiras de celulose sintase (CC), que interagem com CSCs e os microtubulos, promovem formação e dinâmicas dos microtúbulos, que levam ao estabelecimento de um arrajo de microtúbulos

tolerantes ao sal. As CSC reabastecem a membrana plasmática, e a síntese de celulose pode ser restauradas. Na ausência de atividade de CC (mutantes cc1cc2), o arranjo de microtúbulos tolerantes ao estresse não é produzidao, e os CSCs não preenchem novamente a membrana plasmática.

Por outro lado, o influxo de íons transitórios de cálcio no citoplasma induzido pelo estresse salino é um importante mecanismo de resposta. Os resultados de vários estudos mostraram que os MTs podem estar envolvidos na regulação da atividade canais de Ca^{2+} em condições normais. Foi observado um aumento nas concentrações livres de Ca^{2+} no citosol após despolimerização inicial dos MTs induzida pelo estresse salino, e esse aumento foi inibido quando um medicamento para estabilização da MT foi adicionado. Além disso, a recuperação de MTs induzida por sal é regulada pelos aumentos nos níveis de $[Ca^{2+}]$ citosólicos. Assim, se acredita que a despolimerização dos MT desencadeia o influxo de Ca^{2+} e este tem o efeito regulador da remontagem da MT sob estresse salino (THION et al., 1998). Por outro lado, também se acredita que os MT tem um papel ativador da tolerância ao estresse salino através da rota de sinalização SOS (NAKAJIMA et al., 2004).

1.2.3.7 Resposta hormonal

1.2.3.7.1 Ácido abscísico

O ácido abscísico (ABA) é um fito-hormônio envolvido em uma ampla gama de respostas vegetais e é essencial para o desenvolvimento e a sobrevivência das plantas. O hormônio atua como um importante sensor de estresse abiótico, levando a respostas protetoras, como fechamento estomático, dormência das sementes e inibição do crescimento e germinação (MUSTILLI et al., 2002; FUJII & ZHU, 2009). Mesmo nos estágios iniciais do desenvolvimento da planta, ABA aciona mecanismos de tolerância ao estresse e/ou prevenção, ajudando as plantas a sobreviverem em condições adversas (UMEZAWA et al., 2010). As serina-treonina-quinases SnRK2.2/2.3/2.6 (proteinoquinases relacionadas ao SNF1) desempenham um papel central na resposta da via ABA como reguladores positivos da sinalização (MUSTILLI et al., 2002; FUJITA et al., 2009). As quinases regulam a expressão de genes responsivos ao estresse e fatores de transcrição, levando a respostas relacionadas ao ABA. A atividade das quinases é modulada por suas interações com a FOSFATASE 2C (PP2C), que inativa SnRK2s por desfosforilação (UMEZAWA et al., 2009). Na

presença do hormônio, o complexo formado pelos receptores ABA e PYL/PYR/RCAR recrutam e inativam o PP2C, impedindo a entrada do substrato (PARK et al., 2009; ZHANG et al., 2015).

Apesar dos papéis essenciais das proteínas PP2C e SnRK2s na ativação das respostas via ABA, seus efeitos nas células vegetais são influenciados por intercomunicações (*crosstalk*) com outros hormônios. Por exemplo, a dormência das sementes é afetada pela interação de ácido abscísico com giberelinas e etileno (ARC et al., 2013). Além disso, o movimento estomático é regulado sob estresse por ácido jasmônico, citocininas, etileno, auxina e também brassinosteróides (DASZKOWSKA-GOLEC & SZAREJKO, 2013; O'BRIEN & BENKOVÁ, 2013). Em geral, sob condições favoráveis, a sinalização cruzada entre ABA e outros hormônios em relação ao crescimento resulta na atenuação das respostas relacionadas ao ABA por diversos mecanismos moleculares, permitindo o crescimento e desenvolvimento das plantas.

Foi bem caracterizado que o estresse salino ativa rapidamente genes relacionados à biossíntese do ABA, como zeaxantina oxidase, 9-cisepoxicarotenóide dioxigenase, ABA-aldeído oxidase e cofator sulfurase de molibdênio através de uma via de fosforilação dependente de cálcio (KNIGHT et al., 1997; XIONG & ZHU, 2003; VISWANATHAN CHINNUSAMY et al., 2005). As concentrações elevadas do hormônio ABA ajudam as plantas a se acostumarem com a menor disponibilidade de água, fechando os estômatos e acumulando inúmeras proteínas e osmoprotetores para ajuste osmótico (BAUER et al., 2013; SINGH et al., 2015). No entanto, o aumento do nível de ABA inesperadamente leva ao atraso no crescimento das plantas (DELGADO et al., 1994).

Análise da rede global de regulação de genes sob seca e estresse salino mostra que diversos genes responsivos ao estresse são regulados pelo ABA (SHINOZAKI & YAMAGUCHI-SHINOZAKI, 1997; HADIARTO & TRAN, 2011). No entanto, muitos outros genes responsivos ao estresse salino não são controlados pelo ABA, sugerindo que os genes responsivos ao estresse salino poderiam ser controlados de maneira dependente ou independente de ABA.

Fatores de transcrição relacionados com ABA, como ABFs (Fatores de ligação ao elemento responsivo a ABA (ABRE)), MYCs e MYBs são envolvidos na expressão de genes responsivos a estresse salino dependentes de ABA através da ligação direta a ABRE (ACGTGGC), MYCRS (CAnnTG) e MYBRS (YAACR), respetivamente (SHINOZAKI & YAMAGUCHI-SHINOZAKI, 2007). No entanto, os genes da via independente de ABA são regulados pelos fatores de transcrição NAC e DREB2 (AP2 / ERF) via ligação a sequências de DNA de suas regiões promotoras NACRS

e DRE (ligação do elemento responsivo à desidratação; G / ACCGAC) (SHINOZAKI & YAMAGUCHI-SHINOZAKI, 2007).

1.2.3.7.2 *Brassinosteróides*

Brassinosteróides (BRs) são um grupo de hormônios esteróides vegetais que desempenham um papel crítico em uma ampla gama de processos de desenvolvimento das plantas, incluindo divisão e alongamento celular, fotomorfogênese, desenvolvimento reprodutivo, senescência foliar e diversas respostas ao estresse (CLOUSE, 2015). Existe um *crosstalk* entre ABA e BR que inclui dois tipos de regulação: modificação pós-traducional ao nível da proteína e repressão transcricional ao nível do gene. No que se refere à regulação proteína-proteína, a fosforilação e a desfosforilação tem um papel muito importante no *crosstalk* ABA-BR (BELKHADIR & JAILLAIS, 2015).

A presença de brassinosteróide desencadeia a ativação do receptor do tipo BRI1 quinase, da quinase BAK1 e da fosfatase BSU1. Essa fosfatase é responsável pela desfosforilação da quinase BIN2, um importante repressor da sinalização BR (BELKHADIR & JAILLAIS, 2015). As evidências indicam que o BIN2 é um dos principais participantes da conversa cruzada ABA-BR (CAI et al., 2014). Esta quinase pode agir e fosforilar SnRK2.2 eSnRK2.3 de *Arabidopsis, in vitro.* A atividade de BIN2 também afeta outro elemento da via de ABA, corrente abaixo dos SnRK2s, o fator de transcrição básico de zíper de leucina (bZIP), ABA Insensitive 5 (ABI5) (HU & YU, 2014). Na presença de ABA, o ABI5 regula a germinação e o crescimento de sementes, levando a respostas de dormência e parada do crescimento. A ausência de ABA desencadeia respostas de BR ao reprimir o BIN2 através das fosfatases PP2C, ABI1 e ABI2 (UMEZAWA et al., 2009).

Além do BIN2, a quinase BAK1 também parece estar envolvida na reação cruzada ABA-BR. Um estudo mostrou que o BAK1 pode interagir com e fosforilar SnRK2.6 (open stomata 1, OST1) *in vitro* (SHANG et al., 2016), regulando o fechamento estomático (MUSTILLI et al., 2002). A falta de ativação do SnRK2.6 mediada por BAK1 pode explicar o aumento da perda de água por transpiração observada nos mutantes *bak1-3*, mesmo durante o tratamento com ABA (SHANG et al., 2016)

Além das interações proteicas e da modificação pós-traducional, a reação cruzada ABA-BR também compreende mecanismos de regulação no nível transcricional. BES1 tem um papel negativo

na sinalização de ABA. BES1 interage com TOPLESS (TPL)/HISTONE DEACETYLASE 19 (HDAC19). Uma vez ligado ao promotor de ABI3, o BES1 reprime a expressão do ABI3 através da desacetilação de histonas, pela montagem do complexo TPL-HDAC19 (RYU et al., 2014). A inibição direta da expressão de ABI5 parece ser controlada pelo fator de transcrição BZR1. O fator de transcrição induzido por BR se liga às sequências de G-box presentes no promotor ABI5, reduzindo sua expressão (YANG et al., 2016).

1.2.3.8 Resposta epigenética

Foi demonstrado que as plantas podem "memorizar" eventos ambientais passados e podem usar essas "memórias" para ajudar nas respostas quando esses eventos se repetem. A expressão gênica pode ser regulada por mecanismos epigenéticos através de pequenos RNAs, metilação do DNA e modificação de histonas e esses mecanismos são importantes determinantes da aclimatação das plantas aos estresses abióticos (KINOSHITA & SEKI, 2014). Estes são herdados através de divisões celulares mitóticas e, em alguns casos, podem ser transmitidos para a próxima geração.

As histonas são enriquecidas com resíduos de aminoácidos básicos, como lisina e arginina. Os resíduos básicos nas caudas de histonas são modificados covalentemente por metilação, acetilação, fosforilação e ubiquitinação, e essas modificações alteram a atividade dos genes. Os locais de modificação de histonas em *Arabidopsis* foram altamente preservados com outros eucariotos. Geralmente, a acetilação dos resíduos de lisina nas caudas NH_3 e NH_4 neutraliza a carga positiva das caudas de histona, o que diminui sua afinidade pelo DNA e altera a acessibilidade dos fatores de transcrição à cadeia de DNA molde. Como resultado, a acetilação de histonas tende a induzir a ativação gênica (KUO et al., 1996; SHAHBAZIAN & GRUNSTEIN, 2007). Por outro lado, a remoção da acetilação de histonas pode levar à repressão e silenciamento de genes (CHEN & WU, 2010; TO et al., 2011). Os efeitos dos eventos de metilação da histona variam de acordo com o local da modificação. A trimetilação na H3K4 ativa a transcrição, e a dimetilação na H3K9 e H3K27 reprimem a transcrição (CAO & JACOBSEN, 2002). Os modificadores de histonas são bem conservados nas angiospermas e incluem as histonas acetiltransferases (HATs), histona desacetilases (HDACs), histona metiltransferases (HMTs) e histona desmetilases (HDMs). Estudos mostram como algumas histonas deacetilases podem estar envolvidas com o estresse osmótico. Por exemplo,

HDA6, HDA19 mediou a regulação da expressão gênica em estresse salino e tratamento com ABA em *Arabidposis* (CHEN & WU, 2010). Além disso, HD2C foi reprimida no tratamento com ABA em *Arabidopsis* (LUO et al., 2012). Análises de mutantes de *Arabidopsis* demonstraram a função de HDAC na tolerância a estresse salino. As plantas *hda19* exibiram tolerância ao estresse, enquanto as plantas *hda5/14/15/18* exibiram hipersensibilidade ao estresse salino (UEDA et al., 2017). Além disso, em raízes de milho, o estresse salino induziu alterações na acetilação da histona na região promotora de genes do ciclo celular (ZHAO et al., 2014). Foi relatado que inibidores de HDAC como Ky2 e SAHA aumentam a tolerância ao estresse salino em *Arabidopsis* (SAKO et al., 2016) e mandioca (PATANUN et al., 2017), respectivamente. Os inibidores da histona deacetilase (HDI) têm como alvo a deacetilase dependente de Zn^{2+} e agem pelo mecanismo quelante de Zinco.

Além disso, a metilação do DNA é outro dentre os mecanismos epigenéticos que regulam a expressão gênica nas respostas da planta a estresses ambientais. A metilação da citosina no DNA está associada à repressão da cromatina nos promotores dos genes e com repressão da transcrição gênica. Demonstrou-se que o estresse pode induzir hipometilação ou hipermetilação do DNA em plantas como arroz e tabaco (WADA et al., 2004; KARAN et al., 2012). Além disso, a ativação/repressão de alguns fatores de transcrição por metilação do DNA na soja (*Glycine max*) está relacionada à tolerância ao sal (SONG et al., 2013). Por outro lado, o sequenciamento completo do metiloma de *Arabidopsis* indica que linhagens estressadas acumulam 45% mais citosinas diferencialmente metiladas nos locais de GC do que os controles, e 75% delas podem ser herdadas (JIANG et al., 2014). Resultados semelhantes no arroz (*Oryza sativa*) indicam que as alterações induzidas pela salinidade na metilação do DNA podem ser mantidas através de divisões celulares mitóticas (WANG et al., 2015a).

1.3 miRNAs como um componente da regulação gênica

O silenciamento por RNA, mediado por pequenos RNAs não codificadores de 20 a 35 nucleotídeos de comprimento, é uma forma importante e indispensável de regulação de genes entre a maioria dos eucariotos. De acordo com sua origem, modo de processamento e associação de proteínas efetoras, os pequenos RNAs podem ser divididos em quatro categorias principais: microRNA (miRNA), pequeno RNA interferente (siRNA), RNA que interage com PIWI (piRNA,

apenas animais) e pequenos RNA derivados RNAs transportadores (tsRNAs) (BORGES & MARTIENSSEN, 2015; CZECH et al., 2018; ZHU et al., 2018). Ao contrário dos siRNAs e piRNAs, que podem mediar o silenciamento de genes em qualquer nível transcricional (TGS) ou pós-transcricional (PTGS), os miRNAs reprimem predominantemente genes alvo pós-transcricionalmente (BORGES & MARTIENSSEN, 2015).

Os micro RNAs (miRNAs) (com um comprimento entre 20-24 nt) modulam a expressão genética em plantas e animais. Estão envolvidos na regulação gênica de praticamente todas as vias metabólicas celulares, incluindo desenvolvimento da planta e adaptação a estresse biótico ou abiótico. Dessa forma a modulação de sua biogênese/degradação é de suma importância para a manutenção da homeostase celular (AXTELL et al., 2011).

1.3.1 A biogênese dos miRNAs

Os miRNAs são codificados por um gene MIR. Assim de acordo com a localização no genoma, os miRNA podem ser classificados em intergênicos (mais comum em plantas), intrônicos e exônicos. Os miRNA intergênicos são aqueles que se encontram entre dois genes que codificam para proteínas, tem seu próprio promotor, são transcritos como unidades independentes por uma RNA polimerase II e sofrem um processamento por clivagens da proteína Dicer. Enquanto os miRNAs intrônicos estão localizados na região de um íntron de uma unidade transcricional e geralmente usam os promotores de seu hospedeiro. Entre eles há casos particulares onde a sequência precursora dos miRNAs apresenta o tamanho exato do íntron, dependendo da transcrição e processamento do mRNA parental, e não da clivagem pela proteína Dicer. Eles são chamados de "Mirtrons" (MILLAR & WATERHOUSE, 2005; BUDAK & AKPINAR, 2015), este tipo de miRNA é muito mais comum em animais do que em plantas (AXTELL et al., 2011). Existem, no entanto miRNAs localizados em íntrons possuindo seus próprios promotores. Nesses casos, o promotor localiza-se também dentro da região intrônica e o miRNA pode apresentar orientação senso ou antissenso em relação ao mRNA. Os miRNA exônicos, os quais são mais raros, são transcritos dos éxons de genes que codificam proteínas e compartilham os promotores de seu gene hospedeiro (LI et al., 2011b).

Também existem clusters de genes MIR, os quais se encontram agrupados, formando unidades policistrônicas, que podem ser regulados pelo mesmo promotor e, consequentemente, são transcritos simultaneamente (AXTELL et al., 2011). O recrutamento da RNA Pol II envolve a interação de vários ativadores transcricionais e vários motivos de sequência nos promotores de genes MIR (Figura 4). Estes promotores assim como de genes codificantes, apresentam TATA box, modificações em histonas (acetilação) e metilação do DNA, indicando múltiplos componentes no modo de regulação da transcrição dos genes MIR (MEGRAW et al., 2006).

Como produtos canônicos da RNA Pol II, os transcritos primários dos genes MIR (denominados pri-miRNAs) têm uma 7-metilguanosina (CAP) na extremidade 5', são poliadenilados e podem sofrer splicing. Eles são dobrados em estruturas semelhantes a grampos ("hairpin" ou "stem-loop") formando uma fita dupla de RNA (dsRNA), com tronco ("stem") de 33-35 pares de bases, onde se localiza o duplex miR-5p/miR-3p, uma alça terminal, e dois braços (segmentos de fitas simples). O comprimento é muito variável (de 60 nt a mais de 500 nt) e suporta estruturas mais complexas do que suas contrapartes de 70 nt dos animais (AXTELL et al., 2011; ROGERS & CHEN, 2013)

Por outro lado, o tRNA pode originar alguns miRNAs endógenos, fazendo que haja uma participação indireta da RNA pol III nesse processo, já que esta enzima participa na transcrição dos tRNAs (SABLOK et al., 2017). Os pri-miRNAs são reconhecidos e processados por enzimas RNase do tipo III, conhecidas por Dicer (DCLs) e esse processamento pode ocorrer co-transcricionalmente (FANG et al., 2015) (Figura 4). Este processo ocorre em locais subnucleares chamados de corpos de Dicer (corpos-D). A enzima DCL tem dois domínios de Rnase III, um domínio de ligação a dsRNA, um domínio PAZ, e um domínio de helicase DEAD/H box. O tamanho da família das DCL varia de acordo com a espécie. *Arabidopsis* tem 4 membros e arroz 5 (MARGIS et al., 2006; LIU et al., 2009). Da família DCL, o DCL1 é o principal responsável pela clivagem do pri-miRNA em um precursor de miRNA (pré-miRNA) em *Arabidopsis*, a qual é realizada no núcleo com a assistência de um complexo de proteínas acessórias, incluindo a proteína de ligação a fita dupla de RNA *Hyponastic leaves* 1 (HYL1) e a proteína de dedos de zinco Serrate (SE) (DONG et al., 2008). A DCL1 também realiza a subsequente clivagem do pré-miRNA para liberar o duplex miR-5p/miR-3p (AXTELL et al., 2011). Os diferentes membros da família DCL dão origem a miRNAs de diferentes comprimentos. A maioria dos miRNAs da planta têm 21 nucleotídeos, que são processados por

DCL1 ou DCL4. As proteínas DCL2 e DCL3, por outro lado, tendem a gerar miRNAs que são de 22 e 24 nucleotídeos de comprimento, respectivamente (AXTELL et al., 2011).

O duplex miR-5p/miR-3p nascente gerado pelo processamento mediado por DCL exibe 2-nt sobressalentes na extremidade 3' de ambas fitas e cada fita possui um 5' fosfato e dois grupos hidroxila na extremidade 3' (um no carbono 2' e outro no carbono 3'). Embora, os dois grupos hidroxila sejam essenciais, apenas os carbonos 2'-OH são metilados pela metiltransferase HUA Enhancer 1 (HEN1) (YU et al., 2005). Esta metilação ocorre no núcleo e é importante como uma proteção contra degradação. Se acredita que os duplexes de miR-5p/miR-3p metilados são exportados ao citosol pela proteína *Hasty* (HST) (PARK et al., 2005), onde formam o complexo de silenciamento induzido por RNA (RISC) junto com a proteína Argonauta (AGO). *Arabidopsis* possui 10 proteínas AGO, sendo a AGO1 a principal proteína efetora dos miRNAs. Recentemente, BOLOGNA et al., (2018) mostraram que o miRNA é acoplado à proteína Argonauta 1 (AGO1) no núcleo para formar o complexo RISC e é, então exportado para o citosol pela proteína EXPO1. No entanto, os dados atuais não excluem a possibilidade de alguns miRNAs serem exportados como duplexes e acoplados no citosol.

1.3.2 Mecanismo de ação dos miRNAs

O processo começa com a montagem do complexo RISC que requer a chaperona HSP90, facilitado pela Cyclophilin 40/Squint (CYP40/SQN) e inibido pela proteína fosfatase 5 (PP5) (IWASAKI et al., 2010; IKI et al., 2012). Uma fita do duplex miR-5p/miR-3p é seletivamente acoplada na proteína Argonauta (AGO) e a outra é ejetada e degradada. A seleção da fita, 5p ou 3p como guia, é determinada durante o passo de recrutamento da AGO e sua escolha é baseada, principalmente, na estabilidade termodinâmica das duas extremidades do duplex, sendo que a fita que apresenta uma relativa instabilidade na extremidade 5' é selecionada como fita guia (KHVOROVA et al., 2003). Além disso, AGO1 carrega preferencialmente miRNAs com uracila no 5' terminal (MI et al., 2008). Contudo, essa seleção não é completamente rígida e a fita menos favorecida pode ser escolhida como fita guia em algumas situações, sendo o tipo de tecido, o estágio de desenvolvimento da planta ou o tipo de estresse, fatores que podem influenciar nessa escolha (LIU et al., 2017b). Adicionalmente, alguns miRNA parecem ser preferencialmente associados a

diferentes membros da família das AGOs (por exemplo, AGO2, AGO7 ou AGO10) (ZHU et al., 2011b).

Os miRNAs direcionam o RISC para os mRNA alvos através do pareamento de bases e predominantemente medeiam o silenciamento gênico através da desestabilização do RNA alvo ou inibição da tradução. No entanto, estudos recentes também sugerem um papel do RISC/AGO1 na regulação transcricional (YANG et al., 2019). No silenciamento mediado por desestabilização ocorre a clivagem do RNA alvo através da AGO, no sítio do alvo correspondente aos nucleotídeos 10 e 11 do RNA guia, um evento que é mais comum em plantas (LLAVE et al., 2002). O pareamento de bases entre o alvo e o miRNA, em plantas, deve ser quase perfeito na região 5' (não mais que 1 nucleotídeo não pareado ou "mismatch"). O pareamento na região 3' pode conter mais "mismatches" (não mais que 4) (AXTELL & MEYERS, 2018). As proteínas AGO têm três domínios, um PAZ, um MID e um PIWI que são conservados. Os domínios MID e PAZ se ligam aos 5' fosfato e ao terminal 3' dos pequenos RNAs, respectivamente, enquanto o domínio PIWI cliva o RNA alvo através de sua atividade de endonuclease (SWARTS et al., 2014). Os produtos derivados dessa clivagem são rapidamente degradados por exoribonucleases.

Pareamentos imperfeitos entre o alvo e o miRNA, podem promover a inibição da tradução, através de um dos seguintes eventos: competição por ligação ao 5' CAP, inibição da montagem dos ribossomos, deadenilação seguida pelo bloqueio da iniciação da tradução, dissociação prematura de ribossomos, redução da velocidade de elongação ou proteólise durante a fase de elongação.

Tem sido reportado a ocorrência do silenciamento transcricional mediado por microRNAs, que é menos conhecido, sendo observado tanto em animais quanto em plantas, podendo envolver a metilação do DNA em plantas (BAO et al., 2004; WU et al., 2010) ou a modificação da cromatina em mamíferos (YOUNGER & COREY, 2011).

1.3.3   Degradação dos miRNAs

A rotatividade e a degradação dos miRNAs não são apenas importantes para manter a homeostase intracelular do miRNA, mas também fornecem meios para sua liberação em resposta a transições de desenvolvimento e alterações ambientais. Os fatores que afetam a estabilidade do miRNA incluem modificação na extremidade 3', associação a AGO e interação com o RNA-alvo.

Além disso, tem sido caracterizado um grande número de exoribonucleases responsáveis pela degradação de miRNA em vários estágios.

Em relação às modificações na extremidade 3', se sabe que se a proteína HEN1 (encarregada da metilação e estabilização dos miRNAs) sofre mutações ou diminuições na sua expressão, pode ocorrer uma diminuição nos níveis de miRNA, levando a defeitos no desenvolvimento da planta (TSAI et al., 2014). No entanto, não se sabe se o genoma da planta codifica demetilases de miRNA, porque é difícil distinguir se os miRNAs não metilados em plantas são produtos demetilados ou escapes da atividade de HEN1(WANG et al., 2019).

A uridilação desencadeia a degradação de RNA. Em *Arabidopsis*, a HEN1 Suppressor 1 (HESO1) é a principal uridil transferase que catalisa a adição de uracilas à extremidade 3' de miRNAs não metilados (REN et al., 2012). Outra uridil transferase terminal, é a UTP: RNA-uridil transferase 1 (URT1), mas apenas afeta a uridilação de alguns miRNAs (TU et al., 2015).

HESO1 e URT1, não possuem domínios de ligação a dsRNA. Em vez disso, o HESO1 e o URT1 co-localizam e interagem com o AGO1 e podem uridilar os miRNAs ligados ao AGO1 (TU et al., 2015; WANG et al., 2015b). Embora a nuclease que degrada os miRNAs uridilados em plantas ainda precise ser identificada, WANG et al., (2018) sugeriram que as proteínas RRP6-like estão provavelmente envolvidas em degradar miRNAs uridilados.

As pequenas nucleases de degradação de RNA (SDNs), que possuem função semelhante à demetilases, removendo nucleotídeos metilados das extremidades 3', cooperam com HESO1/URT1 e contribuem na degradação de alguns miRNAs metilados. O SDN1 é capaz de degradar pequenos RNAs de fita simples de 17 a 27 nt de comprimento *in vitro*, mas é inativo em duplexes de miRNA/miRNA e RNAs de fita simples longos (RAMACHANDRAN & CHEN, 2008)

As proteínas AGO não apenas servem como proteínas efetoras, mas também influenciam na estabilidade dos miRNAs. Foi observado que a mutação de AGO1 diminui os níveis de miRNA (VAUCHERET et al., 2004). Entretanto, diferentes proteínas AGO podem ter diferentes efeitos na estabilidade dos miRNAs aos quais se ligam, por exemplo a mutação da AGO10 leva a um aumento na quantidade de miR165/166 em *Arabidopsis* (YU et al., 2017).

Considerando que várias enzimas degradativas, como SDN1, HESO1 e URT1, agem sobre os miRNAs associados à AGO, não é surpreendente que as proteínas AGO possam ter, simultaneamente, dois papéis opostos na estabilidade do miRNA (ou seja, proteger os miRNAs da

exposição a várias RNases intracelulares ou por outro lado recrutar fatores de degradação) (WANG et al., 2019).

Em animais, uma alta complementariedade no pareamento de bases entre o miRNA e o RNA alvo pode desestabilizar o miRNA, levando a uma degradação mútua, do alvo e do miRNA (DELA MATA et al., 2015). Além disso, em plantas foi reportado um fenômeno conhecido como *target mimicry* (TM) (FRANCO-ZORRILLA et al., 2007).

Nas plantas, a maioria dos alvos de miRNA são clivados e mostram complementaridade quase perfeita com os miRNAs ao redor do local de clivagem. Um estudo mostrou que um gene não codificador, chamado de IPS1, tem um local de reconhecimento não-canônico do miR399 pela presença de uma protuberância de 3 nt no local de clivagem, que inibe sua clivagem pelo miR399. O IPS1 serve como um alvo mímico (TM) sequestrando o miR399 e impedindo a clivagem dos genes alvos verdadeiros e endógenos (FRANCO-ZORRILLA et al., 2007) . Muitos outros TM têm sido identificados em *Arabidopsis* e arroz por métodos de predição bioinformática (WU et al., 2013). Além disso, TM artificiais tem sido desenhados como estratégia de redução da abundância de miRNA mostrando resultados efetivos (YAN et al., 2012).

1.3.4   Nomenclatura dos miRNAs

Em plantas, os loci genômicos que codificam miRNAs, bem como os pre-miRNAs, são abreviados usando o prefixo **mir**, enquanto o prefixo das formas maduras é grafado com a letra **R** em maiúsculo, **miR**. Ambas designações são seguidas de um hífen e um único número de identificação, sendo, portanto, mir-# e miR-#, respectivamente (AMBROS et al., 2003). Em plantas, sugere-se o uso de letras maiúsculas (**MIR**) para grafar os pre-microRNAs (AMBROS et al., 2003). Assim gma-MIR-482 é um pre-miRNA de *Glycine max* a partir de onde se gera o maduro miR-482.

Alguns genes de microRNAs apresentam várias cópias parálogas no genoma. Quando tais parálogos originam sequências maduras idênticas ou muito similares (de zero a 4 *mismatches*), acrescenta-se mais um hífen e mais uma letra minúscula. Considerando, que os miRNAs maduros devem derivar do mesmo braço do *stem-loop* em todos os casos. Sendo assim, a nomenclatura dos genes é grafada dessa forma mir-172a, mir-172b, mir-172c (MEYERS et al., 2008; AXTELL & MEYERS, 2018). Esses miRNA idênticos ou muito próximos, têm sido agrupados em famílias. No

entanto, existem alguns genes MIR que codificam sequências maduras de miRNA semelhantes que, por razões históricas, receberam identificadores distintos (por exemplo, as famílias miR156/157, miR165/166 e miR170/171) (MEYERS et al., 2008). Quando dois ou mais *stem-loop* produtores de miRNA são dispostos *in tandem* em um único precursor, cada *stem-loop* é tratado como um locus distinto (por exemplo, MIR156b e MIR156c de *Zea mays* (CHUCK et al., 2007)

A partir de 2002 foi criada uma base de dados chamada *the miRNA Registry*, que evoluiu para o que conhecemos hoje como **miRbase** (GRIFFITHS-JONES et al., 2006). Onde hoje (*release* 22, http://www.mirbase.org/) se encontram depositados 684 precursores, e 756 maduros de *Glycine max*. No miRBase, um prefixo de três caracteres indica o nome da espécie, como gma-mir-# para *Glycine max*.

Para diferenciar de qual braço do pre-miRNA provém o miRNA maduro, se usa o sufixo -5p para aquele que provém da porção 5' e -3p para aquele da porção 3' (KOZOMARA & GRIFFITHS-JONES, 2014). Por exemplo, gma-miR-482b-5p ou gma-miR-482b-3p, respectivamente.

Até cinco *mismatches* entre o miR-5p e o miR-3p e até duas protuberâncias assimétricas contendo um total de três nucleotídeos protuberantes, são permitidos nos critérios de anotação (AXTELL & MEYERS, 2018).

Quando um pequeno RNA for anotado como miRNA, deve ser levado em consideração que o tamanho do miRNA não poder ser menor que 20 ou maior que 24 nt. Já que os pequenos RNAs heterocromáticos são majoritariamente de 23 ou 24 nt, e que os miRNAs deste tamanho são raros, se sugere evitar anotações de RNAs nessas condições como miRNA (AXTELL & MEYERS, 2018).

1.3.5   Isoformas de miRNAs (isomiRs)

IsomiRs são variantes do miRNA canônico e já foi demonstrado que podem desempenhar um papel importante na regulação pós-transcricional, em resposta a vários estresses abióticos e bióticos (GOZMANOVA et al., 2017). A via de biogênese dos isomiR em plantas ainda não foi amplamente elucidada, no entanto, considerando que a clivagem de DCL1 não é 100% precisa, vários estudos levantam a hipótese de que as variações na clivagem são a principal causa da biogênese dos isomiRs (MORIN et al., 2008a, 2008b). Estas variantes possuem adição e/ou subtração de nucleotídeos na região das extremidades 5' ou 3' do miRNA maduro canônico. As

características de variações do comprimento, classificam esses isomiRs em isomiRs 5', isomiRs e isomiRs 3' polimórficos (NEILSEN et al., 2012; ROGANS & REY, 2016)

Outra origem especulada para os isomiRs é a ocorrência de uma adição de nucleotídeos na extremidade do miRNA através de uma nucleotidiltransferase PAPD4 (BURROUGHS et al., 2010). Também tem sido demonstrado que substituições de nucleotídeos na parte interna do miRNA podem ter sido originadas através da edição pós-transcricional do RNA (NEILSEN et al., 2012; ROGANS & REY, 2016).

Um mesmo pre-miRNA pode gerar diversas sequências isoméricas irmãs (AHMED et al., 2014). Nestas sequências, as extensões de um único nucleotídeo no terminal 3' são o tipo prevalente de modificação identificada (MORIN et al., 2008a, 2008b). Alterações no terminal 5' são consideradas críticas, já que resultam em mutações na sequência *seed (uma região que corresponde aos primeiros 9 nt da extremidade 5' do miRNA)*, a unidade responsável pela especificidade e funcionalidade de um miRNA sobre seu mRNA alvo. A *seed* é altamente conservada, sendo que a alteração de um único nucleotídeo nesta região pode influenciar na diversidade dos alvos do miRNAs. Em função disso, em animais tem sido reportado que os isomiR 5' são mais raros do que os isomiR 3' (BEREZIKOV et al., 2011).

1.3.6   Os miRNAs conservados e não conservados

Alguns miRNAs são conservados, estando presentes em várias espécies, embora não haja miRNAs estritamente conservados entre todos os organismos. Vários miRNAs ancestrais, como miR156, miR160, miR165/166, miR167, miR319, miR390, miR395 e miR408 estão presentes em plantas terrestres e em musgos, sugerindo que essas famílias de miRNA são universais em linhagens de Embryophyta (TAYLOR et al., 2014). Os miRNAs conservados regulam predominantemente fatores de transcrição ancestrais ou enzimas envolvidas no desenvolvimento básico das plantas ou na tolerância a estresses. Por exemplo, o miRNA 172, que é um repressor da tradução de APETELA (AP1 e AP2), regula o desenvolvimento da flor em várias espécies de plantas (CHEN, 2004). Similarmente, o miRNA 319 regula genes conservados envolvidos com a florescência e morfologia da folha (ZHANG et al., 2006).

Um grande número de miRNAs de plantas são não conservados, sendo que eles e seus alvos correspondentes estão presentes apenas em algumas espécies estreitamente relacionadas ou parecem ser exclusivos de algumas espécies (QIN et al., 2014). Eles têm funções muito específicas, como a regulação da diferenciação das fibras de algodão pelo miR-828 através da clivagem do transcrito de MYB2 (GUAN et al., 2014).

Ao contrário dos miRNAs conservados com alta abundância e baixas variações de sequência, os miRNAs não conservados são frequentemente processados imprecisamente, possuem expressão mais baixa e um menor número de alvos funcionais; assim, em alguns casos, eles foram considerados produtos transitórios e desperdiçadores de energia no genoma da planta. Apesar disso, alguns miRNAs não conservados são expressos abundantemente em tecidos específicos ou muito induzidos em condições particulares, indicando um possível papel fisiológico dos miRNAs não conservados de plantas para adaptações ambientais especiais (QIN et al., 2014)
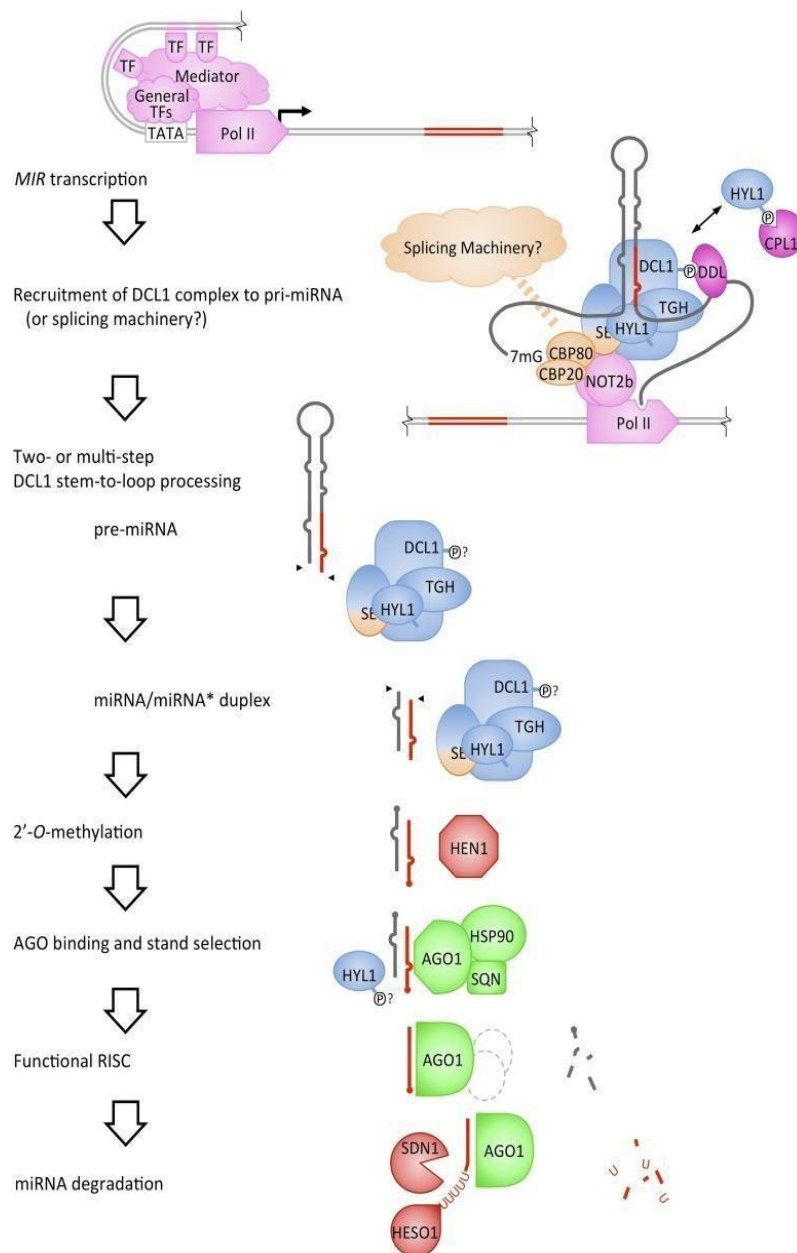
Figura 4. Biogênesis dos miRNAs O miRNA é codificado por um gene MIR, o qual é transcrito por uma RNA pol II, depois do pri-miRNA ser transcrito este será recrutado e processado pelo complexo DCL (Dicer) para gerar um pre-miRNA, que será clivado novamente por DCL até obter um miRNA maduro de fita dupla, o qual será metilado por HEN1. Uma fita será degrada e a outra formará um complexo com AGO para produzir o silenciamento gênico. Uma uridilação do miRNA por HESO1 desencadeia a degradação de RNA com a ajuda da atividade nucleasse de SDN (ROGERS & CHEN, 2013).

1.3.7   Análise da expressão dos miRNAs por RT-qPCR

Uma das limitações da amplificação do miRNA por PCR é a sua sequencia curta em relação ao tamanho dos oligonucleotídeos iniciadores usados. Duas estratégias têm sido implementadas para superar esta restrição: Uma delas é a adição de caudas de poli(A) em todas as moléculas de miRNA da amostra, a qual serve como sítio de ancoramento para um iniciador universal com cauda poli(T) para realizar a síntese de cDNA pela enzima transcriptase reversa, em seguida a amplificação por qPCR é realizada com um iniciador específico para o miRNA desejado e o iniciador reverso universal (DESIGN, 2005). As limitações desta técnica são que todas as moléculas de RNA são poliadeniladas neste passo, e assim não se pode distinguir, confiavelmente, entre pre-miRNAs e miRNAs maduros. Mas deve-se levar em consideração que os pre-miRNAs possuem baixa meia-vida e, muitas vezes, sequer são detectados em plantas por *northern blot* (DESIGN, 2005). Por outro lado, esta técnica não é útil para plantas, devido a sua incapacidade de detectar pequenos RNAs, já que eles possuem a modificação 2´-oximetil no terminal 3´, que impede a poliadenilação (KUMAR et al., 2011)

A outra estratégia, que é utilizada em plantas, emprega iniciadores *Stem-loop* na reação de transcrição reversa (RT) em baixas temperaturas (CHEN et al., 2005). Eles contêm uma sequência com 44 nucleotídeos conservados e seis específicos para cada miRNA, desenhados de modo que sua extremidade 3' seja complementar aos seis últimos nucleotídeos da extremidade 3' do miRNA-alvo. A síntese de cDNA é seguida por PCR com iniciadores *forward* específicos para o miRNA e um iniciador reverso universal, com sequência complementar a uma região da sequência conservada do *stem-loop*. Este método pode ser realizado como multiplex, assim em uma só reação de RT podem ser usados iniciadores para diferentes miRNAs (CHEN et al., 2005).

1.4 Importância da soja como objeto de estudo

Soja (*Glycine max* (L) Merril) é uma planta leguminosa, com aproximadamente 40% de conteúdo de proteína e 20% de óleo na semente. Esta cultura tem o maior conteúdo protéico e a maior produção de óleo vegetal do mundo. Sendo assim uma das culturas mais importantes (SINGH, 2010). O Departamento de Agricultura dos EUA estima que a produção mundial de soja nos anos de

2017 e 2018 foi de 346,02 milhões de toneladas. Prevê-se os países Brasil, Argentina e EUA, produziram mais de 82% da soja do mundo, sendo o Brasil produtor de 112 milhões de toneladas (FAOSTAT, 2018)

Os usos da soja incluem produção de óleo comestível, tinta e biodiesel. Além disso, a proteína de soja é usada em rações animais, suplementos alimentares humanos, salsichas, produção de pães e concentrados proteicos. A produção de fibra proteica também inclui o uso de proteína de soja, sendo misturada com algodão, lã ou fibras químicas (SINGH, 2010).

A soja também é importante por sua capacidade de melhorar as propriedades do solo através de seu sistema radicular profundo e proliferativo e a capacidade de fixar nitrogênio atmosférico no solo em associação com a bactéria *Bradyrhizobium*. Também, a biomassa gerada através da queda das folhas, depois da colheita, serve como adubo e cobertura de campo, conservando a umidade do solo. Tudo isso faz a soja uma cultura útil para muitos sistemas de cultura sequenciais e misturados (SINGH, 2010).

A origem da cultura de soja foi na China. O progenitor da atual soja cultivada (*Glycine max*) é a espécie selvagem *Glycine soja* (DOYLE & BEACHY, 1985) a qual está distribuída através da China, Japão, Coreia e Rússia. Na primeira metade do século 20 a China foi o maior produtor e exportador de soja no mundo, onde é usada para produção de tofu, leite de soja, molho, etc. Contudo, em 1996, a China começou a importar soja, pois, a oferta não satisfazia a demanda; assim a China é hoje o maior importador do mundo. Na década de 1950, a cultura de soja nos EUA se desenvolveu rapidamente e agora este país é o maior produtor do mundo. Na década de 1970 a produção desta cultura se desenvolveu no Brasil, que é agora o segundo maior produtor de soja, seguido da Argentina. Sendo todos eles grandes produtores e exportadores, com uma produção mecanizada (SINGH, 2010). Em 2015, o Brasil exportou cerca de 57 milhões de toneladas de soja (AN & OUYANG, 2016), principalmente para a China (DE LIMA et al., 2018).

A soja é cultivada nos trópicos, subtrópicos e climas temperados, principalmente sob condições dependentes da chuva, mas, a irrigação suplementar é cada vez mais usada (FAOSTAT, 2001). Com respeito às temperaturas, as taxas de crescimento da soja diminuem acima de 35 °C e abaixo de 18 °C. A duração do período total da cultura é de 100 a 130 dias ou mais. Sendo que demora cerca de 10 dias na fase inicial de estabelecimento, 30-40 dias na fase vegetativa, 25-35 na fase de floração, 30-40 na formação do grão, e 10-15 dias na fase de amadurecimento. A cultura pode ser realizada

em uma grande variedade de solos, exceto nos muito arenosos. O pH ideal do solo é de 6 a 6,5. Um lençol freático raso, particularmente durante o período de crescimento inicial, pode afetar adversamente a produção. A planta é sensível ao alagamento, mas moderadamente tolerante à salinidade do solo. A diminuição do rendimento devido à salinidade do solo é: 0% a uma condutividade elétrica (ECe) de 5 dS/m, 10% a 5,5, 25% a 6,2, 50% a 7,5 e 100% a 10 dS/m (FAOSTAT, 2018). Sendo que ECe de 4 dS m^{-1} é aproximadamente 40 mM NaCl.

A soja é um importante modelo de estudo, por isso, a sequência completa de seu genoma já se encontra disponível. O lançamento oficial do genoma de referência da cultivar de soja Williams 82, uma cultivar dos EUA, foi em 2010 (SCHMUTZ et al., 2010), o qual marcou a nova era da pesquisa em soja. Existem no total 56.044 modelos de genes nessa montagem. Depois disso, outras cultivares e sojas silvestres também foram sequenciadas (LAM et al., 2010; QIU et al., 2014; ZHOU et al., 2015).

1.4.1 Soja como modelo de estudo do estresse osmótico

Muitos estudos tentaram avaliar o efeito do estresse osmótico na soja usando diferentes perspectivas (Tabela 1). Alguns estudos foram focados em análises fisiológicas para avaliar o efeito do estresse salino no crescimento da planta, na fixação de nitrogênio, na distribuição de íons e na fotossíntese, usando diferentes espécies selvagens e cultivadas de soja (DELGADO et al., 1994; LIU et al., 2017). Outros estudos focaram a detecção de marcadores de polimorfismos de nucleotídeo único (SNP), usando 104 linhagens de soja, em que foi observada uma correlação entre o genótipo e o fenótipo (clorose das folhas, conteúdo de clorofila e acúmulo de Na$^+$) sob o tratamento com sal (PATIL et al., 2016). Além disso, *Quantitive Trait Loci* (QTLs) foram identificados em cruzamentos de uma cultivar moderadamente sensível com uma tolerante, para mapear genes de tolerância ao sal (WANG et al., 2016; DO et al., 2018). Outros fizeram análises proteômicas de raízes e folhas de soja submetidas a seca e sal. As análises revelaram que as proteínas associadas ao ajuste osmótico, sinalização da defesa, homeostase de Redox e morte celular programada desempenham papéis importantes na adaptação à seca e sal nas plantas de soja (ALAM et al., 2010; JI et al., 2016). Por outro lado, análises de perfis metabólicos revelaram que uma espécie de soja selvagem contém

quantidades significativamente mais altas de aminoácidos, ácidos orgânicos, carboidratos, polióis, ácidos graxos e outros compostos sob estresse do que a soja cultivada (ZHANG et al., 2016).

Perfis de expressão gênica usando dados de RNA-Seq também têm sido feitos. Usando cultivares sensíveis à seca, foram avaliados os genes envolvidos em sinais hormonais, fotossíntese, fixação de carbono e nitrogênio e metabolismo de aminoácidos sob tratamento com sal usando análises de co-expressão (LIU et al., 2019). Também foram comparados genótipos tolerantes a sal com outros sensíveis em diferentes tempos de tratamento (ZENG et al., 2018). O processamento pós-transcricional através de edição do mRNA foi demonstrado ser induzido em cloroplastos de soja sob estresse salino (RODRIGUES et al., 2017). O presente trabalho é o primeiro em produzir transcriptogramas de soja a partir de dados de RNA-seq, para comparação de uma cultivar tolerante a seca e outra sensível, podendo conhecer os patrões de expressão de diferentes processos biológicos sob estresse salino dessas cultivares, e propondo regulação pós-trancricional das rotas de sinalização de ABA e Brassinosteroides.

O Perfil de expressão de miRNAs em nódulos normais ou estressados de soja já foi estudado, onde 110 miRNAs conhecidos e 128 novos miRNAs foram identificados (DONG et al., 2013). O transcriptoma de pequenos RNAs também foi realizado em raízes de soja cultivadas sob condições normais e sob estresse salino. No total, 71 miRNAs, incluindo variantes conhecidas e novas de 59 famílias de miRNA, foram identificados. Curiosamente, foram encontrados elementos cis responsivos à auxina nos promotores de muitos miRNAs responsivos ao sal, implicando que esses miRNAs possam ser regulados pela sinalização de auxina e desempenham um papel fundamental na regulação da plasticidade do miRNAoma e no desenvolvimento da raiz em soja (SUN et al., 2016). miRNAs e seus alvos foram identificados sob estresse por seca, sendo que os alvos incluem genes de fatores de resposta à auxina, superóxido dismutase de Cu/Zn, lacases e plantacianina (ZHENG et al., 2016). Adicionalmente, a superexpressão de alguns miRNAs de *Glycine max* melhorou a tolerância a sal e seca em *Arabidopsis thaliana* (NI et al., 2012; LI et al., 2016; YU et al., 2019). Recentemente, RAMESH et al., (2019) apresentou através de um artigo de revisão, muitos dos miRNAs identificados até agora em Soja, sendo muitos deles responsivos a algum tipo de estresse. No presente trabalho se reportam miRNAs do tecido folhar de soja sendo modulados por estresse salino e os seus potenciais alvos. Alem disso, se propõe regulamento epigenetico de alguns miRNAs de soja.

Tabela 1. Estudos sobre estresse osmótico realizados em soja

Tipo de estudo	Perspectivas	Referencia
Efeito de sal e/ou seca	Análises fisiológicas	(DELGADO et al., 1994; BAI et al., 2019)
	Análises de proteômica	(ALAM et al., 2010; JI et al., 2016)
	Perfis de expressão genica	(LE et al., 2011; LIU et al., 2019)
	Identificação de SNP e QTL	(PATIL et al., 2016)
	Perfil metabolómico	(ZHANG et al., 2016)
	Processamento pós-transcricional	(RODRIGUES et al., 2017)
Estresse salino e miRNAs	Nódulos	(DONG et al., 2013)
	Raiz	(SUN et al., 2016; NING et al., 2019)
	superexpressão do gma-MIR172c	(LI et al., 2016)
Seca e miRNAs	Raiz	(KULCHESKI et al., 2011; ZHENG et al., 2016)
	Superexpressão do gma-MIR394a	(NI et al., 2012)
	Superexpressão do gma-MIR172c	(LI et al., 2016)
	superexpressão do gma-MIR169c	(YU et al., 2019)
miRNAs	Raiz, semente, flor, nódulo	(JOSHI et al., 2010)
	Cadmium, falta de nitrogenio, estresse por aluminio	(FANG et al., 2013; WANG et al., 2013; HUANG et al., 2018)
	Estresse biótico	(KULCHESKI et al., 2011; WONG et al., 2014; RAMESH et al., 2019)
	Simbioses com *Bradyrhizobium japonicum*	(BARROS-CARVALHO et al., 2014)

2. OBJETIVOS

2.1 Objetivo geral

Identificar mecanismos moleculares envolvidos na homeostase do estresse salino em soja.

2.2 Objetivos especificos

2.2.1   Identificar mecanismos moleculares envolvidos na homeostase do estresse salino em soja, comparando a expressão gênica de duas cultivares contrastantes (uma tolerante e outra suceptivel a seca) submetidas ao estresse salino.

2.2.2   Identificar mecanismos de regulação pós-transcricional dos miRNAs como resposta ao estresse salino em soja.

2.2.3   Identificar mecanismos de resposta de soja que possam ser semelhantes nos estresses causados por sal e por PEG.

2.2.4   Avaliar a resposta epigenética sob estresse salino envolvida com a regulação gênica por miRNAs.

3. RESULTADOS

3.1 Capítulo 1. Transcriptomic and post-transcriptional analyses of two soybean cultivars under salt stress.

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Abstract

Soybean is an economically important plant, and its production is affected in soils with high salinity levels. It is important to understand the adaptive mechanisms through which plants overcome this kind of stress and to identify potential genes for improving abiotic stress tolerance. RNA-Seq data of two Glycine max cultivars, a drought-sensitive (C08) and a tolerant (Conquista), subjected to different periods of salt stress were analyzed. The transcript expression profile was obtained using a transcriptogram approach, comparing both cultivars and different times of treatment. After 4 hours of salt stress, Conquista cultivar had 1400 differentially expressed genes, 647 induced and 753 repressed. Comparative expression revealed that 719 genes share the same pattern of induction or repression between both cultivars. Among them, 393 genes were up- and 326 down-regulated. Salt stress also modified the expression of 54 isoforms of miRNAs in Conquista, by the maturation of 39 different pre-miRNAs. The predicted targets for 12 of those mature miRNAs also have matches with 15 differentially expressed genes from our analyses. We found genes involved in important pathways related to stress adaptation. Genes from both ABA and BR signaling pathways were modulated, with possible crosstalk between them, and with a likely post-transcriptional regulation by miRNAs. Genes related to ethylene biosynthesis, DNA repair, and plastid translation process were those that could be regulated by miRNA.

Keywords: abiotic stress, salt, Glycine max, miRNA, transcript, transcriptogram

3.2 Introduction

Soybean (Glycine max L.) is an important crop around the world and used as a source of edible oil, biodiesel, and protein food. Its production is affected in soils with high salinity levels, typically in arid regions and irrigation systems [1]. Under climate change scenarios, salt stress is predicted to increase due to higher temperatures and more variable rainfall, with substantial reductions in precipitation. [2].

Salt stress may cause ion toxicity effect and osmotic stress, triggering photosynthesis reduction, oxidative stress, and reduction of symbiotic nitrogen fixation, limiting the growth and productivity of crops [3]. Plants have developed many morphologic, physiologic, and molecular mechanisms to adapt to this stress [4], including a hormone regulation. Under salt and drought, the abscisic acid (ABA) level increases rapidly to induce protective mechanisms, triggering stomatal closure to decrease water loss by transpiration [5] while brassinosteroids (BRs) signaling pathway, known for having growth regulatory activity, is involve in osmotic stress homeostasis [6]. ABA and BR crosstalk has been well documented in Arabidopsis, where kinase Brassinosteroid-insensitive 2 (BIN2), a negative regulator of BR signaling, can activate Snf1-Related Kinase 2s (SnRK2s) by phosphorylation [7]. SnRK2s are required for activation of ABA-responsive factors (ABF2, ABF3, ABF4, ABI5) and finally leading expression of stress-responsive genes [8]. The SnRK2 activities are inhibited by the clade A-type protein phosphatase 2C (PP2C), ABA-Insensitive 1(ABI1), in the absence of ABA [9]. On the other hand, BRI1-associated receptor kinase 1 (BAK1), a co-receptor of BRs, interacts with and phosphorylates SnRK2.6 (OST1) to regulate guard cell ABA signaling [10].

Soybean is an important crop with many studies that evaluated the salt effect using different perspectives. Some focused on growth and nitrogen fixation effect [11], [12], on the identification of single nucleotide polymorphisms (SNP) markers [13], and Quantitative Trait Locus (QTLs) related to salinity adaptation [14]. Others have performed proteomic analysis [15], metabolic profile [16], gene expression profiles [17], and post-transcriptional processing [18] under salt stress. RNA-Seq based methods have been widely used to generate gene expression profiles of plants under abiotic stresses [19]–[21]. Moreover, gene co-expression profile is an extensive approach to study and understand stress response mechanisms [22]–[24], having into account network of genes

influence gene expression and changes in the expression of many interacting genes may cause measurable effects on cell metabolism [25], [26]. Recently, Liu et al. (2019) [17] carried out a soybean transcriptomic analysis with a co-expression network approach using WGCNA (Weighted Correlation Network Analysis). They evaluated the salt effect in phytohormone signals, photosynthesis, carbon and nitrogen assimilation, and amino acid metabolism.

How those genes are regulated is an important aspect to elucidate. One mechanism of regulation of gene expression is through miRNAs (microRNAs). miRNAs are small (20-24 nt) non-coding molecules that modulate specific mRNA by cleavage or translation inhibition in plants and animals. They are transcribed from a genomic locus (MIR gene) by an RNA polymerase II producing a pri-miRNA, that is processed by an RNase III enzyme into a pre-miRNA and further processed to produce the mature miRNA. This mature form is exported to the cytoplasm, where one strand, 5p or 3p associates to an Argonaute (AGO) protein and compose RISC complex to drive the gene expression silencing [27]. The miRNAs are key molecules in regulatory networks, like plant development, biotic and abiotic stresses. The miRNAs involved in salt stress were evaluated in soybean nodules [28] and roots [29], [30], also in drought stress response in root and leaves [29], [31].

Despite a large amount of data, transcriptomic studies have had to focus on a few genes or particular metabolic pathways. Transcriptogramer method involves an analysis that shows the global status of cellular gene expression in a specific condition and is useful to compare genotypes and treatments and choosing the most interesting pathways and gene members. It requires the definition of gene neighborhood disposed on a line, by clustering together interacting genes [32].

In the present work, we applied the Transcriptogramer method, previously used in studies with Saccharomyces cerevisiae [32], humans [33] and Arabidopsis plants [34] to produce visual patterns of the expression profiles of two soybean cultivars, one drought-tolerant (Conquista) and another one drought-sensitive (C08), using transcriptomic data. This study aimed to improve the current understanding of salt stress adaptation mechanisms by comparing their gene expression profiles. We also investigated potential post-transcriptional regulation mechanisms by miRNAs that could be involved in salinity stress homeostasis using RNA-Seq data of soybean Conquista cultivar.

3.3 Material and Methods

3.3.1 *Plant materials and stress treatment*

Soybean (*Glycine max*) plants from Conquista BR46, a drought-tolerant cultivar [35], were used. Seeds were pre-germinated on filter paper in the dark at 25°C. Seedlings were transferred into a Hoagland´s nutrient solution and grown hydroponically at 25-28°C in 40% relative humidity, under natural daylight for a 16 h day. A modified Hoagland nutrient solution was composed of macroelements (1.0 mM KH_2PO_4, 5.0 mM KNO_3, 5.0 mM Ca $(NO_3)_2$, 2.0 mM $MgSO_4$), microelements (0.046 mM H_3BO_3, 0.009 mM $MnCl_2$ $\cdot 4H_2O$, 0.000765 mM $ZnSO_4$ $7H_2O$, 0.00032 mM $CuSO_4$ $5H_2O$, 0.00011 mM Na_2MoO_4 $2H_2O$) and ferric salts (0.02 mM $FeSO_4$ $7H_2O$, 0.024 M EDTA Na). After 15 days, when the seedling developed the first three leaves, they were submitted to nutrient solution supplemented with 200 mM NaCl. This concentration was used considering that *Glycine max* has an estimated salt tolerance thresholds of 137 mM NaCl for 14 days of treatment [36]. Samples of leaves from six biological replicates per treatment were collected after 4 hours of treatment, and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction for library construction.

3.3.2 *RNA extraction and salt-treated plant library construction*

Total RNA was isolated from Conquista leaves using Trizol (Invitrogen, CA, USA), and the RNA quality was evaluated by electrophoresis on a 1% agarose gel. RNA isolated (10 µg) was sent to Fasteris Life Sciences SA (Plan-les-Ouates, Switzerland) for sequencing through the Illumina HiSeq2000 platform. An RNA-Seq was obtained in duplicate (two control libraries and two salt

libraries). The sequenced library is available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69571 under GSE69569 and GSE69570 access numbers.

3.3.3   Library data download

Additional soybean transcript expression data were obtained from Liu et al. (2019). This RNA-Seq library was produced from primary leaves of soybean seedlings (*Glycine max* [L.] Merr., accession C08; [37]) of a drought-sensitive line [38], subjected to 150 mM NaCl treatment in a time-course experiment (0, 1, 2, 4, 24, and 48 h). Data are available at SRA database: SRP132150 [17] (https://www.ebi.ac.uk/ena/data/search?query=SRP132150). Libraries were obtained in duplicates or triplicates for each time point.

3.2.4   RNA-Seq quality analysis and mapping

Four libraries of mRNA and four of small RNAs were produced using Conquista samples. mRNA libraries were obtained in "pair-end". Conquista (this study) and C08 Liu et al., (2019) libraries were submitted to quality analysis using FastQC. A screening to remove reads with low quality (Q <30) and adapters through Trim Galore was made. An anchorage of library reads on the *Glycine max* Genome deposited in Phytozome v.12.1.6 was made using the TopHat2 software [39].

3.2.5   Statistical analysis and differentially expressed genes (DEGs) identification

The calculation of reads frequency for each gene was obtained by featureCount [40]. Statistical analysis was carried out by the DEseq2 package implemented in R. Genes with a fold change >1.5,

and *p*-value < 0.05 were considered DEGs. The functional annotation of those genes was found in Phytozome v.12.1.6.

3.2.6 Identification of responsive miRNA to salt stress and their potential targets

To examine the Conquista miRNA with differential expression, *Glycine max* pre-miRNA sequences deposited in miRBase (V22) were used. Then, an anchorage of reads of the miRNA library on these sequences was performed by using isomirID [41] was performed. A cutoff of 20 reads anchored to pre-miRNA was used to consider a mature isoform of a miRNA. A table with each mature miRNA frequency in each library was obtained. Then a statistic Student's t-test was conducted to evaluate the significant differences between the two treatments (salt and control) by the DEseq2 package implemented in R. miRNA with a *p*-value < 0.05 were considered. For each differentially expressed gene, miRNAs that may be regulating its expression were examined. Using psRNATarget [42], a Fasta sequence file of each differentially expressed gene and miRNAs were submitted using default parameters, and the expectation value of three was chosen.

3.2.7 Transcriptogram construction and statistical analysis

The analyses began with the classification of the *Glycine max* genes produced by The Transcriptogramer program (V.1.0) (https://lief.if.ufrgs.br/pub/biosoftwares/transcriptogramer) and using a protein-protein interactions file from STRING database (V10) [43], retrieved using a String-score (800). The ordering is used to cluster on a line interacting genes, such that the distance between two genes on the list correlates with the probability that they interact [32]. The term enrichment was produced by Trancriptogramer program using the ordering file and a Gene Ontology file. The outputs

were used to produce a graph that represents the density of genes related to a particular biological process in each position. A peak in these profiles, at a given localization, on the gene list indicates a clustering of the genes belonging to a particular GO-term, averaged over windows of 501 genes (Fig. 1a). All *Glycine max* Gene Ontology categories were taken at AGRIGO database (http://amigo1.geneontology.org).

A transcriptogram profile was produced for each library by transcriptogramer program using zero-hours as a reference for Liu et al. (2019) [17] libraries, and the control condition for Conquista libraries. An expression file (read counting) from RNA-Seq analysis, the ordering gene list, and a 125 radius were used as an input. The obtained transcriptogram file contains an average of the gene expression across 250 genes window centered at the gene and comprising the 125 genes to its left and 125 genes to its right. Through "statistics" tool an average expression value among the libraries replicates for each collection time, and each cultivar was calculated. Besides, a relative expression average for each gene was calculated, using zero-hours as a reference group for C08 and Control condition for Conquista. Using the gene position and the relative expression value of this result file, a graph was plotted using Microsoft excel. Each peak obtained was analyzed by taking the gene list of each one and calculated its expression through DEseq2. The samples analyzed were: four, 24 and 48 hours of salt stress treatment versus zero-hours as a control condition for the C08 access, and four hours versus control condition for Conquista. Genes with a Fold Change > 1.5 and *p*-value < 0.05 were selected.

3.2.8 *Expression validation of microRNAs and predicted targets by RT-qPCR*

RT-qPCR was made to validate the expression pattern of two predicted miRNAs and three targets. The salt treatment was repeated using 6 plants for each treated group and samples of leaves were collected at four h, and then were frozen for RNA extraction using Tryzol. Later, a cDNA synthesis was made using a M-MLV reverse transcriptase enzyme (Promega). For miRNA analysis, stem-loop primers designed according to Chen et al., (2005) were used. These consist of 44 conserved and six variable nucleotides that hybridize miRNA of interest (Table S1). In the case of target genes, primers according to Gadkar and Filion, (2013) were designed (Table S1). Thus, the 3´primer section contents a complemental sequence of each target gen (flanking the miRNA hybridization region) and an additional MYT4 sequence at the 5´end, to avoid the DNA genomic amplification and the use of DNAase I that reduce the RNA quality.

PCR reactions were completed in a volume of 20 µl containing 10 µl of cDNA and a final concentration of 0.1 µM of each reverse and forward primer, 0.4 mM dNTPS, 1X buffer, 3 mM MgCl$_2$, 0.25 U Platinum Taq DNA polymerase (Invitrogen) and 0,1X SYBR green I (Invitrogen). Forward miRNA primers were designed in Oligoanalyzer (v.3.1) based in miRNA sequence and removing the six last nucleotides (Table S1). A universal reverse primer was used in all miRNA RT-qPCR reactions (Table S1). Target gene primers were designed in Primer3 v.0.4.0, aiming to obtain approximately 150 PCR fragments. And a universal reverse primer was used [45] (Table S1). In every case, the melting temperature was adjusted at 60°C.

Samples were analyzed in technical triplicate in a 96-well plate, and a no-template control was included. We used as reference genes from soybean: Actin and Elongation factor B, MIR156b (5'-TGACAGAAGAGAGAGAGCACA - 3'), MIR172ab (5'- AGAATCTTGATGATGCTGCAT - 3')

and MIR1520d (5'- ATCAGAACATGACACGTGACAA - 3'), which has been demonstrated as optimal normalizers for salt stress analysis in *Glycine max* [46]. The thermal cycling conditions consisted of an initial polymerase activation (5 min at 95 ℃) followed by 40 cycles of denaturation (15 s at 95 ℃), annealing (10 s at 60 ℃) and extension (15 s at 72 ℃). A 7500 Real-Time PCR Detection System (Bio-Rad) equipment was used. Afterward melting curves were acquired for PCR products using 0.5°C steps with a hold of 1 s at each step from 65°C to 99°C. Threshold and baselines were manually determined using the ABI 7500 Real-Time PCR System SDS Softwarev2.0.

3.2.9   *RT-qPCR statistical analysis*

The quantification of relative gene expression was complete using the mathematic method $2^{-\Delta\Delta Ct}$ [47] with the control condition expression as a calibrator and the endogenous genes as reference. A Student's t-test was performed to compare differences in expression between treatments. The means were considered significantly different when p-value < 0.05.

3.3 Results

3.3.4   *Gene expression profile*

DEseq2 RNA-Seq analysis revealed 6290 DEGs in Conquista and 17006, 18253 and 22208 in C08 at 4, 24, 48 hours (h) respectively (FC >1.5 and p-value < 0.05) (Table S2). To evaluate the expression profiles of both genotypes in different biological processes, they were compared using a transcriptogram approach. For this aim, a soybean ordered gene list was used (Fig. 1a, Table S3). The transcriptogram profile based on Conquista data showed 26 peaks (P1 to P26) that were

manually delimited (Fig. 1b, Fig. S1). The gene list associated with each peak and their expression level is in Table S4. Among those peaks, fifteen of them represent genes with a tendency to be induced (relative expression values > 1) and eleven to be repressed (relative expression values < 1) under salt stress (Fig. 1b). Each peak denotes a group of genes that share similar biological functions, and have a probable interaction, belonging to cell wall organization, phytohormone response, carbohydrate metabolism, signal transduction and transport, all involved in stress adaptation (Fig 1a, Table 1). Using DEseq2 analysis, from the 20317 genes present in the ordering list, a total of 1400 DEGs were found with FC > 1.5 and a p-value < 0.05 (Table 1, Table S4).

A

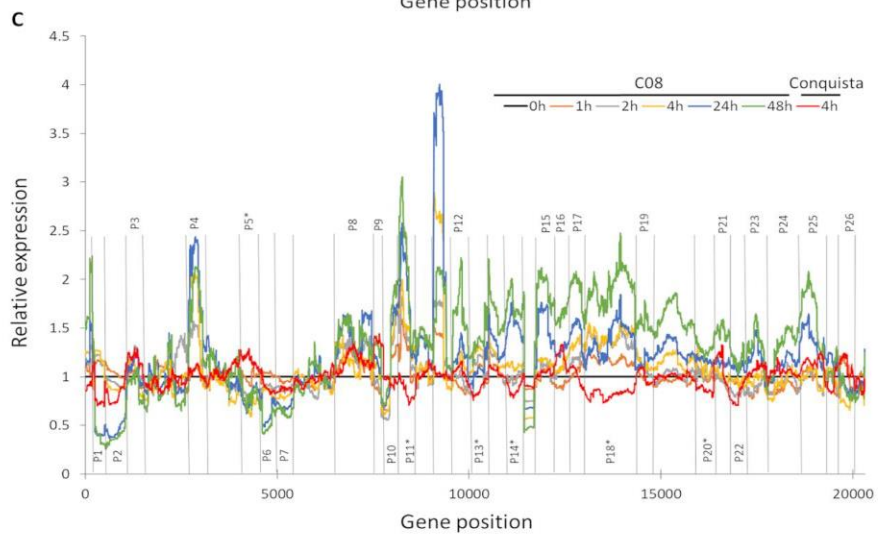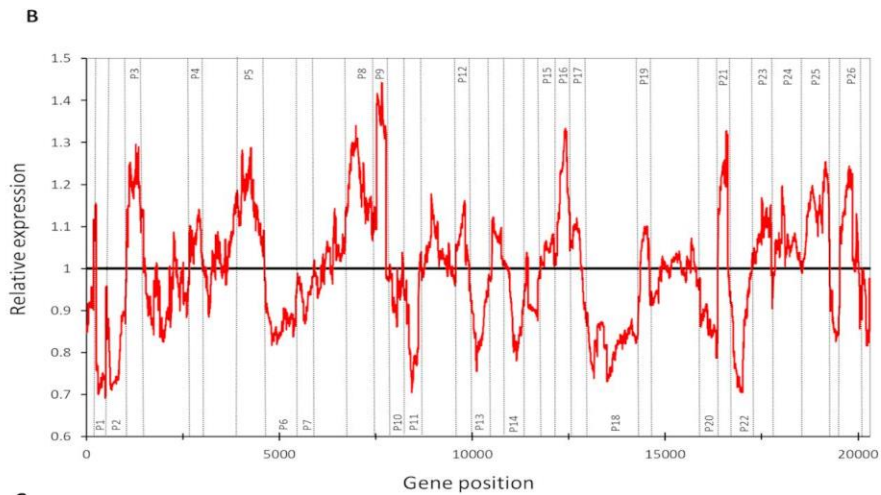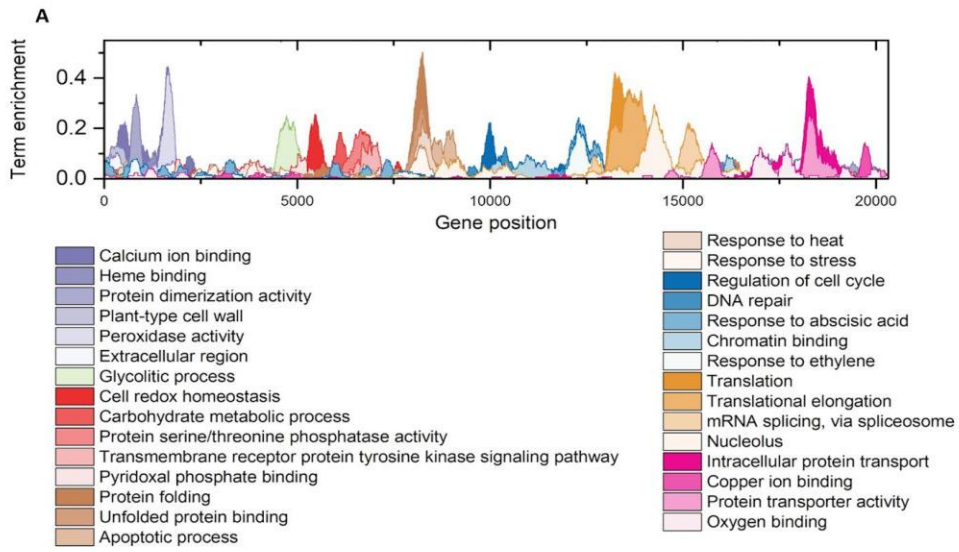| Calcium ion binding | Response to heat |
Heme binding	Response to stress
Protein dimerization activity	Regulation of cell cycle
Plant-type cell wall	DNA repair
Peroxidase activity	Response to abscisic acid
Extracellular region	Chromatin binding
Glycolitic process	Response to ethylene
Cell redox homeostasis	Translation
Carbohydrate metabolic process	Translational elongation
Protein serine/threonine phosphatase activity	mRNA splicing, via spliceosome
Transmembrane receptor protein tyrosine kinase signaling pathway	Nucleolus
Pyridoxal phosphate binding	Intracellular protein transport
Protein folding	Copper ion binding
Unfolded protein binding	Protein transporter activity
Apoptotic process	Oxygen binding

B

C

58

Fig. 1. **The Transcriptogram a.** Ordering of *Glycine max* genes. Closer genes have a higher probability of being associated based on String database information. **b**. Transcriptogram profile from Conquista leaves under 200 mM NaCl for 4 h. Relative expression data of each gene is plotted. Axis *x* represents the gene order. The control group is represented with a horizontal black line. The salt-treated group is represented by a red line **C.** Comparative transcriptograms from C08 soybean leaves under 150 mM NaCl for 0, 1, 2, 4, 24, 48 h and Conquista leaves under 200 mM NaCl for 4 h. Relative expression data of each gene is plotted. Axis *x* represents the gene order. Collection at 0 h is represented with a horizontal black line. Peaks highlighted with an asterisk showed different pattern between different genotypes

Table 1. Peaks, biological process and number of genes differently expressed under salt stress

Peak	Biological function	Genes[b]	Conquista		C08		Conquista and C08[c]	
			Induced	Repressed	Induced	Repressed	Induced	Repressed
P1	Cell wall modification, Carbohydrate metabolic process, Signal transduction	227	18	18	52	70	15	9
P2	Auxin signal transduction, Photosynthesis	383	45	30	93	225	22	28
P3	Carbohydrate metabolism, Signal Transduction, Biosynthetic Process	408	42	31	145	164	24	22
P4	Cellular metabolism	136	17	9	51	40	12	5
P5a	Carbohydrate metabolism, Protein modification, Lipid metabolism, Transport, Signal Transduction, Photosynthesis	580	57	51	239	194	39	29
P6	Carbohydrate metabolism, Photosynthesis, Carbon fixation	708	29	71	253	309	18	36
P7	Signal Transduction, transport, Carbohydrate metabolism	196	12	14	79	63	9	6
P8	Signal transduction, Cell death	547	72	22	185	173	40	12
P9	Protein folding	142	7	7	54	40	7	1

P10	Signal Transduction, Transport, Carbohydrate metabolism	244	16	7	71	73	10	4
P11a	Protein metabolism	241	14	28	93	62	6	7
P12	DNA metabolism, Cell growth	250	8	19	87	80	6	8
P13a	DNA metabolism, Cell growth	346	7	63	135	79	3	10
P14a	DNA metabolism, Protein Modification, Signal Transduction, Cell Growth	455	17	62	199	97	11	15
P15	Protein turnover Signal transduction	477	28	14	184	100	16	7
P16	Signal Transduction	369	20	10	110	84	13	5
P17	Cellular Metabolic Process, Transcription, Protein Modification, Translation	239	12	3	88	48	10	1
P18a	Plastid translation, Growth	968	20	126	599	147	15	29
P19	Translation, Biosynthetic Process	171	12	7	89	36	11	3
P20a	Signal Transduction, Cytoskeleton organization	476	14	37	183	160	10	17
P21	Signal Transduction, Transport, Growth	233	14	13	102	79	7	9
P22	Steroid biosynthesis, Signal Transduction	375	22	38	130	110	9	23
P23	Protein turnover, Signal Transduction, Cell growth	362	19	12	130	90	12	4
P24	Transport, Signal transduction	536	38	21	150	157	21	5
P25	Autophagy, Transport, Cell Growth	638	56	16	224	193	32	11
P26	Metal ion transport	302	31	24	76	123	15	20
Total		**9206**	**647**	**753**	**3801**	**2996**	**393**	**326**

[a] Peaks with different pattern between Conquista and C08 soybean cultivars. [b] Genes associated to each peak are listed in Table S1, Fig. 1B. [c] Number of genes with differential expression under salt stress, shared between Conquista and C08 soybean cultivars are listed in Table S4 and S5

3.3.2 Conquista and C08 access have similar patterns of gene expression profile

C08 expression data [17] were used to produce transcriptograms for each treatment time (Supplemental Fig. S2). Peaks that represent biological process with early response genes (1, 2 and 4 h) were detected (Supplemental Fig. S2), having a differential expression at the first four hours of treatment and kept increasing up to 48 h like P4, P6, P7, P8, P10, P11, P13, P17 and P18. On the other hand, some peaks are formed by genes with a late response at 24 h and 48 h, including P1, P14, P15, P16, P19, P21, P23, and P25 (See the associated biological process in Table 1). Besides, it was possible to perceive how the salt effect increase through time, where 1 h treatment showed the lowest expression levels, and 48 h treatment the highest (Supplemental Fig. S2).

An integrative graph using transcriptograms for both cultivars was obtained (Fig. 1c). Both genotypes have 20 peaks with similar expression patterns and six with a different one. The last could be related to different adaptation mechanisms to salt stress based on the genotype differences.

DEGs in common between Conquista and C08 are presented in Tables S5 and S6. Venn diagram analysis (Fig. 2) showed that several genes were regulated in the same manner at 4 h, 24 h, and 48 h, in both cultivars. Three hundred ninety-three were up and 326 down-regulated at those collection times in both genotypes (Bolded numbers, Fig. 2). Besides, analyzing the early responses (4 hours after stress imposition), 190 up-regulated genes (Table S5) and 224 down-regulated genes (Table S6) were detected in C08 and Conquista (underlined numbers, Fig. 2). Besides, some genes were specifically regulated only in Conquista cultivar, 254 were induced and 427 were repressed (Fig. 2). Venn diagrams for each peak showed the number of genes that were up- or down-regulated in common between both genotypes at different treatment time points (Supplemental Fig. S3).
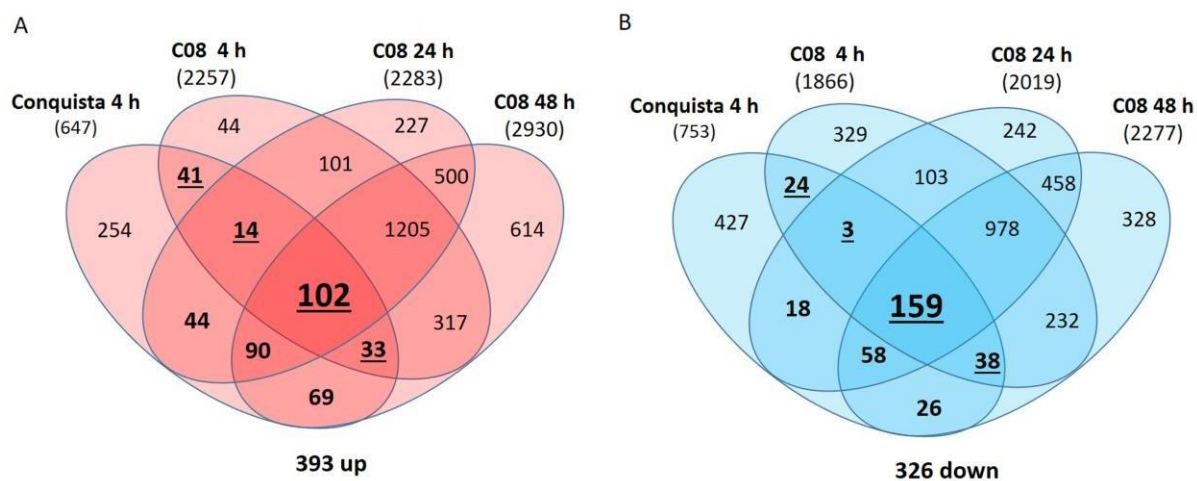
Fig. 2. Venn diagram of differentially expressed genes of both genotypes. **A**. Up-regulated genes, **B**. Down-regulated genes in Conquista and C08 libraries at 4, 24 and 48 h. Bolded numbers represent genes that were up or down-regulated in both cultivars. Underlined numbers represent genes with the early response at 4h of treatment.

3.3.5 *Conquista and C08 access share mechanisms for adaption to salt stress*

It is well-known that many of the components that are responsible for the biosynthesis and signaling of auxin, cytokinins (CK), gibberellin (GA), brassinosteroids (BR), jasmonic acid (JA), abscisic acid (ABA) were identified as taking part in the plant response to drought and salt stress [48]. Many DEGs related to phytohormone signals were found in both genotypes (Table S5 and S6). Some of them from the auxin signaling pathway were up-regulated (P2), such as auxin-responsive protein IAA1, a negative Auxin pathway regulator. Others were down-regulated like auxin response factor 3 and Auxin response factor 11.

Besides, genes from the ethylene biosynthesis pathway were induced (P4), including S-adenosylmethionine synthase 4. Ethylene responsive genes were also up-regulated in both genotypes

(P2 and P3) like Ethylene Insensitive 3-like 1, ethylene-responsive transcription factor 1B and EIN3-binding F-box protein.

Genes from the brassinosteroid signaling pathway (P2) such as BAK1 were induced, and BKI1 (BRI1 kinase inhibitor 1) was repressed (P3). Many genes encoding PP2Cs were regulated (P8); four of them were up-regulated and one down-regulated.

Among genes related to the ABA signaling pathway, key kinases for ABA signaling have their expression modified (P8) such as SRK2c. Transcription factors (TFs) known to be regulated by ABA were induced (P5, P8, P16, P17, P18), like WRKY TF 33, ABA TF 3, ABA-insensitive 5-like protein 6 (ABF3) and MYB like DNA binding protein. The ABA 8' hydroxylase-1 was also induced (P23). This protein is involved in the ABA degradation pathway.

Gibberellins are also involved in the salt response. Genes encoding proteins involved in gibberellin biosynthesis and catabolism such as gibberellin 2-beta-dioxygenase and gibberellin 3-beta-hydroxylase were induced (P1) and repressed (P2), respectively.

Genes related to oxidative stress mitigation were also differentially expressed (P3, P5, P6, Table S5). For chalcone-flavonone isomerase 3-related (CHI3) a flavonoid biosynthesis gene was induced (P3). On the other hand, genes related to photosynthesis process (P1, P2, P6) (Table S6) were inhibited in both genotypes such as Light-harvesting chlorophyll a/b binding proteins. In the same way, genes related to carbon fixation (P1, P6) were inhibited, including carbonic anhydrase. Furthermore, plastid translation genes were repressed (P18). For instance, eukaryotic translation initiation factor 2c and small subunit ribosomal protein S13. On the other hand, genes of chlorophyll degradation were up-regulated (P3) like cytochrome p450 71b21-related.

An expression increment in osmoprotectant genes was found (P1, P3, P5, and Table S5). Genes of sugar metabolism, like glucuronokinases (P1), implicated in the *de novo* synthesis of UDP-

glucuronic acid (UDP-GlcA) were induced. A β-amylase important in the starch catabolic process (P3). Genes related to amino acid biosynthesis and catabolism were also induced (P5) as the branched-chain-amino-acid (BCAA) aminotransferase and 2-oxo-isovalerate dehydrogenase.

Genes related to ion homeostasis were regulated as well, including cyclic nucleotide-gated ion channel 2, sodium/solute symporter, osmotic stress potassium transporter, and heavy metal transports that were induced (P3, P24, P27). The copper transport protein atox1-related that was repressed (P27).

3.3.6   *Genes with different expression pattern between Conquista and C08*

The transcriptogram highlighted six peaks with a different expression profile between the Conquista and C08 cultivars (Fig. 1c). In Conquista, five peaks represent biological processes where the majority of genes were repressed (P11, P13, P14, P18, and P20), and one peak containing mainly the induced genes (P5), while C08 showed the opposite pattern. The whole list of distinct pattern DEGs between both cultivars is presented (Table S7). The most representative genes from those peaks are shown (Fig. 3).

P5 includes 18 regulated genes with an opposite pattern in both cultivars. They are related to plant osmoprotection. One of the primary responses observed in plants exposed to osmotic stress is the accumulation of sugars and amino acids to maintain the cell's osmolarity [49], [50]. Sugar synthesis genes such as trehalose-phosphate synthase (TPS) and sucrose synthase were up-regulated in Conquista and down-regulated in C08. Furthermore, genes involved in amino acid biosynthesis like shikimate dehydrogenase (SDH), adenylyl-sulfate, alanine-glyoxylate transaminase (AGXT) transcripts were also altered in a different manner. Moreover, a key

component of electron shuttling, a flavoprotein encoding gene was induced in Conquista and reduced in C08.

From P11, 19 genes related to protein folding were down-regulated in Conquista and up-regulated in C08. They include chaperonin 60, cyclophilins, HSP10, HSP70, HSP90, and CACYBP. Moreover, genes related to protein turnover like LON protease.

Fifty-one genes from P13 were regulated in a different manner between the cultivars. They are related to DNA metabolism and cell division, for instance, helicase RECQL3, MCMBP, ORC1, CDT1, DNA primase PRI1, DNA polymerase delta subunit, DNA repair recA2, Timeless, CDC7, and Cyclin.

Forty-seven genes from P14 were down-regulated only in Conquista. This peak is involved with nucleosome assembly. Many of those genes encode histone proteins 2A, 2B, 3 and 4. Others encode histone deacetylase 15, histone lysine N-methyltransferase ATX3 and SNF2H. Besides, DNA metabolism genes like DNA helicase (DDM1), DNA polymerase (POLE4), DNA Cytosine specific methyltransferase were also down-regulated in Conquista.

Seventy-five genes from P18 were repressed in Conquista and induced in C08. They are involved with cell division like CDK and GNB2L1 and plastid translation like RP-L10Ae, RP-S30e, RP-L7Ae, EFF2, NACA.

Finally, twenty genes from P20 presented different patterns between cultivars. This peak includes cytoskeleton related genes such as tubulin, kinesin family member, MAPR and Ran GTPase-activating protein 1. Genes involved with gene expression regulation were also included in this peak, like HLH DNA-binding protein, MYC1-TF, and HDT2.

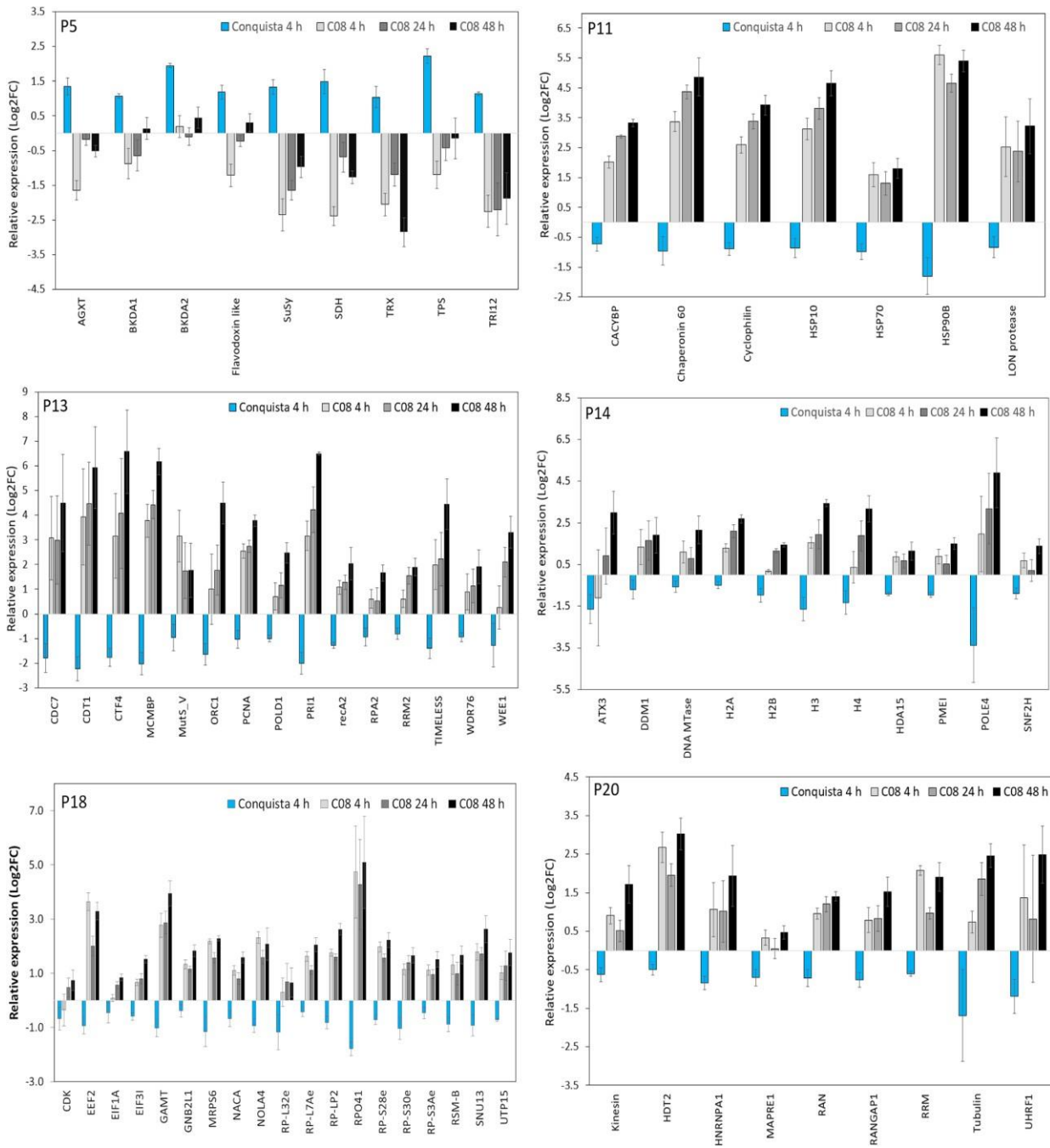**Fig. 3**. **Genes with contrasting expression pattern between both genotypes**. Relative expression of genes belonging to peaks P5, P11, P13, P14, P18 and P20 with different expression pattern between Conquista and C08. Only genes with significant differences among salt treatment and control are shown (*p*-value <0.01). The data (n=4, Conquista and n=9, C08) are means ±SE. The complete list of those genes are shown in Table S7

3.3.7   Identification of miRNAs responsive to salt stress and their putative targets

Fifty-four differentially expressed isoforms of miRNAs belonging to 39 different miRNA were identified in Conquista libraries (p-value < 0.05). They presented a 20-24nt size. From them, 22 were repressed and 32 induced, where miR172cde-5p showed the highest repression and miR4411 the highest induction (Table S8). The analysis of psRNAtarget using the differentially expressed genes from the previous analysis showed that 15 genes could be regulated by miRNAs (Table S9). Twelve miRNAs had an inverse expression pattern concerning their predicted target and could regulate them by mRNA cleavage (Fig. 4, Table S9). Hence, six miRNA were down-regulated (Fig. 4a) while their potential targets were induced (Fig. 4b), and six miRNA were up-regulated (Fig. 4a) with concomitant repression of their targets (Fig. 4b). It is known that one miRNA could regulate different target genes while some genes could be regulated by different miRNAs. For instance, miR319ab-5p could hybridize two different genes and miR4348b-3p three (Fig. 4).

 Genes that exhibited crosstalk between ABA and BR signaling pathway are highlighted in this analysis. Among them BAK1 that could be regulated by miR482be-5p, PP2C by miR4998-5p, BIN2 by miR166-5p, LRRK by miR2118-5p and ABCG29 by miR6300-3p. Besides, genes from the ethylene signaling pathway had hybridization regions for miRNAs. SAM2 could be cleaved by miR9726-3p and the ethylene receptor (ETR) by miR482e (Fig. 4). Interestingly, ten from those genes likely regulated by miRNA (Fig. 4), had different expression patterns between the cultivars (Table S7). On the contrary, guanylate kinase, elongation factor Ts (EF-TS), elongation factor Tu G (EF-Tu G) and BAK1 had the same expression pattern (Table S5 and Table S6).

RT-qPCR confirmed the transcript increment of BAK1 and reduction of miR482be-5p under salt stress. Similarly, expression repression of EF-TS and EF-Tu G genes and induction of their predicted miRNA, miR319ab-5p was confirmed. These results indicate an expression correlation among those genes and miRNA.
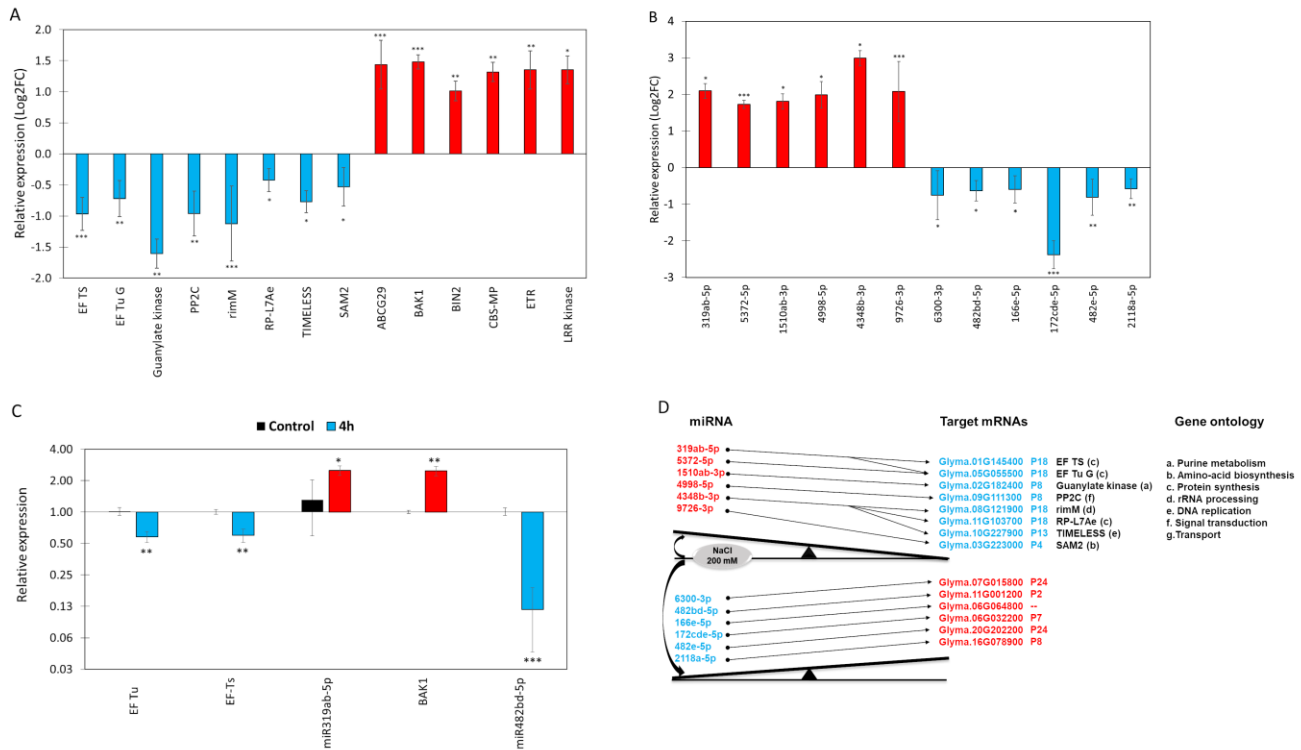
Fig. 4. Relative expression of salt modulated genes and their predicted regulatory miRNAs a. Relative expression of salt modulated miRNAs by RNA-Seq **b**. Relative expression of miRNA target genes by RNA-Seq**. c**. Relative expression of confirmed miRNAs and target by RT-qPCR **d.** Genes with significant differences are labeled with asterisks (*p <0.05, ** p <0.01, *** p <0.001*). The data (n=4) are means ±SE.Opposite expression patterns among miRNAs and their predicted mRNA targets. Genes and miRNAs with increased expression under salt treatment are in red while the reduced ones are in blue

3.4 Discussion

3.4.4   Comparison of two soybean cultivars and their mechanisms to salt stress adaptation

The molecular mechanisms to mitigate salt stress are crucial to study in crops, looking for genes involved in salt tolerance as a step to improve productivity. To better understand the salt effect in soybean gene expression, two contrasting cultivars (Conquista and C08) were compared. Small RNA libraries from Conquista cultivar were also used to identify miRNAs and responsive target genes. A global graphic representation of the soybean gene expression state submitted to different salinity conditions was obtained using transcriptograms. Associated genes, sharing common biological function, were recognized by their ordering. The expression pattern of these genes were represented by peaks. The time course comparison along the salt treatment was performed using the C08 cultivar. We identified gene groups having early or late responses to salt stress. For instance, signal transduction, protein turnover, transcription, translation, and transport pathways presented genes with an early expression pattern, and trigger the initial stress response. The present study compared straightforwardly two genotypes of soybean using genome-wide information. We found many pathways with conserved DEGs under salinity for the two cultivars, such as phytohormone signals, photosynthesis, carbon fixation, oxidative stress, and osmoprotection. They might be interesting to study in more detail and to find potential genes to improve crop production.

Components of ABA signaling were regulated in C08 and Conquista in a similar manner (Table S5). High concentrations of NaCl outside the roots reduce the water potential, making it more difficult for plants to extract water, and results in osmotic stress. ABA signaling pathway helps plants to acclimate under this condition, promoting the stomata closure resulting in transpiration reduction and also promoting the accumulation of numerous proteins and osmoprotectants for an osmotic adjustment [51]. High salinity also induces the formation of reactive oxygen species (ROS) within plant cells, and its over-accumulation results in damage to membrane lipids, proteins, and nucleic acids [52], [53]. Biosynthesis of flavonoids is useful as antioxidants that scavenge oxidative species. Genes related to this pathway were up-regulated in both cultivars, like CHI3s. It suggests an important role of antioxidants in alleviating salt stress-induced oxidative damage. Salt stress causes a decrease in plant growth and productivity by disrupting physiological processes, especially

photosynthesis. The accumulation of intracellular sodium ions at salt stress changes the ratio of K+/Na+, which seems to affect the bioenergetic processes of photosynthesis [54]–[56]. Besides having negative effects on the light reaction of photosynthesis, salt shock also blocked carbon assimilation in the dark reaction of the Calvin–Benson cycle [57]. Therefore, the present results show the salt effect in genes of those biological processes, which were inhibited in both genotypes (P6). Senescence is phenotypically defined by leaf yellowing, which results from loss of chlorophyll. In soybean, chloride accumulation has been associated with the presence of chlorosis and necrosis in leaves and stems, in addition to a general loss in productivity [58], [59]. In agreement with these findings, our results showed an induction of chlorophyll degradation genes.

Genes that represent biological processes with different expression patterns between the cultivars were also found, some induced in Conquista and repressed in C08, and vice versa (Fig. 3). Peak 5 represents the carbon metabolism process, and genes were induced in Conquista and repressed in C08 (Fig. 3). The accumulation of trehalose is reported as a mechanism of osmotic stress tolerance in plants [49], [60]. Although many TPS genes presented up-regulation in both cultivars (Table S5), some of them were strongly induced only in Conquista and repressed in C08 (Table S6). This enzyme is part of the trehalose production pathway [61], [62]. Trehalose has been shown to stabilize dehydrated enzymes, proteins, and lipid membranes efficiently and may also act as a signaling molecule, affecting the transcript levels of genes involved in abiotic stress [63]. Hence, it can be expected that higher gene expression of trehalose biosynthetic genes in Conquista leads to a higher trehalose accumulation and a better osmotic stress adaptation.

Similarly, the sucrose synthase gene was induced in Conquista. It has been reported that adaptive response to salt involves sucrose synthase activity induction and sucrose accumulation in plants [64], [65]. On the other hand, it is well known that many free amino acids present accumulation in plants under osmotic stress [50], [66]. An SDH, involved in the conversion of simple carbohydrates to aromatic amino acids, was also up-regulated in Conquista. Aromatic amino acids serve as precursors of phenolic compounds that are potent scavengers of ROS [67], [68]. The SDH induction in osmotic stress has been reported [69], [70]. Furthermore, despite the expression of BKDA (2-oxoisovalerate dehydrogenase) gene was induced in both cultivars, two genes encoding this enzyme were only induced in Conquista. BKDA participates in BCAA degradation, and interestingly the gene expression was demonstrated to be induced in cold stress [71] and water deficiency [72]. In spite of

BBCA amino acids be shown to have higher levels under osmotic stress, it is likely that degradation is necessary to maintain a balance concentration in the cell. So, a higher activity of this enzyme in Conquista could be related to a higher BBCA accumulation and better osmotic homeostasis. Adenylyl-sulfate reductase (TRX) was also induced only in Conquista. It catalyzes a key reaction in the plant sulfate assimilation pathway, leading to the synthesis of cysteine. This enzyme is related to the production of the antioxidant glutathione, which has been known to accumulate in plants following oxidative stress [73], [74]. Bick et al. (2001) demonstrated TRX is post-translationally activated by oxidative stress. These results suggest TRX gene expression induction could lead to a better ROS adaptation in Conquista. A gene encoding AGXT was also altered. This enzyme participates in the photorespiratory glycolate cycle, particularly in glycine synthesis [76], [77]. Many studies suggested that photorespiration is a salt stress alleviation mechanism [78], [79]. During osmotic stress, closed stomata cause severe CO_2 limitation for the Calvin cycle, resulting in excess reduction power (NAPH) and energy (ATP) from photosynthesis [80]. To prevent oxidative stress under these conditions, photorespiration might assume the role of consuming reducing equivalents and energy in order to prevent ROS accumulation. Besides glycine, generated during photorespiration, can suffer a conversion to serine, releasing CO_2 and leading to mitigation of the CO_2 limitation in the Calvin cycle, due to its intracellular recycling within the cells [81]. Furthermore, glycine can be a substrate for the biosynthesis of glutathione, a key component of antioxidant defense systems [82]. In the same direction, an NADPH-hemoprotein reductase (Flavodoxin-related) transcript increased in Conquista. The reduction of NADPH through this flavoprotein is an alternative mechanism to solve the NADP- availability in the cell. In addition, the importance of flavoproteins to increase tolerance to plant stress was demonstrated before [83].

On the other hand, a reduction in the expression of Chaperonin genes (P11) was found and could be related to Conquista tolerance mechanism (Fig. 3). HSP70 and HSP90 and their co-chaperones, besides folding functions, have been linked to signaling, protein targeting and degradation [84]–[86]. At least six HSP90 proteins from mitochondria and chloroplast were down-regulated in Conquista. Overexpression of different AtHsp90s in A. thaliana reduced tolerance to salt and drought stresses [87]. Moreover, it is known that in non-stress conditions, class A1 HSFs (heat shock transcription factors) are sequestered by HSP90/70. Upon stress application, the high number of misfolded proteins triggers the recruitment of HSP90/70 to its client and frees the HSFA1s. Besides, it was

reported that all HSFA1s are involved in osmotic stress tolerance [88]. Interestingly, in the libraries at least two HSF, one class A (Glyma.08g047400) and another class B (Glyma.10g237800) were up-regulated in Conquista and down-regulated in C08, and other two were induced in both cultivars (P11). As reported in a soybean study, where 14 HSF genes were induced under drought stress [89]. Those results lead to conclude some HSF are necessary for gene expression regulation under salt stress and this could be correlated with a reduction of HSP90 and 70. Moreover, not less than eight chaperonins 60 from mitochondria and chloroplast were repressed in our libraries. Similarly, a previous soybean proteomic analysis of sensitive and tolerant plants showed a reduction in this protein [90]. Curiously, a cyclophilin gene member was repressed. These proteins with peptidyl-prolyl cis-trans isomerase activity are characterized by a wide range of cellular functions like cell division, transcriptional regulation, protein trafficking, protein folding, cell signaling, pre-mRNA splicing, molecular chaperoning and stress tolerance [91], [92]. Cyclophilin expression has been shown to be induced by both biotic and abiotic stresses including salt stress, drought and ABA [93], [94]. Hence, cyclophilin participation in soybean salt stress regulation is still to be elucidated.

Reduction in gene expression related to DNA replication and cell division (P13 and P14) are crucial checkpoints for DNA repair and could be a tolerance mechanism of Conquista (Fig. 3). The ROS can be a product of salinity stress and in high levels can cause damage to critical macromolecules such as DNA. Cells have developed a set of mechanisms to monitor the status and structure of DNA during cell cycle progression. One is by arresting the cell cycle to allow DNA repair to occur before DNA replication or mitosis beginning [95], [96]. Salt stress decreased the transcript levels of CDK (Cyclin-dependent kinase) and cyclin resulting in transient down-regulation of mitotic activity in Arabidopsis plants and cell cycle arrest [97]. Similarly, CDK and Cyclin genes were also down-regulated in Conquista plants. A critical aspect of the DNA damage repair is to inhibit DNA replication during repair to prevent polymerases from encountering DNA damage. Genes related to DNA replication were down-regulated in Conquista and was up-regulated in C08 (Fig. 3). These genes encode proteins that form an initiation replication complex [98], [99]. Overall, we suggest Conquista could use both mechanisms to repair DNA damage. First, a cell division arrest by reduction of CDK and cyclin genes; and second, a DNA replication inhibition by reduction of DNA replication proteins related. Besides, our study suggests a post-transcriptional regulation of

TIMELESS, required to stabilize replication forks during DNA replication, by miRNA 4348b-3p (Fig. 4c).

Peak 14 includes histone deacetylase 15 (HDA15), histone methyltransferase gene ATX3 and DNA cytosine methylation genes related to epigenetic regulation through histone and DNA modifications (Fig. 3). Several studies have reported that histone methylation is involved in repression of gene expression and environmental stress adaptation, including drought stress and ABA treatment [100], [101], and histone deacetylases can also lead to gene repression and silencing [102]. It has been demonstrated that stress modulates hypomethylation or hypermethylation of DNA [103], [104] and associate DNA methylation with gene repression. These genes could be implicated in the epigenetic regulation and the gene expression profile observed between both cultivars. Analyses of changes in chromatin status in both cultivars such as methylation or acetylation levels are necessary to have more information and confirm this possible difference in epigenetic regulation under salinity .

Reduction in plastid translation components (P18) was also observed in Conquista. Several osmotic stresses, including high salt, resulted in a very significant decrease in global translation rates [105]–[107]. A decreasing of ribosomal protein transcripts was exhibited in Conquista (Fig. 3). In agreement with our finding, the RP-S28 gene from Helianthus annuus was repressed in response to osmotic stresses [108] and RP-L32 from the rice was repressed in salt treatments [109]. In soybean, the RP-LP2 gene expression was reduced under pathogen infection [110]. High expression of ribosomal protein genes in plants occurs in actively dividing tissues [111]–[113]. On the contrary, salinity stress has been shown to inhibit cell division [114], [115]. On the other hand, we suggest post-transcriptional regulation of RP-L7Ae, EF-Ts and EF-Tu by miR4348b-3p, miR5372-5p, and 319ab-5p, respectively.

Regulation in microtubule (MT) cytoskeleton protein genes was found (P20). MT primarily functions to control plant growth and cell morphogenesis [116], [117]. Ethylene and ABA synthesis components, that were up-regulated in our experiments, are strong regulators of plant growth, and microtubule organization [118]–[120]. Under salt stress, ethylene signaling played a crucial role in microtubule reassembly [121] and exogenous ABA treatment could also induce microtubule reorganization [120]. Transcripts related to cytoskeleton organization (P18 and P21), were down-regulated in Conquista leaves (Fig. 3). These proteins play important roles in cell division and

directional cell expansion [122], [123]. Cytoskeleton genes repression was observed before in Arabidopsis cell suspension [124] and watermelon roots [125] under osmotic stress. Overall, in Conquista a reduction of DNA synthesis and protein translation transcripts and in the expression of microtubule genes was perceived, as a result, this could lead a cell division inhibition in leaf tissue. This is relevant to osmotic stress, as inhibiting leaf growth can be a strategy to prevent transpiration [126]. Moreover, it is well-known that the process of transient calcium ion (Ca2+) influx into the cytoplasm induced by salt stress is an important response mechanism [127] and several studies have shown cortical microtubules may be involved in regulating the activity of calcium channels [128], [129]. Wang et al., (2007) revealed salt stress induced depolymerization of the cortical microtubules raising the free cytosolic calcium concentration in cells. Therefore, cortical microtubule reorganization is necessary for the plant's ability to withstand salt stress [130].

On the other hand, an HD2 transcript from P20 was induced early and late in C08 and repressed in Conquista (Table S6, Fig. 3). HD2 mediates the deacetylation of histones and gives a tag for epigenetic repression and transcription regulation. The expression of four Arabidopsis HD2 genes was repressed by ABA and salt [100], [131]. Furthermore, overexpression of AtHD2 in Arabidopsis plants enhanced tolerance to salt and drought stresses [132]. Hence, our findings may be related to a Conquista tolerance mechanism. HD2 is likely related to cytoskeleton proteins by the transcriptogram order, leading us to hypothesize that HD2 could be realizing an epigenetic regulation of cytoskeleton reorganization process. All these differences have to be considered with some caution, as both experiments were carried out at different leaf vegetative stages and may also be reflecting this development aspect.

3.4.2 *ABA and BR pathways and their transcriptional and post-transcriptional regulation under salt stress*

Components of ABA and BR signaling crosstalk were regulated and a post-transcriptional regulation by miRNAs was predicted (Fig. 4). BRs have growth regulatory activity, including cell division, vascular differentiation, reproductive development and modulation of gene expression. In the cell's presence of BR, this hormone binds to the extracellular domain of its receptor BRI1, producing its activation. BRI1 recruits BAK1 to form a receptor complex on the plasma membranes [6] (Fig. 5a). Then, a bidirectional transphosphorylation between BRI1 and BAK1 produces a kinase

cascade, resulting in a phosphatase BSU1 activation, which is responsible for BIN2 dephosphorylation and its inactivation. BIN2 is important in the negative control of the BR signaling pathway, through BZR1 and BES1 transcription factors phosphorylation and inactivation (Fig. 5a). In normal conditions, without ABA, PP2Cs dephosphorylate both SnRK2s and BIN2 and inhibits their activity [133], [134] (Fig. 5a). In the presence of BR, dephosphorylation of BZR1 and BES1 by PP2A leads to nuclear accumulation of these proteins and transcriptional regulation of BR-responsive genes [135] (Fig. 5a). The transcription factor BZR1, a positive regulator of the BR signaling pathway, was previously identified as a repressor of ABI5 [136]. Ryu et al. (2014) [137] recently reported that BES1, a homolog of BZR1, forms a transcriptional repressor complex with TOPLESS (HDAC19) to regulate the expression of ABI3 and ABI5, thereby suppressing ABA signaling during early seedling development (Fig. 5a).

Under salt stress, ABA activates the SnRK2s from subgroup III which are positive regulators of ABA signaling. This subgroup, contains three members, SnRK2.2, SnRK2.3, and SnRK2.6 also known as Open stomata 1 (OST1) [138]. SnRK2s phosphorylate ABFs to regulate ABA-responsive gene expression. Some of those kinases and ABFs transcripts were up-regulated under salt stress (Fig. 5b). The activity of SnRK2s is dependent on their phosphorylation. Cai et al. (2014) [7] demonstrated that BIN2 interacts with and phosphorylates SnRK2s enhancing their kinase activity. Transcripts of BIN2 were also increased under salt stress and are potential targets of miR166e-5p which was repressed under salinity (Fig. 5b).

Perception of ABA causes conformational changes of Pyrabactin resistance 1 (PYR1)/PYR1-like(PYL)/regulatory components of ABA receptors (RCAR), which facilitate their binding to PP2Cs to release their inhibition on SnRK2s (Fig. 5b). A down-regulation of PP2C transcripts under salt stress was observed. Besides, we identified a potential PP2C post-transcriptional regulation by miR4998-5p. ABA signal perception is also governed by the ABI5 transcription factor. However, it was not modulated in this study. Under stress, SnRK2s phosphorylate ABI5 triggering conformation changes and enables its further interactions with other proteins [139] (Fig. 5b). Hu and Yu, (2014) [140] showed BIN2 phosphorylates and stabilizes ABI5 protein in the presence of ABA (Fig.5b), whereas BR application represses the regulatory effect of BIN2 on ABI5 (Fig. 5a). SnRK2s and BIN2 phosphorylate ABI5 at different sites [141], and stabilization/promotion of ABI5 by BIN2 phosphorylation is dependent on the phosphorylation of ABI5 by SnRK2s (Fig.5b). BAK1 gene was

induced in Conquista and C08 libraries and this expression pattern under salinity was confirmed by RT-qPCR (Fig. 4c). Shang et al., (2016) [10] demonstrated that OST1/BAK1 complex induced stomatal closure through phosphorylation of SLAC1 and QUAC1 anion channels [142]–[144]. This complex formation is enhanced in ABA presence (Fig. 5b), and reduced in BR treatment (Fig. 5a). Besides, the interaction of ABI1 (PP2C) with OST1 inhibited the interaction of OST1 with BAK1 by OST1dephosphorylation. BAK1 was demonstrated to be localized in guard cells and could interact with OST1 and transphosphorylates it near the plasma membrane (Fig. 5b). BAK1 is a predicted target of miR482be-5p, which was repressed under salt stress conditions by RNA-Seq and RT-qPCR analysis (Fig. 4c and 5b). On the other hand, an LRR kinase was induced in our experiment and could be regulated by mi2118a-5p in Conquista. It is reported BAK1 is a versatile co-receptor, forming complexes with many LRR kinases [145], [146]. For that reason, we proposed a possible interaction between the LRR kinase (Glyma.16G078900) and BAK1, to produce an activation by transphosphorylation and subsequent interaction and activation of OST1 (Fig. 5b).

Guard cell ABA signaling through cytosolic PYR/RCARs is important in regulating basal stomatal opening and rapid stomatal responses to environmental stimuli [147]. Kuromori et al., (2014) [148] demonstrated the major ABA pool is synthesized in the vasculature and from there, ABA is transported to other cells [149]. However, ABA could also be synthesized in guard cells to ensures fast stomatal responses to sudden environmental changes [150] whereas changes in the root environment result in ABA synthesis in the vasculature leading to stomatal closure [147]. When ABA is synthesized it diffuses into guard cells or can be actively taken up to induce a direct effect on guard cell turgor via the cytosolic ABA signaling unit.

In normal conditions, under apoplastic acid pH, ABA can diffuse into cells [151]. Under stress conditions, when ABA diffusion into guard cells decreased because of a basic apoplastic pH, active ABA transport is thought to be very important [152], [153]. Members of the G subfamily of ABC transporters have been shown or suggested to function in ABA transport [154], [155]. ABCG40 is a plasma membrane ABA uptake transporter in guard cells [153]. In this study, ABCG29 gene expression was induced in both cultivars under salinity conditions. In *Arabidopsis*, ABCG29 was reported to be preferentially expressed in guard cells [155]. *GmABCG29* and *AtABCG40* share 58% of aminoacyl sequence identity, and have the same functional domains, meaning that probably they may have redundant functions. In this way, we propose that ABCG29 could be involved in the ABA

influx into guard cells. Despite, Merilo et al. (2015) [155], found low expression of AtABCG29 under low humidity and no change under ABA treatment, suggesting AtABCG29 to be responsible for ABA efflux from guard cells in normal conditions. Additionally the increase of ABCG29 transcripts could also be explained by the presence of a binding site for miR6300-5p that was down-regulated (Fig. 4d and 5b).
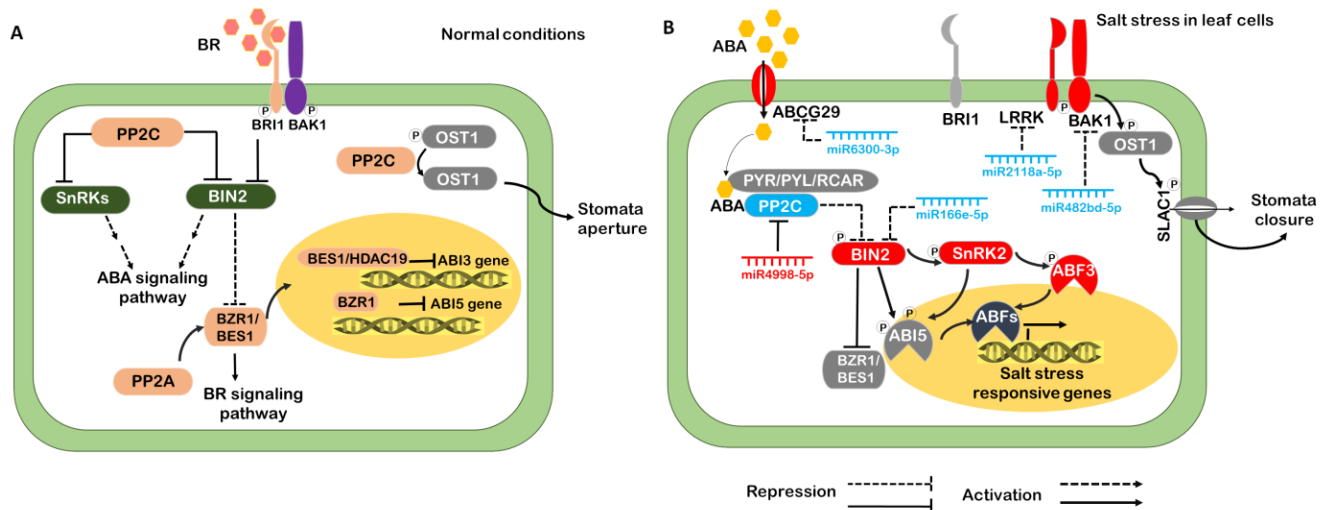

Fig. 5. Proposed model of ABA and BR signaling crosstalk and gene regulation by miRNAs in Conquista cultivar submitted to salt stress. Leaf cells response under **a.** Normal conditions. **b**. salt stress condition. See text for details of the whole process. Proteins encoded by up-regulated genes under salinity conditions are in red. Proteins encoded by down-regulated genes under salinity conditions are in blue. Proteins encoded by genes with unaltered expression under salinity conditions are in gray. Solid lines indicate processes occurring in the represented condition. Discontinue lines represent processes that could occur in the opposed condition

3.5 Conclusions

The present study provides a set of biological functions and genes modulated under salt stress conditions in two soybean cultivars. Their altered expression profiles along time were graphically represented. Genes and pathways with a conserved expression pattern between the two cultivars

were shown. A regulated salt tolerance mechanism in Conquista cultivar was proposed. Our findings corroborate with the main known effects of salt in plant gene expression and highlight the existence of additionally, new post-transcriptional regulation of ABA and BR signaling pathway through the identification of miRNAs with hybridization sequence in genes of those pathways. Taking together, the data presented in this work brings new elements and opens new ways to explore the molecular mechanisms associated with salt stress tolerance in soybean and provides new insights into soybean and gene selection for environmental stress-tolerant improvement.

3.6 Acknowledgments

3.7 References

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3.2   **Capítulo 2. HDAC inhibitor affects soybean miRNA482bd expression under salt and osmotic stress**

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Abstract

MicroRNAs (miRNAs) are small non-coding molecules that modulate gene expression through targeting mRNA by specific-sequence cleavage, translation inhibition, or transcriptional regulation. miRNAs are key molecules in regulatory networks in abiotic stresses such as salt stress and water deficit in plants. Throughout the world, soybean is a critical crop, the production of which is affected by environmental stress conditions. In this study, RNA-Seq libraries from leaves of soybean under salt treatment were analyzed. 17 miRNAs and 31 putative target genes were identified with inverse differential expression patterns, indicating miRNA-target interaction. The differential expression of six miRNAs, including miR482bd-5p, and their potential targets, were confirmed by RT-qPCR. The miR482bd-5p expression was repressed, while its potential HEC1 and BAK1 targets were increased. Polyethylene glycol experiment was used to simulate drought stress, and miR482bd-5p, HEC1, and BAK1 presented a similar expression pattern, as found in salt stress. Histone modifications occur in response to abiotic stress, where histone deacetylases (HDACs) can lead to gene repression and silencing. The miR482bd-5p epigenetic regulation by histone deacetylation was evaluated by using the SAHA-HDAC inhibitor. The miR482bd-5p was up-regulated, and HEC1 was down-regulated under SAHA-salt treatment. It suggests an epigenetic regulation, where the miRNA gene is repressed by HDAC under salt stress, reducing its transcription, with an associated increase in the HEC1 target expression.

Keywords: Abiotic stress, PEG, salt stress, miRNA, SAHA. HDAC

3.3 Introduction

Micro RNAs (miRNAs) are small (20-24 nt) non-coding molecules that modulate gene expression in a post-transcriptional manner, through cleavage or translation inhibition of specific mRNA sequences, in plants and animals (Bartel, 2004). miRNAs also have been related with transcriptional regulation (Yang et al., 2019). miRNAs are transcribed from a genomic locus (MIR gene) by an RNA polymerase II producing a pri-miRNA, that is processed by an RNase III endonuclease (Dicer-like in plants) into a pre-miRNA, and this is processed by the same into mature miRNAs (Axtell et al., 2011; Fang et al., 2015; Voinnet, 2009). In plants, methylation of

miRNAs occurs by an RNA methyltransferase HUA Enhancer 1 (HEN1), in a process that increases their stability (Yu et al., 2005). The mature miRNA molecule is exported to the cytoplasm by Hasty protein (HST) (Park et al., 2005), where it integrates the RNA-induced silencing complex (RISC) with the Argonaute protein (AGO). Recently, Bologna et al., (2018) showed that miRNA is assembled on the Argonaute 1 (AGO1) protein in the nucleus to form the RISC complex and is then exported to cytosol by EXPO1. One strand, 5p or 3p arm, and AGO protein form the RISC to drive the gene expression silencing (Khvorova et al., 2003). 5p/3p arm selection depends on the strand thermodynamic stability but also on the kind of tissue, plant development stage, or stress (Khvorova et al., 2003). miRNAs are involved in regulatory networks, during plant development and under biotic and abiotic stresses (Lima et al., 2012; Kulcheski et al., 2011; Ning et al., 2019).

Environmental stress conditions, such as high-salinity and water deficit, are the main limitations for plant growth, crop productivity, and distribution (Boyer, 1982). Salt stress in plants is associated to arid regions (Malash et al., 2008) where crop irrigation can cause toxicity by $Na+$ and $Cl-$.. The decrease in water uptake by roots (osmotic stress), photosynthesis reduction, oxidative stress, and reduction of symbiotic nitrogen fixation, limit the productivity of crops (Munns and Tester, 2008). Moreover, drought stress is one of the crucial factors affecting crop production in numerous regions worldwide, especially in arid and semiarid areas. This phenomenon causes several deleterious effects on plant metabolic processes, including a decrease in nutrient uptake and metabolism and photosynthetic assimilation (Rouphael et al., 2012). In this context, different studies commonly use polyethylene glycol (PEG) to simulate drought stress (Shi et al., 2015; Zhang et al., 2015), for its ability to reduce the water transport, reducing water absorption and causing desiccation in plants. PEG has limited metabolic interferences because it is less likely to be absorbed by plants and is not phytotoxic (Lawlor, 1970).

Soybean (Glycine max (L) Merril) is an important crop worldwide, being one of the primary sources of edible vegetable oil and protein (Singh, 2010). However, its production is low in arid and semi-arid regions in which salinity and drought are the main problems (Wicks and Klein, 1991). Plants have developed many molecular mechanisms to overcome abiotic stresses (Lamalakshmi Devi et al., 2017) that are important to be studied to identify molecular components useful for crop improvement and to increase the current knowledge about stress homeostasis. Particularly, miRNAs involved in stress were identified, in soybean nodules (Dong et al., 2013) and roots (Li et al., 2011; Sun et al., 2015) under salinity, and in root and leaves under drought

stress (Kulcheski et al., 2011; H. Li et al., 2011).

Several studies have reported that histone modifications, such as methylation and acetylation, can be correlated with gene expression in response to abiotic stresses (Kim et al., 2008; Luo et al., 2012; Patanun et al., 2017). The histone acetylation, performed by histone acetyltransferases (HATs), neutralizes the positive charge of the histone tails (Earley et al., 2007; Zhang et al., 2007). It reduces their affinity for DNA and alters the accessibility of transcription factors. As a consequence, histone acetylation can trigger gene activation (Kuo et al., 1996; Shahbazian and Grunstein, 2007). Conversely, the removal of histone acetylation, by histone deacetylases (HDACs), can lead to gene repression (Chen and Wu, 2010; To et al., 2011). HDACs are categorized into zinc-dependent and NAD+ types based on their catalytic domains. The Reduced Potassium Deficiency3 (RPD3)-like family are zinc-dependent (Seto and Yoshida, 2014; Ueda et al., 2017; Verdin and Ott, 2015). Plants also have evolved a plant-specific HDAC (HD-tuin) family (Brosch et al., 1996; Hollender and Liu, 2008). Members of this family are considered to be zinc-dependent HDACs (Lee and Cho, 2016). HDAC inhibitor (HDI) like Ky2 and Suberoylanilide hydroxamic acid (SAHA) were reported to increase the salt stress tolerance in Arabidopsis (Sako et al., 2016), and Cassava (Patanun et al., 2017), respectively. These HDIs target Zn^{2+}- dependent deacetylase by a chelate mechanism.

The aim of the present study was to evaluate ionic and osmotic stresses (NaCl and PEG) effect in microRNAs and their target expression and to identify post-transcriptional regulation mechanisms under both stresses. Furthermore, miRNA regulation by histone modification was evaluated under combined salt and SAHA treatments to have new insights about the transcriptional regulation of miRNA genes.

3.4 Material and methods

3.4.1   *Plant material and stress treatments*

Soybean (Glycine max) plants from Conquista BR46, a drought-tolerant cultivar (Casagrande et al., 2001), were used. Seeds were pre-germinated on filter paper in the dark at 25°C. Seedlings were transferred into a Hoagland´s nutrient solution (Hoagland and Arnon, 1950) and grown hydroponically at 25-28°C in 40% relative humidity, under natural daylight for a 16 h day.

After 15 days, when the seedling developed the first three leaves, they were submitted to salt, polyethylene glycol (PEG) or Suberoylanilide hydroxamic acid (SAHA) treatment

(Supplementary Fig. S1). For the salt treatment, plants were transferred to a nutrient solution with 200 mM NaCl, considering that soybean suffers yield reduction under this concentration (FAOSTAT, 2018). The control group of plants was maintained in Hoagland´s nutrient solution, and six plants per treatment were used. Samples of leaves of both groups of plants were collected after 4 hours of treatment, and immediately frozen in liquid nitrogen and stored at -80°C until RNA was isolated for RNA-Seq libraries construction. This experiment was repeated once more to confirm the RNA-Seq results by RT-qPCR (Supplementary Fig. S1).

A second treatment was carried out, where plants were transferred to a nutrient solution with 20% PEG-6000 (Zhang et al., 2015), and another group in a solution without PEG was considered as a control condition. Eight plants per treatment were used, and leaf samples of both groups were collected after 4 hours of treatment and immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction for RT-qPCR (Supplementary Fig. S1).

Finally, a SAHA treatment was made where four groups of plants were grown and transferred to a Hoagland nutrient solution. Each group was constituted of four biological replicates. Two groups of them were submitted to 2 µM SAHA for 24 hours, and the remnants groups were left without treatment. After that time, 200 mM NaCl was added in one SAHA pretreated group and one group without treatment, to compare the salt effect with or without SAHA pretreatment. As controls, one group without any treatment and one group with SAHA pretreatment, but not submitted to salinity conditions, were used. Leaves were collected after 4 hours of stress application and then frozen in liquid nitrogen. This material was used for RNA isolation and RT-qPCR analysis (Supplementary Fig. S1).

3.4.2   RNA isolation and sequencing

Total RNA was isolated from leaves using Trizol (Invitrogen, CA, USA), and the RNA quality was assessed by electrophoresis on a 1% agarose gel. RNA isolated (>10 µg) was sent to Fasteris Life Sciences SA (Plan-les-Ouates, Switzerland) for sequencing through the Illumina HiSeq2000 platform.

Libraries were obtained in duplicate, two for each treatment (salt stress and control) and each library had RNA samples from a pool of 3 different plants (Supplemental Fig. S1A). Four paired-end mRNA-Seq libraries were constructed. Briefly, the polyadenylated transcript sequencing (mRNA-Seq) was performed using the following successive steps: Poli-A purification, cDNA

synthesis using Poli-T primer shotgun to generate inserts of 500 nt, 3p and 5p pair-end adapters ligations, pre-amplification, colony generation and sequencing. The Illumina output data were sequence tags of 50 bases. RNA-Seq

Four small RNAs libraries were constructed from leaves, two for each treatment (salt stress and control). Briefly, the construction of the small RNA libraries consisted of the following successive steps: acrylamide gel purification of the RNA bands corresponding to the size range 20-30 nt, ligation of the 3p and 5p adapters to the RNA in two separate subsequent steps, each followed by acrylamide gel purification, cDNA synthesis followed by acrylamide gel purification, and a final step of PCR amplification to generate a cDNA colony template library for Illumina sequencing. The sequenced libraries are available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69571 under GSE69569 (pair-end mRNA sequences are named by letters, GEK37a/b till GEK40a/b) and GSE69570 (microRNAs, GEK 29, 30, 33 and 34) access numbers.

3.4.3   RNA-Seq quality and mapping analysis

Four libraries of mRNA and 4 for small RNAs were analyzed (See flow chart in Supplementary Fig. S2). For mRNA, strand unspecific, "pair-end" libraries with reads of 50-nucleotide length was obtained. Quality analysis of all the libraries was made using FastQC (Andrews, 2010), considering different parameters of Q score, base sequence content, GC content, and adapter content (Supplementary Figure S3). Reads with low quality (Q<30) and adapters were eliminated using Trim galore Software version 0.6.4 (Krueger, 2015). An anchorage of library reads on the Glycine max Genome (Gmax_275_Wm82.a2.v1.gene.gff3) deposited in Phytozome v13 was made using the TopHat2 software (Kim et al., 2013).

. For the small RNA libraries, the quality control was performed by Fasteris Life Sciences SA (Plan-les-Ouates, Switzerland). Reads with low quality (Q < 30) and the adapter sequences were eliminated and the reads with lengths from 18 to 26 nt were used for further analysis (Supplementary Figure S3). The software MultiQC (Ewels et al., 2016) was used to summarize the quality data of all libraries (Figure S3 and S4).

To assess the percentage of small RNAseq reads that map to the soybean genome, the Bowtie software was used (Langmead, 2010), not allowing mismatches or reverse complement alignments

(i.e. options -v 0, -n 0 and –norc).

To identify soybean differentially expressed miRNAs, under salt stress, pre-miRNAs soybean sequences deposited in miRBase V22 (Kozomara et al., 2019) were downloaded. Then, an mapping of miRNA reads on these sequences was made using isomirID version 0.53 (Oliveira et al., 2013). Each read anchored to pre-miRNA with a cutoff of 20 reads was considered to further analysis.

Another offset analysis was made using a mapping of the reads on the miRNA mature sequences from miRBase V22 with ten additional nucleotides from the pre-miRNA sequences, 5nt upstream and downstream of the mature sequence. This was done to analyze the difference of only one type of isoform or a sum of all the possible isoforms of a mature miRNA in the differential expression statistical analysis.

3.4.4 RNA-Seq statistical analysis to identify differentially expressed genes and miRNAs

A frequency table of the reads from each miRNA and mRNA in each library was obtained using isomirID v 0.53 and featureCounts v 1.6.0 (Liao et al., 2014) programs, respectively. For mRNAseq only proper paired pairs were considered for the further analysis, removing chimeric and multi mapped pairs (-p, -B and -C options for featureCounts). To extract the gene count data the gene annotation 'Gmax_275_Wm82.a2.v1.gene.gff3' from phytozome V12 was used and converted to an GTF format using the gffread utility from cufflinks v 2.2.1 (Trapnell et al., 2010). The feature used for counting of read pairs was 'exon' and the meta-feature 'gene_id'.

The DESeq2 package v 1.26.0 (Love et al., 2014) . was used to evaluate significant differences between the two treatments using the standard options (i.e. Benjamini and Hochberg method to calculated adjusted p-value). Genes and miRNAs with a fold change>2 and an adjusted p-value < 0.05 were considered differentially expressed.

To confirm transcriptomic response under salt stress, expression of some control gene was evaluated. Those genes are known to be modulated by saltinity and include: SnRK2.3 (glyma.11g058800), ABF1 (glyma.07g213100), ABF2 (glyma.06g040400), DREB1E (Glyma.05G049900), DREB1A (Glyma.20G155100), Na+/Solute symporter (Glyma.04G123700), P-type ATPase (glyma.19g159900), LEA2 (Glyma.19G260500), RD26 (Glyma.06G248900).

3.4.5 *Prediction of miRNA target with differential expression*

The putative target genes of each miRNA with differential expression were predicted by using the psRNATarget Server V2 (Dai et al., 2018). We used default parameters, a maximum expectation of 4.5 and cleavage as miRNA action mechanism. Therefore, the target with inverse differential expression in the salt treatment in relation to the miRNA was selected. The functional annotation of each gene was extracted from phytozome v12.

3.4.6 *Expression validation of microRNAs and predicted targets by RT-qPCR*

To validate RNA-Seq predicted miRNAs and targets with differential expression by RT-qPCR, the salt treatment was repeated using six plants for each treated group, and samples of leaves were collected at one and four hours after treatment, and then were frozen at -80 °C. Samples from PEG experiments were used to evaluate the miRNA and targets expression from the salt library analysis and to identify common genes affected by both stresses. SAHA experiments were similarly evaluated by RT-qPCR. First, cDNA synthesis was made using an M-MLV reverse transcriptase enzyme (Promega). For miRNA analysis, stem-loop primers designed according to Chen et al., (2005) were used. These consist of 44 conserved and six variable nucleotides that hybridize the miRNA of interest (Table S1). In the case of target genes, primers, according to Gadkar and Filion (2013) were designed (Table S2). Thus, the 3´primer section contents a complementary sequence of each target gene (flanking the miRNA hybridization region) and an additional MYT4 sequence at the 5´end to avoid the DNA genomic amplification and the use of DNAase I treatment that reduces the RNA quality.

PCR reactions were performed in a volume of 20 µl containing 10 µl of cDNA and a final concentration of 0.1 µM of each reverse and forward primer, 0.4 mM dNTPs, 1X buffer, 3 mM MgCl2, 0.25 U Platinum Taq DNA polymerase (Invitrogen) and 0.1 X SYBR green I (Invitrogen). Forward miRNA primers were designed in Oligoanalyzer v.3.1 based in the miRNA sequence and removing the last six nucleotides (Table S1). The universal reverse primer 5'GTGCAGGGTCCGAGGT 3' was used in all miRNA RT-qPCR reactions. Target genes primers were designed in Primer3 v.0.4.0, aiming to obtain approximately 150 nt PCR fragments. The universal reverse primer 5´CAGCTTGGTAGAATCGATCAGCTAC3´was used (Gadkar and Filion, 2013). In every case, the melting temperature was adjusted at 60°C.

Samples were analyzed in technical triplicate in a 96-well plate, and a no-template control was included. We used as reference genes from soybean: Actin and Elongation factor B, MIR156b (5'-TGACAGAAGAGAGAGAGCACA - 3'), MIR172ab (5'- AGAATCTTGATGATGCTGCAT - 3') and MIR1520d (5'- ATCAGAACATGACACGTGACAA - 3'), which has been demonstrated as optimal normalizers for salt stress analysis in Glycine max (Kulcheski et al., 2010). The thermal cycling conditions consisted of an initial polymerase activation (5 min at 95 °C) followed by 40 cycles of denaturation (15 s at 95 °C), annealing (10 s at 60 °C) and extension (15 s at 72 °C). A 7500 Real-Time PCR Detection System (Bio-Rad) equipment was used. Afterward, melting curves were acquired for PCR products using 0.5°C steps with a hold of 1 s at each step from 65°C to 99°C. Threshold and baselines were manually determined using the ABI 7500 Real-Time PCR System SDS Softwarev2.0.

3.4.7   *RT-qPCR statistical analysis*

The quantification of relative gene expression was complete using the mathematical method $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) with the control condition expression as a calibrator and the endogenous genes as references. A Student's t-test was performed to compare differences in expression between treatments. When more than two conditions were compared, a two way ANOVA, Tukey test was made using R. The means were considered significantly different when *p*- value < 0.05.

3.4.8   *Conservation analysis of miRNA binding site*

We compared the mature and precursor miRNA sequence of *Glycine max* with of other species using the searching for similarity tool of miRBase release 22, to know how the identified miRNAs are conserved in other species. Besides, to evaluate if the miRNA hybridization site is conserved among different species, orthologous of target genes were searched in Phytozome V12, and those hybridization sites were evaluated by psRNAtaget software.

3.5 Results

3.5.1 Effects of salt stress in soybean transcriptional level

After trimming, the number of reads of the mRNA-Seq libraries varied from 24.4 to 30.8 million with about 85% of the paired reads mapping concordantly to the soybean genome (Table 1). Excluding chimeric paired reads (i.e., paired reads that mapped in different chromosomes) and multi mapped paired reads, ~ 66% of the total reads were considered properly paired and were used to further analysis (Table 1 and Fig. S3). The number of reads from small RNA-Seq libraries, with 18–26 nt in length, varied from 8.2 to 11.1 million (Table 2 and Fig. S4). From those, ~ 70% mapped without mismatches into the soybean genome (Table 2). The percentage of reads (~ 30%) that did not map likely belongs to different pathogenic, symbiotic and free-living organisms (Molina et al., 2012) or RNA plant virus integration in the soybean genome that leads to the production of small RNAs (da Fonseca et al., 2016d). When mapped to miRNA precursors from miRBase v22, around 14% of the total reads mapped with perfect matches (Table 2). As a control check of the effect of salt treatment in soybean   transcripts modulation, the expression of genes known to be responsive to salt stress, in ABA-dependent (SnRK2.3, ABF1, ABF2, Na+/Solute symporter, p-type ATPase, LEA2) and independent (DREB1E, DREB1A) manner or both (RD26) were identified in the data set (Supplementary Fig. S5). The differential gene expression analysis confirmed them as up-regulated, corroborating salt stress observation in other plants (Chen et al., 2019; Freitas et al., 2019; Kulik et al., 2011; Serrano and Rodriguez-Navarro, 2001; Zandkarimi et al., 2015)

Table 1. Mapping data from mRNAseq libraries.

Libraries	Total reads	Mapped to genome concordantly		Proper paired reads*	
		Total	%	Total	%
GEK-37	25,601,239	21,829,754	85.3	17,251,216	67.4
GEK-38	24,418,115	20,689,863	84.7	16,159,471	66.2
GEK-39	30,784,398	26,016,040	84.5	20,041,752	65.1
GEK-40	28,968,130	24,930,043	86.1	19,687,552	68.0

* Excluding chimeric and multimapping pairs

Table 2. Mapping data from small RNAseq libraries

Libraries	Total reads*	Mapped to genome		Mapped to miRNA precursors	
		Total	**%**	**Total**	**%**
GEK-29	8,187,784	5,945,408	72.6	1,108,915	13.5
GEK-30	8,414,443	6,065,107	72.1	1,069,002	12.7
GEK-33	9,206,530	6,557,491	71.2	1,321,451	14.4
GEK-34	11,133,156	7,852,637	70.5	1,670,191	15.0

*Reads from 18 to 26 nt in length

3.5.2   *Salt stress modulates miRNA expression*

Based on small RNA-Seq libraries, 17 isoforms of miRNAs have a differential expression (Fold change >2, p-value <0.01) (Fig. 1A, Table 1). Their miRNAs sizes range from 18 to 23 nt, being 21 nt the most abundant (Table 3). Some soybean specific miRNAs were identified: miR5372-5p, miR5373-3p, miR4411-3p, miR9726-3p, miR6300, miR4382-3p (Supplementary Fig. S6). Sequences from miR172 (c,d,e), miR482 (b,d), miR156 (h,k), and miR319 (a,b) were originated from multiple-locus. In those cases, the mature miRNA share the same nucleotide sequence and could derive from one among several pre-miRNAs; so far, they received a multiple letter designation, as miR172cde. Additionally, mature miRNAs with different 5p/3p arm selection, not previously reported, were identified. For instance, miR172cde-5p, miR156hk-3p, miR156k-3p, miR319b-5p and miR319ab-5p exhibited the opposite arm in relation to the canonic sequence. Other findings were the isoforms of microRNAs (isomiRs), miRNA with different sequences from canonic. As an example, miR482bd-5p, miR4414a-5p, miR166i-5p, mir1510a-3p, miR5373-3p and miR4411-3p present slightly different sequences compared to the canonical miRNA (Supplementary Fig. S7). The isomiRs biogenesis is related to the inaccurate cleavage of the Dicer-like ribonuclease enzyme, which may lead to alternative pre-miRNA processing (Morin et al., 2008b, 2008a). Among the identified miRNAs, miR9726-3p showed the highest increase under salt treatment, and the most significant reduction was in miR172cde-5p (Fig. 1A). A complementary miRNA offset analysis was performed to evaluate the effect on differential expression results when considering the sum of reads from all possible miRNA isoforms (Supplementary Fig. S8A). This analysis identified 11 out of the 17 miRNA originally described as differentially expressed, considering the mature miRNA sequences separately (Supplementary Fig. S8B).
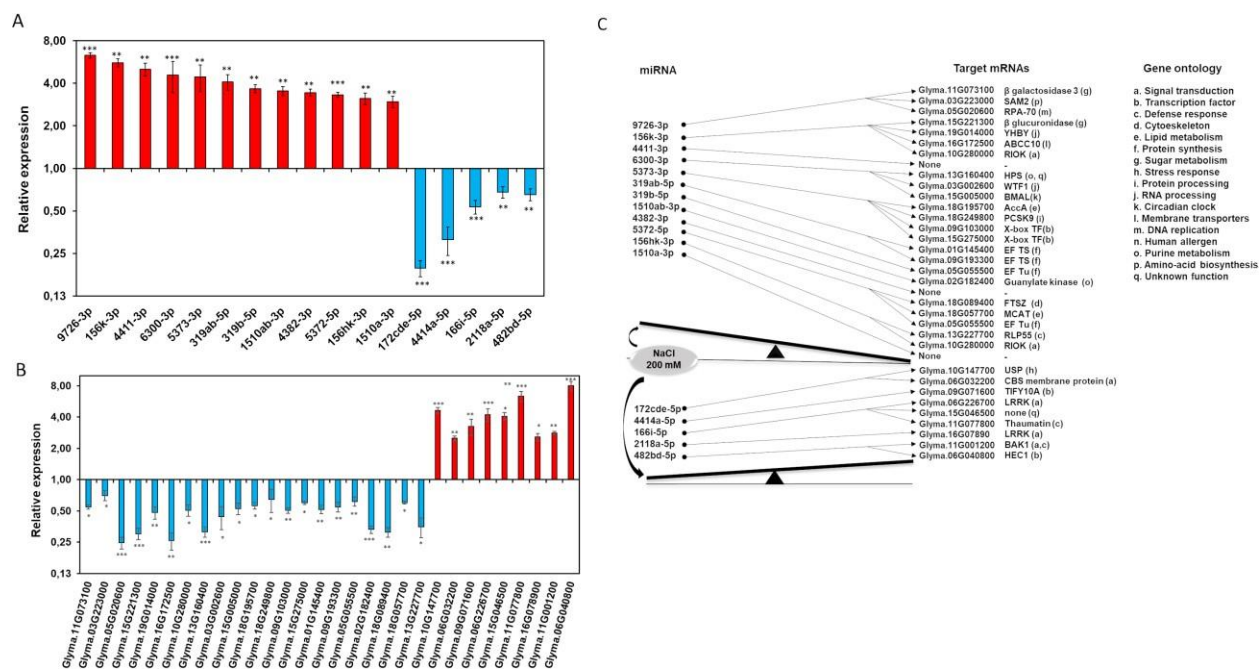
Figure 1. **Differential expression analysis by RNA-Seq from soybean leaves under 200 mM NaCl for 4 h A.** Fold change values of differentially expressed miRNAs **B**. Fold change values of predicted miRNA targets with differential expression. A and B. Values are based on salt and control condition comparison (T-test, *p <0.05, **p <0.01, ***p <0.001), data are means ± SE with n=4. Relative expression was plotted using a log2 scale. Genes and miRNAs expression in black bars were increased, and those in gray were reduced **C.** MiRNA and targets with an inversed expression relation.

Table 3. miRNA and predicted targets with differential expression under salt stress in soybean leaves by RNA-Seq analysis

miRNA	Sequence	size	FC miRNA	P-value miRNA	Targets	FC Target	P-value target	Exp	Functional annotation
9726-3p	TATAGGCATTATTTTTTTCTTC	22	3.143	0.0002	Glyma.11G073100	0.51	0.002	3	Beta-galactosidase 3
					Glyma.03G223000	0.62	0.014	3	S-ADENOSYLMETHIONINE SYNTHASE 2
					Glyma.05G020600	0.25	0.000	4.5	Replication protein a 70 kda dna-binding subunit
156k-3p	GCTCACTTCTCTTTCTGTCAA	21	2.562	0.0052	Glyma.10G280000	0.47	0.001	2.5	Serine/threonine-protein kinase RIO
					Glyma.15G221300	0.29	0.000	4.5	glucosyl/glucuronosyl transferases
					Glyma.19G014000	0.45	0.003	4.0	RNA-binding protein YHBY
					Glyma.16G172500	0.30	0.000	4.5	ABC transporter C family member 10

4411-3p	TATTGTAACTAATTTGTCGGTA	22	2.599	0.0024	none				
6300-3p	GTCGTTGTAGTATAGTGG	18	2.548	0.0003	Glyma.13G160400	0.30	0.000	2.5	Hydrophobic seed protein
					Glyma.03G002600	0.41	0.004	3.0	RNA recognition domain-containing protein WTF1
					Glyma.15G005000	0.49	0.003	4.0	Circadian protein clock
5373-3p	CTTGATTCTAGATGATGTTGA	21	2.299	0.0052	Glyma.18G195700	0.52	0.003	2.0	Acetyl-coenzyme a carboxylase carboxyl transferase subunit alpha
					Glyma.18G249800	0.48	0.003	3.0	Proprotein convertase subtilisin/kexin
					Glyma.09G103000	0.45	0.001	2.5	X-BOX transcription factor-related
					Glyma.15G275000	0.54	0.002	2.5	X-BOX TRANSCRIPTION FACTOR-RELATED
319ab-5p	AGAGCTTTCTTCAGTCCACT	20	2.400	0.0012	Glyma.01G145400	0.47	0.001	3.0	Elongation factor TS
					Glyma.09G193300	0.48	0.005	3.0	Elongation factor TS
319b-5p	AGAGCTTTCTTCAGTCCACTT	21	2.242	0.0030	Glyma.01G145400	0.47	0.001	3.0	Elongation factor TS
					Glyma.09G193300	0.48	0.000	3.0	Elongation factor TS
					Glyma.05G055500	0.55	0.002	3.5	Ribosome-releasing factor 2
1510ab-3p	TGTTGTTTTACCTATTCCAC	20	2.124	0.0086	Glyma.02G182400	0.37	0.001	2.5	Guanylate kinase
4382-3p	TATGTTAACTGATTTCATGGAT	22	2.204	0.0012	none				
5372-5p	TTGTTCGATAAAACTGTTGTG	21	2.172	0.0008	Glyma.18G089400	0.32	0.001	3.5	Cell division protein FTSZ homolog 1
					Glyma.05G055500	0.55	0.002	3	Elongation factor Tu
					Glyma.18G057700	0.54	0.003	4.5	Acyl-carrier-protein S-malonyltransferase
156hk-3p	GTGCTCACTTCTCTTTCTGTCA	22	2.001	0.0052	Glyma.10G280000	0.47	0.001	3.5	Serine/threonine-protein kinase RIO
					Glyma.13G227700	0.39	0.001	5.0	Receptor like protein 55
1510a-3p	TGTTGTTTTACCTATTCCACCC	22	1.949	0.0036	Glyma.02G182400	0.37	0.001	2.5	Guanylate kinase
172cde-5p	GGAGCATCATCAAGATTCACA	21	0.190	0.0000	Glyma.10G147700	3.63	0.000	2.5	Universal stress protein family (Usp)
					Glyma.06G032200	2.00	0.003	3.0	CBS domain membrane protein
4414a-5p	AGCTGCTGACTCGTTGGCTCG	21	0.324	0.0003	Glyma.09G071600	2.39	0.001	5.0	Protein TIFY 10A-RELATED
166i-5p	GGAATGTCGTCTGGTTCGAGA	21	0.427	0.0003	Glyma.06G226700	3.07	0.000	4.0	Protein tyrosine kinase (Pkinase_Tyr)
					Glyma.15G046500	2.77	0.001	4.0	Non annotation
					Glyma.11G077800	4.29	0.000	4.5	Pathogenesis-related thaumatin family protein
2118a-5p	GAGAAGAGCTTGAGGAAGTGATG	23	0.528	0.0056	Glyma.16G078900	1.86	0.028	2.5	Leucine-Rich Repeat Kinase (LRRK)

482bde -5p	TATGGGGGGGATTGGGAAGGAA	21	0.510	0.0032	Glyma.11G001200 0	2.23	0.001	2.5	Brassinosteroid insensitive 1-associated receptor kinase 1-related
					Glyma.06G040800 0	7.94	0.000	4.5	Transcription factor HEC1

3.5.3   Differentially expressed miRNA targets under salt stress

The identification of an inverse correlation between microRNAs and their target expression was possible by comparing miRNA and mRNA-Seq libraries. It was necessary to identify differentially expressed genes (DEGs) from mRNA libraries, to find the candidate genes post-transcriptionally regulated under salt stress. Besides, a target prediction analysis was conducted for each differentially expressed miRNA (Supplementary Table S3). Thirty-one predicted targets were found to have a differential expression (FC > 2, p-value < 001) and an inverse expression in comparison to the miRNA (Table 1, Fig. 1B). These genes are involved in stress homeostasis. Nine of them were up-regulated, including transcription factors (TFs) such as TIFY motif (TIFY) and HECATE 1 (HEC1), and signaling protein kinases as Brassinosteroid Insensitive 1-associated receptor kinase 1 (BAK1). Twenty-two were down-regulated, including proteins from photosynthesis, circadian clock, cellular division, DNA replication, and translation (Fig. 1C). These results indicate a miRNA regulation through mRNA cleavage, with miRNA cleavage sites in the 5′UTR, 3′ UTR, or CDS. Ten miRNAs, such as miR156k-3p, miR5372-5p, miR5373-3p, miR9726-3p, miR166i-5p, miR319ab-5p, miR6300-3p and miR482bd-5p have multiple targets (Fig. 1C), indicating that these miRNAs could present different functions in the cell.

3.5.4   miRNA and target expression are modulated under osmotic and ionic stresses

The differential expression of six miRNAs, identified as regulated under salt stress by mRNA-Seq, was validated by RT-qPCR (Fig. 2A, Supplementary Fig. S9). Kinetic analysis for salt treatment showed that some miRNAs had an early response at one hour of treatment, such as miR 9726-3p, miR4414a-5p and miR482bd-5p. The remaining miRNAs have differential expression only at four hours (Fig. 2A). Expression analysis of the predicted miRNA targets by RT-qPCR confirmed that seven targets have the same expression pattern as identified by the RNA-Seq analysis, with an inversed expression pattern in comparison to their regulatory miRNAs. Four were down-regulated and three up-regulated (Fig. 2B). The stress response analysis indicates that the transcription factor HEC1, and the receptor BAK1 genes had an early response at one-hour

105

treatment, such as miR482bd-5p. All the down- regulated genes were modulated after four hours of treatment. Under PEG treatment, three miRNAs showed an inverse expression pattern regarding their putative targets, such as miR166i-5p, miR156hk- 3p, and miR482bd-5p (Fig. 3A). They were down-regulated, while the five target genes were up-regulated (Fig. 3B). Osmotic and ionic stresses, induced by PEG and salt, were investigated to determine if they share the same miRNA expression patterns after four hours of each stress treatment. From the six evaluated miRNAs, miR319b-5p and miR482bd-5p exhibited the same expression pattern (Supplementary Fig. S10A), which indicates that they could be important in the osmotic stress response mechanism. Concerning the target genes, six of them showed a similar expression pattern between both stresses (Supplementary Fig. S10B), such as Circadian Protein Clock (BMAL) that was down-regulated. The other genes were up-regulated, such as Thaumatin protein, Leucine rich protein tyrosine kinase (LRRK), HEC1, and BAK1. In summary, seven genes might be regulated by miRNAs under salt stress based on an RNA-Seq and RT-qPCR analysis, considering the miRNA/mRNA inversed expression pattern (Fig. 2C). Meanwhile, under PEG stress, three miRNAs might regulate five stress-responsive genes (Fig. 3C). It is interesting to highlight that miR482bd-5p and their potential targets HEC1 and BAK1 had the same expression pattern in both osmotic stresses (Supplementary Fig. S10).
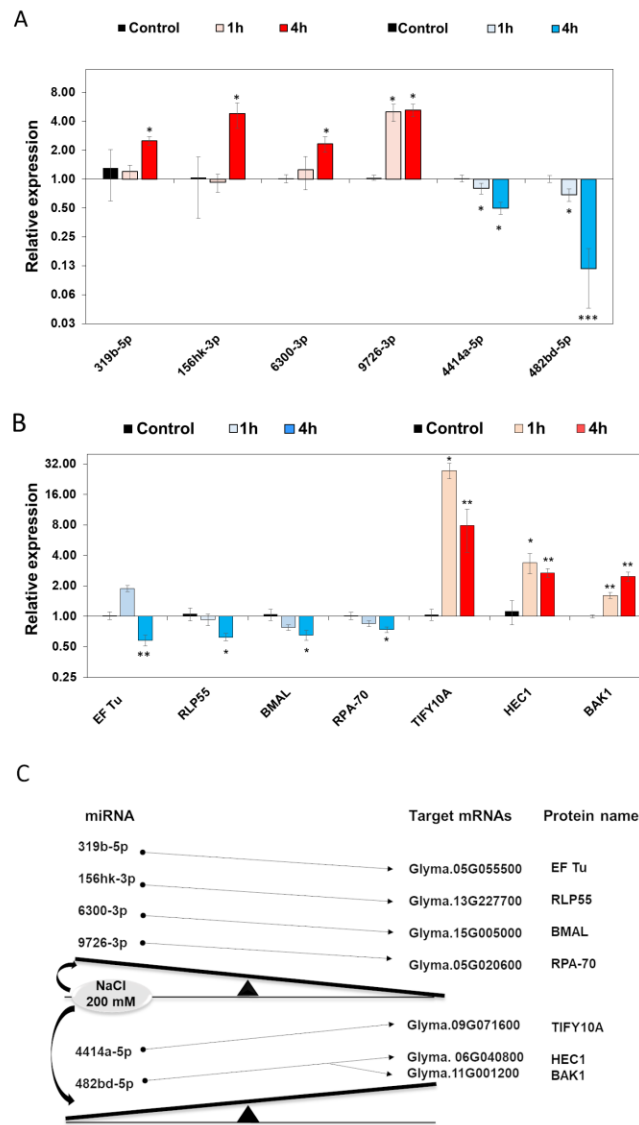
Figure 2. Kinetic analysis of miRNAs and targets expression by RT-qPCR in leaves of soybean under salt stress for one and four hours. A. Relative expression values of differentially expressed miRNA. **B**. Relative expression values of differentially expressed miRNA targets. Values are based on salt and control condition comparison (Unpaired T-Test, *p <0.05, **p <0.01, ***p <0.001), data are means ± SE with n=4. Relative expression was plotted using a log2 scale. Genes and miRNAs expression in red were increased, and in blue were reduced C. miRNA and targets with an inversed expression relation.
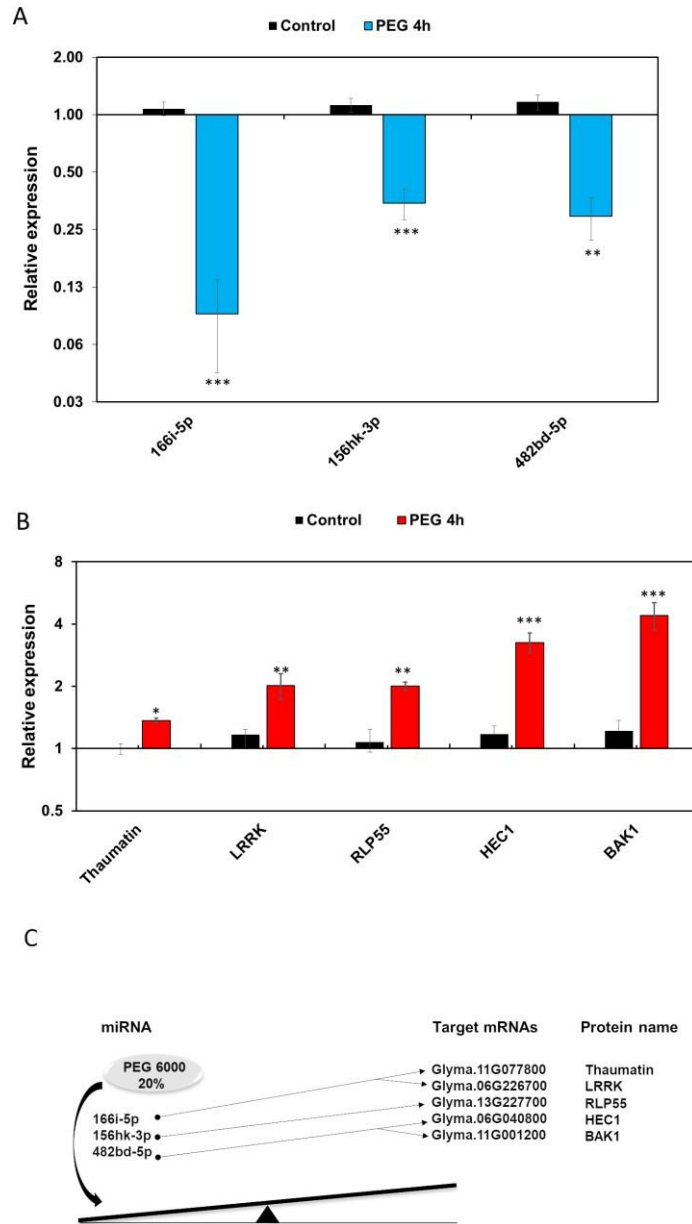
Figure 3. **RT-qPCR analysis of miRNAs and targets expression from soybean leaves under PEG stress for 4 h. A.** Relative expression values of differentially expressed miRNAs. **B**. Relative expression values of differentially expressed miRNA targets. Values are based on PEG and control condition comparison (Unpaired T-Test, *p <0.05, **p <0.01, ***p <0.001). The data are means ±SE with n=8. Relative expression was plotted using a log2 scale. Genes and miRNAs represented in black and gray bars correspond to an induced and reduced expression, respectively.

3.5.5   HDAC inhibitor affects miRNA482bd and target expression under salt stress

To inspect the possibility of epigenetic regulation by histone acetylation under salt stress, a 24 h SAHA treatment, an histone deacetylase inhibitor (HDI), was carried out before appling the stress. The effect of SAHA in miRNA expression was evaluated after four hours of salt stress. SAHA treatment triggered the up-regulation of miR482bd, as can be observed when the treated group was compared to the control condition, but the remaining miRNA was not affected (Fig. 4A). The SAHA effects was compared not only against control but also for salt and SAHA-salt treated plants (Fig. 4A). SAHA triggered induction of, miR482bd and miR4414a expression. At the same time, these miRNAs were down-regulated under salt stress, meaning that likely some HDACs are related to the expression inhibition of those miRNAs under salt stress, which was prevented by the addition of an HDI. The miR482bd-5p was the only one down-regulated under both salts, and PEG stress and its predicted targets (BAK1 and HEC1) were up- regulated. Besides, the HDI induced the miRNA expression even under salt stress. For that reason, we found it interesting to analyze HEC1 and BAK1 expression under SAHA treatment. When BAK1 was analyzed, variable expression patterns were obtained, with no definite conclusion; expected patterns were observed only for HEC1. As previously reported, in this experiment, miR482bd-5p and HEC1 presented an opposite expression pattern under salt stress (Fig. 4B). Under the SAHA effect, the miR482bd-5p expression did not present modulation, and HEC1 expression increased in comparison to the control condition. Interestingly, the combination of SAHA pretreatment with a further salt treatment triggered a miR482bd-5p induction and a HEC1 significant reduction (Fig. 4B) what allows to establishment of correlation in the miRNA and target expression.
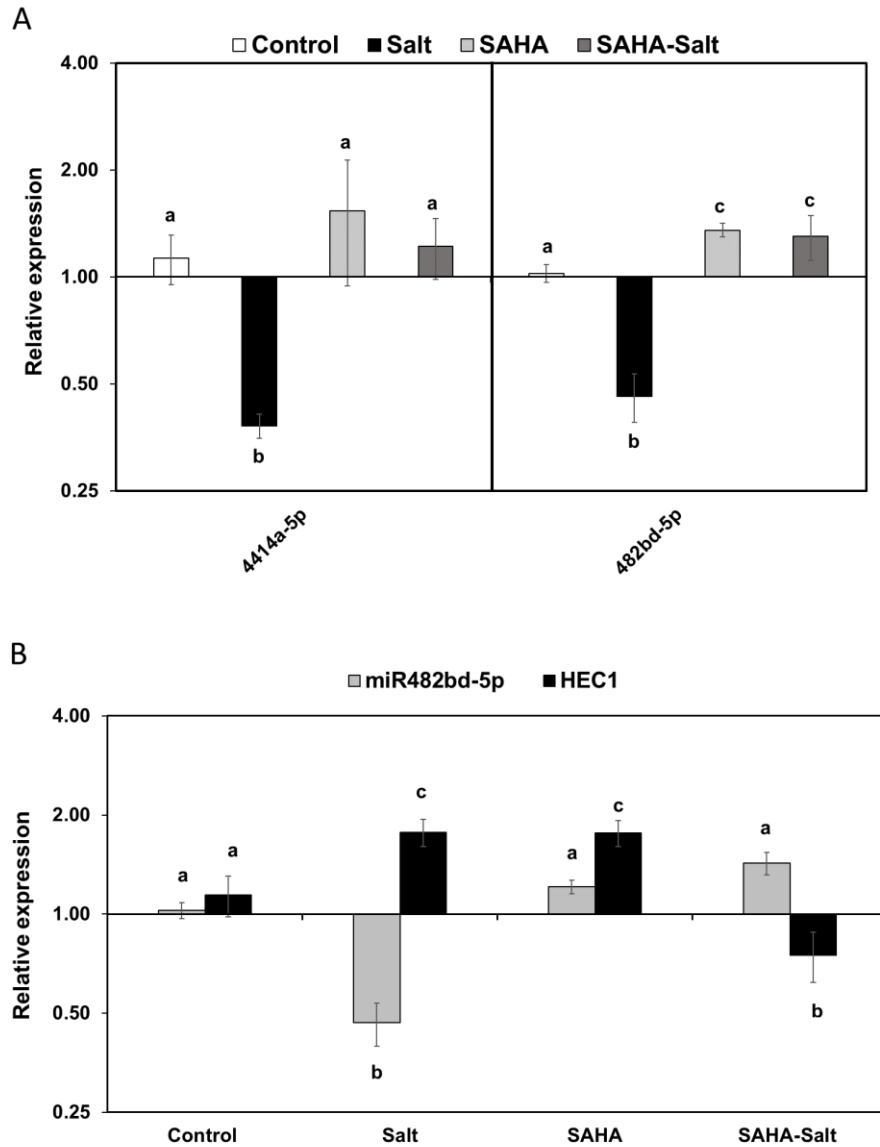
Figure 4. RT-qPCR analysis of Histone deacetylase inhibitor treatment effect from soybean leaves under 24 h SAHA and 4 h salt treatment. A. Relative expression values of differentially expressed miRNA. Values with significant differences are labeled with letters (ANOVA, Tukey test, p<0,05). The data are means ±SE with n=4. **B**. Relative expression values of miRNA482bd-5p and HEC1. Values with significant differences are labeled with asterisks (ANOVA, Tukey test, *p <0.05, **p <0.01). The data are means ±SE. Relative expression was plotted using a log2 scale.

3.5.6   *Conservation of miRNA regulation among species*

Considering the induced genes under salinity stress that were predicted as targets of miRNAs, one encoding a TIFY10A-related protein (Glyma.09G071600) was found. Its transcript has a binding site for the miR4414a-5p, which was down-regulated. TIFY10A (also known as JAZ1) belongs to a large family of transcription factors that are specific to plants, and it is known to be a transcriptional repressor of the JA signaling pathway (Chini et al., 2007; Fern´andez-Calvo et al., 2011; Melotto et al., 2008). Taking all the miRNAs deposited in the miRBase v 22, the miR4414a-5p of soybean presented sequence similarity only with the Medicago truncatula miR4414a-5p with a single mismatch at the 18 nucleotide position, (which likely does not influence the target specificity, Supplementary Fig. S11A). The psRNATarget v2 allowed the identification of an mtr-miR4414a-5p binding site on the MtTIFY10A (Medtr4g124960.1) transcript, with an expectation value of 5 and a likely inhibition by cleavage (Supplementary Fig. S11A). These results lead us to hypothesize that the post-transcriptional regulation of TIFY10A by this miRNA seems to be conserved among Fabaceae. On the other hand, GmTIFY10A paralogous in the Conquista cultivar (Glyma.01G204400, Glyma.13G112000, Glyma.16G010000, Glyma.15G179600) were up-regulated under salt stress (Supplementary Table S4). However, miR4414a-5p does not have binding sites on those genes. The conservation of miR482bd-5p binding site on the transcription factor HEC1 was evaluated. It was found, by BLAST analysis in NCBI, that HEC1 of Vigna radiata has a similar binding site (with 1 mismatch) to Glycine max (Supplementary Fig. S11B). miR482bd-5p from V. radiata was not available in the miRBase v22. When Vr HEC1 transcript region was submitted to psRNAtarget analysis, a gma-miR482bd-5p binding site was found with an expectation value of 3.5. This result allows us to consider this miRNA/target relation seems to be conserved among Fabaceae. Besides, Glyma.17G173100 also encodes HEC1 protein in soybean and is up-regulated under salt stress (Table S4). However, it does not exhibit a binding site for miR482bd-5p. Interestingly, Glyma.04G039500, which has a miR482bd-5p binding site, was not modulated (Table S4). The conservation of miR482bd-5p binding site on the BAK1 transcript in other species was evaluated. However, no similar binding regions were found in other plant genomes. Using wild and cultivated soybean genome sequences of an earlier study (Lam et al., 2010), it was found that the Conquista binding site of BAK1 is conserved among 11 different cultivars of Glycine max and 12 accessions of Glycine soja, from China, Japan, USA, and Brazil

(Supplementary Fig S11C). Other BAK1 paralogous of Conquista were up-regulated under salt stress (Glyma.10G258800, Glyma.20G132400, Glyma.15G051600, and Glyma.05G119500). However, miR482bd-5p does not have a binding site on those genes. Among the down-regulated genes, Receptor-like protein 55 (RLP55, Glyma.13g227700) was found to be a putative target of the induced miR156hk-3p. Receptor-like protein (RLP) family contains leucine-rich repeats (LRRs) and are likely mediators of protein-protein interactions. RLP family, including AtRLP55, are involved in development and disease resistance in many plants (T¨or et al., 2009; Wang et al., 2010; Zhang et al., 2010). Based on miRBase information, the mature miRNA sequence is conserved among other species, such as Medicago truncatula, Vriesea carinata, Zea mays, Oryza sativa, and Arabidopsis thaliana (Supplementary Fig S11D). However, we did not found a miR156hk-3p binding site on the RLP55 transcript of those species. Under salt stress, miR319b-5p was up-regulated while its predicted target Elongation factor Tu (EF Tu), was down-regulated. This gene mediates the disassembly of ribosomes from mRNA at the end of mitochondrial protein biosynthesis and allows the "ribosome recycling". miR319b-5p is known to be conserved among other species such as Malus domestica, Arabidopsis lyrata, and M. truncatula (Supplementary Fig S11E). Sequences of EF Tu of those species were analyzed by psRNAtarget using miR319b-5p. Results indicate that the EF Tu transcript of M. domestica (MDP0000290453) might be a target of md-miR319b-5p by cleavage with an expectation value of 4.5 (Supplementary Fig S11E). These results suggest that this regulation is likely to be present in other Families such as Rosaceae. Some mechanisms seem to be soybean specific. For instance, The miR6300 was up-regulated, while its predicted target Circadian protein clock/BMAL (Glyma.15G005000.3) was repressed under salt stress. Additionally, Replication protein A 70 KDa DNA-binding (RPA-70) was also modulated, likely being negatively controlled by the soybean specific miR9726-3p under salinity (Supplementary Fig. S6). These miRNAs are non-conserved, being reported only in Glycine max plants (Supplementary Fig. S6), which means that these regulations are likely specific to soybean.

3.6 Discussion

The understanding of the stress regulation mechanisms of crop plants is crucial not only to expand the current knowledge of plant stress homeostasis but also to identify critical components to improve crop production. Plant miRNAs have been reported as having a strong tendency

towards regulating responses to abiotic stress, including dehydration, freezing, salinity, alkalinity, and other stresses (Jones-Rhoades and Bartel, 2004). Hence, our study aimed to identify miRNAs and their putative targets involved with ionic and osmotic stress response in soybean. By RNA-Seq, seventeen responsive miRNA were identified, and post-transcriptional regulation of 31 salt-responsive genes was proposed. We confirmed a salt-expression correlation among seven genes and their corresponding miRNAs by RT-qPCR. Nevertheless, the inhibitory actions of these miRNAs have to be established by additional analysis, such as Rapid amplification of cDNA ends (RACE 5′) or functional genomics.

Interestingly, all the performed analyses (RNA-Seq, offset RNA-Seq, and RT-qPCR) presented a reduction in the miR482bd-5p expression under salt stress. Similarly, this miRNA was also repressed under the PEG condition. These indicate that miR482bd-5p miRNA has a role in soybean osmotic stress homeostasis. When a target prediction was made, two up-regulated genes under osmotic stress, HEC1, and BAK1, were found to have binding sites for this miRNA. Besides, the genes and the miRNA exhibited a modulation at one and four hours of salt treatment, confirming that there is a correlation among stress responses.

The transcription factor HEC1 (Glyma.06G040800), a member of the bHLH gene family, is known for controlling the development of transmitting tract and stigma (Pires and Dolan, 2010). During reproductive tissue development, HEC1 controls auxin distribution by the direct regulation of auxin transporters (PIN1 and PIN3) and is a negative regulator of cytokinin signaling (Schuster et al., 2015). Additionally, the application of an ethylene precursor strongly induced the HEC1 gene expression in Arabidopsis (Boex-Fontvieille et al., 2016). Moreover, the comparison of wild-type Anthurium andraeanum plants with a mutant version that is involved in cold and drought tolerance showed higher levels of HEC1 transcripts in the stress-tolerant mutant (Jiang et al., 2019). Those suggest that HEC1 has varied roles being implicated with different hormone pathways and stress responses. We hypothesize that HEC1 can be involved in soybean salt homeostasis. The present results indicate its post-transcriptional regulation by miR482bd-5p. The conservation of this mechanism among species was scrutinized, and the result shows that it might be conserved only among Fabaceae species.

The up-regulated BAK1 also has a binding site for miR482bd-5p. This gene is responsive to brassinosteroid hormones, and it is implicated in plant immunity (Chinchilla et al., 2007), cell death (Liu et al., 2017), and ABA signaling response (Rodrigues et al., 2017; Shang et al., 2016). It

corresponds to a versatile co-receptor or adaptor protein. For instance, in the presence of BR, this hormone binds to its receptor Brassinosteroid insensitive 1 (BRI1), inducing its activation. BRI1 recruits BAK1 to form a receptor complex on the plasma membranes triggering the expression of BR-responsive genes (Nam and Li, 2002). Furthermore, BAK1 activates plant defense responses upon recognition of bacterial flagellin, through forming a complex with flagellin insensitive 2 (FL2) (Chinchilla et al., 2007). Moreover, Shang et al. (2016) demonstrated that BAK1 forms a complex with open stomata 1 (OST1) and induced stomatal closure through phosphorylation of SLAC1 and QUAC1 anion channels (Geiger et al., 2009; Imes et al., 2013; Vahisalu et al., 2008), leading to transpiration reduction. This complex formation is enhanced in ABA presence and is reduced in BR treatment. Hence, we proposed that miR482bd-5p repression under salt stress could be necessary for a BAK1 (Glyma.11G001200) expression induction, and a concomitant stomata closure in soybean plants (Cadavid et al., 2020). Conservation analysis of this miRNA regulation mechanism suggests this is likely an ancestral soybean specific miRNA.

On the other hand, the HDI treatment analysis showed that miRNA482bd-5p was up-regulated, and HEC1 was down-regulated under SAHA and salt stress treatment (Fig. 4B). This may be explained by an epigenetic regulation, where miRNA482bd-5p gene expression is controlled directly or indirectly by an HDAC under salt stress to reduce its transcription with an associated increase in the expression of the target (Fig. 5A). This process was reverted by using an HDI (Fig. 5B), triggering an increase in miRNA482bd transcript levels and a reduction of HEC1 transcripts. We also suggested miRNA epigenetic regulation by histone acetylation in soybean. DNA methylation and histone modification were previously reported in human miRNA genes involved with diseases (reviewed by (Sato et al., 2011)).

Earlier studies revealed that HDAC genes are involved in ABA and stress response (Sridha and Wu, 2006; Ueda et al., 2017; Yang et al., 2018; Zheng et al., 2016). We hypothesize that an up-regulation of HDAC genes may be implicated in miRNA482bd-5p salt responsive regulation. However, from 28 HDAC of the soybean genome, only three of them were modulated under salinity in our study, being HDAC17 (Glyma.17G120900), HDT4 (Glyma.12G181400) and HDT2 (Glyma.11G189500) repressed, which is in agreement with a previous study in soybean under salt treatment (Yang et al., 2018). Hence, more analyses are needed to elucidate the regulation mechanism of miRNA482bd-5p expression.

It is known that the miR482 gene family is present in different plant species and can regulate

NBS-LRR disease resistance proteins in many plants, including soybean (Li et al., 2012, 2010; Shivaprasad et al., 2012; Zhu et al., 2013). However, those reports are associated with the miR482-3p that is the opposite arm from the one analyzed in this work. Alignment analysis of Glycine max miR482 precursor with other species in miRBase showed that miR482-5p is conserved between Glycine max and Glycine soja. At the same time, miR482-3p is conserved among different plants from Fabaceae, sharing the same mature sequence with Vigna unguiculata and Phaseolus vulgaris (Supplementary Fig. S12). This data corroborates the conserved function of miR482-3p.

A Blast sequence search was carried out to evaluate the genomic localization of this miRNA, using pre-miR482bd as a query against the Glycine max genome. It was found that the pre-miR482 locus b and d aligned on an exonic region of the gene Glyma.20G122000 and Glyma.10G269100 (both with no annotated domains), additionally, other two pre-miR482b intergenic copies, were found (Fig. S13). This data indicates that miR482bd has an intergenic and exonic origin. The exonic origin of miRNAs is quite rare in plants (Axtell et al., 2011). There are reports of other miRNAs with this genomic localization. For instance, in rice, the osa-miR3981 is located in the last exon of a glyoxalase mRNA. More interestingly, miR3981 is predicted to target its mRNA host (Li et al., 2011a, 2011b).

Additionally, osa-miR6256 was also located in the exonic region of an mRNA that encodes a flavonol synthase/flavanone 3hydroxylase (FLS/F3H), and osa-miR6256 was predicted to target its host gene (Liu and Zhang, 2012). To assess the possibility that exonic miRNAs can target its own host, miR482bd-5p and miR482bd-3p binding sites were identified in the transcripts of Glyma.20G122000 and Glyma.10G269100 using the psRNAtarget server. The results suggest that miR482bd-5p could regulate Glyma.20G122000 by translation inhibition with an expectation value of 4.5 (Supplementary Fig. S13).
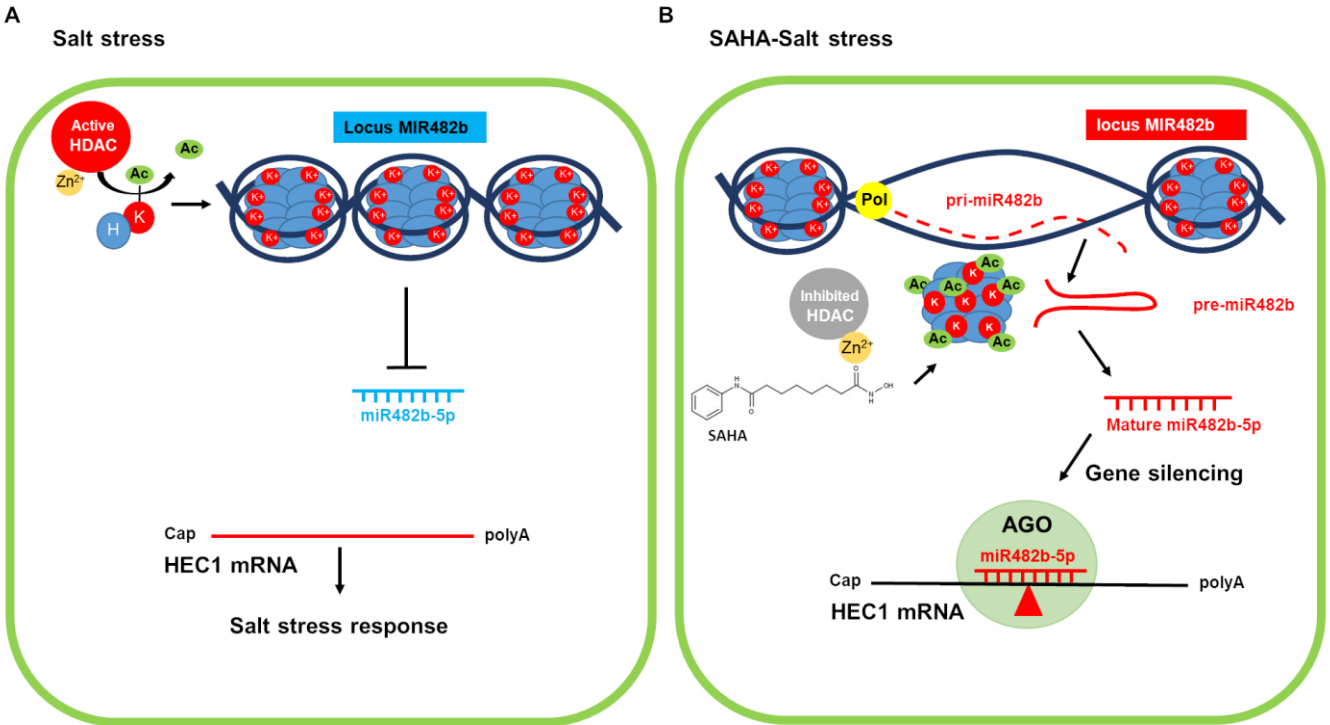
Fig. 5. Proposed model of regulation of miRNA482bd gene by Histone Deacetylase (HDAC).
A. Under salt treatment the miRNA482bd expression is regulated by histone deacetylation activity (Zinc dependent) in the miRNA gene, reducing the transcript level of the mature miRNA. HDAC function results in positive charged Lysine residues (K+), improving DNA and histone affinity and a chromatin closed configuration. The HEC1 gene is responsive to salt stress, and its tanscript level increased under this condition likely triggering a salt stress homeostasis B. An inhibition of histone deacetylases occurs after a SAHA treatment by Zinc chelation, leading to histone acetylation increase in miRNA482bd gene. In consequence, with a subsequent salt stress, the active expression of miRNA482bd gene leads to a higher miRNA transcript level and as a result a HEC1 transcripts reduction and gene silencing.

3.7 Conclusions

Seventeen salt-responsive miRNAs identified by small RNA-Seq analysis had their expression confirmed by RT-qPCR under salt and osmotic stresses. Seven miRNA targets were predicted, and their inversed expression pattern in relation to the miRNA was confirmed by RT-qPCR. The miRNA482bd-5p and its predicted targets (HEC1 and BAK1) were modulated under both stresses. Results indicate that miRNA482bd-5p may have an important role in osmotic stress through modulation of these genes, which are known to be involved in stress response and homeostasis. The miRNA HEC1 regulation is likely conserved among Fabaceae species, and its potential to regulate BAK1 expression is ancestral and soybean-specific. Additionally, the use of SAHA revealed a possible transcriptional regulation of the miRNA482bd-5p gene by histone modifications. A previously unknown exonic localization of the miRNA482bd gene was also reported.
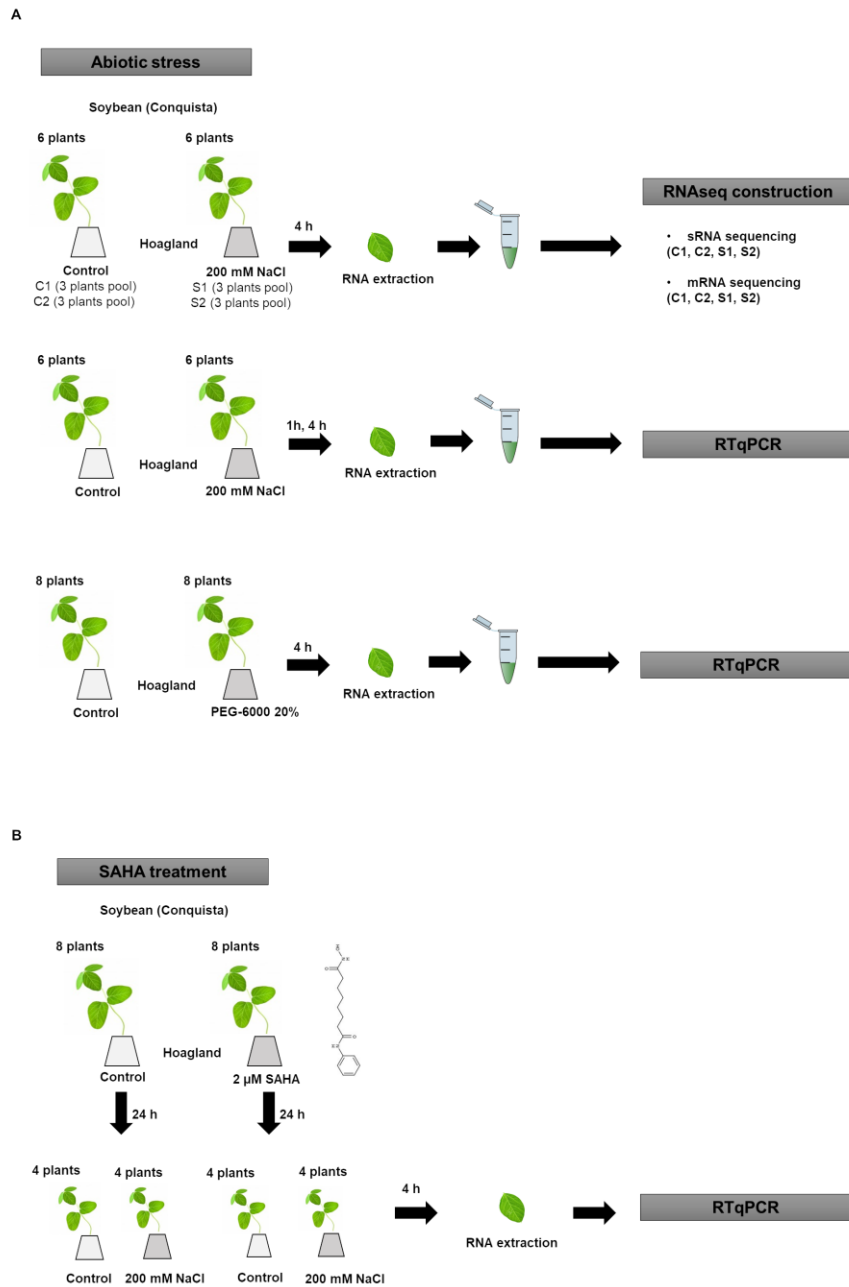
Figure S1. Flow chart of soybean experiments. A. Abiotic stress (Salt and PEG) followed by miRNAs and gene expression analysis by RNA-Seq or RTqPCR B. SAHA treatment followed by miRNA and target expression analysis by RTqPCR.
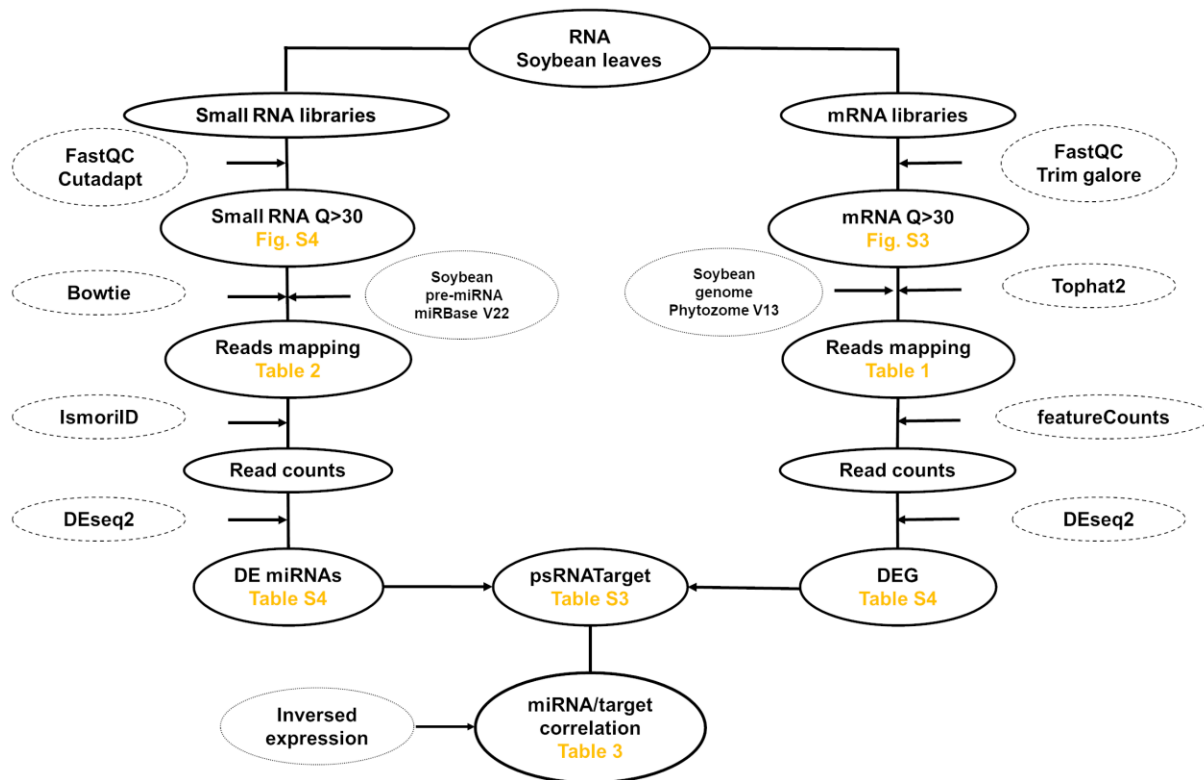
Figure S2. Flow chart of RNA-Seq data analysis. Inputs (RNA-Seq data) and outputs (differentially expressed miRNA and targets) are presented. Programs that were used in each part of the analysis are presented in circles with discontinuous lines.

Figure S3 and S4. Trimming and processing results for mRNA-Seq libraries and smallRNA-Seq libraries. Please find in https://www.sciencedirect.com/science/article/abs/pii/S0176161720301516
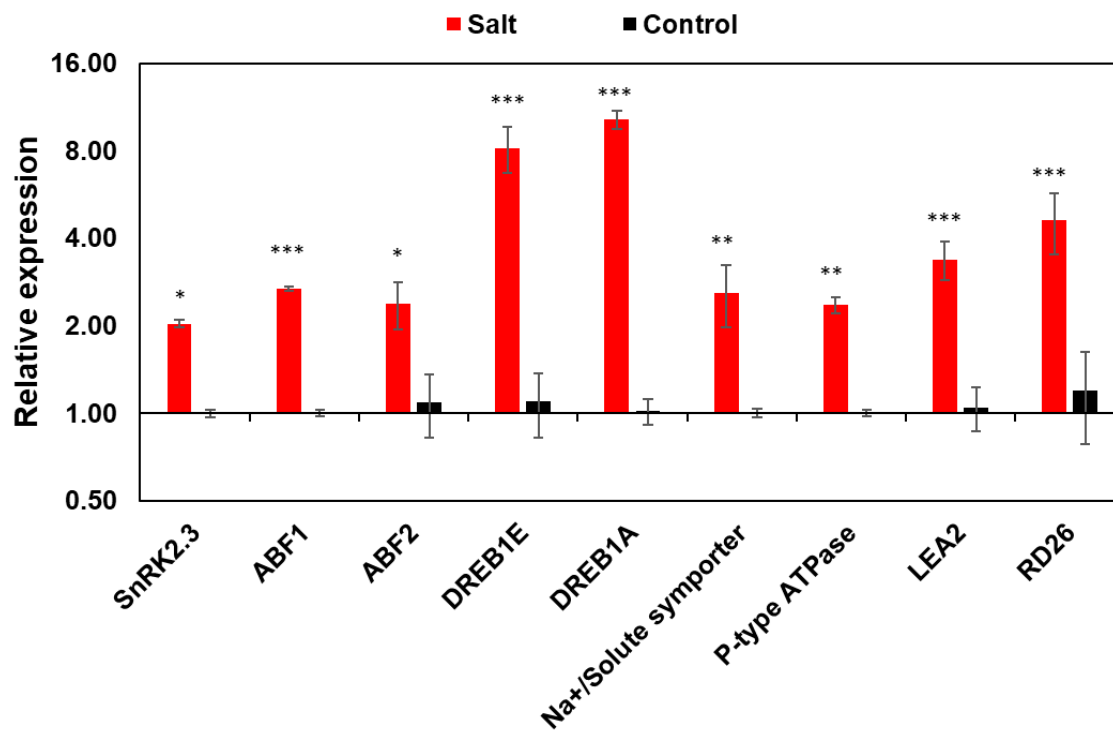
Figure S5. Control genes expression pattern under 200 mM NaCl for 4 h by RNA-Seq analysis. Fold change values are presented based on salt and control condition comparison of respective reads counts (*p <0.05, **p <0.01, ***p <0.001), data are means ± SE with n=2. Relative expression was plotted using a log2 scale.

Figure S6. Soybean specific MiRNAs. MiRNA sequences were used as an input for BLAST analysis in miRBase 22 release. Results showed 6 differentially expressed miRNA from our analysis are only reported for soybean until now.

UAUGGGGGGAUUGGGAAGGAAU   miR482bd-5p
UAUGGGGGGAUUGGGAAGGAA   miR482bd-5p

AGCUGCUGACUCGUUGGCUC   miR4414a-5p
AGCUGCUGACUCGUUGGCUCG   miR4414a-5p

GGAAUGUCGUCUGGUUCGAG   miR166i-5p
GGAAUGUCGUCUGGUUCGAGA   miR166i-5p

UUGUUGUUUUACCUAUUCCACCC   miR1510a-3p
UGUUGUUUUACCUAUUCCACCC   miR1510a-3p
UGUUGUUUUACCUAUUCCAC   miR1510ab-3p

UCUCUUGAUUCUAGAUGAUGU   miR5373 3p
CUUGAUUCUAGAUGAUGUUGA   mir5373 3p

UUAUUGUAACUAAUUUGUCGGU   miR4411 3p
UAUUGUAACUAAUUUGUCGGUA   miR4411 3p

Figure S7. miRNAs isoforms detected from RNA-Seq. The pink sequence corresponds to the canonic sequence deposited in miRBase release 22 and black sequence to miRNA sequence found in this work under salt stress.

A

gma-MIR482b premiR sequence

GG`TATGGGGGGGATTGGGAAGGAAT`ATCCATAAGCAAAATATGCTATTTCTTCCCTACACCTCC
CATACC

| 5nt|	Mature miR482b-5p	|5nt		Read number	
				Control	Salt
GG`TATGGGGGGGATTGGGAAGGAAT`ATCCA					
TATGGGGGGGATTGGGAAGGAA				1403	911
TATGGGGGGGATTGGGAAGGAAT				2247	1863
TATGGGGGGGATTGGGAAGGA				3818	3293
ATGGGGGGGATTGGGAAGGA				118	90
TATGGGGGGGATTGGGAAGG				123	101
ATGGGGGGGATTGGGAAGGAA				23	15
ATGGGGGGGATTGGGAAGGAAT				68	62
TATGGGGGGGATTGGGAAG				12	8
TATGGGGGGGATTGGGAAGGAATA				16	14
				7828	6357
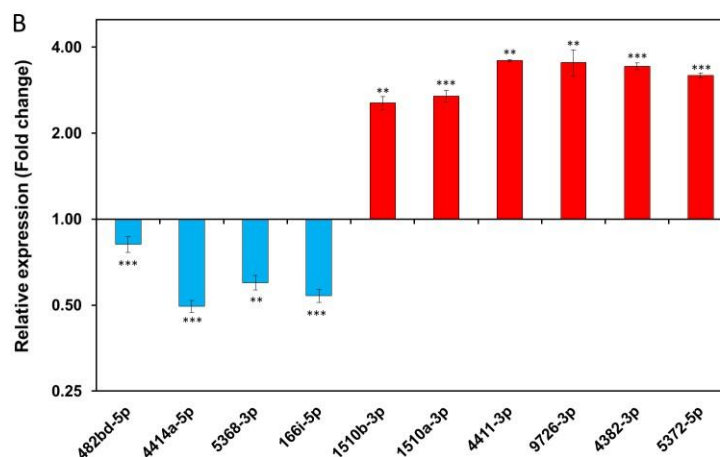
Figure S8. **Analysis of offset by RNA-seq.** A. The mature miRNA sequences were taken, and five nt upstream and downstream of the corresponded pre-miRNA were added. This new sequence was used to anchor the library reads and to detect and include different isoforms in the counting analysis. B. Fold change values of differentially expressed miRNAs identified by offset analysis (T-test, *p <0.05, **p <0.01, ***p <0.001).
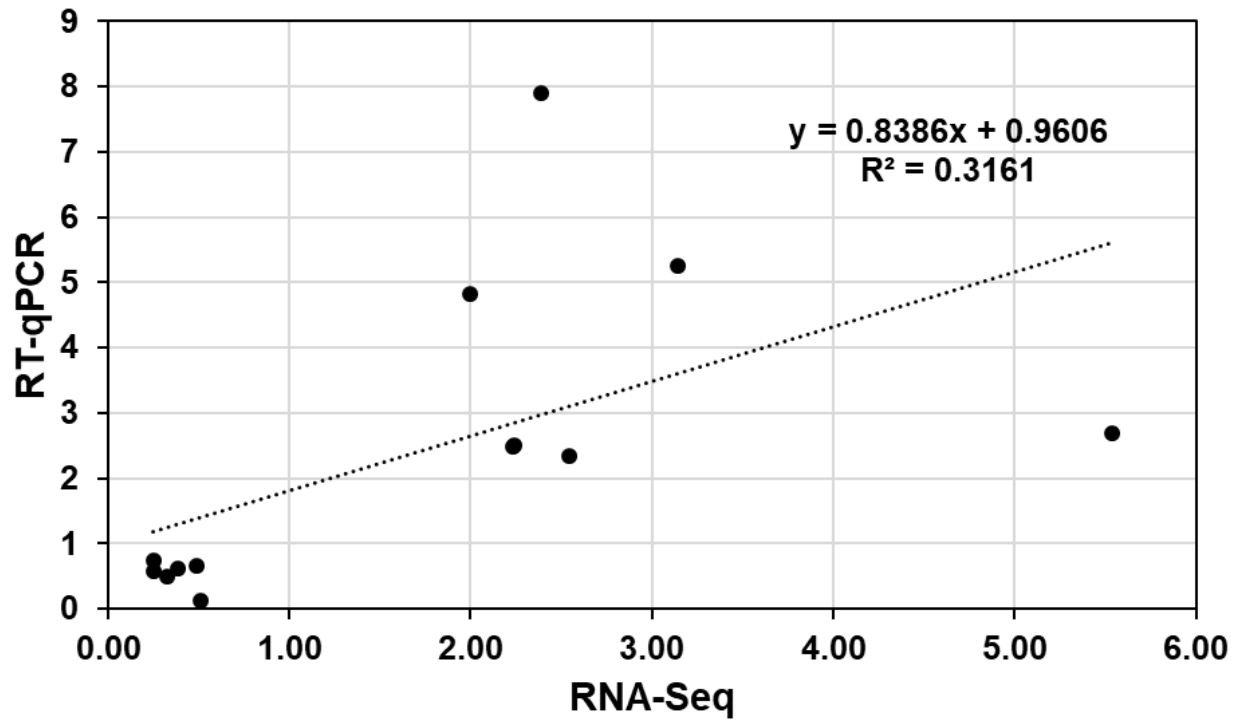
Figure S9. Correlation analysis between RT-qPCR and RNA-seq fold change values for different pairwise comparisons.
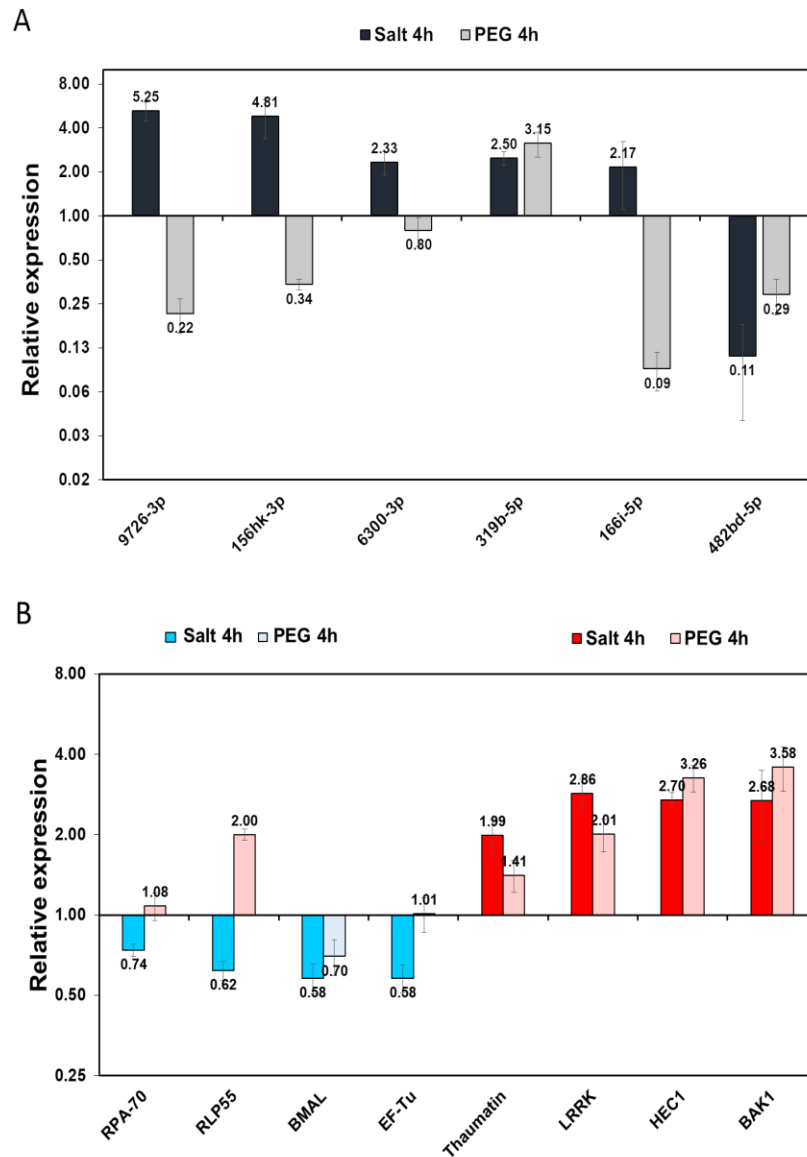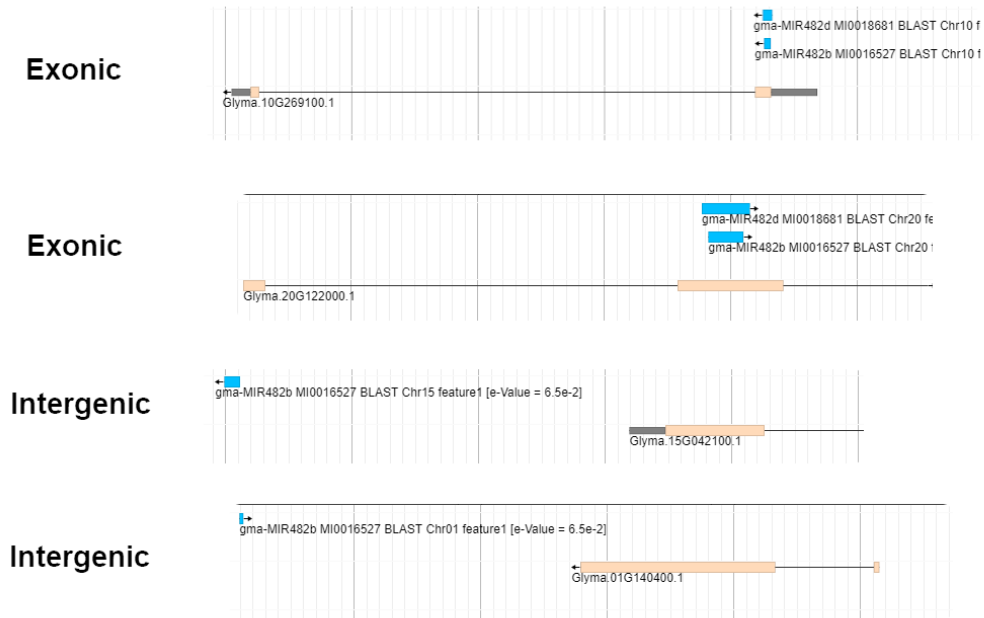
Figure S10. RT-qPCR analysis of miRNA and targets with differential expression in soybean leaves under both PEG and salt stress for 4 h. **A.** Relative expression values of differentially expressed miRNAs. **B.** Relative expression values of differentially expressed miRNA targets. All data have a significant difference between control and treatment (Unpaired T-Test, $p < 0{,}05$). The data are means ±SE with n=6. Relative expression was plotted using a log2 scale.

Figure S11. Please find in

https://www.sciencedirect.com/science/article/abs/pii/S0176161720301516v

Figure S12. Conservation analysis of pre-mir482. The pre-mir482 alignment with different species is shown. In yellow are identified mature miRNA482-5p (on the left) or 3p (on the right). This analysis was made in miRBase release 22 using precursors information and gma-pre-mir482a, b, and c as a query sequence.

Figure S13. pre-mir482 has an exonic and intergenic genomic localization. The pre-mir482 alignment with Glycine max genome is shown. This analysis was made using Phytozome blast tool and gma-pre-mir482b and d sequences. Blue boxes represent pre-mir482 sequence; pink boxed represent exonic regions of different host genes.

3.8 Acknowledgments

Brazilian government.

3.9 References

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4. DISCUSSÃO GERAL

Elucidar os mecanismos moleculares da homeostase ao estresse salino em plantas é um passo importante para melhorar a produtividade. Por esta razão, neste trabalho se usou cultivares de soja, conhecida por sua importância alimentar e econômica, como modelo de estudo para aprofundar o conhecimento da expressão e regulação gênica sob estresse salino.

Com esse objetivo, dados de RNA-Seq e ferramentas de análises foram usadas para entender o efeito do estresse salino na modulação dos genes de soja, além disso se propôs uma regulação gênica por meio de miRNAs. A expressão gênica de duas cultivares contrastantes de soja, Conquista (tolerante à seca) e C08 (sensível à seca), foi comparada usando transcriptogramas. Adicionalmente, bibliotecas de pequenos RNAs da cultivar Conquista foram usadas para identificar miRNAs que poderiam regular genes responsivos ao estresse salino.

Através do uso de transcriptogramas foi obtida uma visão global do estado dos transcritos na situação de estresse salino para cada cultivar, e diferentes tempos de tratamento. Assim, foram encontrados padrões de expressão semelhantes e contrastantes entre as duas cultivares. Entre os padrões semelhantes foram observados: respostas hormonais, diminuição da expressão dos genes da fotossíntese e da fixação de carbono, aumento da osmoproteção e resposta a estresse oxidativo.

Os resultados demonstram que Conquista e C08 compartilham vários mecanismos de homeostase a estresse. Contudo, em alguns processos biológicos, como aqueles da sinalização hormonal e osmoproteção, Conquista apresentou respostas mais rápidas, o que pode indicar uma maior tolerância ao estresse.

A respeito dos mecanismos divergentes entre as cultivares, em Conquista foi observado um maior número de transcritos relacionados ao acúmulo de açúcares (sacarose, trealose) e aminoácidos, que podem ajudar a manter a osmolaridade da célula (GARG et al., 2002; HUANG & JANDER, 2017). Também se observou maior expressão nos genes envolvidos na produção de antioxidantes, como a Glutationa, e aumento na expressão de genes da fotorrespiração. Estes últimos são importantes tanto para aliviar o efeito do estresse oxidativo, quanto para liberar CO2 útil no ciclo de Calvin, que se encontraria em deficiência pelo fechamento estomático (BUSCH et al., 2013). Por outro lado, em Conquista foi observado uma redução na expressão de genes, que foram induzidos em C08 como por exemplo os genes que codificam chaperonas HSP70, HSP90, HSP10 e 60. Esses

resultados corroboram com estudos de estresses osmóticos em outras plantas, os quais reportaram uma diminuição da expressão desses genes, sendo que a superexpressão de HSP90 em *Arabidopisis* incrementou a sensibilidade ao estresse salino (SONG et al., 2009; PI et al., 2016). Adicionalmente, Conquista teve uma repressão dos genes envolvidos com replicação do DNA e ciclo celular, o que pode estar relacionado com um controle para reparação do DNA, evitando sínteses de DNA e divisão celular com moléculas prejudicadas pelo estresse oxidativo (HARPER & ELLEDGE, 2007; CICCIA & ELLEDGE, 2010; HU et al., 2016). Similarmente, Conquista apresentou diminuição em transcritos de proteínas ribossomais, fatores de tradução e proteínas de microtúbulos, a qual poderia levar a uma inibição da divisão celular no tecido foliar, podendo ser um dos possíveis mecanismos que aumentam a tolerância dessa cultivar ao estresse osmótico. Essa regulação é particularmente relevante, pois a inibição do crescimento foliar pode ser uma estratégia conservadora para impedir que a transpiração exceda o suprimento de água disponível (BHASKARA et al., 2017). Além disso, a remodelação do citoesqueleto está relacionada com o aumento dos níveis de Ca^{2+} no citosol, necessário para a transmissão de sinais e para homeostase a estresse (XIONG et al., 2002; CHINNUSAMY et al., 2005). Adicionalmente, genes de modificação de cromatina e de metilação de DNA foram reprimidos em Conquista e induzidos em C08, o que demonstra que estas cultivares têm mecanismos de regulação epigenética contrastantes.

A regulação pós-transcricional através de miRNA, faz parte do mecanismo de adaptação das plantas aos estresses abióticos (JONES-RHOADES & BARTEL, 2004). Por esta razão, neste estudo, esse tipo de regulação foi analisado. Trinta e nove miRNAs responsivos a estresse salino foram identificados em Conquista, e entre os alvos preditos para esses miRNAs alguns deles são genes com expressão diferencial e estão representados no transcriptograma, incluindo genes da via de sinalização de ABA, brassinosteróides e etileno e da tradução plastídica. Neste estudo se propõe uma resposta da soja ao estresse salino através do *crosstalk* entre as vias de ABA e BR, com uma modulação por miRNAs, sendo que componentes da regulação positiva de ABA (SnRK2) e da regulação negativa de BR (BIN2) foram induzidos e seus miRNAs reprimidos. Interessantemente, o gene BAK1, que é alvo do miRNA482bd-5p, foi significativamente induzido nas duas cultivares de soja. Este gene é conhecido por seu papel de co-receptor para a percepção de BR na célula (NAM & LI, 2002) e na ativação do fechamento estomático, na presença de ABA (Shang et al., 2016).

Outros genes do genoma de soja preditos como alvos dos miRNAs e com expressão diferencial na situação de estresse, não foram incluídos na análise de transcriptograma, mas foram analisados no segundo capítulo da tese. Nesse capítulo o padrão de expressão inverso entre alguns alvos e miRNAs foi confirmado por RT-qPCR em dois tipos de estresse osmótico (sal e PEG).

Vários miRNAs de soja responsivos a diferentes condições já haviam sido identificados (revisado em RAMESH et al., 2019). No entanto, este trabalho é o primeiro a identificar miRNAs de folhas de soja sob tratamento com sal e PEG. Dos miRNAs responsivos achados, alguns deles já foram reportados como envolvidos em estresses abióticos e bióticos. Por exemplo, miR482bd-5p, o qual foi reprimido neste estudo sob sal e PEG, também foi reprimido sob estresse por seca (KULCHESKI et al., 2011; LI et al., 2011). Outros membros desta família também já apresentaram regulação por estresse em outros trabalhos, como o miR482a-3p que foi induzido em resposta ao sal (DONG et al., 2013) e o miR482a-5p que foi reprimido em resposta por infeção de *Soybean mosaic virus* (CHEN et al., 2016). O miR1510a-3p, que foi induzido por sal com base em análises de RNAseq, também já foi previamente reportado como induzido por seca (LI et al., 2011) e por altas concentrações de sal (DONG et al., 2013). O miR4411-3p que foi identificado como aumentado sob estresse salino nas análises por RNAseq, já foi mostrado ser induzido sob seca e sal (LI et al., 2011) e reprimido em resposta por a infecção de *Soybean mosaic virus* (CHEN et al., 2016). Esses resultados sugerem que esses miRNAs têm papéis importantes durante o estresse osmótico, embora parecem ter papéis divergentes sob estresse biótico. No presente estudo o miR319ab-3p foi regulado positivamente em folhas tanto em resposta ao tratamento com sal quanto em PEG. Embora, outro estudo mostrou resultados contrastantes, sendo ele reprimido em nódulos de soja submetidas ao estresse salino (DONG et al., 2013), esses dados podem indicar que a função desse miRNA pode ser tecido-específica. O miR2118a-5p foi induzido sob sal, no presente estudo, e sob seca em outro estudo (ZHENG et al., 2016). Adicionalmente, ele parece também estar envolvido em estresse biótico, sendo que foi induzido em infeção por *Soybean mosaic virus* (CHEN et al., 2016). Os alvos para esses miRNAs não tinham sido descritos ainda e neste estudo foi apresentado uma lista de possíveis alvos.

Entre os genes induzidos sob estresse salino e previstos como alvos de miRNAs, sete tiveram o padrão de expressão inverso ao miRNA confirmado por RTqPCR. Entre eles, foi encontrado um gene que codifica para o fator de transcrição TIFY10a (Glyma.09G071600), cujo transcrito possui

um sítio de ligação do miR4414a-5p que foi regulado negativamente. TIFY10a é conhecido por ser um repressor transcricional da via de sinalização de JA (CHINI et al., 2007; FERNÁNDEZ-CALVO et al., 2011; MELOTTO et al., 2008). Esse gene foi induzido sob estresse salino e sob tratamento com o hormônio JA, em *Glycine soja* (ZHU et al., 2011; ZHU et al., 2013) e em linhagens celulares de videira (ISMAIL et al., 2012). Além disso, a superexpressão do GsTIFY10a promoveu tolerância alcalina na alfafa (*Medicago sativa* L.) (ZHU et al., 2014) e tolerância ao sal e alcalina em *Arabidopsis* (ZHU et al., 2012). Esses resultados demonstraram a importância do TIFY10a como componente da adaptação ao estresse salino e a implicação da sinalização de JA na regulação da resposta ao estresse abiótico. Portanto, nossos achados na cultivar Conquista de soja estão de acordo com o que já foi demonstrado. Além disso, sugerimos que uma regulação pós-transcricional deste gene possa ocorrer pela clivagem do miR4414a-5p e que a mesma possa ser conservada em outras espécies de Fabaceae.

Entre os genes reprimidos, o gene do receptor RLP55 (Glyma.13g227700) foi predito como alvo do miR156hk-3p, que foi induzido sob estresse. A família de proteínas do tipo receptor (RLP) contém repetições ricas em leucina (LRRs) e acredita-se que medeia as interações proteína-proteína. A família RLP, incluindo AtRLP55, está envolvida na resistência a doenças em muitas plantas (TÖR et al., 2009; WANG et al., 2010; ZHANG et al., 2010). Por outro lado, as plantas contam com quinases do tipo receptor (RLKs) para mediar transduções de sinal através de eventos de fosforilação e, eventualmente, estabelecer uma resposta celular a um ligante específico (CHINCHILLA et al., 2007). Para isso, as RLPs dependem do domínio da quinase citoplasmática dos RLKs, com quem interagem. Foi demonstrado que várias RLPs de *Arabidopsis* funcionam em conjunto com as RLKs para regular a imunidade das plantas. Por exemplo, o receptor imune RLP23, que interage constitutivamente com SUPRESSOR DE BIR1-1 (SOBIR1) também requer uma associação dependente de ligante com BAK1 para reconhecer a nlp20, um fragmento conservado de 20 aminoácidos de várias espécies de patógenos (ALBERT et al., 2015). Portanto, nesse estudo é hipotetizado que RLP55 poderia estar envolvida na resposta ao estresse biótico da soja, através da formação de um complexo com BAK1, que sendo reprimido sob estresse salino, o BAK1 pode formar um complexo com outras proteínas para desempenhar diferentes papéis nas respostas ao estresse abiótico, como fechamento de estômatos (SHANG et al., 2016). Além disso, sugerimos uma regulação pós-transcricional do RLP55 por miR156hk-3p que possivelmente é conservada. Da

mesma forma, a expressão inversa entre miR156hk-3p e RLP55 foi demonstrada sob estresse por PEG. No entanto, o padrão de expressão foi oposto ao encontrado sob estresse salino. Sendo miR156hk-3p reprimido, enquanto o RLP55 foi induzido. Esta descoberta sugere que o papel do RLP55 sob estresse osmótico pode ser diferente do papel do estresse iônico e ainda precisa ser melhor compreendido.

Sob estresse salino, o miR319b-5p foi regulado positivamente, enquanto o alvo predito, Fator 2 liberador de ribossomo, foi regulado negativamente. Esse gene medeia a desmontagem de ribossomos do RNA mensageiro no término da biossíntese de proteínas mitocondriais. Isso permite a "reciclagem do ribossomo". Além disso, também foi modulado negativamente um gene de replicação de DNA, Rep A 70 KDa, provavelmente reprimido pelo miR9726-3p, específico de soja. Esse resultado indica que o estresse salino pode inibir à divisão e o crescimento celular (WEST et al., 2004; OGAWA et al., 2006).

Além disso, a expressão de miRNAs e alvos sob PEG e estresse salino foi analisada. Se sabe que estes dois tipos de tratamentos produzem um estresse hiperosmótico na planta, por isso se considera que existem rotas que são igualmente responsivas tanto ao sal quanto à seca (XIONG et al., 2002). Em termos de regulação pós-transcricional, o miR482bd-5p parece ter um papel importante nas duas condições. Curiosamente, todas as análises que foram feitas (RNA-Seq, offset-RNA-Seq e RT-qPCR, tratamento com sal e tratamento com PEG) apontam para uma redução da expressão de miR482bd-5p sob estresse e aumento da expressão dos genes HEC1 e BAK1, os quais foram preditos como seus alvos. Finalmente, as análises mostraram que o miR482bd pode ter uma origem exônica, o que significa que é controlado pelo promotor do seu gene hospedeiro. Além disso, esse promotor pode ter uma regulação epigenética através da desacetilação de histonas, quando a planta é submetida ao estresse salino. Esses resultados precisam de estudos adicionais para ajudar a esclarecer os componentes deste mecanismo.

Como perspectivas futuras, ensaios para confirmar os mecanismos propostos neste trabalho deveriam ser feitos. Entre eles uma validação experimental dos alvos de miRNAs deve ser realizada, como por exemplo através de amplificação de terminações de cDNA (RACE 5'), ou de genômica funcional. Este último pode ser por ensaios de bioluminescência com co-expressão de miRNA/mRNA, por avaliação do efeito da superexpressão do miRNA na proteína do alvo e efeito do miRNA na função biológica (KUHN et al., 2008).

Para confirmar que o miRNA482bd-5p está sendo regulado por um mecanismo epigenético, ensaios de *inmunoblot* permitiriam ver se SAHA aumentou a acetilação de histonas. Além disso após um tratamento com sal um ensaio de CHIPseq perimitiria conhecer se o promotor do miRNA482bd e de outros genes envolvidos estão sendo deacetilados como resposta ao estresse. Também um ensaio de mono-híbrido de levedura permitiriam conhecer quais proteínas estão se ligando ao promotor do miRNA482bd, podendo identificar HDAC relacionadas com estresse salino em soja.

5. REFERÊNCIAS DA INTRODUÇÃO E DISCUSSÃO

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6. APÊNDICES

7.1 Tabelas e figuras suplementares do capítulo 1

Disponivel em: https://link.springer.com/article/10.1007/s11033-020-05398-3

Fig. S1. Conquista Transcriptogram. Transcriptogram profile from Conquista leaves under 200 mM NaCl for 4 h. Relative expression data of each gene is plotted. Axis *x* represents the gene order. The control group is represented with a horizontal black line. The salt-treated group is represented by a red line. Standard deviation is represented by pink shadow for salt treatment and a gray shadow for control condition.

Fig. S2. C08 Transcriptogram. Transcriptogramer profile from C08 leaves under 150 mM NaCl for 0, 1, 4, 24 and 48 h. Data of relative expression of each gene is plotted, axis *x* represents the gene ordering list. Collection at 0 h is represented with a black line.

Fig. S3. Venn diagram of differentially expressed genes of both genotypes. A. Up-regulated genes B. Down-regulated genes in Conquista and C08 libraries at 4, 24 and 48 h. For peaks with similar expression pattern, Bolded numbers represent genes that were up or down-regulated in both cultivars. Underlined numbers represent genes with early response at 4 h-treatment. In the case of P10, P12, P17, P18, and P20, underlined and bolded numbers represent genes that were up or down- regulated only in Conquista and are listed in Table S6.

7.2 Tabelas suplementares do capítulo 2

Disponivel em: https://www.sciencedirect.com/science/article/abs/pii/S0176161720301516

Table S1. List of miRNA primers that were designed and used in the RT-qPCR.

Table S2. List of miRNA target primers were designed and used for RT-qPCR.

Table S3. miRNA and predicted targets with differential expression under salt stress in soybean leaves by Deseq2 analysis.

7.3 Carta de aceite do artigo do capítulo 1

Date:	24 Mar 2020
To:	"Rogerio Margis" rogerio.margis@ufrgs.br
From:	"Molecular Biology Reports (MOLE)" Abishek.Sundaram@springernature.com
Subject:	Decision on your manuscript #MOLE-D-19-03898R1

Dear Dr. Margis:

I am pleased to inform you that your manuscript, "Transcriptional analyses of two soybean cultivars under salt stress." has been accepted for publication in Molecular Biology Reports.

Please remember to always include your manuscript number, #MOLE-D-19-03898R1, whenever inquiring about your manuscript.

When you receive the proofs for your article, please check all names and affiliations very carefully, as these cannot be corrected once the article is published.

Thank you.

Sincerely yours,
Rodrigo Guimarães
Editor-in-Chief
Molecular Biology Reports

COMMENTS TO THE AUTHOR:
Reviewer #1: Thank for corrections. All questions have been answered well. I think paper can be accepted as it is now. Because salinity and other stress response regulated mainly epigeneticaly and this regulation is dependent form age and even different in neighbour cells with different fate, it is recommend to focus on epigenetic chnages induced by stress with in situ methods, like chromatin accessibility, histone modifications etc.
My best wishes!

7.4 Carta de aceite do artigo do capítulo 2

Date: Aug 07, 2020
To: "Rogerio Margis" rogerio.margis@ufrgs.br,rogerio.margis@gmail.com
From: "Journal of Plant Physiology" eesserver@eesmail.elsevier.com
Reply To: "Journal of Plant Physiology" esubmissionsupport@elsevier.com
Subject: Your Submission
Ms. Ref. No.: JPLPH-D-20-00203R3
Title: HDAC inhibitor affects soybean miRNA482bd expression under salt and osmotic stress.
Journal of Plant Physiology

Dear Rogerio,

I am pleased to inform you that your paper
"HDAC inhibitor affects soybean miRNA482bd expression under salt and osmotic stress." has been accepted for publication in
Journal of Plant Physiology.

Please note that your manuscript will be published online within 3 weeks after the corrected proofs have been returned by the
corresponding author. The online version of your article will have a DOI code* and can be legitimately cited.

* A digital object identifier (DOI) can be used to cite and link to electronic documents. A DOI is guaranteed never to change, so
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Thank you for submitting your work to Journal of Plant Physiology.

With best wishes,

Herbert

Prof. Herbert J. Kronzucker
Distinguished Professor & Editor-in-Chief
Journal of Plant Physiology (Elsevier)
Visiting Professor, University of British Columbia
Honorary Professor, University of Melbourne
Honorary Professor, Shanghai Jiao Tong University

<p align="center">**7.5 CURRICULUM VITÆ**</p>

<p align="center">**CADAVID, IC.**</p>

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3. ESTÁGIOS:

- Bolsista de Ensino: Curso de Genética. Liderado pelo professor Juan Bautista Lopez. Universidade Nacional da Colômbia, Medellín. 2007-2009

- Iniciação científica**:** Avaliação citotóxica e genotóxica de isoespintanol. Grupo de Pesquisa em Biotecnologia Animal. Liderado pelo Professor Benjamin Rojano e Juan Bautista Lopez. Universidad Nacional de Colombia, Medellín. 2007-2008.

- Iniciação científica**:** Avaliação da capacidade antioxidante da graviola. Grupo de Pesquisa em química de produtos e alimentos naturais. Liderado pelo Professor Benjamin Rojano. Universidad Nacional de Colombia, Medellín. 2006-2007.

- Dourtorado sanduíche: Avaliação do efeito da deficiência de ferro em *Oryza sativa* superexpressando bHLH35. Grupo de pesquisa sobre estresse abiótico. Liderado pelo Professor Michael Frei. Universidade de Bonn, Bonn, Alemanha. 2018-2019

4. PRÊMIOS E DISTINÇÕES

- Jovem pesquisador. "Virginia Gutierrez de Pineda", Dezembro 2011. COLCIENCIAS.

- Jovem pesquisador."Virginia Gutierrez de Pineda", Dezembro 2009. COLCIENCIAS.

5. EXPERIÊNCIA PROFISSIONAL OU DIDÁTICA ANTERIOR

Pesquisador:
- Projeto: Identificação de borboletas de alta montanha com base no gene COI. Grupo de pesquisa em sistematica molecular. Universidad Nacional de Colombia. Agosto de 2013- Julho de 2015.

- Projeto: Enterobactérias (Enterobactereacea) isoladas de moscas sinantrópicas (Diptera, Calyptratae) em uma área urbana de Medellín (Antioquia, Colômbia)". Grupo de pesquisa forense e de saúde. Tecnólogico de Antioquia, Medellín. Fevereiro de 2010 - Março de 2011

Professor:
- Aulas de biologia geral, gestão de literatura cientifica, manejo de evidencia biologica. Tecnologico de Antioquia, Medellín. Julho de 2012-Julho de 2015.

- Aula de biologia. Ensino medio, Instruimos, Medellín. 2012

- Aula de metodologia da pesquisa. Tecnologico de Antioquia, Medellín. Agosto de 2011-Novembro de 2011.

6. ARTIGOS COMPLETOS PUBLICADOS

CADAVID IC, ROSERO D, URIBE S. Comparison of two DNA extraction methods for plants belonging to *Solanum* genus *Leptostemonum* subgenus. (2013). *Revista Colombiana de biotecnología.* XV(2): 186-192

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