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VALDIR GOMES NETO

CARACTERIZAÇÃO DE GENES DE MAMONA (*Ricinus communis* L.) ASSOCIADOS A TOLERÂNCIA A ESTRESSES ABIÓTICOS

Salvador

2021

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Tese apresentada junto ao Programa de Pós-Graduação em Biotecnologia (PPGBiotec), como requisito para a obtenção do título de doutor em Biotecnologia pela Universidade Federal da Bahia.

Orientador: Prof. Dr. Renato Delmondez de Castro

Co-orientador: Prof. Dr. Paulo Roberto Ribeiro

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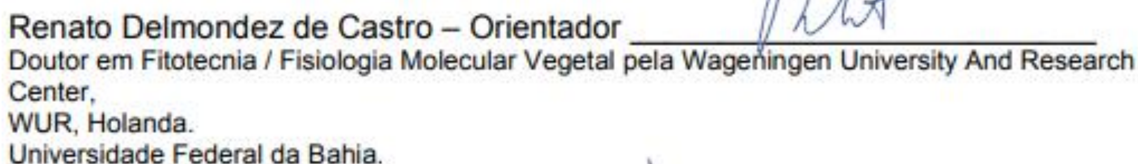
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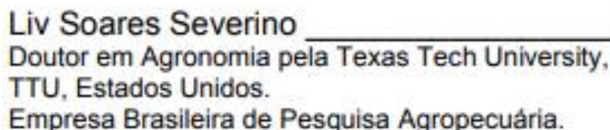
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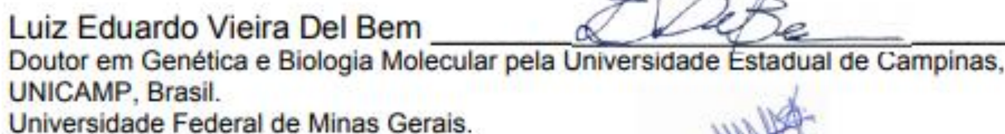
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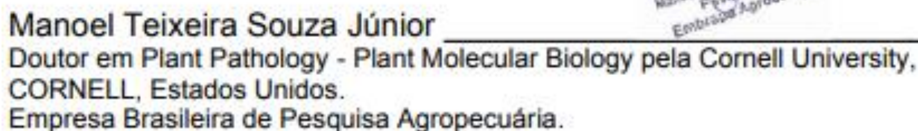
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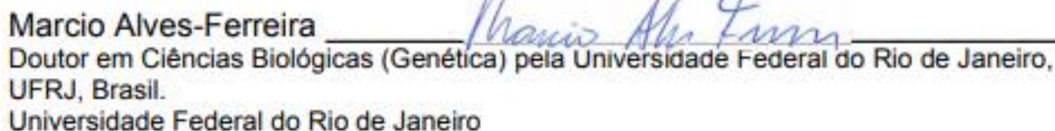
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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota". (Madre Teresa de Calcuta)

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RESUMO

A mamona (*Ricinus communis* L.) é uma espécie oleaginosa reconhecida globalmente pelas inúmeras aplicações industriais e elevado valor de mercado global do óleo extraído de suas sementes. Além disso, tem significativa importância socioeconômica no que se refere à sua produção ser tradicional e majoritariamente realizada no Brasil por agricultores familiares na região do semiárido nordestino brasileiro onde predominam os cultivos rudimentares sob condições adversas típicas da região. O objetivo da presente pesquisa foi a caracterização e superexpressão de genes de mamona associados à melhor tolerância a estresses abióticos durante a germinação e desenvolvimento inicial de plântulas. Foram analisadas três famílias de genes relacionadas às proteínas superóxido dismutase (SOD); proteínas de choque térmico (small heat shock proteins – sHSP), e do fator de transcrição nuclear Y subunidade B (NF-YB), conforme a importância dos referidos genes na literatura, e da seleção por análise prévia dos genes induzidos em microarray de genes expressos em mamona sob estresse térmico. A identificação e caracterização dessas famílias em mamona foi comparada com genomas de angiospermas, obtendo o perfil de expressão gênica em condições de estresses abióticos durante a a embebição e germinação de sementes, e pós-germinação (plântulas jovens), e caracterização funcional dos genes alvo quanto a tolerância a estresses abióticos através da superexpressão em *Arabidopsis thaliana*. No **CAPÍTULO 1** (artigo publicado), identificamos a família de genes da SOD em mamona (*RcSOD*), e os genes ortólogos em angiospermas, mostrando o perfil de expressão dos genes de *RcSOD* em embriões durante a embebição e germinação sob diferentes potenciais osmóticos (restrição hídrica). Os genes *RcCuZnSOD1* e *RcFeSOD8* demonstraram serem induzidos durante a embebição pelo estresse osmótico indutor de *priming* de sementes de mamona, estando associados às respostas benéficas ao vigor de sementes e plântulas em diferentes genótipos de mamona. No **CAPÍTULO 2** (artigo a ser submetido), identificamos possíveis elementos regulatórios de genes de *RcSOD* sob estresses abióticos e regulação por ABA, e o mecanismo de regulação pelo microRNA 398 para os genes *RcCCuSOD4* e *RcCuZnSOD3*. Identificamos os genes *RcCuZnSOD1* e *RcFeSOD8* como responsivos ao estresse térmico (35 °C) durante a embebição e germinação. Além disso, identificamos que outros genes *RcSOD* (*RcCCuSOD4, RcFeSOD7, RcCuZnSOD3*) foram induzidos em sementes sob o estresse térmico durante a protrusão radicular (germinação *per se*) e em plântulas com radículas de 2 cm. Foi possível oberservar a localização subcelular dos genes de *RcSOD* em folhas de *Nicotiana benthamiana*, em que genes da *RcSOD* (*RcMnSOD5* e *RcFeSOD8*) podem ter diferenças comparado a localização subcelular em *A. thaliana*. No **CAPÍTULO 3** (artigo publicado), identificamos inicialmente 41 genes da família de proteínas de choque térmico preditos em mamona (*RcsHSP*), mostrando que a duplicação em tandem parece ser uma das possíveis causas para o maior número de

genes em mamona comparado a *A. thaliana*. Ademais, mostramos o padrão de expressão gênica de 10 genes *RcsHSP* induzidos durante a protrusão radicular e estágio inicial de plântulas. Porém, demonstrando especificidade de expressão em raízes, cotilédones e folhas em diferentes estádios de desenvolvimento de plântulas sob estresse térmico. Por fim, fizemos a caracterização funcional por meio da superexpressão de dois genes *RcsHSP* (*RcsHSP12* e *RcsHSP19*) em *A. thaliana*, resultando em maior porcentagem de germinação das sementes sob estresses térmico, osmótico e salino; maior potencial antioxidante enzimático de SOD; e maior concentração de carboidratos protetores (frutose e rafinose). No **CAPÍTULO 4** (artigo submetido), identificamos a família de genes do fator de transcrição nuclear NF-YB em mamona, e fizemos a caracterização funcional do gene *RcNF-YB8* quanto a indução do florescimento precoce em *A. thaliana*. A comparação filogenética nos permitiu identificar genes ortólogos em angiospermas e os padrões de motifs que possam estar associados a diferenças entre as subfamílias dos *RcNF-YB*. A indução dos genes de *RcNF-YB* foi observada como sendo maior durante a embebição e germinação comparado à fase pós-germinativa, também apresentando perfis diferentes de indução e supressão por estresse térmico. O gene *RcNF-YB8* demonstrou supressão por estresse térmico e um padrão de maior expressão em folhas, sendo que a superexpressão em *A. thaliana* demonstrou também induzir o florescimento precoce, impactando no tamanho da planta e frutos, e consequente produtividade. Os resultados envolvem ampla caracterização de genes de três famílias importantes na resposta de sementes e mudas (plântulas) a estresses abióticos e subsequente desenvolvimento da planta, em que a caracterização dos genes mostrou significativa relevância, indicando genes-alvos potencialmente úteis em programas de melhoramento genético visando variedades superiores de mamoneira, que possam ter melhor vigor e sucesso na implantação e desenvolvimento da lavoura, e consequente produtividade de sementes de mamona pelo mundo e pelos agricultores familiares no semiárido nordestino brasileiro.

Palavras-chave: *Ricinuns communis*, estresses abióticos, marcadores moleculares, transgenia, bioinformática, melhoramento genético

GOMES NETO, Valdir. Characterization of Castor bean (*Ricinus communis* L.) genes associated with tolerance to abiotic stresses. 2021. Thesis advisor:: Renato Delmondez de Castro. 199 s. ill. Thesis (Doctor in Biotechnology) - Institute of Health Sciences, federal university of Bahia, Salvador, 2021

ABSTRACT

Castor bean (*Ricinus communis* L.) is an oilseed species globally recognized for an uncounted number of industrial applications and high market value of the oil extracted from its seeds. Besides, it has significant socioeconomic importance concerning its production being traditional and mostly carried out in Brazil by family farmers in the region of the Brazilian Northeastern semiarid region, where rudimentary crops predominate under adverse conditions typical of the region. Therefore, the present study aimed the characterization and overexpression of castor bean genes associated with better tolerance to abiotic stresses during germination and initial seedling development. Three families of genes related to superoxide dismutase (SOD), small heat shock proteins (sHSP), and the nuclear transcription factor Y subunit B (NF-YB) were analyzed, according to the importance of these genes in the literature, and their selection by complementary analysis of within a castor bean microarray for genes expressed under heat stress. The identification and characterization of these families in castor bean was compared with angiosperm genomes, obtaining the profile of gene expression under conditions of abiotic stresses during imbibition, radicle protrusion (germination *per se*) and post-germination (young seedlings), and functional characterization of the target genes for tolerance to abiotic stresses through overexpression in *Arabidopsis thaliana*. **In CHAPTER 1** (published article), we identified the SOD gene family in castor bean (*RcSOD*), and the orthologous genes in angiosperms, showing the expression profile of *RcSOD* genes in embryos during imbibition and germination under different osmotic potentials (water restriction). The *RcCuZnSOD1* and *RcFeSOD8* genes were induced during imbibition by osmotic stress that induces 'priming' in Castor bean seeds as being associated with beneficial responses to seeds and seedlings vigor in different Castor bean genotypes. **In CHAPTER 2** (article to be submitted), we identified possible regulatory elements of *RcSOD* genes under abiotic stresses and regulation by ABA, and the mechanism of regulation by the microRNA 398 for the *RcCCuSOD4* and *RcCuZnSOD3* genes. We identified the *RcCuZnSOD1* and *RcFeSOD8* genes as responsive to heat stress (35 °C) during imbibition and germination. Besides, we identified that other *RcSOD* genes (*RcCCuSOD4*, *RcFeSOD7*, *RcCuZnSOD3*) were induced in seeds under heat stress during radicle protrusion and in seedlings with 2 cm roots. It was possible to observe the subcellular localization of the *RcSOD* genes in *Nicotiana benthamiana* leaves, in which *RcSOD* genes (*RcMnSOD5* and *RcFeSOD8*) may have differences compared to the subcellular location in *A. thaliana*. **In CHAPTER 3** (published article), we initially identified 41 genes of the heat shock protein family predicted in Castor bean (RcsHSP), showing that tandem duplication seems to be one of the possible causes for the largest number of castor genes compared to *A. thaliana*. Besides, we showed the pattern of

gene expression of 10 *RcsHSP* genes induced during radicle protrusion and early seedling stage. However, demonstrating the specificity of expression in roots, cotyledons, and leaves at different stages of seedling development under heat stress. Finally, we performed the functional characterization through the overexpression of two *RcsHSP* genes (*RcsHSP12* and *RcsHSP19*) in *A. thaliana*, resulting in (a) higher percentage of seed germination under heat, osmotic and saline stresses; (b) greater enzymatic antioxidant potential of SOD; (c) and higher concentration of protective carbohydrates (sucrose and raffinose). In **CHAPTER 4** (submitted article), we identified the family of genes of the nuclear transcription factor subunit B (NF-YB) in Castor bean and made the functional characterization of the *RcNF-YB8* gene regarding the induction of early flowering in *A. thaliana*. The phylogenetic comparison allowed us to identify orthologous genes in angiosperms and motif patterns that may be associated with differences between the *RcNF-YB* subfamilies. The induction of *RcNF-YB* genes was observed to be greater during imbibition and germination compared to the post-germinative phase, also showing different profiles of induction and suppression by heat stress. The *RcNF-YB8* gene demonstrated suppression by heat stress and a pattern of greater expression in leaves, while the overexpression in *A. thaliana* also demonstrated to induce early flowering, therefore, impacting the size of the plant and fruits, and consequent productivity. The results involve a broad characterization of genes from three important families in the response of seeds and seedlings to abiotic stresses and subsequent plant development, in which the characterization of the genes showed significant relevance indicating target genes potentially useful in breeding programs towards varieties of Castor bean with superior vigor aimed at better stand stablishment and crop development, and consequently better productivity of Castor bean around the world and by the family farmers in the Brazilian Northeast semiarid.

Keywords: *Ricinuns communis*, abiotic stresses, molecular markers, transgenics, bioinformatics, plant breeding

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LISTA DE ABREVIATURAS E SIGLAS

AAB – Ácido **A**bscísico
AG – Ácido giberélico
AJ – Ácido **J**asmônico
APX – Ascorbato **P**eroxidase
BZIP – Fator de transcrição **B**asic (region) **L**eucine **ZIP**pers
CAT – Catalase
CBF – CCAAT **B**inding **F**actor
CTE – Cadeia **T**ransportadora de **E**létrons
Cu/Zn – Cobre/Zinco
DNA – Deoxyribo**n**ucleic **A**cid
ERO – Espécie **R**eativa de **O**xigênio
Fe – Ferro
FT – Fator de transcrição
FSI – Fotossistema 1
FSII – Fotossistema 2
GPX – Guaicol **P**eroxidase
GR – Glutationa **R**edutase
H_2O_2 – Péroxido de hidrogênio
HSF – Heat **S**hock **F**actor
HSP – Heat **S**hock **P**rotein
KDa – kilo**d**altons
MAPK – Mitogen-**A**ctivated **P**rotein **K**inase
MDHAR – Mono**d**e**h**ydro**a**scorbate **r**eductase
Mn – Manganês
MYB – Fator de transcrição v-myb avian **my**elo**b**lastosis viral oncogene homolog
MYC – Fator de transcrição **My**elo**c**ytomatosis oncogenes
NAC – Fator de transcrição – Nome com origem com os FTs (**N**AM, **A**TAF1 e **C**UC2)
NFY – Nuclear factor Y
NFY-A – Nuclear factor Y subunidade A
NFY-B – Nuclear factor Y subunidade B
NFY-C – Nuclear factor Y subunidade C
$O_2^{\cdot -}$ – Radical superóxido
PEG – Poli**E**tileno**G**licol
sHSP – Small **H**eat **S**hock **P**rotein
SOD – Super**ó**xido **D**ismutase
WRKY – Fator de transcrição com motif conservado **WRKY**GQK

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1. INTRODUÇÃO GERAL

A espécie *Ricinus communis* L., conhecida como mamona, é uma espécie de interesse econômico e social, cultivada principalmente por pequenos agricultores em regiões semiáridas da região Nordeste do Brasil, onde é comum a ocorrência de estresses abióticos tais como seca, solos salinos e temperaturas elevadas. Apesar de ser reconhecida por tolerar o clima do semiárido nordestino, a mamoneira tem sua produtividade comprometida sob tais condições. A perda na produção decorrida de estresses abióticos afeta também espécies comerciais diversas em todo o mundo. Nesse sentido, inumeras pesquisas vem sendo realizadas com o intuito de entender melhor os mecanismos envolvidos na tolerância de plantas a estresses bióticos.

A prospecção de genes relacionados a tolerância de plantas a estresse abióticos visa entender os mecanismos moleculares e bioquímicos em busca de marcadores moleculares passíveis de uso por meio de transformação genética e de melhoramento genético em inumeras espécies vegetais. Muitos genes podem ser modulados quando plantas são expostas a situações de estresses abióticos, porém alguns genes desempenham papel relevante e essencial para que a planta se adapte a condição estressante e seja capaz de reestabelecer a homeostase celular, levando a planta a melhor tolerância aos diferentes estresses abióticos.

O aumento da produção de espécies reativas de oxigênio (ERO) é uma característica observada em planta expostas a estresses abióticos. Quando a planta não é capaz de manter o balanço de ERO, é então instalado o chamado estresse oxidativo. As ERO são produzidas por plantas em diversas rotas metabólicas e compartimentos celulares, principalmente nos processos de respiração, fotossíntese e degradação de lipídeos, sendo importantes principalmente no processo de sinalização celular. As ERO são produzidas a partir do oxigênio molecular (O_2), e podem ser o radical superóxido ($O_2^{-\cdot}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($^{\cdot}OH$).

A expressão de enzimas antioxidantes constitui um dos principais mecanismos que plantas utilizam para manter os níveis de ERO em equilíbrio. Dentre as quais a enzima superóxido dismutase (SOD) é relatada como enzima antioxidante chave na tolerância de plantas a estresses abióticos. Os genes que codificam a enzima SOD tem sido caracterizados em várias espécies, com intuito de entender como esses genes são expressos no desenvolvimento da planta e quais podem conferir melhor tolerância aos estresses abióticos.

A classe de proteínas de choque térmico (HSP) também demonstram papel relevante na tolerância de plantas a estresses abióticos, constituindo família conhecida de proteínas chaperonas, as quais desenpenham papel de proteção a outras proteínas/enzimas e outras móleculas na célula quando em situações de estresses abióticos. Dentre as HSP, as sHSP são proteínas pequenas, constituindo menos que 43 KDa caracterizadas por apresentarem o domínio alfa cristalino. Muitos estudos demonstraram a indução dos genes de sHSP em situações de estresses abióticos, mostrando que esses genes possuem padrão de expressão gênica diferentes, dependendo do tecido, estágio de desenvolvimento e estresse abiótico. As sHSP são classificadas de acordo a sua localização subcelular na planta modelo *Arabidopsis thaliana* e o número de sHSP demonstra ser bem diferente dentre as espécies vegetais.

Fatores de transcrição (FT) são reguladores de expressão gênica e são ativados por estresses abióticos desencadeando a expressão de genes importantes na tolerância de plantas a estresses abióticos. Dentre as classes de FT, o fator de transcrição nuclear Y (NF-Y) se destaca por conter genes induzidos sob condições de estresses abióticos, e na regulação de eventos como o florescimento. Essa classe de fator de transcrição forma um complexo com três subunidades denominadas A, B e C. Cada subunidade pode ser codificada por muitos genes. A caracterização desses genes vem sendo realizada para entender melhor o papel dessa família de FT na tolerância das plantas a estresses abióticos.

Nesse contexto, este estudo tem como alvo a prospecção e caracterização de genes que possam ser importantes na melhor tolerância de *Ricinus communis* a estresses abióticos. Foram conduzidas abordagens por filogenia para identificação das família de genes em *Ricinus communis* e seus respectivos genes ortológos em *Arabidopsis thaliana*, expressão relativa de genes e a caracterização funcional dos respectivos genes de *Ricinus communis*.

2. REVISÃO DE LITERATURA

2.1 A espécie *Ricinus communis* (mamona)

A espécie *Ricinus communis,* conhecida como mamona, é uma oleaginosa pertencente à família Euphorbiaceae e distribuída em regiões tropicais e subtropicais em todo o mundo. Hoje, Índia, China, Moçambique e o Brasil são os principais produtores de mamona. O produto principal é o óleo extraído das suas sementes, geralmente por meio de prensa mecânica, extração por solventes ou combinação dos dois métodos. Trata-se de um óleo de alto valor tradicionalmente utilizado na chamada 'indústria ricinoquímica', com aplicações na produção de medicamentos, cosméticos, tintas, dentre outros inumeros setores tecnológicos, incluindo mais recentemente a indústria de biocombustíveis (biodiesel). Com isso, a demanda pelo óleo de mamona tem se demonstrado crescente a cada dia devido a não concorrência outras oleaginosas dos setores alimentícios, sua natureza renovável e biodegradabilidade. Estima-se que o óleo de mamona tenha mais de 700 diferentes usos na indústria. Sua qualidade e demanda em inúmeras aplicações, vem do óleo de mamona ter em sua composição mais de 90% de ácido ricinoléico, o qual possui uma hidroxila no seu carbono 12, que lhe confere opções de reações químicas comparado a outros óleos (MUBOFU, 2016; PATEL *et al*., 2016; YEBOAH *et al*., 2020).

A mamona também é reconhecida no Brasil por sua importância socioeconomica, como fonte de renda para os pequenos agricultores familiares que tradicionamente cultivam rudimentarmente a mamoneira na região semiárida do nordeste brasileiro, onde estão a mercê das condições de estresses abióticos, como característica natural e comum do semiárido. Os estresses impõem grande desafio para a germinação de sementes, estabelecimento e desenvolvimento de plântulas de modo a formar um estande de plantas que permita bons rendimentos da lavoura (DA SILVA CÉSAR; OTÁVIO BATALHA, 2010; MITTLER, 2006; RIBEIRO *et al*., 2014; WANG, YINGNAN *et al.*, 2019).

A mamona é conhecida por sua alta variabilidade fenotípica, como altura de planta, morfologia foliar, duração do ciclo, tamanho, forma e coloração das sementes,

além de perfis fisiológicos de sementes distintos que influenciarão significativamente no desempenho de plântulas e estabelecimento da cultura (AGYENIM-BOATENG *et al.*, 2019; YEBOAH *et al.*, 2020). Sementes podem apresentar colaração única ou secundária variando em peso e formato. Apresenta sistema radicular fistiloso, constituído de raiz principal pivotante e raízes secundárias bem desenvolvidas e folhas do tipo simples e alternadas. É uma planta monóica que apresenta como inflorescência padrão do tipo racemos com flores femininas no ápice e masculinas na base (Figura 1 A-H).

Figura 1: *Ricinus communis* L. (A-E) Sementes de genótipos com diferentes composições de cores; F) Plântulas com 7 dias de germinação; G) Planta jovem de mamona em campo. H) Frutos. Fonte: Laboratório de Bioquímica, Biotecnologia e Bioprodutos (LBBB).

A temperatura ótima de desenvolvimento da mamoneira está em torno de 25 a 30 °C, onde a temperatura de 35 °C demonstrou beneficiar maior rapidez no crescimento inicial das plântulas. Mudanças no metabolismo foram detectadas em 8 h de embebição em 35 °C comparado com temperaturas de 20 e 25 °C. Em sementes

embebidas em 20 e 25 °C foi observado o acúmulo de carboidratos enquanto em 35 °C demonstrou o aumento na concentração de alguns aminoácidos. Temperaturas acima de 35 °C demonstraram afetar o vigor de plântulas, tamanho da planta, queda na taxa fotossintética, transpiração e carboidratos solúveis nas raízes. Temperaturas acima de 38 °C causam diminuição na produção de sementes pela lavoura (RIBEIRO *et al.*, 2015; RIBEIRO *et al.*, 2015; RIBEIRO *et al.*, 2015; RIBEIRO *et al.*, 2014; SEVERINO *et al.*, 2012).

A seca ou restrição hídrica em nível moderado demonstra não afetar a mamoneira quanto a altura de planta, peso fresco e peso seco de folhas e hastes, mas o número e a área de folhas pode ser afetado enquanto que estresse severo por seca demonstra afetar todos os parâmetros citados, além das folhas perderem água e diminuição do potencial de turgor (PAPAZOGLOU *et al.*, 2020). Foi observado em testes *in vitro* com restrição hídrica induzida por polietilenoglicol (PEG), que após 15 dias de restrição houve perda de água em folhas, assim como diminuição na porcentagem de plantas vivas. Assim como aumentaram os níveis de peróxido de hidrogênio (H_2O_2), acompanhado do aumento da atividade das enzimas antioxidantes superóxido dismutase (SOD), ascorbato peroxidase (APX) e catalase (CAT) (DE ARAÚJO SILVA et al., 2016). Ademais, o ajuste osmótico foi associado a melhor capacidade de tolerar o estresse por restrição hídrica, e usado para distinguir variedades de mamona mais tolerantes a seca. Variedades mais adaptadas a seca demonstraram manter o potencial osmótico, acumularam mais prolina, açucares solúveis, aminoácidos livres e potássio, além de maior rendimento em sementes (MAHESWARI *et al.*, 2010). Enquanto que o aumento na taxa de germinação de sementes de mamona recém colhidas e secas e submetidas ao osmocondicionamento em PEG mostrou relação com o aumento no acúmulo de tubulinas, como marcador da reativação do metabolismo e evolução do ciclo celular durante a embebição e germinação *per se* das sementes (protrusão da radícula embrionária) (DE CARVALHO TEIXEIRA VASCONCELOS *et al.*, 2017).

O estresse salino é conhecido por afetar o crescimento e o peso seco de mamona, sendo possível identificar diferenças no nível de tolerância ao estresse salino entre cultivares (SÁ *et al.*, 2016). O estresse salino também demonstra afetar o acúmulo de massa seca da parte área da planta em relação às raízes, em que os cultivares avaliados também exibiram diferentes níveis de tolerância a estresse salino (PRESOTTO *et al.*, 2016).

Dessa forma, estudos que visem entender mecanismos moleculares e bioquímicos pelos quais as plantas utilizam para se adaptar a condições de estresses abióticos tornam-se relevantes para possível aplicação em programas de melhoramento genético da mamona.

2.2 Processo de Germinação de sementes

A germinação de sementes pode ser definida como uma sequência de eventos fisiológicos que ocorrem antes do momento da protrusão radicular em sementes embebidas não dormentes. O processo de germinação de sementes em geral pode ser dividido em três fases, a embebição (fase-I), onde ocorre inicialmente uma rápida absorção de água, a fase lag (fase-II), onde ocorre uma diminuição da absorção de água, e a germinação propriamente dita (fase-III), marcada pelo momento da protrusão radicular (NONOGAKI et al., 2008).

A germinação e estabelecimento inicial de plântulas são ditas as fases mais críticas das plantas, onde a semente passa de um estado metabólico basal nas sementes maduras para um estado de reativação metabólica ao iniciar o processo de embebição de sementes ocorrendo a mobilização de reservas e mRNA já existentes. Logo subsequentemente ocorre a transição do metabolismo com a síntese *de novo* de mRNA, ocorrendo a transcrição de genes relacionados aos processos de germinação em função do desenvolvimento e estabelecimento de uma plântula saudável com suas estruturas aereas e radicular (Figura 2). A respiração é ativada logo subsequente ao início da embebição dando suporte energético para todos os processos metabólicos ativados, já o processo de expansão do embrião está atrelado a diminuição da

resistência imposta pelos tecidos de cobertura e o processo de divisão celular normalmente só irá iniciar após o término do processo germinativo (RAJJOU *et al*., 2012)

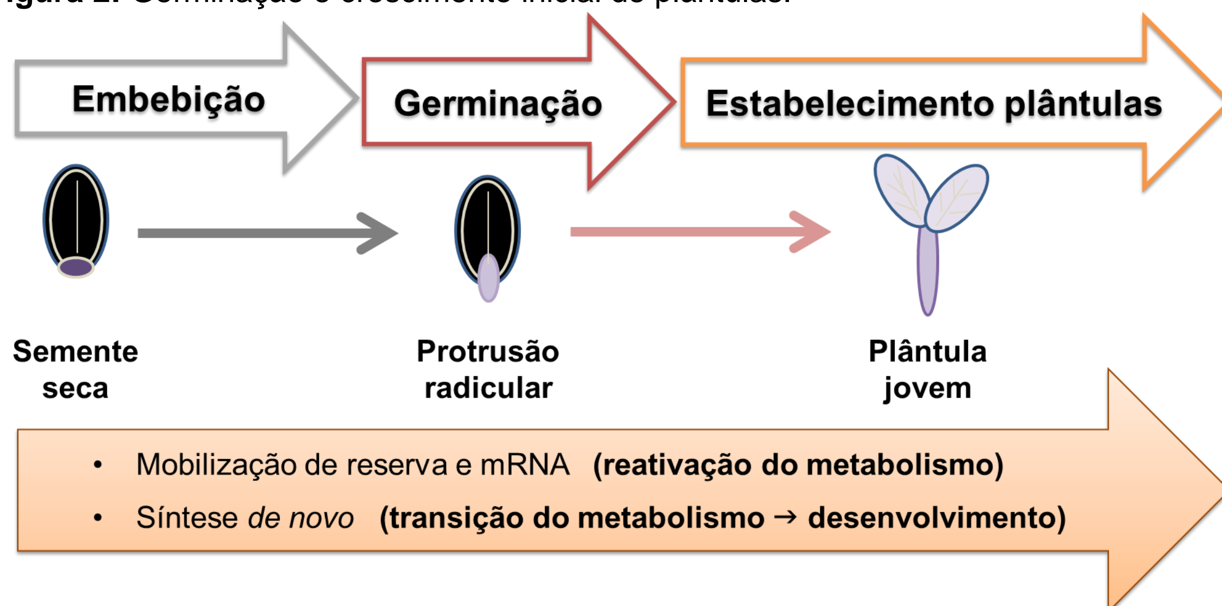
Durante a maturação de sementes ocorre o acúmulo dos componentes de reserva da semente. Essas reservas podem ser acumuladas nas formas de carboidratos, lipídeos e proteínas. Durante a germinação ocorre a mobilização das reservas estocadas no tecido de reserva para dar suporte ao crescimento inicial de plântulas (NONOGAKI *et al*., 2008). É conhecido que os hormónios vegetais como o ácido giberélico (AG) e ácido abscísico (AAB) agem como antagonistas no processo de germinação, onde o AAB induz a dormência de sementes e o AG induz o processo de germinação. O balanço hormonal entre AG e AAB pode ser alterado por variações ambientais ou estresses abióticos. Além disso é observado a diminuição dos níveis de AAB e aumento dos níveis de AG durante o processo de germinação. Os AG possuem um papel importante nas fases iniciais e finais da germinação. Nas fases iniciais as AG induzem a expressão de genes que codificam para enzimas modeladoras da parede celular como as alfa-expansins e no final da fase de germinação estão envolvidas no estímulo direto do crescimento do embrião (DASZKOWSKA-GOLEC, 2011; YAMAGUCHI; KAMIYA; NAMBARA, 2018).

Estresses abióticos causam grandes efeitos sobre o crescimento de plantas podendo inibir ou retardar o crescimento a depender da fase de desenvolvimento da planta, intensidade do estresse e duração. A capacidade de resistir e tolerar estresses abióticos como osmótico, salino, altas temperaturas durante a germinação é essencial para a sobrevivência da planta. A exposição a estresses abióticos podem induzir uma interrupção do processo de germinação, onde este processo está coordenado por um complexo sistema de fatores de transcrição, onde a retomada da germinação ou continuação do processo de interrupção da germinação é decidido por eventos onde diferentes vias metabólicas, transdução de sinais, hormônios vegetais, espécies reativas de oxigênio e MicroRNA participam (NONOGAKI; BASSEL; BEWLEY, 2010; DASZKOWSKA-GOLEC, 2011).

Germinação máxima, uniformidade, rapidez na germinação são requisitos fundamentais para um bom estabelecimento do cultivo, principalmente em condições

ambientais adversas. Existem técnicas que podem ser usadas para melhorar os parâmetros de germinação, como a técnica de *priming* de sementes, que é uma técnica pre-germinativa que consiste na embebição controlada das sementes que permite a ativação parcial do metabolismo mas não o suficiente para as sementes germinarem. Após o *priming* sementes demonstram um envigoramento, demonstrando maior uniformidade na germinação e vigor de plântulas por estarem metabolicamente preparadas para iniciar o processo de germinação (PAPARELLA, S. *et al.*, 2015).

Figura 2: Germinação e crescimento inicial de plântulas.



Fonte: Adaptado de Rajjou *et al.*, 2012.

2.3 Resposta das plantas a estresses abióticos

Estresses abióticos impactam diretamente no desenvolvimento e vigor de plantas, e consequemente na produção de inumeras lavouras comerciais no Brasil e mundo afora. Dentre eles, temperaturas elevadas, seca e salinidade do solo são os principais fatores ambientais que afetam a distribuição geográfica das plantas na natureza, assim como diretamente a produtividade (ZHU, 2016; HE *et al.*, 2018). Entender como plantas percebem os sinais ambientais e se adaptam é uma questão de extrema relevância tendo em vista obter plantas mais tolerantes a estresses e aumentar a produtividade.

A resposta das plantas a estresses abióticos envolve um sistema interconectado que se inicia com a percepção dos sinais ambientais, e dispara uma cascata de sinalizações para ativação de genes resposivos a estresses, regulados por fatores de transcrição específicos. As plantas ativam diferentes mecanismos bioquímicos, moleculares e alterações morfofisiológicas buscando se adaptar e manter ou estabelecer um novo patamar de homeostase celular (ZHU, 2016). Alterações bioquímicas podem ser observadas através de mudanças em rotas metabólicas pela variação em determinados metabólitos produzidos, tanto no metabolismo primário quanto no secundário, além de alterações na morfologia e fisiologia das plantas (AUSTEN *et al.*, 2019; BAKTHISARAN; TANGIRALA; RAO, 2015; FÀBREGAS; FERNIE, 2019; FRAIRE-VELAZQUEZ; EMMANUEL, 2013; ISAH, 2019).

Os estresses abióticos são considerados estresses primários, os quais desencadeiam na célula estresses secundários, podendo ser iônico, oxidativo ou osmótico. A seca ocasionada pela escassez de água leva ao estresse osmótico enquanto que o estresse salino leva tanto ao estresse osmótico quanto ao estresse iônico (toxicidade por íons). Enquanto que o estresse por altas temperaturas, osmótico e salino induz o aumento da produção de ERO (ASHRAF *et al.*, 2018; WANG, WANGXIA; VINOCUR; ALTMAN, 2003; ZHU, 2016). O estresse oxidativo, pelo aumento de ERO pode causar danos em componentes celulares como DNA, proteínas e lipídeos, levando a disfunções de vias metabólicas e da membrana celular (Figura 2).

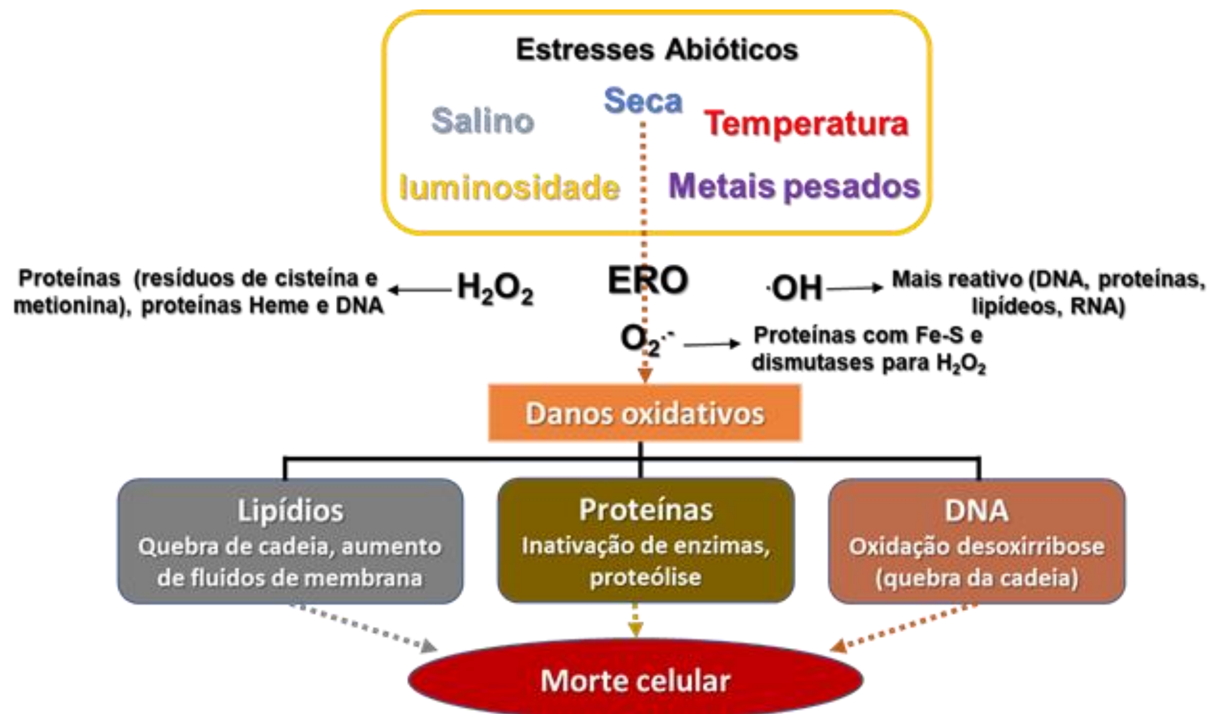


Figura 3: Estresses abióticos aumentam a produção de espécies reativas de oxigênio (ERO). Fonte: Adaptado de Wang *et al*., (2003).

Embora danosas à célula, as móleculas de ERO (radicais livres superóxido, radical hidroxil e o peróxido de hidrogênio (H_2O_2)), também desepenham funções de sinalização celular. O H_2O_2 ativa a cascata de sinalização das proteínas Cinases (*mitogen-activated protein kinase* - MAPK) regulando a expressão de genes relacionados a estresses (DUMANOVIĆ *et al*., 2021; VERMA; RAVINDRAN; KUMAR, 2016). Os hormônios vegetais são descritos por mediarem a resposta das plantas aos estresses abióticos. Alterações nos níveis de hormônios vegetais como ácido jasmônico (AJ), ácido abscísico (ABA), etileno e auxinas são outra forma de propagar o sinal de estresse em um tecido para toda a planta, assim como sinais elétricos, cálcio e ERO, dentre outros. O ABA é produzido pelas plantas quando estão em situação de estresses abióticos induzindo na planta alterações morfofisiológicas como o fechamento de estômatos e ativação de fatores de transcrição (FT) e consequente regulação de genes de estresse. Análise da região promotora de genes responsivos ao ABA tem mostrado que genes possuem a presença de múltiplos elementos responsivos ao ABA, chamados de (ABREs; PyACGTGG/TC), sendo regulados pelas proteínas que se ligam

aos elementos responsivos ao ABA. Alguns FT como o NAC, MYB e MYC são descritos por serem estímulados pelo ABA. Além disso, tem sido demonstrado que a sinalização por hormônios vegetais induz um padrão de repostas cruzadas em resposta a estresses abióticos, compondo um esquema complexo de regulação de genes pelos hormônios (Figura 3) (CHOUDHURY *et al.*, 2017; SAH; REDDY; LI, 2016; TUTEJA, 2007; VERMA; RAVINDRAN; KUMAR, 2016).

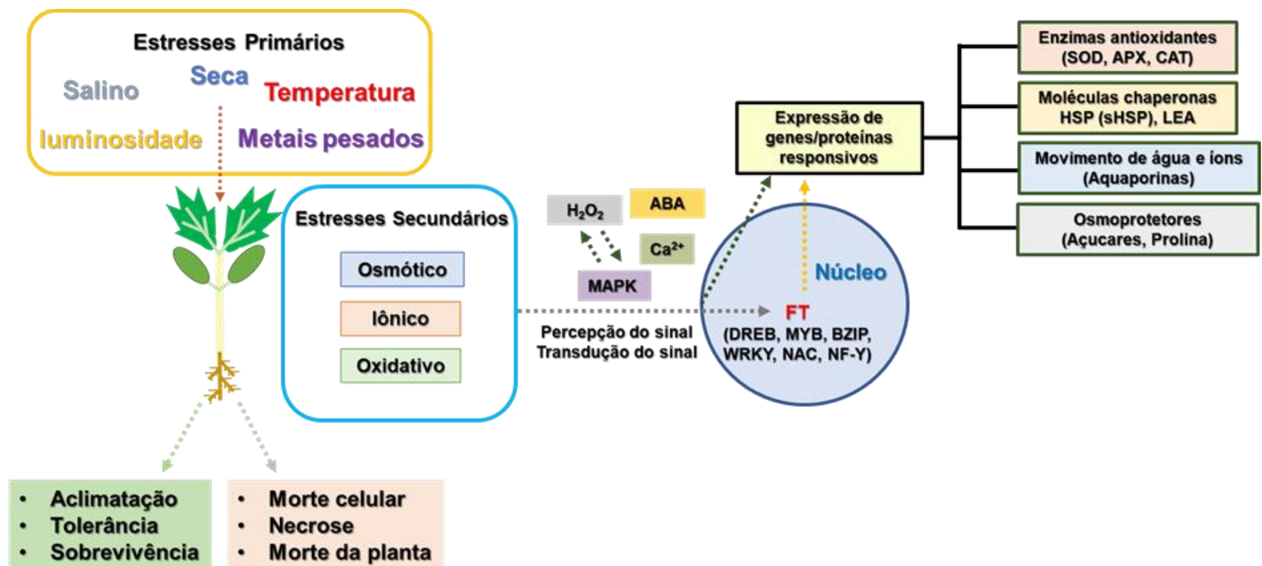


Figura 4: Estresses primários e secudários, e percepção dos sinais de plantas sob estresses abióticos. Fonte: Adaptado de Wang *et al.*, (2003), He *et al.*, (2018) e Verma *et al.*, (2016).

O uso de técnicas moleculares de alto rendimento como microarrays e RNAseq, tem sido utilizadas para identificar genes responsivos a estresses abióticos e muitos deles estão sendo funcionalmente caracterizados quanto a tolerância a estresses abióticos. Genes responsivos a estresses abióticos podem desempenhar funções essenciais na planta quanto a melhor tolerância a estresses abióticos, como atuar na transdução de sinais, proteção da célula sob estresses, expressão de novos genes, produção de enzimas e proteínas/enzimas de rotas metabólicas essenciais, dentre outras funções. Genes relacionados a várias funções tem sido associados a melhor tolerância a estresses abióticos, tais como enzimas antioxidantes, proteínas chaperonas, FT, microRNAs, genes relacionados a vias de hormónios vegetais, e genes

que codificam para enzimas chaves em rotas metabólicas essenciais para as plantas (GUJJAR; AKHTAR; SINGH, 2014; LIU *et al.*, 2018; MANGELSEN *et al.*, 2011; RASHEED *et al.*, 2016; RIBEIRO, PAULO R. *et al.*, 2018; SHRIRAM *et al.*, 2016; SHU *et al.*, 2018; XU *et al.*, 2013; ZANDALINAS *et al.*, 2017, 2020).

Enzimas antioxidantes desenpenham papel crucial na detoxificação de ERO, impedindo assim que o DNA, proteínas e lipídeos venham a ser danificados. Tem sido observada a modulação de genes que codificam para enzimas antioxidantes e atividade total enzimática em plantas expostas a estresses abióticos . Os genes que codificam para essas enzimas podem desempenhar papel importante em diferentes estádios do desenvolvimento da planta, assim como para tecido e compartimento celular específico (CAVERZAN; CASASSOLA; BRAMMER, 2016; ROSSATTO *et al.*, 2017; ZANDALINAS *et al.*, 2017).

Proteínas chaperonas são importantes por desempenhar um papel de proteção às enzimas, membrana e DNA na célula, demonstrando serem relevantes para as plantas tolerarem condições de estresses abióticos. Uma classe extremamente importante de proteínas chaperonas são proteínas de choque térmico (*heat shock protein* – HSP). Essas proteínas foram inicialmente identificadas na resposta de plantas ao estresse térmico. Porém foi demonstrado que as HSP são reguladas também por outros estresses abióticos podendo ser expressas em tecidos espécificos a depender do estádio de desenvolvimento da planta (JACOB; HIRT; BENDAHMANE, 2017).

Os FT são proteínas centrais reguladoras da expressão de genes. Os FT reconhecem uma sequência de DNA específica se ligando fisicamente nessas regiões do DNA ativando assim um conjunto de genes específicos. Além de se ligar ao DNA, os FT podem interagir com diferentes proteínas em complexos transcricionais regulando a expressão de um vasto número de genes. Os FT podem modular a expressão de gênica por estímulo ambientais, hormônios, diferenciação e desenvolvimento de órgãos. Vários FT são descritos por terem um papel relevante na tolerância de plantas a estresses

abióticos e exercerem funções essenciais em diferentes estádios de desenvolvimento de plantas (BAILLO *et al.*, 2019; GUJJAR; AKHTAR; SINGH, 2014; GUO *et al.*, 2016).

2.4 Espécies reativas de oxigênio (ERO) e enzimas antioxidantes

As ERO são inevitáveis produtos de reações de redução, como também do metabolismo aérobico de plantas, podendo serem formadas em diversos compartimentos celulares como na mitocôndria, cloroplasto, peroxissomo, membrana plasmática, apoplasto, e no retículo endoplasmático. As ERO são geradas na célula principalmente pelas reações envolvendo a cadeia transportadora de elétrons (CTE), fotossíntese e fotorespiração. Os fotossistemas (FSI e FSII) juntamente com a CTE geram o radical superóxido ($O_2^{\cdot -}$) por meio da chamada reação de Mehler (MEHLER, 1951; MEHLER; BROWN, 1952). As ERO podem ser formadas nos complexos I e III da CTE. Também podem ser produzidos no processo de fotorespiração na via do glicolato nos peroxissomos (DEL RÍO; LÓPEZ-HUERTAS, 2016). No apoplasto, enzimas peroxidases de classe III e oxalato oxidase também podem formar ERO. Adicionalmente, enzimas NAD(P)H oxidase são formadoras de ERO em vários compartimentos, a exemplo do retículo endoplasmático, citoplasma e mitocôndria (Figura 4) (DUMANOVIĆ *et al.*, 2021; MITTLER, 2017; SHARMA *et al.*, 2012).

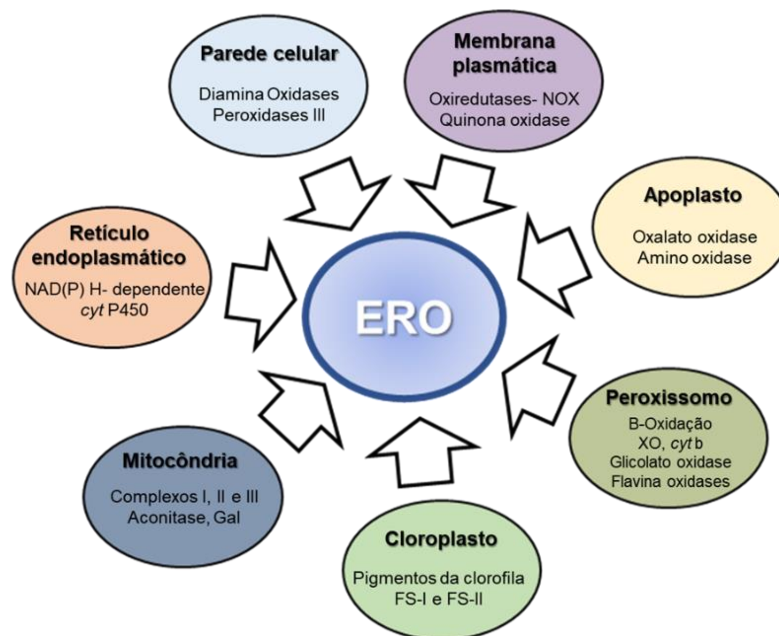


Figura 5: Formação de espécies reativas de oxigênio (ERO) em compartimentos de células vegetais. Fonte: Adaptado de Dumanovic *et al*., (2021).

As células vegetais desenvolveram sofisticados mecanismos de defesa contra as ERO produzidas em plantas sob estresses abióticos, seja limitando sua formação ou promovendo sua detoxificação. Em condições normais de crescimento, a formação e remoção de ERO são balanceadas de modo que o organismo atinja um estado homeostático. No entanto, sob condições estressantes, a formação de ERO aumenta drasticamente e o sistema de defesa pode ser sobrecarregado, o que possibilita a interação das ERO com outras moléculas biológicas causando danos celulares (SHARMA *et al*., 2012). O sistema de defesa antioxidante em plantas compreende componentes enzimáticos e não enzimáticos. O maquinário enzimático inclui a superóxido dismutase (SOD - EC 1.15.1.1), catalase (CAT - EC 1.11.1.6), ascorbato peroxidase (APX - EC 1.11.1.11), monodeidroascorbato redutase (MDHAR - EC 1.6.5.4), dehidroascorbato redutase (DHAR - EC 1.8.5.1), glutationa redutase (GR - EC 1.6.4.2) e guaiacol peroxidase (GPX - EC 1.11.1.7) (GILL *et al*., 2015a; GILL; TUTEJA, 2010; HASANUZZAMAN *et al*., 2012).

2.5 Enzima superóxido dismutase (SOD)

Dentre os genes que codificam para enzimas antioxidantes, os genes da família SOD tem sido estudados devido a sua importância no metabolismo oxidativo, sendo a primeira linha de defesa enzimática no combate a ERO, desempenhando um papel importante na homeostase de ERO através da conversão do radical $O_2^{\cdot -}$ a H_2O_2. Por ser um radical livre, o $O_2^{\cdot -}$ não pode atravessar a membrana plasmática e é acumulado no compartimento em que é produzido. Trata-se de um radical de tempo de vida curto, mas que pode causar danos em proteínas (CHOUDHURY *et al.*, 2017; DEL RÍO *et al.*, 2018; DEL RÍO; LÓPEZ-HUERTAS, 2016; GILL *et al.*, 2015b).

A SOD é uma família de metaloenzimas que diferem quanto ao metal que compõem os grupos prostéticos do sítio ativo. Os genes que codificam para a SOD são classificados de acordo com o cofator que se liga à enzima, que em plantas podem ser o Cu/Zn, Mn ou Fe. Os cofatores são essenciais para a reação enzimática de óxido-redução e detoxificação do radical $O_2^{\cdot -}$ (BOWLER *et al.*, 1994). Os genes dos grupos Mn e Fe demonstram ter grande similaridade nas suas estruturas primária, secundária e terciária, e são mais antigos evolutivamente, sendo encontrados em eucariotos e também em procariotos, enquanto que o grupo Cu/Zn são encontrados apenas em eucariotos (SMITH; DOOLITTLE, 1992). Além disso, os genes de SOD demonstram possuir padrões de expressão gênica diferentes a depender do tecido e estádio de desenvolvimento (ASENSIO *et al.*, 2012; FENG, XIN *et al.*, 2015; GIANNOPOLITIS; RIES, 1977; PILON; RAVET; TAPKEN, 2011).

O H_2O_2 é um produto da reação enzimática da SOD e descrito por ser um transdutor de sinais na interação planta-patógeno, e em resposta a estresses osmótico e por excesso de luz. Assim como pode modular a expressão de genes relatados como responsivos a estresses abióticos. Essa importância do H_2O_2 acentua ainda mais o papel da SOD na homeostase de celular (CHOUDHURY *et al.*, 2017; DEMIDCHIK, 2015; INZÉ *et al.*, 2012).

As isoformas Cu/Zn-SOD tem sido encontradas no citosol, cloroplastos e peroxissomos, enquanto que as isoformas Fe-SOD estão localizadas principalmente em cloroplastos e em menor extensão nos peroxissomos e no apoplasto, enquanto as isoformas Mn-SOD estão localizadas principalmente nas mitocôndrias (GILL *et al.*, 2015). Em geral, a ocorrência de SOD é reportada em raízes, folhas, frutos e sementes de muitas espécies de vegetais, e demonstraram estarem envolvidas na proteção contra estresses abióticos como calor, frio, seca e salinidade (ASENSIO *et al.*, 2012; FENG, XIN *et al.*, 2015; GIANNOPOLITIS; RIES, 1977; PILON; RAVET; TAPKEN, 2011).

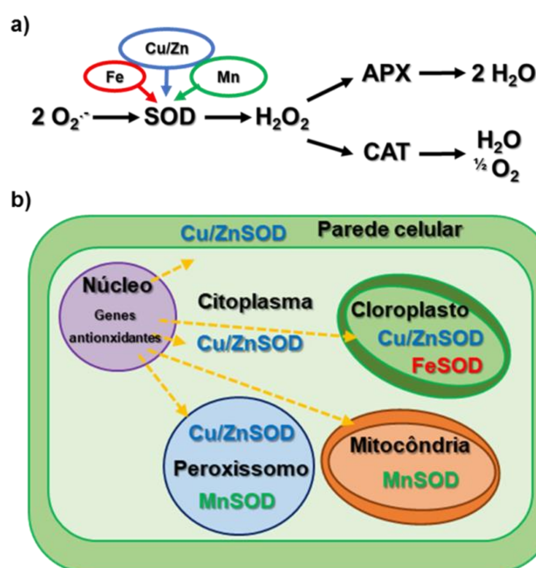


Figura 6: Isoformas da enzima superóxido dismutase (SOD). **a)** Reação enzimática da SOD. **b)** Padrão de localização subcelular de isoformas de SOD em plantas. Fonte: Adaptado de Gill *et al.*, (2015).

As famílias dos genes da SOD são descritas para algumas espécies, tais como em *Arabidopsis thaliana* (KLIEBENSTEIN; MONDE; LAST, 1998), *Oryza sativa* (NATH *et al.*, 2014), *Sorghum bicolor* (FILIZ; TOMBULOĞLU, 2015), *Populus trichocarpa* (MOLINA-RUEDA; TSAI; KIRBY, 2013) e *Solanum lycopersicum* (FENG, KUN *et al.*, 2016). No entanto, ainda não existem relatos do perfil filogenético e regulação dos genes da família de SOD em mamona.

2.6 Proteínas Pequenas de Choque Térmico (*small heat shock protein* - sHSP)

As HSP são uma importante classe de proteínas chaperonas, que estabilizam a estrutura de outras proteínas e protege a membrana plasmática, mediando a montagem, conformação, translocação e degradação de proteínas e polipeptídeos redundantes em condições celular normais, e também mantendo processos metabólicos fazendo com que a planta tolere de forma mais eficiente as mudanças ambientais. Dentre os alvos da HSP estão enzimas do metabolismo primário. (JACOB; HIRT; BENDAHMANE, 2017; USMAN *et al.*, 2014).

Figura 7: Função das Proteínas de Choque Térmico (Heat Shock Proteins, HSP). Fonte: Adaptado de Usman *et al.*, (2014).

As HSP são classificadas de acordo ao seu peso molecular em cinco famílias, sendo as HSP100, HSP90, HSP70, HSP60 e as sHSP. A expressão das HSP são controladas pelos fatores de choque térmico (heat shock factor -HSF). Dentre as classes de HSF, a classe HSFA1 é considerada o maior regulador de genes responsivos a estresses incluíndo as HSP, aparentemente regulando positivamente enzimas antioxidantes como SOD, CAT e POD (LI *et al.*, 2017; UL HAQ *et al.*, 2019; WANG, MINGLE *et al.*, 2017).

Dentre esses subgrupos, as sHSP também tem recebido maior atenção pela sua relação com a tolerância de plantas a estresses abióticos (FENG, XIAO HUI *et al.*, 2019; NAGARAJU *et al.*, 2020; ZHANG *et al.*, 2018). As sHSP são baixo peso molecular, menor que 43 KDa e são caracterizadas por terem o domínio alfa cristalino. Essa família foi caracterizada em *A. thaliana* e classificada de acordo com a localização

subcelular e homologia das sequências de aminoácidos. Foram identifcados seis grupos citoplasmático-nuclear (CI, CII, CIII, CIV, CV, CVI), um grupo cloroplástico, um grupo endoplasmático, um grupo no retículo endoplasmático, dois grupos mitocondriais e um grupo no peroxissomo. A caracterização dos genes das sHSP tem sido realizadas em várias espécies como *A. thaliana*, *Oryza sativa*, *Solanum licopersycum*, *Populus trichocarpa*, *Capsicum annum, e Glycine max*, e a identificação do padrão de expressão desses genes tem revelado que existe grande divergência na indução desses genes em tecidos e estádio específicos de desenvolvimento da planta, assim como para estresses abióticos (LOPES-CAITAR *et al.*, 2013; MANI; RAMAKRISHNA; SUGUNA, 2015; MU *et al.*, 2013; SCHARF; SIDDIQUE; VIERLING, 2001; YU *et al.*, 2016).

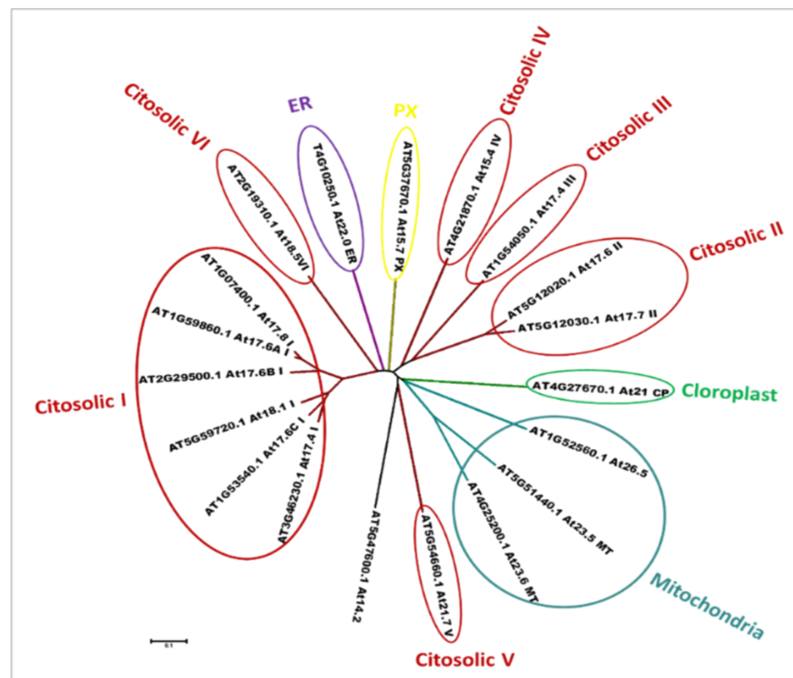


Figura 8: Árvore filogenética da família das Pequenas Proteínas de Choque Térmico (Small Heat Shock Proteins, sHSP) em Arabidopsis thaliana e a classificação em subgrupos de acordo a localização subcelular. Fonte: Classificação de acordo Scharf *et al.* (2001).

2.7 Fatores de transcrição e a família de fator nuclear Y (NF-Y)

Cerca de 10% dos genes codificam para FT em plantas, sendo que um grande número de banco de dados estão disponíveis fornenendo informações sobre famílias de FT em diferentes espécies. E como tal, os FT são candidatos promissores para aplicação em melhoramento genético vegetal por serem reguladores da expressão de grupos de genes envolvidos na tolerância a estresses abióticos e desenvolvimento de plantas. Muitos genes de diversas famílias de FT são responsivos a estresses abióticos, dentre essas famílias de FT podemos destacar os NFY, WRKY, NAC, MYB e BZIP. Cerca de 30 famílias de FT foram identificadas em *Arabidopsis thaliana*, sendo que cerca de 1922 genes tem sido relacionados a diferentes funções (BAILLO *et al.*, 2019; GUJJAR; AKHTAR; SINGH, 2014; GUO *et al.*, 2016; ZHAO *et al.*, 2017).

Dentre os FT, a classe dos NF-Y demonstra atuar em vários eventos durante o ciclo de vida da planta, sendo encontrados em animais e outros eucariotos. Dentre os vários papeis dos NF-Y no desenvolvimento da planta, citam-se a dormência e germinação de sementes, fotomorfogênese e desenvolvimento inicial de plântulas, crescimento de raiz, florescimento dependente do fotoperíodo, maturação de sementes e embriogênese, e tolerância a seca. Os genes que codificam para a subunidade B (NFY-B) são descritos por serem responsivos a estresses abióticos e estarem relacionados a regulação do florescimento, e tolerância a seca (KUMIMOTO *et al.*, 2008; NELSON *et al.*, 2007).

O NF-Y é um complexo com três subunidades denominadas NFY-A, NFY-B e NFY-C e reconhece a sequência CCAAT no DNA, sendo por isso denominado de fator de ligação a CCAAT (CCAAT Binding Factor - CBF). Cada subunidade pode ser codificada por vários genes, onde esses genes podem possuir múltiplas formas de splicing e vários modificações pos-traducionais. Porém, as subunidades não podem regular genes independentemente, precisam estarem em heterodímeros ou heterotrímeros. As subunidades NFY-B e NFY-C tem sido apontadas por formarem complexos sem a subunidade NFY-A , podendo interagir com outras proteínas tais como Constans (CO) e BZIP formando novos complexos. Os NF-Y tem sido descritos por serem responsivos por uma série de processos do desenvolvimento fisiológicos da

planta, exercendo funções no ciclo de vida e resposta de plantas a estresses abióticos (MYERS; HOLT, 2018; SWAIN *et al.*, 2017; ZHAO *et al.*, 2017).

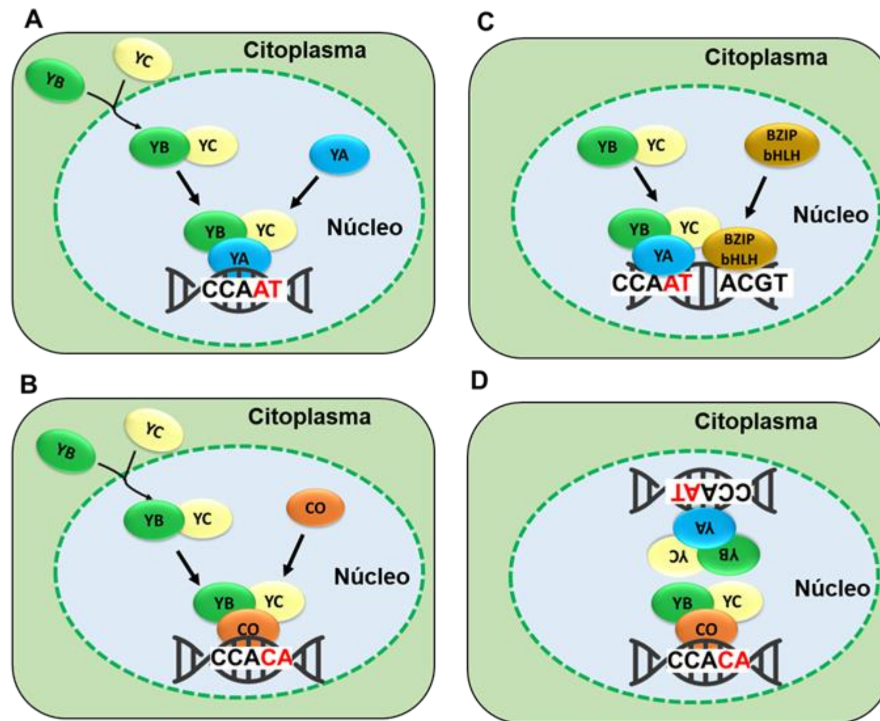


Figura 9: Mecanismos de como o complexo de Fator nuclear Y (*Nuclear factor Y*, NFY) pode modular a expressão de genes. Fonte: Adaptado de Myers & Holt (2018).

Nesse contexto, o foco dessa tese é voltado à caracterização da família de genes da família SOD, sHSP e NF-YB por demonstrarem relação com a regulação de processos de desenvolvimento e de resposta de plantas a estresses abióticos e, por meio da caracterização dos referidos genes e acúmulo de conhecimentos relevantes e que demonstram o uso potencial dos mesmos em eventuais programas de melhoramento genético da mamona.

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3. OBJETIVOS

3.1 Geral

Prospectar e caracterizar genes de *Ricinus communis* associados a tolerância a estresses abióticos

3.2 Específicos

- Identificar e caracterizar genes da família SOD de *Ricinus communis* (*RcSOD*) através de comparação filogenética com *A.thaliana* e perfil de expressão gênica na germinação sob estresse osmótico.

- Obter a expressão relativa de genes da família SOD durante a germinação e crescimento de plântulas sob diferentes condições de estresse térmico, e tecidos diferentes e identificar a localização subcelular dos genes de *RcSOD* através da fusão com a proteína fluorescente verde (GFP) em folhas da planta *Nicotiana benthamiana*.

- Identificar e caracterizar genes da família sHSP de *Ricinus communis* (*RcsHSP*) através da comparação filogenética com *A.thaliana*, perfil de expressão gênica e caracterização funcional através da superexpressão dos genes em plantas transgênicas de *A. thaliana*.

- Identificar e caracterizar genes de *Ricinus communis* que codificam para o fator de transcrição nuclear Y subunidade B através da comparação filogenética com *A. thaliana*, perfil de expressão gênica sob estresse térmico durante a germinação e caracterização funcional do gene *RcNF-YB8* quanto a regulação do florescimento através da superexpressão dos genes em plantas transgênicas de *A. thaliana*.

CAPÍTULO 1: Characterization of the superoxide dismutase gene family in seeds of two *Ricinus communis* L. genotypes submitted to germination under water restriction conditions.

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Characterization of the superoxide dismutase gene family in seeds of two *Ricinus communis* L. genotypes submitted to germination under water restriction conditions

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Characterization of the superoxide dismutase gene family in seeds of two *Ricinus communis* L. genotypes submitted to germination under water restriction conditions

ABSTRACT

Abiotic stresses are the most important environmental factors that negatively affects crop production worldwide, whereas germination is the most critical phase in the plants life cycle. Water availability is essential for proper seed imbibition and vigorous germination and often scarce for *Ricinus communis* (castor bean) cultivated in semiarid regions of Brazil. Water restriction generally leads to the generation of reactive oxygen species (ROS) and one of the major families of enzymes that counteract ROS are superoxide dismutases (SODs). The objective of this study was to identify and characterize the SOD gene family of *R. communis* by phylogenetic, biochemical and gene expression analysis and link it to the effect of osmoconditioning on seed vigor. We characterized the effect of osmotic conditioning treatments of seeds of two contrasting *R. communis* cultivars, EBDA MPA34 and PARAGUAÇU, on germination performance. We also searched the *R. communis* genome for SOD genes and analyzed them phylogenetically and evaluated their activity and expression in embryos of seeds imbibed under different PEG concentrations (water restriction stress). We could show that some of these treatments improved germinability and vigor of seeds and seedlings in a cultivar-dependent manner. Additionally, *R. communis* seeds turned out to be very sensitive to the inhibition of germination by osmotic stress even at relative low concentrations. Eight SOD genes were found in the *R. communis* genome and classified based on phylogenetic analysis, protein domains and prediction of subcellular localization. SOD gene expression was mostly induced during seed imbibition, although their expression was generally inhibited in osmotic potentials below -0.6 MPa and showed partly different gene expression profiles between contrasting cultivars. Cv. PARAGUAÇU showed increased seed vigor after osmoconditioning as compared to cv. EBDA MPA34 which could be related to higher responses of some cv. PARAGUAÇU SOD genes to stress. Our results indicate that SOD genes might be of special interest

for *R. communis* genetic engineering aimed at the increase of production in semiarid regions.

Keywords: Castor bean, germinability, oilseeds, oxidative stresses, pre-germination treatments, seed priming, water restriction.

1. INTRODUCTION

Ricinus communis L. (Castor bean) is an oilseed crop that belongs to the Euphorbiaceae family and it is distributed in tropical, sub-tropical and warm temperate regions worldwide. The oil extracted from its seeds, has several pharmaceutical and industrial applications (SEVERINO *et al*., 2012). This oilseed crop species has an optimal growth temperature ranging from 20 to 26°C, although it shows efficient vegetative growth at temperatures up to 35°C (RIBEIRO *et al*., 2014a). Due to its tolerance to higher temperatures and drought, this species can be and is grown in arid and semiarid regions of countries such as China, India and Brazil (ATABANI *et al*., 2013). Its cultivation in Brazil occurs mainly by small-scale family farmers in the semiarid region of the Northeastern Brazil (NEVES; SANTOS; DONATO, 2013).

Large-scale cultivation of oilseed crops in arid and semiarid regions imposes great challenges for seed germination, seedling development and proper crop establishment, mainly due to water restriction conditions (CAMPOS, 2015; MACHADO; LA ROVERE, 2018). Poor seed germination and seedling establishment are major factors for suboptimal crop growth and productivity, especially under drought stress imposed by water restriction conditions in early stages of plant development (SAUSEN; ROSA, 2010). Severe drought can lead to complete inhibition of germination (RADHAMANI *et al*., 2012) as well as detrimental changes in development and physiology of plants (BRITO *et al*., 2016). It severely impairs the reactivation of metabolism along with cell elongation and expansion during seed imbibition and early plant development (KUSAKA; OHTA; FUJIMURA, 2005). Moreover, exposure to drought stress leads to the generation of reactive oxygen species (ROS), which can cause lipid peroxidation, protein degradation, DNA damage and ultimately lead to cell death (ZHANG *et al*., 2015).

R. communis is well known for its plasticity in respect to major plant and seed phenotypic variability among genotypes, since plant height, leaf morphology and life cycle duration, to seed size and shape, multicolored seeds, and seed physiological profiles which may significantly influence seedling performance and crop establishment

(BABITA, 2010; SEVERINO *et al.*, 2012 SOWMYA *et al.*, 2016). Seed osmoconditioning may be used to simulate water restriction stresses during seed imbibition and germination by allowing seeds to imbibe in osmotic solutions which limit hydration to levels that allow reactivation of seed metabolism, but without allowing seed radicle protrusion or germination *sensu stricto*, i.e. as described by the triphasic pattern of seed water uptake and respective metabolic/physiological status during imbibition and gemination (BEWLEY *et al.*, 2013). Such germinative pretreatments are in fact also used by the seed industry as a technological means to improve seed and seedling performance of many crops under field conditions named seed priming or osmopriming (HUSSAIN *et al.*, 2015). In fact, osmoconditioning in PEG at -1.0 MPa has been shown to result in osmopriming by enhancing seed germination speed and uniformity in *R. communis* as evidenced by the reactivation of the cell cycle as the result of *de novo* synthesis of tubulins and assembly of a cortical microtubular cytoskeleton (VASCONCELOS *et al.*, 2017). The same osmoconditioning approach may also be used to study the biochemical and molecular mechanisms that may regulate metabolic/physiological profiles and improve the performance of *R. communis* seeds and seedlings under adverse field conditions.

Plants have developed sophisticated defense mechanisms against ROS produced under such conditions, either by limiting their formation or promoting their detoxification. Under normal growth conditions, the formation and removal of ROS are balanced so the organism reaches a homeostatic state. However, under stressful conditions, ROS formation increases dramatically and the defense system can be overwhelmed leading to cellular damage (SHARMA *et al.*, 2012). Plant antioxidant defense system comprises enzymatic (Hasanuzzaman et al., 2012) and non-enzymatic components (GILL; TUTEJA, 2010). The enzymatic machinery includes superoxide dismutase (SOD), catalase, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione peroxidase, guaiacol peroxidase, peroxidase, and glutathione-S-transferase (HASANUZZAMAN *et al.*, 2012).

SOD is one of the most effective components of the antioxidant defense system in plant cells against ROS toxicity. It constitutes the first line of defense against ROS produced in response to abiotic stresses. SOD catalyzes the dismutation of $O_2^{\bullet -}$ to H_2O_2 and O_2. and it can be further classified in plants according to its different cofactors, namely Cu/Zn$^-$, Fe$^-$ and Mn-SOD. Cu/Zn-SOD can be found in the cytosol, chloroplasts, and peroxisomes, whereas Fe-SOD is mostly localized in chloroplasts and to a lesser extent in peroxisomes and apoplast and Mn-SOD is mainly localized in mitochondria (GILL *et al.*, 2015). Overall, SODs have been detected in roots, leaves, fruits, and seeds of many plant species (GIANNOPOLITIS; RIES, 1977) and have been shown to be involved in protection against abiotic stresses such as heat, cold, drought and salinity (WANG *et al.*, 2004; PILON *et al.*, 2011; ASENSIO *et al.*, 2012; FENG *et al.*, 2015). In general, most of those studies focused on the SOD enzymatic antioxidant system in respect to diverse environmental stress conditions, genotype, tissue and phase of development, besides SOD cofactors and subcellular localization (FENG *et al.*, 2016, GÓMEZ *et al.*, 1999; WU *et al.*, 1999; BAEK; SKINNER, 2003; MYOUGA *et al.*, 2008; SAIRAM *et al.*, 2005). The families of SOD genes are described for a few species like *Arabidopsis thaliana* (7 genes; KLIEBENSTEIN; MONDE; LAST, 1998), rice (8 genes; NATH *et al.*, 2014), sorghum (8 genes; FILIZ; TOMBULOĞLU, 2015), poplar (12 genes; MOLINA-RUEDA *et al.*, 2013) and tomato (9 genes; FENG *et al.*, 2016). However, the SOD gene family in *R. communis* has not been described previously both in terms of their phylogenetic profile as well as in respect to their regulation.

Hence, the objectives of this study were to identify and characterize the phylogenetic relation of the SOD gene family in *R. communis* and their possible role during early stages of seed imbibition under water restricted conditions. For this purpose, SOD activity and expression of the different SOD genes was determined in embryo axis of seeds submitted to imbibition under different osmotic potentials from two genotypes that presented contrasting initial germination profiles.

2. MATERIAL AND METHODS

2.1 Plant material

R. communis cultivars EBDA MPA34 and PARAGUAÇU were sown in February 2013 and harvested in July-August of the same year at the Field Experimental Station of the Agricultural Research Company of Minas Gerais (EPAMIG-CEMC), located in the municipality of Montes Claros (coordinates: 16°66'S 43°73'W), situated within the northern region of the state of state of Minas Gerais which is geographically part of the main Brazilian semiarid geographical region (Machado & La Rovere, 2018), at an altitude of 602 m and with an average temperature of 23.55°C and average rainfall of 950 mm.

2.2 Seed osmoconditioning

Seeds were first surface sterilized with 0.5 % of active chlorine for 15 min and rinsed four times with distilled water. Water restriction stress was induced by imbibing the seeds in polyethylene glycol solutions (PEG 8000, Sigma-Aldrich), i.e. osmoconditioning, at decreasing osmotic potentials (-0.2, -0.6 and -1.0 MPa) (VILLELLA; BECKERT, 2001; VASCONCELOS *et al.*, 2017). As control, seeds were imbibed in distilled water (0.0 MPa). Seed imbibition was performed by using germination paper as substrate (Germilab) in plastic boxes incubated in a germination chamber (Panasonic MLR-351H) for 7 d at 25°C in the dark. For imbibition curves, seeds were weighted three times a day up to 168 h at 25°C in the dark (data are shown for up to 72 h) and the water uptake was expressed as percentage of initial dry seed weight based on the average weight of three pools of 10 seeds for each treatment. The initial seed water content and dry weight was determined by the oven method as described by the Brazilian rules for *R. communis* seed analysis (BRAZIL, 2009)

2.3 Recovery of seed germinability

After 7 days of osmoconditioning, seeds were subsequently washed four times with distilled water and transferred for incubation in water using germination paper (Germilab) as substrate for 7 d at 25°C in the dark. As control, non-osmoconditioned

seeds were also imbibed in distilled water (0.0 MPa). Germination was monitored for 7 d and seeds were considered germinated when the embryonic radicle had protruded the seed coat by at least 2 mm. Four biological replicates of 25 seeds each were used for each condition.

The following seed germinability parameters were evaluated using the 'GERMINATOR' software package (JOOSEN *et al.*, 2010): maximum germination percentage (G_{max}), germination uniformity (U_{84-16}), i.e. as the mean time between 16 and 84% germination, germination speed as the mean time for 50 % germination (T_{50}), and the overall seed performance expressed as the 'area under the curve' after 100h of imbibition (AUC_{100}) which represents the integration value that enumerates the germinability parameters and discriminative power between samples. After 7 d imbibition in water, the following seedling vigor parameters were analyzed: mean percentage of normal seedlings, seedling dry mass, seedling length and seedling dry mass /length ratio (mg/cm). Seedling length and dry mass refers to the hypocotyl plus primary root. All seed germinability and seedling vigor parameters were analyzed with the SISVAR statistical software package (FERREIRA, 2011) and means were compared Statistical significance was evaluated by Scott-Knott test ($p < 0.05$) for overall means comparison and *t*-test for pairwise least significant difference between both genotypes at each data point (LSD, $p < 0.05$).

2.4 Sample collection

Embryo axes were collected from seeds at four-time points: non-osmoconditioned dry seeds (0 h) and seeds imbibed for 12 h (middle of Phase-I), 24 h (early Phase-II), and 40 h (late Phase-II, i.e. prior to radicle protrusion), either in water (0,0 MPa, control) or in PEG osmotic solutions at potentials of -0.2, -0.6 and -1.0 MPa (Vasconcelos et al., 2017). Samples were collected in 2-mL tubes, immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. Three biological replicates of 50 seeds each were used.

2.5 Protein extraction and quantification

For protein extraction, frozen embryo axes were ground in liquid nitrogen with glass canes and homogenized in 0.1 M potassium phosphate buffer (pH 7.8). The homogenate was centrifuged at 14.000 g for 10 min at 4°C. The supernatant, hereafter referred to as crude SOD extract, was collected and stored at -20°C for further analysis. Extracts were diluted 10 times for protein quantification and SOD activity assays. Total water-soluble protein was quantified in the crude SOD extracts, using bovine serum albumin as standard ((BRADFORD, 1976).

2.6 Superoxide dismutase (EC 1.15.1.1) activity in embryo axes

Total SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT) by $O_2^{\bullet-}$, as described by (GIANNOPOLITIS; RIES, 1977). The initial rate of the reaction was determined through absorbance at 560 nm using a Multiskan FC® Microplate Photometer (Thermo Scientific). Initially, 175 µL of the reaction mixture (50 mM PPB (pH 7.8), 13 mM methionine, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA) was mixed with 25 µL of enzyme extract. Then, the reaction was initiated by placing samples underneath a 30 W fluorescent lamp at 25°C for 15 min. Enzyme activity was expressed as units of SOD activity (U) per mg of total protein. One unit of SOD activity is expressed as the amount of enzyme required to cause 50 % inhibition of NBT reduction under the experimental conditions. Statistical significance was evaluated by Scott-Knott test ($P < 0.05$) for overall means comparison and *t*-test for pairwise least significant difference between both genotypes at each data point (LSD, $P < 0.05$).

2.7 Phylogenetic analysis and prediction of subcellular localization

The complete set of *Arabidopsis thaliana* SOD protein sequences (KLIEBENSTEIN; MONDE; LAST, 1998) was used as query in blastp searches (e-value cut off < e^{-04}) against the complete predicted proteome of *R. communis* v0.1 from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). To investigate the phylogenetic relationships of *R. communis* superoxide dismutase genes (RcSODs) with other angiosperms, we constructed a phylogenetic tree together with the previously

described complet SOD sets from *A. thaliana* (KLIEBENSTEIN; MONDE; LAST, 1998), rice (DEHURY *et al*., 2013; NATH *et al*., 2014), sorghum (FILIZ; TOMBULOĞLU, 2015), poplar (MOLINA-RUEDA; TSAI; KIRBY, 2013), and tomato (FENG *et al*., 2016). The amino acid sequences of SOD proteins were aligned using mafft version 7 (https://mafft.cbrc.jp/alignment/software/) with parameters --thread 10 --threadtb 5 --threadit 0 --reorder --leavegappyregion –auto. The phylogenetic analysis was conducted using MEGA 7.0 (http://www.megasoftware.net/). Topology was calculated by Neighbor-Joining method with distances calculated by p-distance. Bootstrapping was performed with 1000 replicates. The tree was rooted at the midpoint.

Predictions of subcellular localizations of RcSOD proteins were performed with Wolf Psort (https://wolfpsort.hgc.jp/) and Multiloc2 (https://abi.inf.uni-tuebingen.de/Services/MultiLoc2). Prediction of subcellular localizations of SOD proteins from *A. thaliana* (KLIEBENSTEIN; MONDE; LAST, 1998), rice (DEHURY *et al*., 2013; NATH *et al*., 2014), sorghum (FILIZ; TOMBULOĞLU, 2015), poplar (MOLINA-RUEDA; TSAI; KIRBY, 2013), and tomato (FENG *et al*., 2016) were retrieved from the literature. Protein domains were identified using Pfam HMM search (http://pfam.xfam.org/search). Protein schemes were drawn using IBS 1.0.3 software (http://ibs.biocuckoo.org/). Molecular weight and isoelectric point (pI) of RcSOD proteins were estimated using ProtParam (http://web.expasy.org/protparam/).

2.8 RNA extraction, cDNA synthesis and gene expression analysis

Total RNA was extracted from 5mg of the lyophilized and ground embryo axes samples using the hot borate method with modifications (WAN; WILKINS, 1994). RNA was quantified in a Ultrospec^{TM700} spectrophotometer (GE Healthcare) and RNA integrity was further inspected on a 1% agarose gel. First strand cDNA was synthesized with 1 µg of total RNA using the ImProm-II™ Reverse Transcription System cDNA synthesis kit (Promega) according to the manufacturer’s instructions. The cDNA was diluted 20 times and stored at -20 °C prior to further analysis by qRT-PCR.

Primers were designed using primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) with melting temperatures (Tm) of 59-61 °C, primer lengths of 18-22 bp

and amplicon lengths of 120-250 bp (Supplementary table S1). Primer efficiency was evaluated based on a standard curve generated by qRT-PCR analysis of a two-fold serial dilution series of a pooled cDNA sample. The primer specificity was verified by separating the products on a 2 % agarose gel and by melting curve analysis. Serine/threonine phosphatase 2A (PP2AA1) gene was used as internal reference gene (Cassol et al., 2016; Ribeiro et al., 2014b). qRT-PCR was performed in a total volume of 20 µL containing 5 µL of cDNA (20x diluted), 1 µL of primers (10 µM), 10 µL of iQ SYBR Green Supermix (Applied Biosystems) and 4 µL of water. qRT-PCR experiments were run on a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). The following program was used for all PCR reactions: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melting curves were obtained after this program by heating samples from 65 to 95°C, increasing the temperature stepwise by 0.5°C every 5 s (RIBEIRO *et al.*, 2014). Statistical significance was evaluated by Scott-Knott test ($p < 0.05$) for overall means comparison and *t*-test for pairwise least significant difference between both genotypes at each data point (LSD, $p < 0.05$).

3. RESULTS & DISCUSSION

3.1 Diminished water uptake caused by osmotic stress inhibits *R. communis* seed germination

We first intended to understand the dynamics of water uptake in *R. communis* seeds under normal and water restriction conditions, i.e. osmoconditioning. For this goal, we first determined the seed water content of both genotypes, i.e. 4,86 % ± 0,31 for EBDA MPA34 and 4,74 % ± 0,07 for PARAGUAÇU, and further compared the water uptake of seeds imbibed in water (0.0 MPa) and seeds imbibed in PEG solutions with different osmotic potentials (Figure 1).

The effect of water restriction promoted by PEG solutions on the water uptake of *R. communis* seeds could already be observed within six hours of imbibition, although it became more prominent after 72 h of imbibition. Within six hours, EBDA MPA34 seeds imbibed in control conditions took up 14.26, 29.98 and 35.60 % more water than seeds imbibed in osmotic potential of -0.2, -0.6 and -1.0 MPa, respectively. Besides this, a

similar pattern occurred within 72 h of imbibition, when EBDA MPA34 seeds imbibing in control conditions took up 25.26, 41.93 and 47.89 % more water than seeds imbibing in osmotic potential of -0.2, -0.6 and -1.0 MPa, respectively (Figure 1). Both cultivars presented a very similar imbibition pattern, except for the fact that cv. PARAGUAÇU needed slightly more water to enter the Phase-III of the triphasic imbibition curve (Figure 1), i.e. radicle protrusion or germination *strictu sensu*, where Phase-I is generally characterized by fast water uptake and resumption of energy metabolism; Phase-II starts when water uptake decreases, and reserve mobilization begins; and Phase-III begins with radicle protrusion and completion of germination into seedlings (NONOGAKI; BASSEL; BEWLEY, 2010).

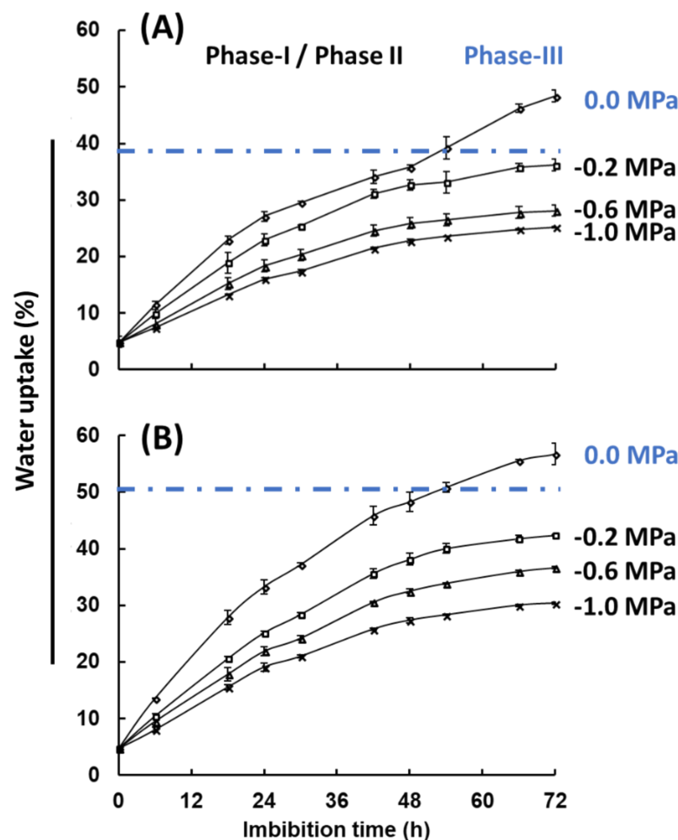

Figure 1. Water uptake by *Ricinus communis* seeds during imbibition in different osmotic potentials. Data are shown as percentage of seed dry weight of seeds imbibed up to 72 h. (A) cv. EBDA MPA34; (B) cv. PARAGUAÇU. Mean values with standard deviations are shown for 3 biological replicates of 10 seeds. Horizontal dashed

blue lines show the water content above which radicle protrusion / germination *sensu stricto* occurred when seeds were imbibed in water (0.0 MPa), i.e. when seeds entered into Phase-III, and below which seeds did not germinate (or only at a low percentage) and were kept at Phase-I / Phase-II.

Germination was majorly observed for seeds imbibed in water, whereas a minor percentage germinated in osmotic potential of -0.2 MPa, i.e. 16.7 % for EBDA MPA34 and 3.3 % for PARAGUAÇU (Figure 2). None of the seeds germinated at -0.6 and -1.0 MPa even after 168 h imbibition period (data not shown). This shows that at an early stage of its life cycle *R. communis* is very sensitive to water restrictive conditions as compared to other crop species. This may represent an ecological means of preventing the reactivation of metabolism and germination under unfavorable water restriction conditions, i.e. at osmotic potentials below -0.2 MPa, which may be coupled to the well know tolerance of *R. communis* to drought stress (ANJANI, 2012; BABITA *et al.*, 2010).

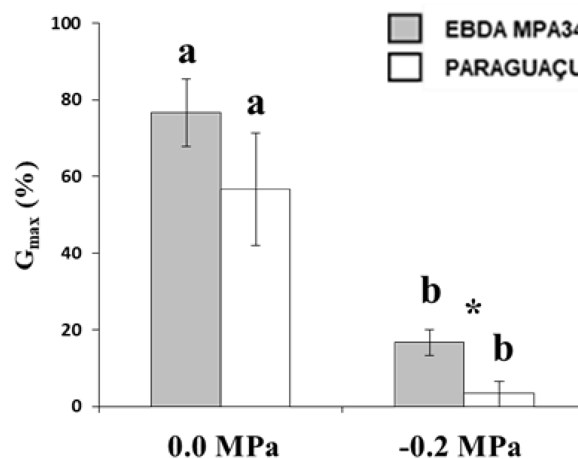

Figure 2. Percentage of maximum germination (G_{max}) of *Ricinus communis* seeds (cv. EBDA MPA34 and PARAGUAÇU) imbibed for 72 h in water (0.0 MPa) and in PEG (-0.2 MPa). Different letters indicate significant differences between data points as evaluated by Scott-Knott test ($p < 0.05$), whereas (*) indicates significant differences between the

genotypes for each imbibition medium according to least significant difference (LSD, t-test at $p < 0.05$).

3.2 Osmoconditioning improves *R. communis* seed germinability and seedling vigor in a cultivar-dependent manner

To address the possible effects of seed osmoconditioning on seed and seedling performance of EBDA MPA34 and PARAGUAÇU, we osmoconditioned seeds from both cultivars for 7 d in a variety of osmotic potentials (-0.2, -0.6 and -1.0 MPa) and tested the effect on seed germination and seedling establishment. Our data showed that osmoconditioned seeds of cv. PARAGUAÇU performed better than those of cv. EBDA MPA34 in most physiological parameters, with significant priming effects for PARAGUAÇU for all osmoconditioning treatments in terms of an higher percentage (G_{max}) and speed of germination (as shown by a lower T_{50}) and higher values for AUC_{100} (Figure 3A,B,D). Additionally, osmoconditioning also resulted in a higher percentage of normal seedlings and although there was no significant difference on seedling dry mass and seedling length (data not shown), osmoconditioning resulted in a significant higher ratio between dry mass and length of seedlings for PARAGUAÇU (Figure 3E,F). These results are in agreement with publications for other species since osmoconditioning is a well-known technique for priming seeds and has been shown to increase seed/seedling physiological quality and improve tolerance to abiotic stresses in various species such as among others *Physalis angulata* (DE SOUZA *et al.*, 2016), *Vigna unguiculate* (SINGH *et al.*, 2014), *Zinnia elegans* (SZOPIŃSKA; POLITYCKA, 2016), *Glycine max* (SADEGHI *et al.*, 2011), *Moroccan alfalfa* (MOURADI *et al.*, 2016), *Psidium guineense* (SANTOS *et al.*, 2016), *Eucalyptus* sp. (JOSÉ; CRISTINA; SILVA, 2016), *Oryza sativa* (SHETEIWY *et al.*, 2016), *Solanum lycopersicum* (LIGTERINK *et al.*, 2007) and *Sorghum bicolor* (ZHANG, FEI *et al.*, 2015).

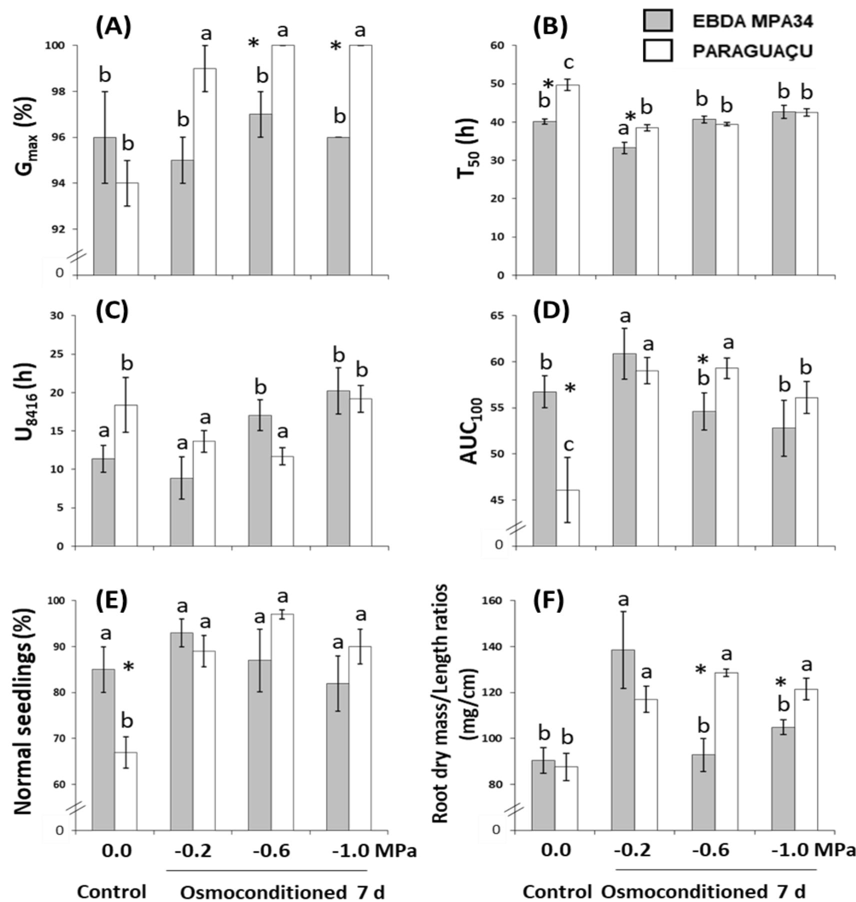

Figure 3. Germinability and vigor of *Ricinus communis* seeds and seedlings upon (re)imbibition in water. Data are shown for non-osmoconditioned seeds imbibed for 7 d in water (0,0 MPa; control) and for seeds that were osmoconditioned for 7 d in PEG solutions (-0.2, -0.6 and -1.0 MPa) and then re-imbibed in water for 7 d. (A) Percentage of maximum germination after 7 d imbibition (G_{max}); (B) Time for 50% germination (T_{50}); (C) Seed germination uniformity as the time between 16 and 84% germination (U_{84-16}); (D) Area under the curve (AUC_{100}) after 100h of imbibition as the integration value that enumerates the germinability parameters and discriminative power between samples, (E) Percentage of normal seedlings after 7 d of (re)imbibition. (F) Seedling dry mass/length (mg/cm) after 7 d of (re)imbibition. Mean values with standard error are shown for 4 biological replicates of 25 seeds each. Different letters indicate significant differences between data points (Scott-knott test at $p < 0.05$), whereas (*) indicate significant differences between the genotypes at each imbibition medium according to least significant difference (LSD, *t*-test at $p < 0.05$).

In conclusion, osmoconditioning was more effective and induced priming in PARAGUAÇU seeds and seedlings as seen in improvement of several physiological parameters (Figure 3). Interestingly, the physiological parameters of EBDA MPA34 were in general not affected by osmoconditioning at any osmotic potential (Figure 3), suggesting that the beneficial effects of "osmopriming" is dependent on the genetic background of *R. communis*. Similar results were previously reported in other species (HUSSAIN, M. *et al*., 2006), although the molecular mechanisms underlying this phenomenon are not well understood yet.

3.3 Total SOD activity in embryo axes depends on the *R. communis* genotype and is affected by osmotic stress

SOD acts by catalyzing the dismutation of superoxide $O_2^{\bullet-}$ radical into either H_2O_2 or ordinary molecular O_2 to control the cellular level of ROS (GILL *et al*., 2015). It is very responsive to increased level of ROS produced during early seed imbibition and germination (BAILLY; BAILLY, 2004). The process of germination naturally produces ROS (BAILLY; BAILLY, 2004; KRANNER *et al*., 2010), although abiotic stresses such as extreme temperatures, drought and salinity can dramatically increase ROS production through increased oxidative stress (CHOUDHURY *et al*., 2017; GILL; TUTEJA, 2010). Thus, seeds able to better balance the level of ROS are more likely to survive and produce vigorous seedlings (BAILLY *et al*., 2008; KRANNER *et al*., 2010). Therefore, we aimed to understand the variation in total SOD activity in seeds of two *R. communis* cultivars in response to osmotic stress.

Considering that most of the metabolism that is reactivated during seed imbibition and germination is concentrated in the embryo axis (WOJTYLA *et al*., 2006; NONOGAKI *et al*., 2010; ROSENTAL *et al*., 2014) we, therefore, choose it as target tissue for all biochemical and molecular analysis (Supplementary Figure S1). Based on our results, four-time points (0, 12, 24 and 40 h) were selected within the specific physiological profiles that characterize the triphasic imbibition curves in water (0,0 MPa) and in different PEG solutions (-0.2, -0.6 and -1.0 MPa) for further analysis of SOD activity and gene expression in embryo axes of *R. communis* seeds. Total SOD activity was higher in embryo axes of non-osmoconditioned dry seeds (0 h) of cv. PARAGUAÇU than of

EBDA MPA34, (Figure 4). This might be due to a greater production of ROS during seed development and maturation in cv. PARAGUAÇU leading to a greater SOD protein activity. However, total SOD activity in embryo axes of cv. EBDA MPA34 reached comparable levels to cv. PARAGUAÇU after 24 h of imbibition in water (0.0 MPa) (Figure 4A), whereas comparable levels were reached only after 40 h of imbibition at -0.2 MPa (Figure 4B).

Total SOD activity showed significant increase in EBDA MPA34 seeds after 12 h of imbibition at -0.6 and -1.0 MPa, whereas in PARAGUAÇU seeds a significant decrease was seen after 12 h of imbibition in the same osmotic potentials (Figure 4C,D). This might suggest that cv. EBDA MPA34 seeds perceive stronger osmotic stress than PARAGUAÇU seeds at the lower osmotic potentials and might be related to the higher moisture content of PARAGUAÇU seeds (Figure 1).

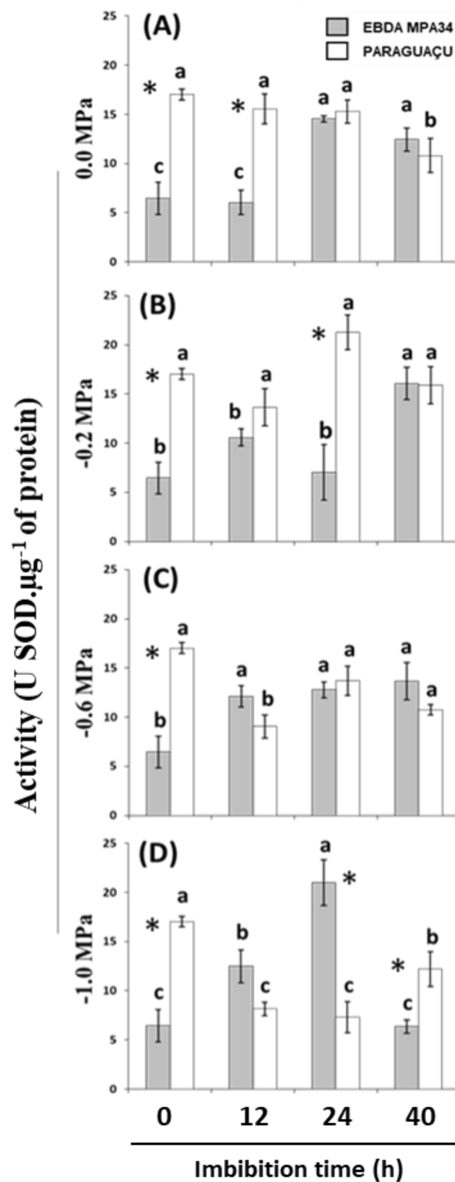

Figure 4. Total superoxide dismutase (SOD) activity in *Ricinus communis* embryo axis during seed imbibition in different osmotic potentials. Activity is shown for intact non-osmoconditioned dry seeds (0 h) and seeds imbibed in water (12, 24 and 40 h at 0.0 MPa) (A), and for seeds imbibed in PEG solutions (12, 24 and 40 h osmoconditioning at -0.2 (B), -0.6 (C) and -1.0 MPa(D)). Mean values with standard error are shown for 3 biological replicates of 50 seeds each. Different letters indicate significant differences between data points (Scott-knott test at $p < 0.05$), whereas (*) indicates significant differences between the genotypes at each imbibition time according to least significant difference (LSD, *t*-test at $p < 0.05$).

The reactivation of mitochondrial activity commonly leads to the accumulation of ROS, in which approximately 2-3 % of the oxygen used by the mitochondria is converted into superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (BAILLY; EL-MAAROUF-BOUTEAU; CORBINEAU, 2008). ROS generated during seed imbibition could be harmful to proteins and other cell components leading to decreased seed vigor (WOJTYLA *et al.*, 2006). SOD is the first line of defense against ROS and it is therefore a key enzyme contributing to the acquisition of tolerance to oxidative damages caused by abiotic stresses during seed germination and seedling establishment (GILL *et al.*, 2015).

Total SOD activity increased in embryo axes of *Lupinus luteus* (Fabaceae) up to the protrusion of the radicle (GARNCZARSKA; WOJTYLA, 2008). The increase in total SOD activity during imbibition and germination is expected in normal conditions because the seed metabolism is activated, and ROS is produced by respiration and metabolism (BAILLY; BAILLY, 2004). As such, enough energy reserves acquired during seed maturation can be additionally beneficial for the adjustment of cell metabolism in order to mount a proper acclimation response into a new degree of homeostasis and better tolerance of plants against abiotic stresses (CHOUDHURY *et al.*, 2017). Overall, our results suggest that when mature dry seeds have higher SOD activity, less variation is observed during imbibition under osmotic stress conditions, as was observed for PARAGUAÇU (Figure 4). However, when mature dry seeds have lower SOD activity, higher variation seems to occur during imbibition, perhaps in an attempt to overcome the osmotic stress conditions during imbibition, at least up to a certain time point, as was observed for EBDA MPA34 seeds (Figure 4).

3.4 Evolutionary analysis of SOD genes in angiosperms

To better understand the genes underlying the SOD activity, we mined the *R. communis* genome through a blastp search using the complete set of SOD genes from *Arabidopsis thaliana* (KLIEBENSTEIN; MONDE; LAST, 1998), which allowed the identification of eight *R. communis* SOD genes. To reveal their evolutionary origins and further classification, we performed a phylogenetic analysis integrating the complete sets

of SOD genes from the angiosperms *Arabidopsis thaliana* (KLIEBENSTEIN; MONDE; LAST, 1998), rice (NATH *et al.*, 2014), sorghum (FILIZ; TOMBULOĞLU, 2015), poplar (MOLINA-RUEDA; TSAI; KIRBY, 2013) and tomato (FENG *et al.*, 2016) (Figure 4). In the *R. communis* genome, we identified three *CuZnSOD*, one chaperone *CuSOD* (CCuSOD), two *MnSOD* and two FeSOD genes (Figure 5). We found a slight variation in the copy number of SOD genes in different plant species that may be attributed to a few lineage-specific gene duplications (Figure 5). Gene duplications can be attributed to *tandem* duplication (PANCHY *et al*., 2016), such as *RcMnSOD5* and *RcMnSOD6* (neighbor genes that are most likely recent paralogs), and segmental or whole genome duplications.

The predicted length of RcSOD proteins varied between 152 and 330 amino acids, which is very similar to the variation of protein length in *Arabidopsis* (between 137 and 320 aa) (WANG *et al*., 2016). Furthermore, RcSOD proteins had a calculated molecular mass ranging from 15.2 to 34.9 kDa and a theoretical pI from 4.84 to 7.82 (Supplementary Table S2). According to studies in other species, all CuZnSODs have an acidic character, while Fe-MnSODs have a basic or acidic character (FENG *et al*., 2016; DEHURY *et al*., 2013; ZHOU *et al*., 2017). We found in the present study that most RcSODs have an acidic character, except for RcMnSOD5 (29729.m002354) and RcFeSOD8 (29799.m000633) with theoretical pIs of 7.82 and 7.01, respectively (Supplementary Table S2).

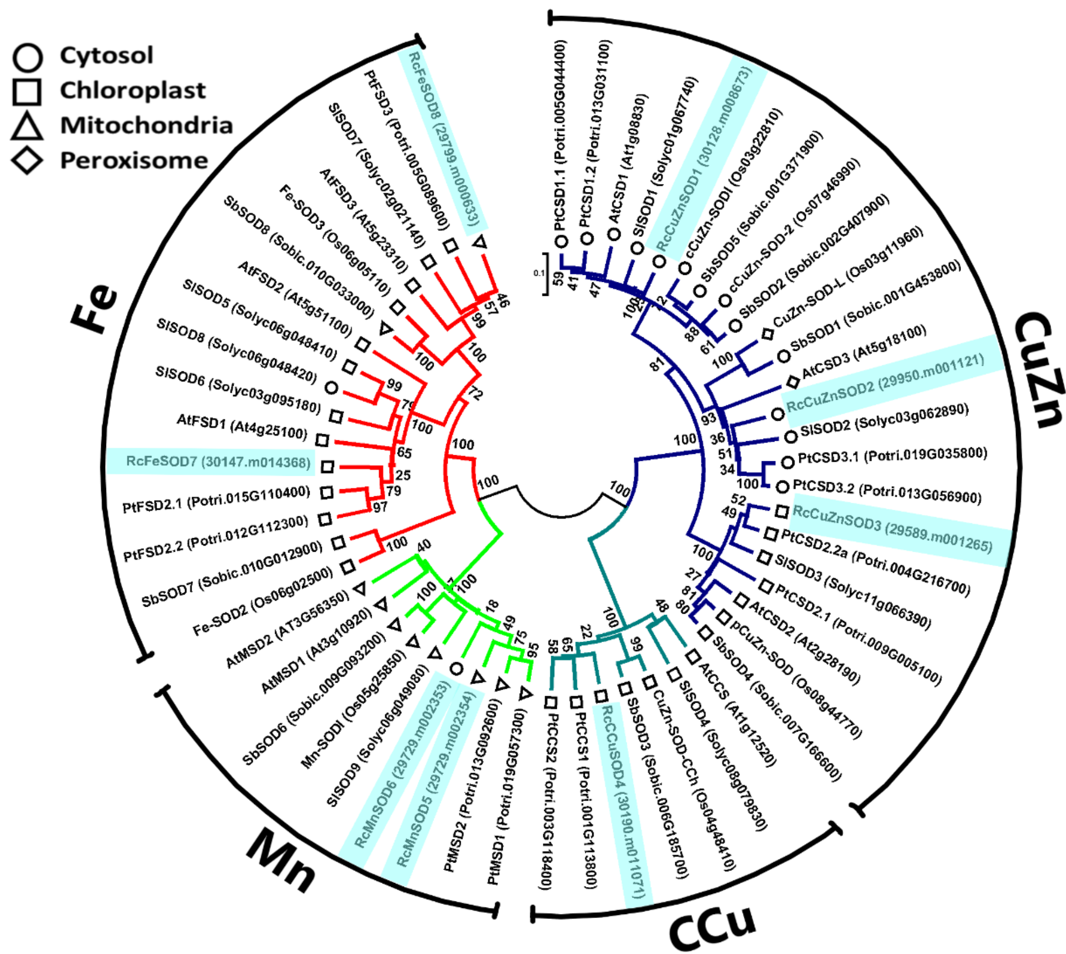

Figure 5. Phylogenetic relationships of SOD genes in angiosperms. SOD groups are classified and distinguished by color. Topology was calculated by Neighbor-Joining method with distances calculated by p-distance. Bootstraps are shown for 1000 replicates. *Ricinus communis* genes are highlighted with blue rectangles. Predicted subcellular localizations are presented for *Ricinus communis* (Rc; this work), *Arabidopsis thaliana (At;* Kliebenstein et al., 1998*), Oryza sativa (Os;* Nath et al., 2014*), Sorghum bicolor (Sobic;* Filiz and Tombuloğlu, 2015*), Populus trichocarpa (Potri;* Molina-Rueda et al., 2013*)* and *Solanum lycopersicum (Solyc;* Feng et al., 2016*)* according to the key on the upper left.

To further characterize RcSODs, we predicted their subcellular localizations. The results showed that RcCuZnSOD3 (29589.m001265), RcCCuZnSOD4 (30190.m011071), and RcFeSOD7 (30147.m014368) are most likely located in the chloroplast, RcCuZnSOD1 (30128.m008673), RcCuZnSOD2 (29950.m001121), and RcMnSOD6 (29729.m002353) in the cytosol, and RcMnSOD5 (29729.m002354) and RcFeSOD8 (29799.m000633) in the mitochondria (Figure 5). We compared our results with the predicted subcellular locations of SOD proteins from *Arabidopsis thaliana* (KLIEBENSTEIN; MONDE; LAST, 1998), rice (NATH *et al.*, 2014), sorghum (FILIZ; TOMBULOĞLU, 2015), poplar (MOLINA-RUEDA; TSAI; KIRBY, 2013) and tomato (FENG *et al.*, 2016). Overall, there was a great consistency of the inferred phylogenetic groups with the subcellular predictions of the proteins (Figure 5). All CCu proteins in the six angiosperm genomes were predicted to be localized in chloroplasts. MnSOD and FeSOD proteins were mostly predicted to be localized in mitochondria (8 out of 9 proteins) and chloroplast (13 out of 16 proteins), respectively. We identified 2 phylogenetic groups of CuZnSOD proteins with distinct subcellular locations. All orthologs of *A. thaliana* AtCSD1 and AtCSD2 were predicted to be localized in the cytosol (9 out of 9) and chloroplast (7 out of 7) respectively. Nearly all orthologs of *A. thaliana* AtCSD3 were predicted to be localized in the citosol (5 out of 7 proteins).

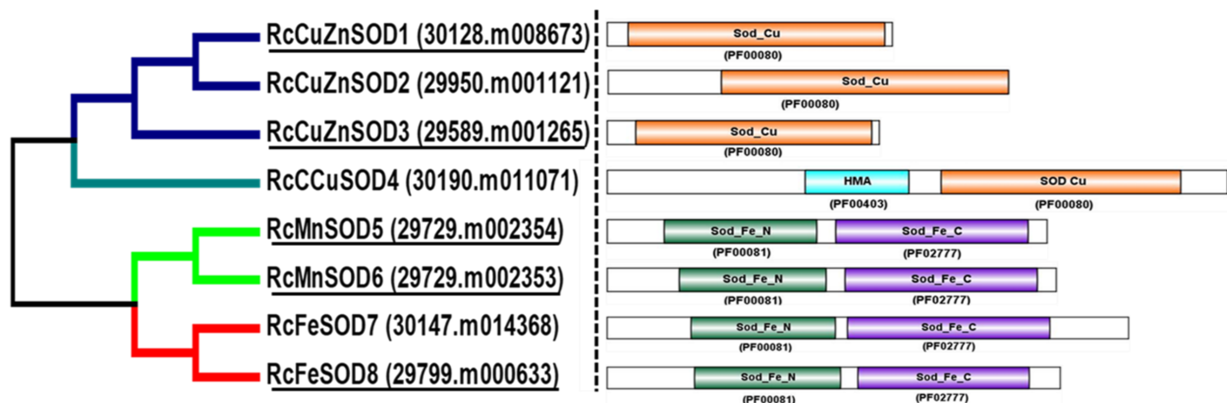

Figure 6. *Ricinus communis* classification of SOD proteins by prediction and functional domains. Cladogram of *Ricinus communis* SOD proteins. SOD groups are classified and highlighted with different colors. Genes whose expression was analyzed are underlined. Functional domains are represented by colored boxes and classified

according to PFam database (http://pfam.xfam.org/). Protein schemes were drawn using IBS 1.0.3 software (http://ibs.biocuckoo.org/).

We then analyzed the *RcSOD* proteins for the presence of Pfam domains (http://pfam.xfam.org/search). The results showed that RcFeSODs and RcMnSODs contain the C-terminal iron/manganese SOD domain (PF02777) and the N-terminal iron/manganese SOD alpha-hairpin domain (PF00081). RcCuZnSODs and RcCCuSOD contain the SOD copper-zinc domain (PF00080) and RcCCuSOD contains an additional heavy-metal associated domain (PF00403) (Figure 6). Taken together, the phylogenetic consistency of protein domains and predicted subcellular locations further support the gene classification present in our phylogenetic analysis (Figure 6).

3.5 Osmotic stress generally decreases the expression level of SOD gene in *R. communis* embryo axis

To have a deeper understanding of the expression dynamics of the RcSOD genes we performed gene expression analyses for a set of RcSOD genes belonging to the 3 main groups of the SOD gene family (Cu/Zn, Fe and Mn) in response to different osmotic potentials during the imbibition time course up to 40 h.

Three RcSOD genes were up-regulated during imbibition in water (0.0 MPa) (Figure 7A,C,D), except for *RcCuZnSOD3* whose expression was not significantly affected (Figure 7B) and *RcMnSOD5* that was repressed during the progression of imbibition in water (Figure 7E). RcMnSOD5 is predicted to be located in the mitochondria and a knockdown of its closest homolog in *Arabidopsis* (*AtMSD1*) showed retarded root growth even under control growth conditions, suggesting that reduced MnSOD affects mitochondrial redox balance and plant growth (MORGAN *et al.*, 2008). Mitochondria are one of major sources of ROS (BAILLY; BAILLY, 2004) and in contrast to Arabidopsis, *R. communis* has two types of SOD proteins predicted to be localized in the mitochondria (RcFeSOD8 and RcMnSOD5) which can be an advantage for reestablishing mitochondrial redox homeostasis. Nevertheless, only *RcFeSOD8* was induced upon imbibition in both *R. communis* genotypes, whereas *RcMnSOD5* was highly repressed in both genotypes (Figure 7C,E). This might indicate that *RcFeSOD8*

as a mitochondrial *RcSOD* gene may be developmentally regulated during seed imbibition to counteract ROS production as a result of reactivation of metabolism and respiration, whereas *RcMnSOD5* might have a function during late stages of seed development and therefor show a sharp decrease during imbibition.

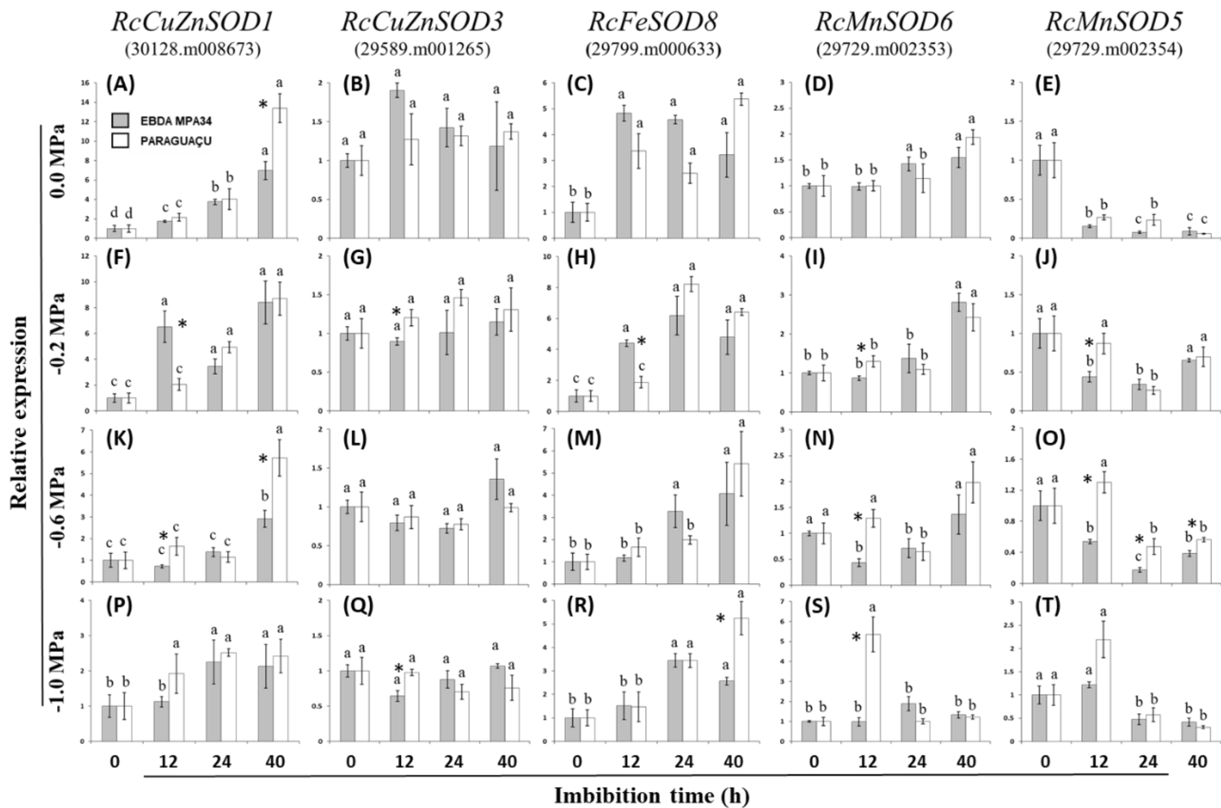

Figure 7. Relative expression of *RcCuZnSOD1*, *RcCuZnSOD*3, *RcMnSOD*5, *RcMnSOD6*, and *RcFeSOD8* in *Ricinus communis* embryo axis during seed imbibition in different osmotic potentials. Expression data for target genes are presented for intact non-osmoconditioned dry seeds (0 h) and seeds imbibed in water (12, 24 and 40 h, at 0.0 MPa) (A-E), and for seeds imbibed in PEG solutions (12, 24 and 40 h, osmoconditioning at -0.2 (F-J), -0.6 (K-O) and -1.0 MPa (P-T)). Expression values were calculated by $2^{-\Delta\Delta CT}$ method and are relative to the expression in dry seeds (0 h). Mean values with standard error are shown for 3 biological replicates of 50 embryo axis each. Different letters indicate significant differences between data points (Scott-knott

test at $p < 0.05$), whereas (*) indicates significant differences between the genotypes at each imbibition time according to least significant difference (LSD, *t*-test at $p < 0.05$).

The exposure of seeds to osmotic stress generally limited the ability of RcSOD genes to be induced, leading to lower expression values as seen for the cytosolic *RcCuZnSOD1*, for which the limitation of gene induction was related to the intensity of the stress (Figure 7A,F,K,P). Overexpression of the homologous tobacco *CuZnSOD* promoted seed germination and enhanced tolerance to mild water stress (FAIZE *et al.*, 2015), suggesting that the overexpression of *RcCuZnSOD1* could have beneficial effect on germination and water restriction stress tolerance also.

Different from its repression in water, *RcMnSOD5* was not progressively repressed during the imbibition time course under osmotic stress (Figure 7J,O,T). *RcCuZnSOD3* was not much affected by the osmotic stress, showing no significant expression differences in any of the conditions (Figure 7B,G,L,Q).

In general, the patterns of expression were similar for both cultivars except that PARAGUAÇU often reached higher expression level differences (Figure 7), leading to most of the significant variations between cultivars. *RcCuZnSOD1* and *RcFeSOD8* were more quickly induced in EBDA MPA34 after 12 h when seed were exposed to imbibition at lighter osmotic stress of -0.2 MPa (Figure 7F,H). This quicker induction was inhibited by the lower osmotic potentials of -0.6 and -1.0 MPa (Figure 7K,M and P,R).

Osmopriming can induce the expression of antioxidant enzymes that help seeds in the germination process (BAILLY *et al.*, 2000; BAILLY; BAILLY, 2004; CHEN; ARORA, 2013). We found that PARAGUAÇU was able to achieve a better physiological profile when seeds were osmoprimed (-1.0 MPa), whereas the analysis of gene expression in the same conditions revealed that the SOD genes were in general repressed (Figure 7P,Q,R,S,T) when compared to the imbibition of non-osmoprimed seeds (Figure 7A,B,C,D,E). The overall expression of SOD genes was repressed when seeds were imbibed in PEG-solutions (Figure 7) which was coupled with a higher total SOD activity for some data points during imbibition, i.e. at the lowest osmotic potential of -1,0 MPa (Figure 4D). Herein, we suggest that the better performance of cv.

PARAGUAÇU seedlings in response to seed osmopriming and the absence of this response in EBDA MPA34 might be related to a higher expression of *RcFeSOD8* at 40h of imbition under osmoticum especially in comparison to its expression in EBDA MPA34 (Figure 7R).

5. CONCLUSIONS

We showed that *R. communis* seed germination is highly susceptible to disruption of water uptake caused by osmotic stress. This stress is known to increase the formation of ROS in seeds and because of that we investigated total SOD activity, gene repertoire and expression in response to different levels of osmotic stress. Our findings highlighted differences between genotypes in terms of SOD activity, gene expression and physiological parameters of germination and seedling vigor. We demonstrate that seed osmoconditioning was able to increase seedling physiological parameters in cv. PARAGUAÇU but not in cv. EBDA MPA34, suggesting that genetic factors are involved in seed osmoconditioning response. One of these factors can be the increased ability of some cv. PARAGUAÇU SOD genes to respond to stress. This makes SOD genes an interesting possible target for future biotechnological improvement of this important oilseed crop. Further studies are needed to better clarify the molecular basis of the effect of seed osmoconditioning in *R. communis* and its possible ecological relevance. However, our results highlight the agricultural benefits of osmoconditioning when inducing priming effects for better success for small-scale family farmers cropping systems in semiarid regions worldwide as well as for large-scale crop production and opens the possibility to further improvement of this technique for the *R. communis* industry.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DECLARATION OF EACH AUTHOR'S CONTRIBUTIONS

VGN executed all the experimental assays, data acquisition, statistical analysis, and wrote the manuscript; PRR, DTB and LGF designed the biochemical and molecular experiments, interpretation and analysis; LEDB and VGN designed all the bioinfomatics approaches, interpretation and analysis; STCL and RDC designed all the physiological experiments, interpretation and analysis; RDC was responsible for the conception, integrity and funding of the work as a whole; WL and all authors read, critically revised, and agreed with the content of the manuscript.

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CAPÍTULO 2: The *Ricinus communis* superoxide dismutase gene family response to heat stress during germination and seedling stages

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The *Ricinus communis* superoxide dismutase gene family response to heat stress during germination and seedling stages

ABSTRACT

High temperatures affect crop production in many countries across the globe. Abiotic stresses such as heat can increase the production of reactive oxygen species (ROS), which then lead to subsequent oxidative stress. Plant's response to high-temperature stress involves the antioxidant enzymes system. The antioxidant Superoxide dismutase (SOD) enzyme is the first to act in the line against oxidative stresses by catalyzing the reaction of superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2). SOD can be classified according to its metal cofactors (Cu/Zn, Mn, or Fe) and respective specific subcellular localizations of SOD genes in the cytosol, mitochondria, chloroplast, peroxisome, or the nucleus. *Ricinus communis* have eight SOD genes (*RcSOD*), three Cu/Zn, one Chaperone Cu, two Mn, and two Fe, while being an important oilseed crop species cultivated in Brazil mainly by small family farmers in its Northeast semiarid region, where abiotic stress conditions are common. Therefore, our objective in the present study was to investigate how *RcSOD* genes are modulated n different tissues by heat stress aimed at the adjustment of the ROS balance and promote abiotic stress tolerance during seed imbibition and germination and further development of *R. communis* seedlings by analyzing the structure of RcSOD genes, promoter regulation sites and miRNA. The expression of *RcSOD* genes was evaluated along imbibition and germination (dry seeds, early imbibition, radicle protrusion, and seedlings with 2 cm root), and of growing seedlings (in roots and cotyledons at 3, 6, and 9 days; and in different dimensions of leaves: L1, L2, and L3) under different temperatures (20, 25°C, and 35°C). Green fluorescent protein (GFP) fusion in *Nicotiana benthamiana* leaves was applied for subcellular localization and better understanding of how *RcSOD* genes act in cells. *RcSOD* genes demonstrated a pattern of regulation under heat stress that varies among tissues during germination. *RcCuZnSOD1* and *RcFeSOD8* genes were upregulated during early imbibition under heat stress (35°C) while the *RcCuZnSOD3, RcCCuSOD4, RcFeSOD7* genes were regulated during radicle protrusion (germination *per se*) and young seedlings. The *RcMnSOD5* and *RcMnSOD6* genes were downregulated according to the time point of development. In seedling roots, the *RcCuZnSOD* group and *RcFeSOD8* showed upregulation, while the *RcMnSOD5*, *RcMnSOD6,* and *RcFeSOD7* did not change or were downregulated at 35°C, whereas a similar pattern was observed in cotyledons. In general, *RcCuZnSOD* showed an increase in the expression levels according to time point in development of cotyledons. *RcMnSOD5*, *RcMnSOD6,* and *RcFeSOD7* demonstrated high levels of expression in cotyledons of 3 days but were downregulated at 6 and 9 days, while the expression of *RcFeSOD8* not change. In leaves, the *RcCuZnSOD* group and *RcFeSOD8* were downregulated according to the size of leaves while the genes *RcMnSOD5, RcMnSOD6*, *RcFeSOD7* demonstrated an increase of expression or no change. The promoter analysis showed that expression of *RcSOD* genes can be influenced by

hormones (ABA, Gibberillin, MeJa, Salicylic acid), by Transcription factors as the MYB and MYC, circadian control, meristem expression, light, anaerobiosis, low temperature, and defense against stress. The miRNA analyzis showed that *RcCuZnSOD3* and *RcCCuSOD4* can be repressed by Rcmi-RNA398a-b. Overall, the different patterns of *RcSOD* expression under heat stress in different tissues can be explained by the different forms of regulation in the promoter region and regulation by miRNAs, whereas the subcellular localization showed that the *RcSOD* genes have cytosol, chloroplast, nucleus and mitochondria localization. Our results contribute to understanding the SOD family in *R. commmunis* and their possible use as molecular markers in *R. communis* breeding.

Keywords: Castor bean, heat stress, superoxide dismutase, transient expression, GFP fusion, agroinfiltration.

INTRODUCTION

Abiotic stresses are a major cause of loss of production in commercial crops (CALANCA, 2017). Plants under stress conditions may lead to the overproduction of reactive oxygen species (ROS) causing damages to cells (CHOUDHURY *et al.*, 2017; PANDEY *et al.*, 2017). Various metabolic pathways generate ROS in different compartments as in mitochondria, chloroplasts, and peroxisomes (DEL RÍO *et al.*, 2006; FILIZ *et al.*, 2019; NAVROT *et al.*, 2007). Plants have different mechanisms to abiotic stress response, involving physiological, morphological, biochemistry, and molecular changes (FRAIRE-VELAZQUEZ; EMMANUEL, 2013; HASANUZZAMAN *et al.*, 2013). Transcriptomic analysis in *Arabidopsis thaliana* reveals that antioxidant enzymes are regulated by heat stress and can help plants in abiotic stress tolerance (FILIZ *et al.*, 2019). The antioxidant enzyme superoxide dismutase (SOD) constitutes one of the major mechanisms of response to ROS, being a metalloprotein and the first in the line of action against ROS by catalyzing the reaction of superoxide (O_2-.) transformation into hydrogen peroxide (H_2O_2). SODs are known to have a natural role in plant growth and development and it is associated with tolerance against abiotic stress conditions (GILL *et al.*, 2015; SINGH *et al.*, 2019).

The SOD family in plants can be classified into three groups according to the metal cofactor (Cu/Zn, Mn, and Fe). The genes coding to Fe and Mn are more ancient than Cu/Zn genes. *CuZnSOD* genes are found only in eukaryotes (BOWLER *et al.*, 1994), being commonly found in cytosol, chloroplast, peroxisome, and nucleus, while *MnSOD* genes are commonly found in mitochondrias and *FeSOD* in chloroplasts (KLIEBENSTEIN; MONDE; LAST, 1998). Some studies of prediction of the regulation in the promoter region of SOD genes showed sites resonsive to light, hormones as ABA, gibberellin, salicylic acid, jasmonic acid, and sites to MYB and MYC transcription factors (FENG, KUN *et al.*, 2016; FENG, XIN *et al.*, 2015a; VERMA; LAKHANPAL; SINGH, 2019; WANG *et al.*, 2017; ZHOU, YONG *et al.*, 2017). The large and diverse types of

regulation for SOD genes suggest that these genes have a complex form of regulation involving hormones and transcription factors signalization. The *CSD1*, *CSD2,* and *CCS* SOD genes are localized in three different compartments in *A.thaliana* and are repressed by micro RNA (miR398)(ZHU; DING; LIU, 2011), possibly linked to the regulatory mechanisms to plant response to abiotic stress (GUAN *et al.*, 2013; JIA *et al.*, 2009; JOVANOVIĆ *et al.*, 2014; TRINDADE *et al.*, 2010; YAMASAKI *et al.*, 2007; ZHU; DING; LIU, 2011).

Ricinus communis have eight SOD genes, three *CuZn-SOD*, one *CCu-SOD*, two *Mn-SOD,* and two *Fe-SOD*. The *RcCuZnSOD1* and *RcFeSOD8* showed to be upregulated by imbibition time and osmotic stress during the germinative process suggesting that these genes can be involved in oxidative stress response during germination. Besides, the cv. Paraguaçu of Ricinus communis (wich responded positively to priming osmotic pretreatment) showed high levels of *RcCuZnSOD1* and *RcFeSOD8* expression when compared to the MPA34 genotype (wich did not respond to the osmotic priming pretreatment) during germination and during the osmotic priming pretreatment seeds of *R. communis* (GOMES NETO *et al.*, 2018).

Ricinus communis is a worldwide important oilseed crop, which in Brazil has also a relevant socio-economic role since it is mostly cultivated rudimentarily by small family farmers in the Northeastern semiarid region of Brazil where heat stress is a common condition. Thereby, the objectives of the present study were to investigate the regulation of *RcSOD* genes by prediction of cis-elements in the promoter region, prediction of miRNA in *RcSOD* coding region, subcellular localization by GFP fusion in *Nicotiana benthamiana* leaves, and analysis of *RcSOD* gene expression in embryonic radicles during seed germination and in cotyledons and leaves of growing seedlings under heat stress conditions. Therefore, expecting a better understanding of the regulation of *RcSOD* genes and their possible application as molecular markers in Ricinus communis genetic engineering or breeding programs of *Ricinus communis*.

2. MATERIAL AND METHODS

2.1 Gene structure and conserved motif analysis

The complete set of genomic, CDS, and protein sequences of *RcSOD and AtSOD* were used from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html#). The gene structure analysis was performed in Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/). Conserved motifs analysis was performed by the Multiple Em for Motif Elicitation (MEME Suite 5.0.1) (http://meme-suite.org/). The parameters used were minimum motif width = 6, maximum motif width = 200, and the maximum number of motifs to identify was 10.

2.3 Prediction of *RcSOD* regulatory elements

The 1000bp sequence from ATG obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#) was used for the cis-element analysis of *RcSOD* genes performed by the Plant care database (http://bioinformatics.psb.ugent.be /webtools/ plantcare/html/). For miRNA, the CDS of *RcSOD* genes was loaded and submitted against *R. communis* (63 published miRNA) by psRNATarget (http://plantgrn.noble.org/psRNATarget/). The miRNA database was from a previously published article (XU *et al.*, 2013).

2.3 Sample collection for gene expression analysis

The complete set of *RcSOD* genes was analyzed by gene expression in seeds and seedlings submitted to different temperatures (20, 25 and 35 °C) for 7d (RIBEIRO *et al.*, 2015a), by collecting samples in different stages of seed imbibition and germination, i.e. dry seeds, 6h of imbibition, radical protrusion (RP), and 2 cm roots of young seedlings (R2) (RIBEIRO *et al.*, 2015b). And also in root and cotyledon samples collected after 3, 6 and 9 days of seedling growth along with samples from leaves of different dimensions: L1 (3x2 cm), L2 (7x5 cm) e L3 (11x7 cm) (RIBEIRO *et al.*, 2014b).

2.3 RNA extraction and cDNA synthesis

The total RNA was obtained from 5 mg of the lyophilized and ground samples using the hot borate method with modifications (GOMES NETO *et al.*, 2018). The RNA

integrity was evaluated on 1% agarose gel, and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesized using the Bio-Rad iScript™ cDNA synthesis kit according to the manufacturer's instructions. For qRT-PCR analysis, the cDNA was diluted 20 times.

2.4 Gene expression analysis

Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) was used for primer design using melting temperatures (Tm) of 59–61°C, primer lengths of 18–22 bp and amplicon lengths up to 150 bp (Supplementary Table S1). The standard curve by two-fold serial dilution using a pooled cDNA sample was used to evaluate primer efficiency. The primers specificity was verified by normal PCR and the amplicon size on 2% agarose gel. qRT-PCR was performed in a total volume of 10 µL containing 2.5 µL of cDNA (20x diluted), 0.5 µL of primers (10 µM), 5 µL of IQ SYBR Green Supermix (Bio-Rad), and 2 µL water. qRT-PCR assays were run on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). All PCR reactions were run at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melting curves were obtained after this program by heating samples from 65 to 95 °C, increasing the temperature stepwise by 0.5 °C every 5 s (RIBEIRO; LIGTERINK; HILHORST, 2015). Serine/threonine phosphatase 2A (PP2AA1) and Actin genes were used as an internal reference (CASSOL *et al.*, 2016; RIBEIRO *et al.*, 2014a). Statistical significance was evaluated with test t at $p<0.05$.

2.5 Cloning of *RcSOD* genes

Cloning of *RcSOD* genes was performed using the Gateway® technology according to the manufacturer's instructions. Full-length coding regions were amplified from *R. communis* cDNA samples without stop codon. Primers used and additional information about the cloned genes are presented in Supplementary Table S2. Amplified products were recombined in the donor vector pDONr207 (supplementary Figure 1). These plasmids were transferred to *Escherichia coli* (DH5α) cells by electroporation. Transformed single colonies were inoculated in 4 mL LB (Lysogeny Broth) medium containing gentamycin (25 µg/mL) overnight at 37 °C. PCR was used to select positive

colonies to pDONr207-*RcSOD* and the plasmids were isolated using the NucleoSpin Plasmid EasyPure (Macherey-Nagel) according to the manufacturer's instructions and the Correct reading frame was confirmed by sequencing. Thereafter, the plasmids were recombined by LR reaction (Invitrogen) in the gateway binary vector pGWB605 carrying CaMV35S (CaMV—cauliflower mosaic virus), and vectors pGWB605-*RcSOD* were transferred to *Escherichia coli* (DH5α) cells by electroporation. pGWB605-*RcSOD* were digested by restriction enzymes and *A. tumefaciens* (Agl0) cells were transformed by electroporation (supplementary figure 2; Nakamura et al., 2010). Transformants were selected on LB (Lysogeny Broth) medium supplemented with spectinomycin (100 µg/mL) and rifampicin (25 µg/mL) for 48 h.

2.6 Subcellular localization of *RcSOD* genes fused by GFP in *Nicotiana benthamiana* leaves

Four weeks grown *N. benthamiana* plants were selected for the agroinfiltration process in two plant repetitions to each *RcSOD* genes (Supplementary Figure 3). *A. tumefaciens* culture with pGWB605-*RcSOD* was grown up in 5 mL of LB (Lysogeny Broth) medium supplemented with spectinomycin (100 µg/mL) and rifampicin (25 µg/mL) for 24 h and inoculated in 15 mL of the same LB medium to more 24 h. Cultures were centrifuged 15 min 4000 RPM and the supernatant was discarded. Pellets were resuspended in buffer with $MgCl_2$ (500mM), MÊS-KOH (500mM), acetosyringone (100mM) in pH 5.7, and OD was adjusted to 0.5 nm. The cultures were shaked for 2 h before agroinfiltration, and 1 mL syringe was used to infiltrate *A.tumefaciens* in the abaxial area of *N. benthamiana* leaves. Infiltrated leaves were colletc after two days and leaf samples prepared and analyzed in a Leica fluorescent microscope (Supplementary Figure 3; ARTUR *et al*., 2019).

3. RESULTS AND DISCUSSION

3.1 Gene structure and conserved motif analysis

The number of introns and exons and UTR's region of *RcSOD* and *AtSOD* genes were analyzed by submitting the complete set of genomic and CDS sequences to Gene

Structure Display Server 2.0 software (http://gsds.cbi.pku.edu.cn/). *RcSOD* genes contain different intron/exon numbers and positions between groups (Cu/Zn, Mn, and Fe). In general, the exon/intron number of *AtSOD* and *RcSOD* genes were similar in the Mn and Fe groups, while similar exon/intron numbers were found in *RcCuZnSOD3* and *RcCCuSOD4* genes representing the CuZn group. The genes *RcCuZnSOD1* (6 exons) and *RcCuZnSOD2* (5 exons) presented different exon/intron numbers compared to *A. thaliana* orthologs *AtCSD1* (7 exons) and *AtCSD3* (7 exons). The exons four and five of *AtCSD1* were fused in *RcCuZnSOD1* genes, while the exons five, six, and seven in *AtCSD3* were fused in *RcCuZnSOD2*. The *RcMnSOD6* gene presented a split in the exon one compared to *AtMSD1* and *RcMnSOD5* genes. The AtFSD1 gene presented the exons two and three excluded compared to *RcFeSOD7* and *AtFSD2*. The *PtCSD1.1* and *PtCSD1.2* in *Populus trichocarpa* present the same fusion in exon four of *RcCuZnSOD1* gene showing that the fusion does not occur only in *R. communis* species but the number of the exons of the *AtCSD3* gene is similar to the *PTCSD2* gene in *P. trichocarpa* is similar showing that the fusion of the exons five, six and seven in *RcCuZnSOD2* is possible to be exclusive to *R. communis* (MOLINA-RUEDA; TSAI; KIRBY, 2013).

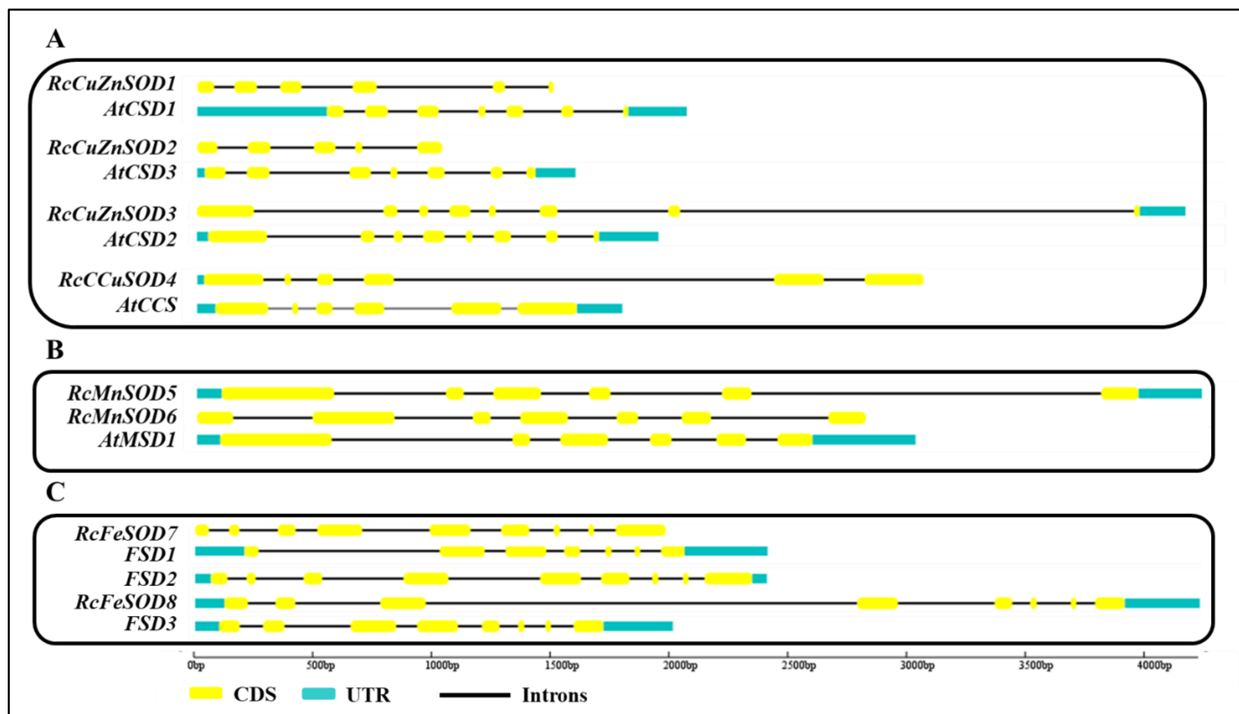

Figure 1. Gene structure analysis of *SOD* genes for *R. communis* and *A.thaliana*. Genomic and CDS sequences of *RcSOD* SOD genes were analyzed in Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/). **A** Gene structure of *CuZnSOD* genes. **B** Gene structure of MnSOD genes. **C** Gene structure of FeSOD genes.

Conserved motifs were identified by using The Multiple Expectation Maximization for Motif Elicitation (MEME). Ten putative motifs were found in *RcSOD genes* (Figure 2). The genes of *RcCuZn* group contained motifs 1, 2, and 5 except for the *RcCuZnSOD2* gene in which the motif 1 was excluded. The gene *RcCCuSOD4* contained motif 2 similar to the CuZn group and motif 10 similar to *MnSOD* genes. In *A. thaliana* the CCS gene is a chaperone Cu gene, described to deliver Cu ions to CuZn genes (HUANG *et al.*, 2012). The genes of *RcMnSOD* group contained motifs 1, 3, 4, 7, 8 e 10. The genes of *RcFeSOD* group present motifs 1, 3, 4, 6, 8, and 9. The pattern of motifs was different when comparing the presence of motifs in all SOD groups. However, motifs 1 to 7 were identified within three SOD domains by the Pfam database. Motif 1, 2, and 5 presented the SOD Cu domain (PF00080), motif 1, 3 presented the domain SOD_Fe_(C -PF02777), and the motifs 4, 6, and 7 presented the domain SOD Fe (N -PF00081) (Supplementary Table 4).

In general, all SOD genes presented motif 1 except for a group of chaperone Cu genes (*AtCCS* and *RcCCuSOD4*). Motif 1 was identified by two Cu (PF00080) and one Fe (C -PF02777) domains suggesting that motif 1 is important to all SOD groups. The genes of the Mn and Fe groups shared motifs 1, 3, 4, and 8. The genes of Mn and chaperone Cu genes shared the motif 10. In general, the *AtSOD* and *RcSOD* genes shared the same motifs except for *RcCuZnSOD2* gene in which motif 1 was absent (Figure 2).

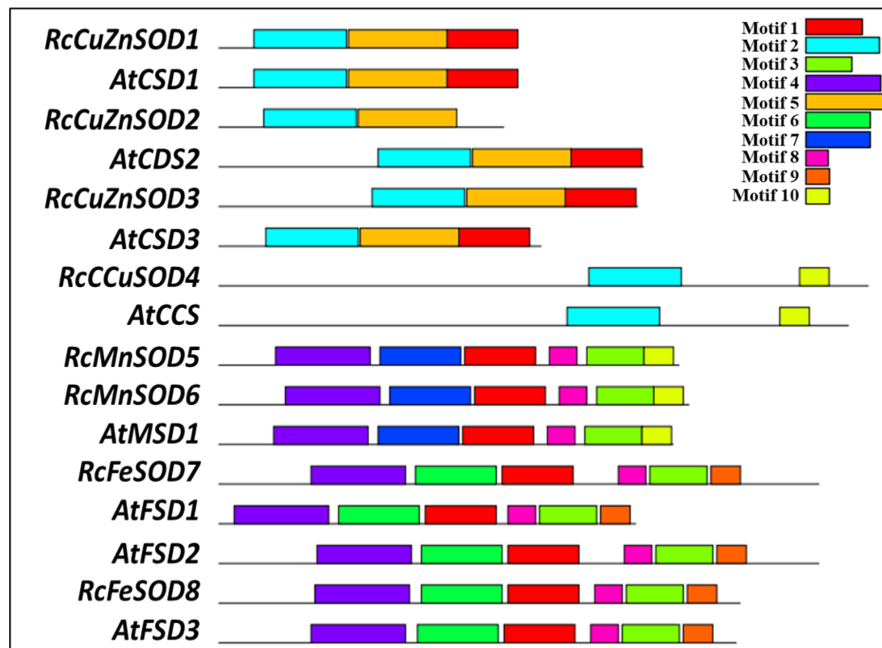

Figure 2. Conserved motif analysis of *RcSOD* and *AtSOD* genes. The full length of amino acid sequences was analyzed by The Multiple Expectation Maximization for Motif Elicitation (MEME). Different motifs are shown in different colors.

3.2 Prediction of *RcSOD* regulatory elements

The promoter region of *RcSOD* genes was analyzed by the PlantCARE database. Possible sites of regulation to transcription factors were investigated using 1kb from ATG. The possible sites of regulation were separated into subgroups: low temperature, salicylic acid, circadian control, defense and stress, gibberellin, MYB, MIC, meristem expression, light, methyl jasmonate (MeJa), anaerobic induction, and abscisic acid (ABA) (Figure 3). In general, the light response showed in 8 to 8 genes, and the number of light sites change between 1 to 9. The large amounts of light-responsive cis-elements on the promoter region of *RcSOD* may contribute to light stress resistance. The transcription factors MYB and MYC were found in 7 to 8 and 6 to 8 *RcSOD* genes. The MYB and MYC transcription factors are associated with abiotic and biotic stress responses (LI; NG; FAN, 2015; ROY, 2016). The abundant presence of MYB-related cis-elements on the *RcSOD* promoter region indicates that it may involve *RcSOD* gene expression regulation. The MYC transcription factor is a positive regulator of jasmonate

biosynthesis genes in *A. thaliana* (ZHANG; MEMELINK, 2016). MeJa is described to interact with other phytohormones involved in growth, development, and abiotic stress response (Per et al., 2018). MeJa was predicted to regulate the *RcCuZnSOD1, RcMnSOD5,* and *RcMnSOD6* showing to be possible involvement in SOD regulation.

The *RcCuZnSOD1, RcMnSOD5, RcFeSOD7* and *RcFeSOD8* have sites for ABA, which is an important hormone involved in abiotic stress response, regulating TF and miRNAs involved in abiotic stress response (CHO; VON SCHWARTZENBERG; QUATRANO, 2018). The genes *RcCuZnSOD2, RcCuZnSOD3,* and *RcCCuSOD4* presented sites for gibberellins in the promoter region, which are hormones involved in plant developmental processes, including seed germination, stem elongation, leaf expansion, pollen maturation, and the development of flowers, fruit, and seeds (ACHARD *et al.*, 2004; HEJÁTKO; HAKOSHIMA, 2018; ZHOU, CHENGZHE *et al.*, 2019).

Only the gene *RcFeSOD7* presented the salicylic acid regulation site. The genes *RcCuZnSOD2, RcMnSOD5, RcFeSOD7,* and *RcFeSOD8* presented the meristem expression regulation and the genes *RcCCuSOD4* and *RcFeSOD8* presented the circadian control regulation. The presence of a large number of elements in the promoter region of SOD genes has been described in other species as *Solanum lycopersycum* (FENG, KUN *et al.*, 2016), *Musa acuminata* (FENG, XIN *et al.*, 2015b), *Gossypium hirsutum* (WANG *et al.*, 2017), *Cucumis sativus* (ZHOU, YONG *et al.*, 2017), *Brassica juncea* and *B. rapa* (VERMA; LAKHANPAL; SINGH, 2019), suggesting that *SOD* genes should be involved in distinct regulatory mechanisms in response to stress and during development.

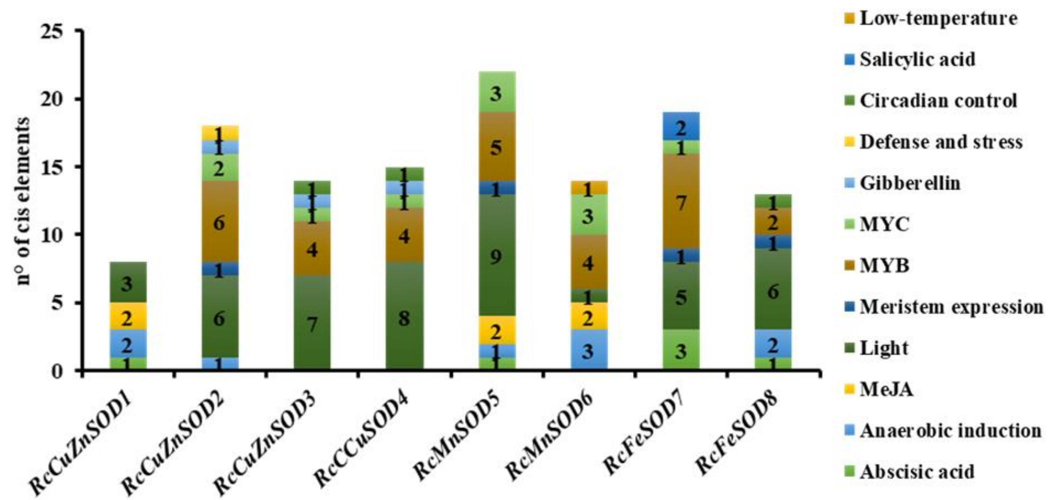

Figure 3. Cis element analysis of *RcSOD* genes. The promoter region of the *RcSOD* was analyzed in the Plant care database. Different cis-elements with the same or similar functions are shown in the same color.

The miRNA prediction was analyzed to CDS sequence of *RcSOD* genes using the RcmiRNAs for understanding the *RcSOD* gene expression regulation pattern at the posttranscriptional level. The prediction shows that four miRNAs (miR319a-d, miR159, miR398a-b and miR408) regulate four *RcSOD* genes (*RcCuZnSOD2, RcCuZnSOD3, RcCCuSOD4* and *RcFeSOD7*) (Figure 4; Supplementary Table 5). The miR319a-d showed a target to the *RcCuZnSOD2* gene. The miR159 showed a target to *RcCuZnSOD2* and *RcCuZnSOD3*. The miR398a showed target only to *RcCCuSOD4* while miR398b showed target to *RcCCuSOD4* and *RcCuZNSOD3*. The miR408 showed a target to *RcFeSOD7*. The miRNAs of plants can be involved in the regulation of the plant growth processes, hormone signaling, and biotic/abiotic stress response by repressing the target gene's expression (ZHANG, 2015).

In *A. thaliana*, the expression of miRNAs appears to depend on the type stress treatment. Drought stress-induced the miRNA (miR319, miR408) while salinity stress induced the expression of miR319, miR159, while significantly inhibiting the expression of miR398 (LIU, HAN HUA *et al*., 2008). Some studies suggest that miR159 can be regulated by ABA, whereas gibberellins (GA) regulated miR159 expression and

controlled floral organ development (ACHARD *et al.*, 2004), while downregulating miR398 (JIA *et al.*, 2009). In rice, miR319 was induced in ABA-mediated responses (LIU *et al.*, 2009). The miRNAs (miR169, miR319a.2, and miR408-5p) were demonstrated to be H_2O_2-responsive (YAMASAKI *et al.*, 2007). Heat stress-induced the expression of miRNA398 and a reduced level of miR398 led to improved tolerance of transgenic lines compared with the wild type plants under oxidative stress conditions (GUAN *et al.*, 2013; ZHU; DING; LIU, 2011).

The miR398 is described in *A. thaliana* to repress three SOD genes, two *CuZnSOD,* and one chaperone SOD (*CSD1, CSD2,* and *CCS*). Transgenic plants expressing miR398-resistant versions of *CSD1*, *CSD2,* or *CCS* were hypersensitive to high temperatures and the expression of some genes of HSP and HSF genes was reduced by heat stress. In contrast, in mutant plants, *csd1,* and *csd2* the expression levels of HSF and HSP genes increased under heat stress, and *csd1*, *csd2,* and *ccs* plants were tolerant to heat stress compared to wild type (GUAN *et al.*, 2013).

The RcmiR398b demonstrated to repress the *RcCuZnSOD3* and *RcCCuSOD4* genes (Figure 4). The results indicate that the regulation of miRNA398 was similar in both species, but only two *RcSOD* genes showed to be regulated by miRNA398, while three SOD genes were regulated in *A. thaliana* (*CSD1, CSD2,* and *CCS*) (ZHU; DING; LIU, 2011). The isoform Rc-miR398b in *R.communis* was higher than isoform Rc-miR398a in leaves, roots, and seeds, suggesting one evidence that these isoforms can be acting regulating the *RcCuZnSOD3* and *RcCCuSOD4* genes (XU *et al.*, 2013).

Figure 4. Cleavage sites mapping of *RcSOD* genes by miRNAs. *RcSOD* genes that present a target to miRNAs are indicated**.** Blue rectangles are exons, black lines are introns and red rectangles are UTR's regions. **A)** rco-miR319a-d and rco-miR159 Target *to RcCuZnSOD2***. B)** rco-miR159 and rco-miR398b target to *RcCuZnSOD3***. C)** rco-miR398a-b target to *RcCCuSOD4***. D)** rco-miR408 target to RcFeSOD7**.**

3.3 Modulation of *RcSOD* genes during germination and seedling establishment in response to abiotic stress

The *RcSOD* genes showed different patterns of gene expression during germination under the different temperature conditions (cold stress at 20°C, normal condition at 25°C, and heat stress at 35°C). The genes *RcCuZnSOD1* and *RcFeSOD8*

were upregulated during early imbibition under heat stress (35 °C). The upregulation of *RcCuZnSOD1* and *RcFeSOD8* were also observed in *R. communis* embryos of seeds imbibed in osmotic solutions (GOMES NETO *et al.*, 2018). The genes *RcCuZnSOD2*, *RcCCuSOD4, and RcFeSOD8* were upregulated during radicle protrusion (germination *per se*). And the genes *RcCuZnSOD3, RcCCuSOD4, RcMnSOD5, RcMnSOD6,* and *RcFeSOD7* were induced in young seedlings with 2 cm root (Figure 5). In tomato, the expression profile of *SlSOD1, SlSOD2, SlSOD5, SlSOD6, SlSOD7,* and *SlSOD8* genes were upregulated by drought stress while the *SlSOD1*, *SlSOD3,* and *SlSOD4* genes were upregulated by salt stress (FENG *et al.*, 2016).

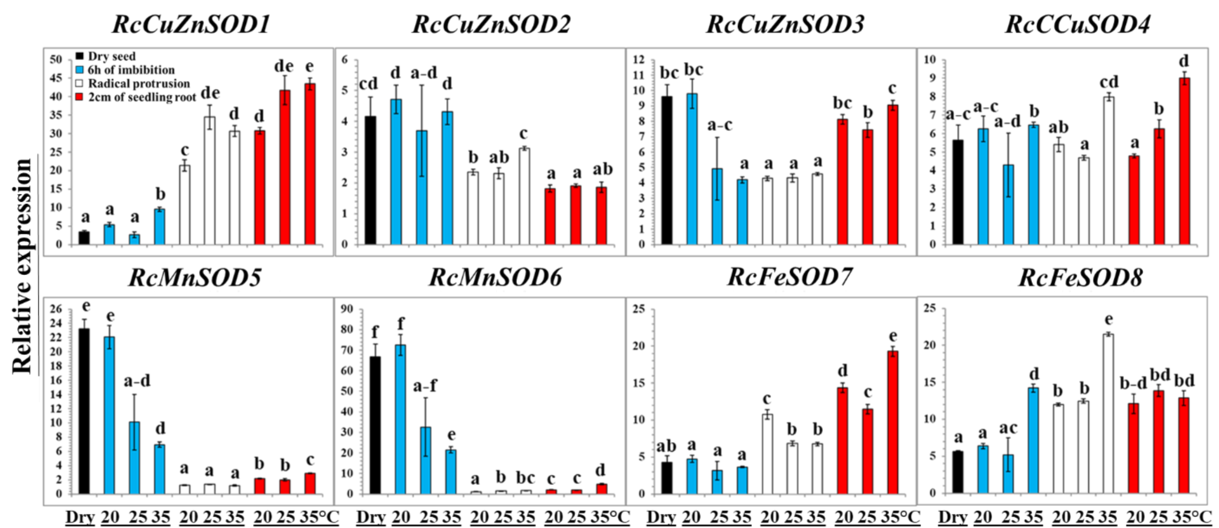

Figure 5. *RcsSOD* gene expression during germination at different temperatures. Samples were collected at dry seeds, early imbibition, radicle protrusion (RP, blue bars), and early seedling development (R2, white bars) at three different temperatures (20, 25, and 35°C). Means and standard errors of three biological replicates containing 15–20 seeds each are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p < 0.05$).

We also assessed the *RcSOD* gene expression in the roots of seedlings under the same temperatures (20, 25, and 35°C). In general, all *RcCuZnSOD* genes were upregulated, whereas genes *RcMnSOD5* and *RcFeSOD7* were not induced, while *RcMnSOD6* was downregulated at 35°C. The *RcFeSOD8* was the only one of Fe group induced at 35°C (Figure 6). The specific SOD genes were upregulated in *A.thaliana*

(*AtCSD2*, *AtMnMSD1*, *AtFSD1*) and *O. sativa* (*OsCSD4*, *OsMSD1,* and *OsFSD1*) in two weeks seedlings in response to cold, heat, drought, and salt stresses. In Arabidopsis, the gene *CSD3* and *CCS* genes showed to be upregulated in roots under heat stress while the major part of OsSOD genes was upregulated in heat stress compared to other stresses (YADAV *et al.*, 2019).

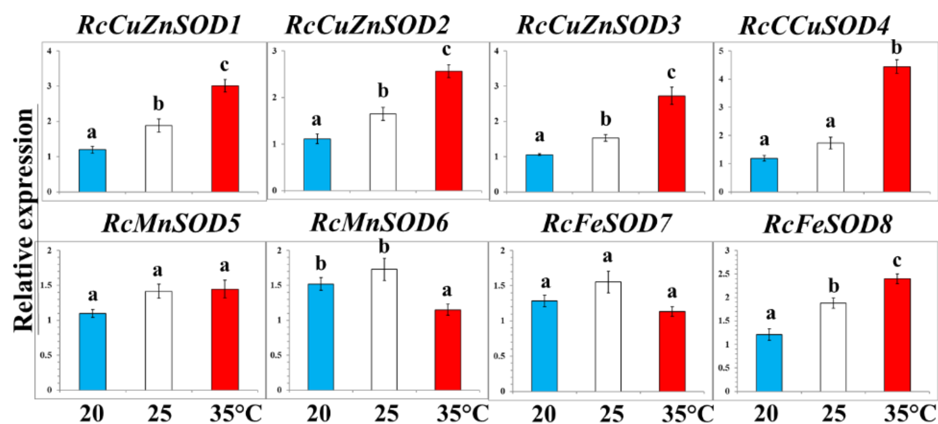

Figure 6. *RcSOD* gene expression in roots of seedlings grown in different temperatures. Means and standard errors of three biological replicates containing 15–20 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p < 0.05$).

We also assessed the *RcSOD* gene expression in cotyledons (3, 6 and 9 days) and in leaves of different dimensions (L1, L2, L3) during seedling growth under the same different temperatures (20, 25, and 35°C). The genes of *CuZnSOD* group showed an increase of the expression in cotyledons while the genes *RcMnSOD5, RcMnSOD6,* and *RcMnSOD7* were repressed according to time of development. The gene *RcFeSOD8* did not change expression levels. The *CuZnSOD* and *RcFeSOD8* genes showed repression in leaves L3 compared to L1 and L2 while the genes *RcMnSOD5*, *RcMnSOD6,* and *RcFeSOD7* showed high levels of expression in L3 compared to L1 and L2.

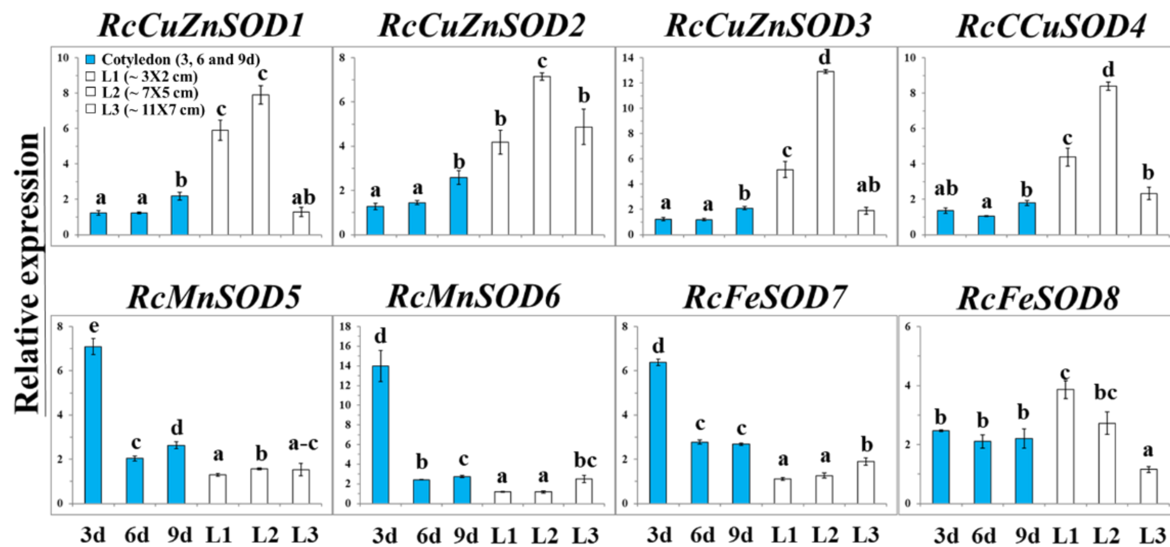

Figure 7. Gene expression of *RcSOD* genes in cotyledons and leaves at different developmental time points. Samples comprised 3-, 6- and 9-day-old cotyledons (C1, C2, and C3) and the first real leaves of the different dimensions: L1 (3x2 cm), L2 (7x5 cm) e L3 (11x7 cm). All samples were collected at 25°C Means and standard errors of three biological replicates containing 15–20 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p < 0.05$).

In general, germination of *R.communis* under heat stress (35°C) showed upregulation of the *RcCuZnSOD1* and *RcFeSOD8* genes during early imbibition, while the genes *RcCuZnSOD2, RcCuZnSOD3, RcCCuSOD4, and RcFeSOD7* were upregulated during radicle protrusion and at the stage of 2cm root young seedlings. The genes *RcMnSOD5* and *RcMnSOD6* were downregulated according to development time. In seedlings roots, the genes of the CuZn group and the genes *RcSOD8* demonstrated to be responsive to heat stress (35ºC). In cotyledons, at different time points, the induction of SOD genes was observed in the genes of the CuZn group according to the time point. While there was no specific patterns observed in leaves.

3.4 Subcellular localization of *RcSOD* genes in *N. benthamiana* leaves

We use GFP fused to *RcSOD* genes (*RcCuZnSOD1, RcCuZnSOD3, RcCuZnSOD4, RcMnSOD5, RcFeSOD7,* and *RcFeSOD8*) in control of the 35S promoter to investigate the subcellular localization and correlate with prediction and

gene expression results. Young *N. benthamiana* leaves were used in the agroinfiltration process. The fluorescence of GFP fusion was observed and compared with the chlorophyll signal (Overlay) to prove the chloroplast localization. The nuclei and cytosol localization of *RcSOD* genes were obtained compared to the GFP signal and cell structure (NELSON; CAI; NEBENFÜHR, 2007).

The gene *RcCuZnSOD1* showed subcellular localization of GFP expression in the cytosol and nucleus, while *RcCuZnSOD3* showed the GFP expression in chloroplast and cytosol, while *RcCCuSOD4* showed GFP expression in chloroplasts, cytosol, and in the nucleius. The gene *RcMnSOD5* showed cytosol subcellular localization and *RcFeSOD7* in chloroplasts, and *RcFeSOD8* showed possible mitochondrial subcellular localization (Figure 8). In general, the prediction of subcellular localization by Cello and Sherloc2 (Supplementary Table 4) is similar to that found by GFP fusion except by the gene *RcMnSOD5* that showed to be located in the cytosol. Comparing the orthologs *AtSOD* genes the subcellular localization is similar in *RcSOD* genes except to the *RcMnSOD5* genes and the *RcFeSOD8* genes that showed localization by GFP in cytosol and mitochondria respectively.

RcMnSOD5 did not show mitochondrial subcellular localization as in prediction of subcellular localization, instead the subcellular localization by GFP showed cytosol localization, whereas it showed repression of gene expression during germination. On the other hand, the gene *RcFeSOD8* showed prediction and possible GFP expression to mitochondrial localization (NELSON; CAI; NEBENFÜHR, 2007). Besides that, *RcFeSOD8* showed to be upregulated during the early stages of germination and radical protrusion under heat stress (35ºC). Mitochondria is the major generator of ROS and is expected that during germination the gene expression levels of mitochondrial SOD to be increased.

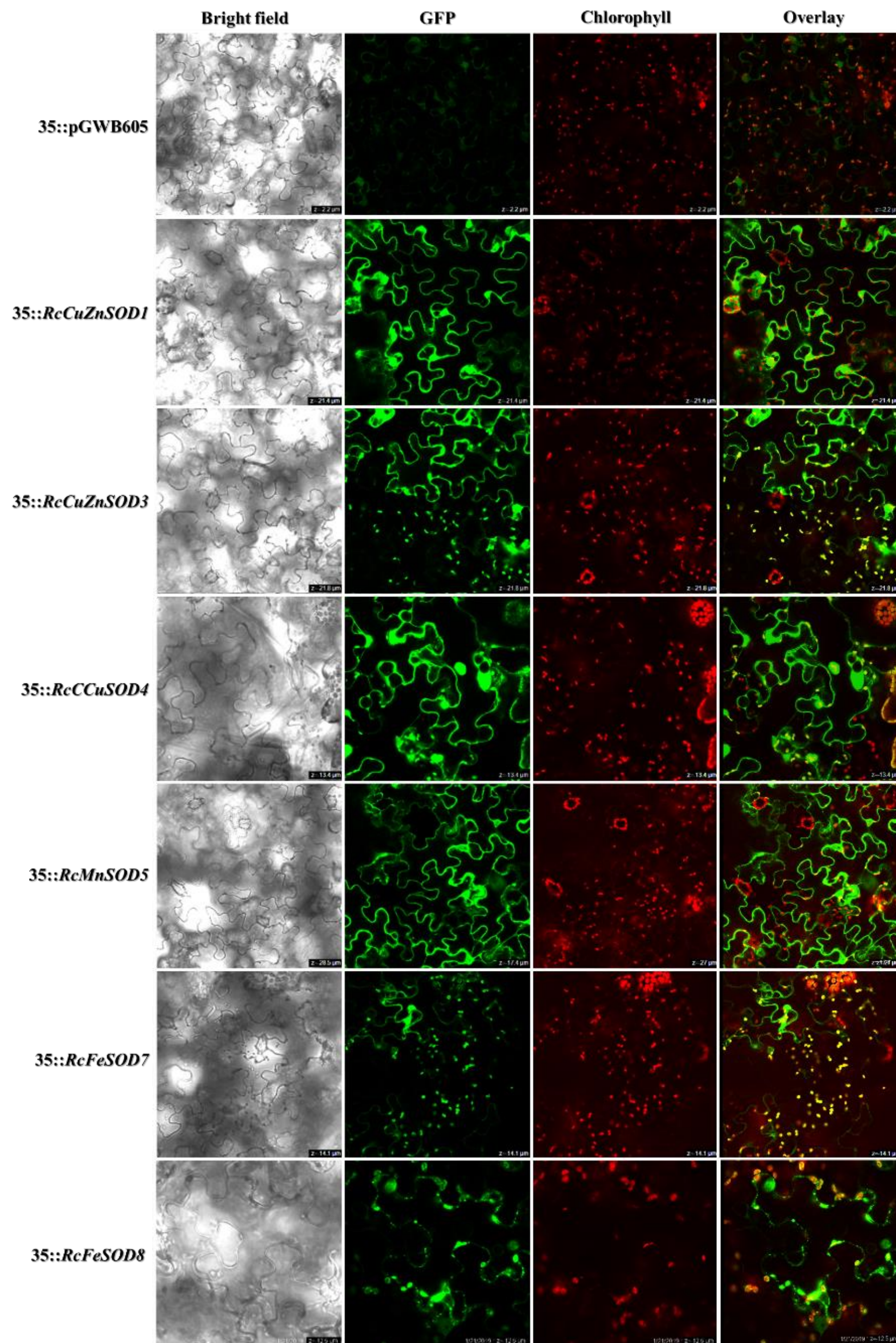

Figure 8. Subcellular localization of the GFP-*RcSOD* fusion protein in Transgenic *N. Benthamiana* plants. GFP is presented in green and the autofluorescence of chlorophyll is indicated in red. Leaves cells of transgenic tobacco plants were visualized using laser confocal scanning microscopy.

4. CONCLUDING REMARKS

In conclusion, we showed that *RcSOD* genes have a large amount of regulation in promoters involved in the development and response to abiotic stresses, and possibly regulated by different mechanisms involving hormones, transcription factors, and heat abiotic stress by prediction of the promoter region. The genes *RcCuZnSOD1*, *RcCuZnSOD2*, *RcMnSOD6* present a difference in the number of exon/intron compared to *AtSOD* genes explained by fusion or split of exons. Four miRNA demonstrated prediction to repress the *RcSOD* genes. The miRNA398 appears to possibly be involved in *RcSOD* regulation of *RcCuZnSOD3* and *RcCCuSOD4* genes similar to observed in *A. thaliana* for CuZn SOD genes. The *RcSOD* gene expression shows a different pattern of induction under heat stress during germination. In general, the *CuZnSOD* and *RcFeSOD8* genes showed to be induced in roots by heat stress, and *CuZnSOD* genes shown to be induced by development stages in cotyledons while the *MnSOD* and *FeSOD* genes were downregulation or did not change. The subcellular localization shows that *RcMnSOD5* was not located in mitochondria and the *RcFeDOD8* demonstrated possible involvement in ROS response in mitochondria, whereas the results show possible molecular markers which may be used in *R. communis* genetic engineering or breeding aimed at superior genotypes with better tolerance to heat and other possible abiotic stresses.

5. ACKNOWLEDGMENTS

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary material

Tab. 1. Primers used in gene expression of *RcSOD* genes by qPCR.

Gene	Gene Identifier	Forward primer	Reverse primer	Amplicon (bp)
RcCuZnSOD1	**30128.m008673**	GAAGGGCAGTCGTTGTTCAT	GCAAACCAATAATACCACATGC	**114**
RcCuZnSOD2	**29950.m001121**	GCTCCATTCCATGAAGAACG	GTCAGCGGTATCTGCATGTC	**104**
RcCuZnSOD3	**29589.m001265**	GCCAGATACCGTTGAGTGGT	AAACCAACAACACCACATGC	**148**
RcCCuSOD4	**30190.m011071**	GGAAACATGGTTGGTCCATT	TCACCCTGTGGCTCTTTTTC	**112**
RcMnSOD5	**29729.m002354**	CTGTTCGTGAAGGAGGTGGT	ACAGCAGCGCCTTCTGTATT	**115**
RcMnSOD6	**29729.m002353**	TCATCACCAGAAGCATCACC	TGGTTGATATGACCTCCTCCA	**146**
RcFeSOD7	**30147.m014368**	CAACTCGTCGATCACAATGG	GCTCCAAAGCATCCAAGG	**117**
RcFeSOD8	**29799.m000633**	CGAGCCTATGTGGAAGGATT	TCATGATTCCAGACCTGAGC	**146**
ACT	**30206.m000761**	TCCCTCAGTACGTTCCAGCA	CACCTCCATACTCCTCCCT	**198**
PP2AA1	**28883.m000734**	GTTGGGTGTTGGGTTCTTTG	ATTCTTCAGCAAGGCGTTT	**119**
POB	**29724.m000826**	TCCTCCGCTACTTCGTCT	ACAATCCATCTTCCTCGTTACT	**177**

Tab. 2. Cloning primers used to amplify the full length of *RcSOD* to subcellular localization.

Gene	Sequência	Tamanho	TM
RcCuZnSOD1_Fw	**ATG**GTGAAGGCTGTTACC	18	56
RcCuZnSOD1_Re	GGTATTATTGGTTTGCAAGGA	21	55.6
RcCuZnSOD2_Fw	**ATG**GCAGCAACAGGTACA	18	58.5
RcCuZnSOD2_Re	ATTTGCAGATATTTTGCAAACCG	23	58.3
RcCuZnSOD3_Fw	**ATG**GCAGCTCATACTATCATTG	22	57.5
RcCuZnSOD3_Re	GTTGGTTTGACGCCAGTG	18	58.4
RcCCuSOD4_Fw	**ATG**GCATTTCTAAGGTCTGT	20	56.1
RcCCuSOD4_Re	ATTTTGTTACCAGCAAGGTT	20	55.3
RcMnSOD6_Fw	**ATG**GCTCTCCGCTCTCTT	18	59.8
RcMnSOD6_Re	TATGCGAAAGAATGCCCGTC	20	60.1
RcMnSOD5_Fw	**ATG**CAGTACCCCATCACC	18	58.3
RcMnSOD5_Re	TGAGAGCATATGCCCTTCAT	20	58.3
RcFeSOD7_Fw	**ATG**GTTGCAGTAGCTGCT	18	58.5
RcFeSOD7_Re	AATGATGAATCTGAGGCTGAC	21	57.3
RcFeSOD8_Fw	**ATG**AGCTGGTGTTGTAATCT	20	56.1
RcFeSOD8_Re	CCAAAAATTCCGGTTGCT	18	55.5

Tab. 3. *In silico* digestion with enzyme restriction of pGWB605 vector with *RcSOD* genes.

Gene	ID	Vector	En. Restrição	Fragmentos
RcCuZnSOD1	30128.m008673	PGWB605	HINDIII+BAMHI	9669 +1401
RcCuZnSOD3	29589.m001265	PGWB605	HINDIII+BAMHI	9669+1584
RcCCuSOD4	30190.m011071	PGWB605	HINDIII	9774 +1830
RcMnSOD5	29729.m002354	PGWB605	HINDIII	9909 +1407
RcFeSOD7	30147.m014368	PGWB605	HINDIII	9876 +1653
RcFeSOD8	29799.m000633	PGWB605	HINDIII	10416 + 993
Empty	-	PGWB605	HINDIII	12274

Tab. 4. Motif sequences of RcSOD proteins identified by MEME.

Motifs	Sequence	Pfam
Motif 1	VVHAIEDDFGSGEHFLEKFTGNAATRFGSGWVWLAL	Sod_Cu -PF00080 Sod_Fe_C -PF02777
Motif 2	RFTQEDDGPTTVTGRISGLSPGLHGFHIHEFGDTTNGCMSTGPHYNP	Sod_Cu -PF00080
Motif 3	PLLTIDVWEHAYYLDYKNRRPDYIKTFMN	Sod_Fe_C -PF02777
Motif 4	ATFTLKPPPYPLDALEPYMSRETLELHWGKHHRAYVDNLNKQLEGTDL	Sod_Fe_N -PF00081
Motif 5	GKTHGAPEDENRHAGDLGNIVAGADGVAEITIVDSQIPLSGPNSIIGRAV	Sod_Cu -PF00080
Motif 6	EEVVKVTYNNGBPLPAFNNAAQAWNHEFFWESMQPGGGGKP	Sod_Fe_N -PF00081
Motif 7	DSSSVVKLQSAIKFNGGGHINHSIFWKNLAPVSEGGGEPPH	Sod_Fe_N -PF00081
Motif 8	VVKTTNAVBPLVWD	-
Motif 9	VSWEAVSARLEAAEA	-
Motif 10	VANWKYASEVYEKLC	-

Tab. 5. Prediction of *R. communis* miRNA in *RcSOD* genes. The CDS of *RcSOD* genes was loaded and submitted against 63 published miRNA of *R. communis* by psRNATarget (http://plantgrn.noble.org/psRNATarget/).

miRNA	Target (CDS)	Target_start	Target_end	miRNA_aligned_fragment	Target_aligned_fragment	Inhibition	Multiplicity
rco-miR319a	***RcCuZnSOD2***	57	77	UUGGACUGAAGGGAGCUCCCU	UAAAGGCUCUCUUCAGUUUAU	Cleavage	1
rco-miR319b	***RcCuZnSOD2***	57	77	UUGGACUGAAGGGAGCUCCCU	UAAAGGCUCUCUUCAGUUUAU		
rco-miR319c	***RcCuZnSOD2***	57	77	UUGGACUGAAGGGAGCUCCCU	UAAAGGCUCUCUUCAGUUUAU		
rco-miR319d	***RcCuZnSOD2***	57	77	UUGGACUGAAGGGAGCUCCUU	UAAAGGCUCUCUUCAGUUUAU		
rco-miR159	***RcCuZnSOD2***	58	78	UUUGGAUUGAAGGGAGCUCUA	AAAGGCUCUCUUCAGUUUAUA		
rco-miR159	***RcCuZnSOD3***	357	377	UUUGGAUUGAAGGGAGCUCUA	AGGGGCACAUUUCAAUCCUAA		
rco-miR398b	***RcCuZnSOD3***	417	438	UGUG-UUCUCAGGUCGCCCCUG	UGCGGGCGACCUGGGAAACAUA		
rco-miR398a	***RcCCuSOD4***	732	752	UGUGUUCUCAGGUCACCCCUU	ACAGGGUGACUUGGGAACACU		
rco-miR398b	***RcCCuSOD4***	732	752	UGUGUUCUCAGGUCGCCCCUG	ACAGGGUGACUUGGGAACACU		
rco-miR408	***RcFeSOD7***	93	113	CUGCACUGCCUCUUCCCUGGC	GACAGAGAAGAGGAAAUGCAC		

Tab. 4. Prediction of subcellular localization in *RcSOD* genes by Cello (http://cello.life.nctu.edu.tw/) and by Sherloc2 (https://abi-services.informatik.uni-tuebingen.de/sherloc2/webloc.cgi).

Gene	Gene Identifier	Cello	SherLoc2
RcCuZnSOD1	**30128.m008673**	Cytoplasmic	Cytoplasmic
RcCuZnSOD2	**29950.m001121**	Extracellular	Cytoplasmic
RcCuZnSOD3	**29589.m001265**	Chloroplast	Chloroplast
RcCCuSOD4	**30190.m011071**	Chloroplast	Chloroplast
RcMnSOD5	**29729.m002354**	Mitochondrial	Mitochondrial
RcMnSOD6	**29729.m002353**	Extracellular	Cytoplasmic
RcFeSOD7	**30147.m014368**	Chloroplast	Chloroplast
RcFeSOD8	**29799.m000633**	Cytoplasmic/Mitochondrial/Chloroplast	Mitochondrial

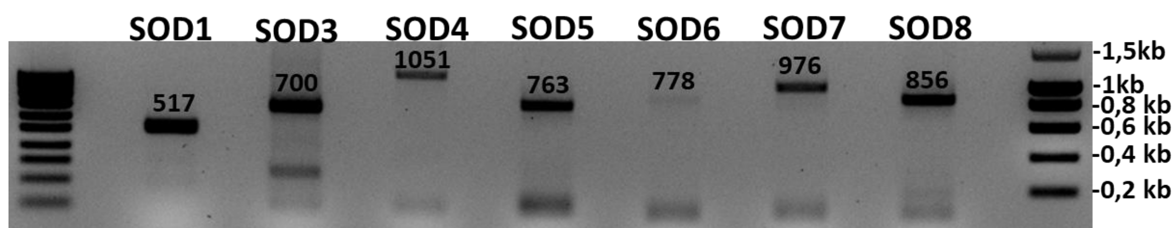

Fig. 1. Amplification of full-length *RcSOD* genes with Q5 high fidelity enzyme.

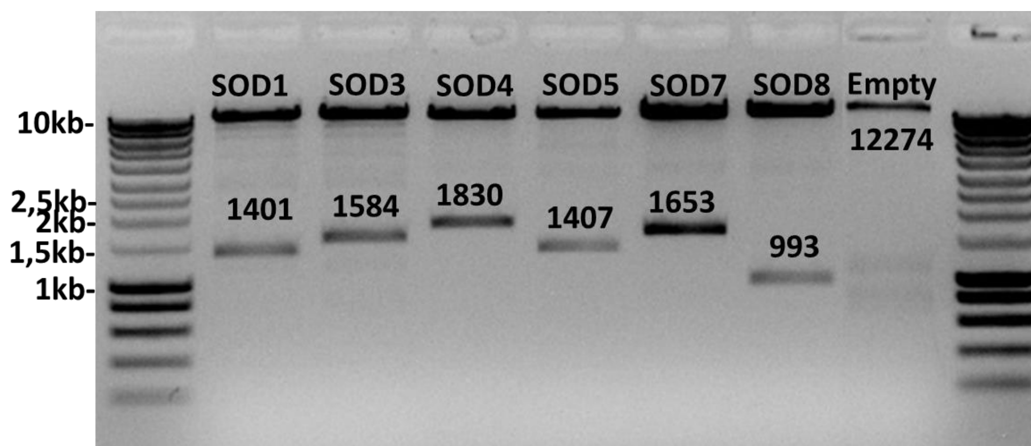

Fig. 2. Digestion of pGWB605-*RcSOD* with HindIII and HINDIII+BAMHI restriction enzyme.

A) Four weeks old plants

B) After 48 h agroinfiltration

Fig. 3: *N. benthamiana* plants of four weeks old used in the agroinfiltration process.

CAPÍTULO 3: Sequence analysis of the small heat shock protein (sHSP) subfamily in *Ricinus communis* and their role in abiotic stress responses.

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Sequence analysis of *Ricinus communis* small heat-shock protein (sHSP) subfamily and its role in abiotic stress responses

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Sequence analysis of the small heat shock protein (sHSP) subfamily in *Ricinus communis* and their role in abiotic stress responses

ABSTRACT

Small heat shock proteins (sHSP) play major roles in plant defense mechanisms towards abiotic stresses. sHSP acts as molecular chaperones providing the necessary tools to sustain cellular homeostase under adverse conditions. sHSP genes possess specific expression signature, which depends on tissue-specificity, developmental stage and the nature of the abiotic stress. Despite the fact that *Ricinus communis* is an important oilseed crop with large socioeconomic impact on small family farmers in semi-arid regions worldwide, the characterization of *RcsHSP* genes and their possible contribution to plant survival under harsh environmental conditions has not been addressed. Hence, this study aimed at characterizing *R. communis* sHSP subfamily, through phylogeny, gene structure, duplication, and expression profile analysis, as well as by characterizing *Arabidopsis thaliana* plants overexpressing RcsHSP genes. We identified 41 RcsHSP genes that possessed the α-crystallin domain and compatible molecular weight (< 43 KDa). RcsHSP subfamily showed different homology levels with sHSP genes from other plant species, suggesting the occurrence of specific gene expansion and loss. RcsHSP subfamily was classified according to the cellular locations of the genes, which included cytosolic, chloroplastic, mitochondrial, and endoplasmic reticulum groups. Ten putative motifs were found amongst RcsHSP genes, but only motifs 4, 6 and 8 were sHSP protein domains. *RcsHSP* subfamily showed 19 genes produced by tandem duplications events, which might have been crucial for RcsHSP diversification and *R. communis* acquisition of tolerance. Gene expression analysis showed that RcsHSP subfamily possesses different regulatory mechanisms in response to various abiotic stress. Additionally, overexpression of RcsHSP genes in *A. thaliana* was followed by enhanced SOD activity and higher content of osmoprotectants molecules, which ultimately led to enhanced seed germination under a variety of abiotic stresses. Our results will be very helpful for breeding programs aiming at developing high tolerant *R. communis* plants, providing economic and social support for farmers in semiarid areas worldwide.

Keywords: abiotic stress, oilseed crop, regulatory mechanisms, tolerance.

1. INTRODUCTION

Abiotic stresses are one of the major environmental cues that compromises large scale production and high yields of agricultural crops worldwide (LIPIEC *et al.*, 2020). Plants are able to modulate morphological, physiological, biochemical and molecular mechanisms to cope with harsh environmental conditions imposed by abiotic stresses (GAO; CHAO; LIN, 2008; MEENA *et al.*, 2017; RASUL *et al.*, 2017; SHARMA *et al.*, 2019; ZHANG, JINGXIAN *et al.*, 2000). Heat Shock Proteins (HSP) are expressed in all kingdoms and they play essential roles not only during normal plant growth, but also act as the main biochemical and molecular responses to many forms of cellular stress (GRIGOROVA *et al.*, 2011; MU *et al.*, 2013; MUTHUSAMY *et al.*, 2017; SEWELAM *et al.*, 2019). In plants, they act in important processes such as peptide-folding, refolding of stress-denatured proteins, oligomeric assembly, protein trafficking, and assistance in proteolytic degradation (KOTAK *et al.*, 2007; PARK; SEO; PARK, 2015). HSP family show great diversity with molecular size varying from 10 to 100 kDa, and distribution in various cellular compartments (CHEN *et al.*, 2018; HELM; ABERNETHY, 1990). Due to its great diversity, HSP family has been subdivided into five subfamily based on their molecular weight and sequence homology: HSP100, HSP90, HSP70, HSP60 e small HSP (sHSP) (AL-WHAIBI, 2011; PARK; SEO; PARK, 2015). sHSP show molecular weight lower than 43 kDa and are characterized by the presence of the α-crystallin domain (ACD), which contain approximately 90 amino acids. The ACD is flanked by an N-terminal domain and a short C-terminal extension. Additionally, some members of the sHSP subfamily may form homo-oligomeric complexes of 200-750 kDa (9 to >24 subunits) (BASHA; NEILL; VIERLING, 2012; WATERS, 2020). sHSP are expressed in all developmental stages during the plant life cycle, including seed maturation and germination, pollen development and fruit maturation (WATERS, 2020). Overexpression of sHSP in *Arabidopsis thaliana* induce high level of expression of antioxidant enzymes resulting in an increased tolerance to abiotic stresses, such as heat, salinity, osmotic, and oxidative stresses (JIANG *et al.*, 2009; MU *et al.*, 2013; SUN, WEINING *et al.*, 2001; ZHANG, LU; GAO; PAN, 2013; ZHONG *et al.*, 2013). It seems that there is a crosslink between sHSP subfamily and the redox system involving Heat Shock Factors (HSF). HSF can be induced either by abiotic stress or via the accumulation of reactive oxygen

species (ROS). HSF modulates HSP and superoxide dismutase gene expression via miRNA398 induction. Fast ROS accumulation induces HSF expression leading to upregulation of HSP genes, but downregulation of superoxide dismutase genes. Nevertheless, long-term ROS accumulation can induce ROS scavenger and chaperone activity via induction of HSP genes (DRIEDONKS *et al.*, 2015; JACOB; HIRT; BENDAHMANE, 2017). Additionally, sHSP bind to unfolded proteins to facilitate further refolding by HSP complexes (WANG, WANGXIA *et al.*, 2004). The sHSP subfamily have been characterized in several plant species, i.e. *Arabidopsis thaliana* (MU *et al.*, 2013; SCHARF; SIDDIQUE; VIERLING, 2001), *Oryza sativa* (MANI; RAMAKRISHNA; SUGUNA, 2015; MU *et al.*, 2013), *Solanum licopersycum* (YU *et al.*, 2016), *Populus trichocarpa* (MU *et al.*, 2013), *Capsicum annuum* (GONG; WANG; LU, 2015) and *Glycine max* (LOPES-CAITAR *et al.*, 2013), and were classified in subfamilies according to their cellular locations and sequence homology.

Ricinus communis L. is an important oilseed crop worldwide due to the peculiar oil that is extracted from its seeds, which has many industrial applications (CONEJERO *et al.*, 2017; GONZALEZ-CHAVEZ *et al.*, 2014; PATEL *et al.*, 2016). It presents high levels of tolerance to a diverse range of abiotic stresses (BAUDDH; SINGH, 2012; DE CARVALHO TEIXEIRA VASCONCELOS *et al.*, 2017; OGUNNIYI, 2006; RIBEIRO, PAULO R.; FERNANDEZ; *et al.*, 2014; VELASCO, LEONARDO; ROJAS-BARROS; FERN, 2005). Therefore, it possess great socioeconomic importance for developing countries, in which frequently this species is cultivated by small family farmers in semiarid areas (FALASCA; ULBERICH; ULBERICH, 2012; GARCEZ; VIANNA, 2011; LEITE *et al.*, 2013; SRINIVASARAO *et al.*, 2011; WANG, CHAO *et al.*, 2013). In this study, we identified sHsp homologs in *R. communis* and examined their expression pattern during different developmental stages and during abiotic stress treatment. Furthermore, we prepared *A. thaliana* plants overexpressing *R. communis* sHSP genes and showed their contribution to abiotic stress responses. Our results lay the basis for functional analysis of the sHSP in stress tolerance of *R. communis*.

2. MATERIALS AND METHODS

2.1 Phylogenetic analysis and prediction of cellular locations

Initially, three known sHSP protein sequences from *Arabidopsis thaliana* (At2G29500, At4G25200, and At5G12020) were used as queries in a blastp search against the complete predicted proteome of *R. communis* v0.1 from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Then, the potential sequence candidates were loaded into Pfam protein database (http://pfam.xfam.org/search) and only those with the known sHSP protein domains were selected as *R. communis* sHSP.

A phylogenetic tree was constructed by using the the complete set of sHSP sequences from *A. thaliana* (SCHARF; SIDDIQUE; VIERLING, 2001) and the putative *RcsHSP* genes identified in this work. For that, the sHSP amino acid sequences were aligned using MUSCLE and the phylogenetic analysis was conducted using MEGA 7.0 (http://www.megasoftware.net/). Topology was calculated by the neighbor-joining method with distances calculated by p-distance. Bootstrapping was performed with 1000 replicates.

Prediction of cellular locationss were performed with Wolf Psort (https://wolfpsort.hgc.jp/) and Cello (http://cello.life.nctu.edu.tw/), whereas molecular weight and isoelectric point (pI) were estimated using ProtParam (http://web.expasy.org/protparam/). Analysis of the conserved motifs wwere performed by the Multiple Em for Motif Elicitation (MEME Suite 5.0.1) (http://meme-suite.org/). The parameters used were minimum motif width = 6, maximum motif width = 200, and maximum number of motifs to identify was 10. Intron/exon configurations were assessed by using the Gene Structure Display Server 2.0 (http://gsds.gao-lab.org/). Tandem duplications were identified in PTG Base (http://ocri-genomics).

2.2 Sample collection for gene expression analysis

We chose ten *RcsHSP* sequences for gene expression analysis. For that, we used *R. communis* samples in different developmental stages during seed germination and seedling establishment. The first group comprised samples collected during seed germination at different temperatures (20, 25 and 35 °C) at two developmental stages:

at radicle protrusion and at young seedlings with roots of ca. 2 cm (RIBEIRO, PAULO R.; ZANOTTI; *et al.*, 2015). The second group comprised root samples collected from seedlings grown at different temperatures (20, 25 and 35 °C) for 7d (RIBEIRO, PAULO R.; LIGTERINK; HILHORST, 2015). The third group comprised 3-, 6- and 9-day-old cotyledons and real leaves of the different dimensions: L1 (3x2 cm), L2 (7x5 cm)e L3 (11x7 cm) (RIBEIRO, PAULO R.; FERNANDEZ; *et al.*, 2014).

2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from 5 mg of the lyophilized and ground samples using the hot borate method with modifications (GOMES NETO *et al.*, 2018) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The RNA integrity was further inspected on 1% agarose gel. First strand cDNA was synthesized with 1 µg of total RNA using the Bio-Rad iScript™ cDNA synthesis kit according to the manufacturer's instructions. The cDNA was diluted 20 times and stored at -20 °C prior to further analysis by qRT-PCR.

2.4 Gene expression analysis

Primers were designed using primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) with melting temperatures (Tm) of 59–61 °C, primer lengths of 18–22 bp and amplicon lengths up to 150 bp (Supplementary Table S1). Primer efficiency was evaluated based on a standard curve generated by a two-fold serial dilution series of a pooled cDNA sample. The primer specificity was verified by separating the products on a 2 % agarose gel and by melting curve analysis. Serine/threonine phosphatase 2A (PP2AA1) and Actin genes were used as an internal references (RIBEIRO *et al.*, 2014). qRT-PCR was performed in a total volume of 10 µL containing 2.5 µL of cDNA (20x diluted), 0.5 µL of primers (10 µM), 5 µL of iQ SYBR Green Supermix (Bio-Rad) and 2 µL water. qRT-PCR experiments were run on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The following program was used for all PCR reactions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Melting curves were obtained after this program by heating samples from 65 to 95 °C, increasing the temperature stepwise by 0.5 °C every 5 s (RIBEIRO *et al.*, 2014). Statistical significance was evaluated with test t at $p<0.05$.

2.5 Cloning of *RcsHSP12* and *RcsHSP19* genes

Cloning of *RcsHSP* genes was performed using the Gateway® technology according to the manufacturer's instructions. Full-length coding regions were amplified from *R. communis* cDNA samples. Primers used and additional information about the cloned genes are presented in Supplementary Table S2. Amplified products were recombined in the donor vector pDONr207, to produce pDONr207-*RcsHSP12* and pDONr207-*RcsHSP19*. These plasmids were transferred to *Escherichia coli* (DH5α) cells by electroporation. Transformed single colonies were inoculated in 5 mL LB (lysogeny broth) medium containing gentamycin (25 µg/mL) for 24h. pDONr207-*RcsHSP12* and pDONr207-*RcsHSP19* were isolated using the QIAprep Spin Miniprep High-Yield kit (Qiagen) according to the manufacturer's instructions. Thereafter, the plasmids were recombined by an LR reaction (Invitrogen) in the gateway binary vector pGD625 carrying CaMV35S (CaMV—cauliflower mosaic virus). Expression vectors pGD625-sHSP were transformed to *Agrobacterium tumefaciens* (Agl0) cells by electroporation. Transformants were selected on LB broth supplemented with kanamycin (100 µg/mL) and rifampicin (25 µg/mL). Colony PCR, digestion with restriction enzymes and sequencing were used to confirm the presence of the desired gene sequences in the isolated plasmids.

2.6 *RcsHSP12* and *RcsHSP19* overexpression in *Arabidopsis thaliana*

Arabidopsis thaliana plants were transformed with *A. tumefaciens* (strains CaMV35S::*RcsHSP12* and CaMV35S::*RcsHSP19*). For plant transformation, a modified protocol of the floral-dip method was used (LOGEMANN *et al.*, 2006). *A. tumefaciens* strains carrying the target genes were obtained from a 100 mL liquid culture. The bacterial pellet was re-suspended in 100 mL 5% sucrose solution with Silwet L-77 (0,05%). Newly formed *A. thaliana* (Col-0) flowers were dipped in this suspension and subsequently covered with a plastic bag over night. Plants were allowed to develop further to form siliques after which the seeds (T1) were harvested.

2.7 Plant transformant selection

T1 seeds of infiltrated plants were surface sterilized with 70% ethanol for 5 min, followed by rinsing three times in milliQ water. Seeds were plated on 1/2 strength MS medium supplemented with 50 μg ml^{-1} kanamycin. After storing the plates at 4°C for 48 h, they were transferred to a chamber with controlled temperature (24ºC; 16h/8h photoperiod). Plants that were able to grow on the media were considered to be transformed and were used to obtain T2 seeds. For T2 selection, approximately 100 seeds were plated on 1/2 strength MS medium supplemented with 50 μg ml^{-1} kanamycin. After two weeks, T2 lines that showed a 75/25 ratio for resistant/sensitive seedlings were selected and were used to obtain T3 seeds. For T3 selection, approximately 100 seeds were plated on 1/2 strength MS medium supplemented with 50 μg ml^{-1} kanamycin. After two weeks, T3 lines that showed a 100% of for resistant seedlings were selected and were used to obtain T4 seeds.

2.8 Germination under abiotic stress

Seeds of *A. thaliana* plants overexpressing *RcsHSP* genes *(RcsHSP12.1, RcsHSP12.2, RcsHSP19)* and Col-0 (wild line -WT) were germinated under abiotic stress conditions. Prior to germination, seeds were sterilized with 70% ethyl alchohol solution, sown on petri dishes with germination paper moistened with 6 mL water, PEG or saline solutions followed by incubation at 4°C in the dark for 72 h. Heat stress assay was performed by incubating stratified seeds at two different temperatures (22 and 34°C). Osmotic stress was performed by incubating stratified seeds in 5 different polyethylene glycol (PEG) potentials (-0.2, -0.4, -0.6, -0.8, -1.0 MPa), whereas saline stress was performed by incubating stratified seeds at different NaCl solution concentrations (0.25; 0.50; 0.75 and 1.0 mg/L). Germination percentage was scored on daily basis and germinability data was processed using the GERMINATOR software package (JOOSEN *et al.*, 2010).

2.9 Protein extraction, quantification and superoxide dismutase activity assay

Protein quantification and superoxide dismutase activity assays were performed as described in Gomes Neto *et al.*, (2018). For protein extraction, frozen germinated

seeds were homogenized in 0.1M potassium phosphate buffer (pH 7.8). The homogenate was centrifuged at 14.000 g for 10 min at 4 °C. The supernatant, hereafter referred to as crude SOD extract, was collected and stored at−20 °C for further analysis. Extracts were diluted 20 times for protein quantification and SOD activity assays. Total protein content was determined by using bovine serum albumin as standard as described previously (BRADFORD, 1976; GOMES NETO *et al.*, 2018). Total SOD activity was determined by the capacity to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT) by O^{2-}, as described by (GIANNOPOLITIS; RIES, 1977; GOMES NETO *et al.*, 2018). Enzyme activity was expressed as units of SOD activity (U) per mg of total protein. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of NBT reduction under the experimental conditions. Statistical significance was evaluated by Scott-Knott test ($p < 0.05$).

2.10 Nuclear Magnetic Resonance (NMR) analysis

Extracts (10 mg) were dissolved in D_2O (700 µL), filtered and transferred to 5-mm NMR tubes. ^{1}HNMR spectra were measured at 20°C using Bruker AVANCE III spectrometer (400 MHz). 3-(Trimethylsilyl)propionic acid-D4 sodium salt (TMSP-d4) was used as internal standard. Concentrations were normalized based on the TMSP-d4 signal.

2.11 Data processing and compound identification

^{1}H-NMR spectra were loaded into NMRProcFlow web-tool (https://nmrprocflow.org/) (DEBORDE; MAUCOURT, 2017; RIBEIRO, PAULO R.; WILLEMS; *et al.*, 2015). Baseline correction and binning alignment where performed, after which signal integrations were automatically performed on user-defined regions of interest (buckets). Compound identification was performed by comparing the NMR signals with those of authentic samples and data from the literature. Normalized data were uploaded at MetaboAnalyst 2.0; a web-based analytical pipeline for high-throughput metabolomics studies (RIBEIRO, PAULO R.; WILLEMS; *et al.*, 2015; XIA; WISHART, 2011). Row-wise normalization allowed general-purpose adjustment for differences among samples. Cube root transformation and auto-scaling allowed comparison of features. Multivariate analyses were performed using log transformed and

auto-scaled data. ANOVA was performed to assess overall variation in metabolite levels, followed by post-hoc analyses (Bonferroni correction, FDR < 0.05).

2.12 Statistical analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences Statistics® (International Business Machines - IBM) and Microsoft® Excel 2010 programs (Microsoft). Analysis of variance was used to identify statistically significant differences between the samples ($P < 0.05$) followed by Tukey's multiple comparison tests. The results are presented as the mean of replicates ± standard deviations.

3. RESULTS AND DISCUSSION

3.1 Identification and phylogenetic analysis of the sHsp gene families in *R. communis* (RcsHSP)

The homology search using three *A. thaliana* sHSP sequences (At2G29500, At4G25200, and At5G12020) as queries resulted in the identification of 72 sHSP loci candidates in *Ricinus communis*. Among them, only 41 presented the α-crystallin domain (ACD) and compatible molecular weight (< 43 KDa) and, therefore were putatively identified as *R. communis* sHSP genes, thereafter *RcsHSP* (Supplementary Table S3). RcsHSP gene molecular weight varied from 10223.06 to 33560.98 Da, with length varying from 84 and 305 bp and theoretical pI varying from 5.03 to 10.37 (Supplementary Table S3). The *RcsHSP* subfamily showed homology with 27 genes from *Arabidopsis thaliana* (MU *et al.*, 2013; SCHARF; SIDDIQUE; VIERLING, 2001), 29 genes from *Oryza sativa* (MANI; RAMAKRISHNA; SUGUNA, 2015; MU *et al.*, 2013), 33 genes from *Solanum licopersycum* (YU *et al.*, 2016), 37 genes from *Populus trichocarpa* (MU *et al.*, 2013), 35 genes from *Capsicum annuum* (GONG; WANG; LU, 2015), and 51 genes from *Glycine max* genes (LOPES-CAITAR *et al.*, 2013), suggesting specific gene expansion and gene loss occurred in these plant species. Comparatively, *R. communis* has more sHSP proteins than other plant species, indication that they might have been crucial in adaptation to adverse land environments (TANG *et al.*, 2016).

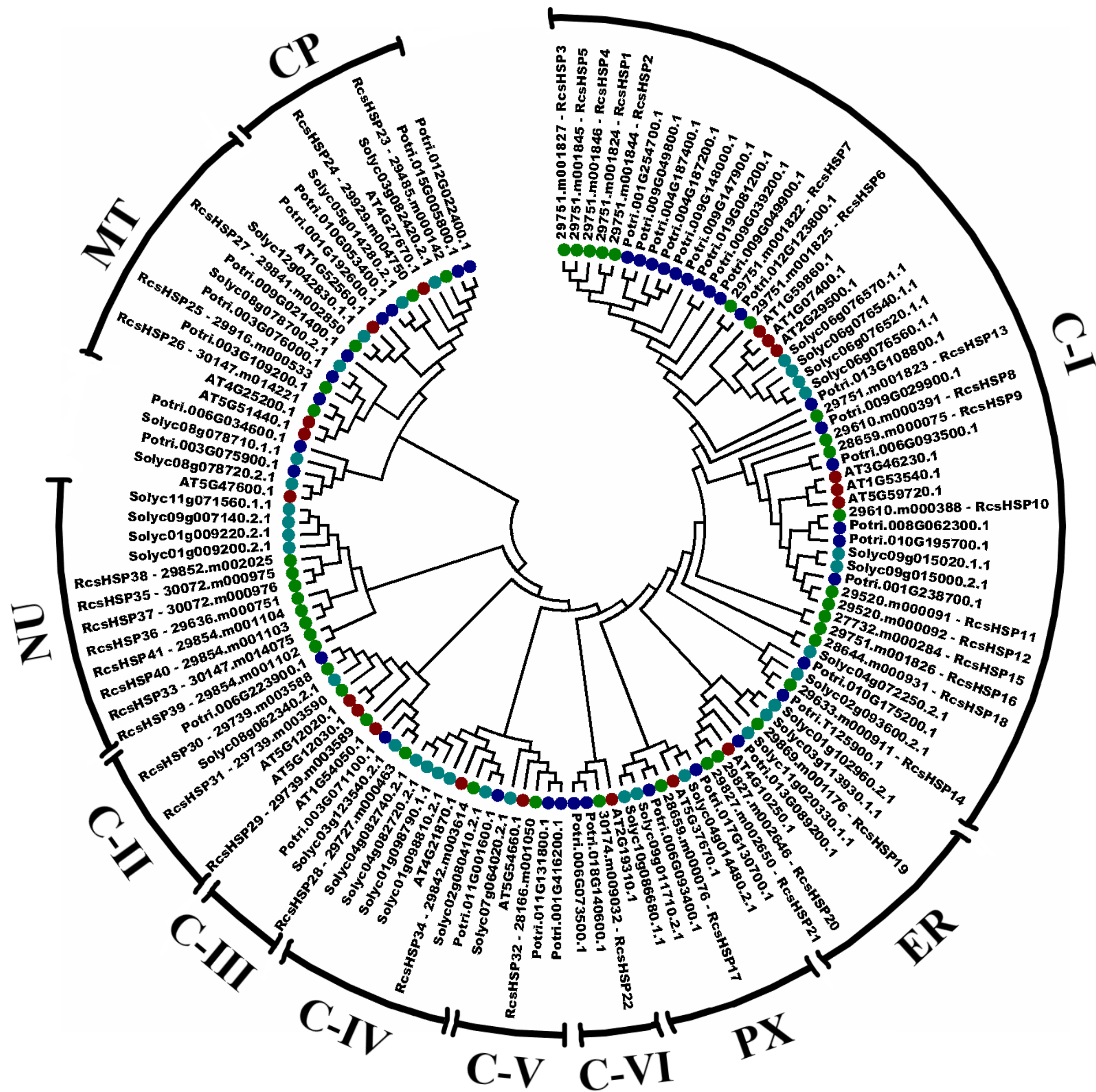

Figure 1. Small heat shock protein subfamily in *Ricinus communis*, *Arabidopsis thaliana*, *Solanum licopersycum*, and *Populus trichocarpa*. Group classification is based on the homology between sHSP genes and their predicted subecellular localization. Circle colors depicted in the figure represent different plant species: *Ricinus communis* (green), *Arabidopsis thaliana* (red), *Solanum licopersycum* (light blue), and *Populus trichocarpa* (dark blue).

To investigate the evolutionary relationship between *sHSP* genes from *Ricinus communis*, *Arabidopsis thaliana*, *Solanum licopersycum*, and *Populus trichocarpa*, we

constructed a neighbour-joining (NJ) phylogenetic tree using their full-length amino acid sequences (Figure 1). In Arabidopsis the sHSP classification is based on the gene cellular locations: cytoplasmic-nuclear (CI, CII, CIII, CIV, CV, CVI), chloroplastic (CP), endoplasmic reticulum (ER), mitochondrial (MTI and MTII), and peroxisomal (PX) (SCHARF; SIDDIQUE; VIERLING, 2001). Accordingly, the classification of RcsHSP was performed based on the homology with *A. thaliana*, *S. licopersycum*, and *P. trichocarpa* genes and their cellular locations. Six groups were classified as cytosolic, encompassing 76 members including 24 *R. communis* homologs. C-I was the largest subfamily within the cytosolic group, with 48 members including 17 *R. communis* homologs. The chloroplastic group (CP) showed eight members (including two *R. communis* homologs), the mitochondrial group (MT) showed 12 members (including three *R. communis* homologs), the nuclear group (Nu) showed 13 members (including eight *R. communis* homologs), the peroxisomal (PX) showed seven members (including one *R. communis* homologs), whereas nine members (including three *R. communis* homologs) are likely found in the *endoplasmic* reticulum (ER). *R. communis* shows the same number of genes as Arabidopsis in groups C-III, C-VI and MT (Figure 1). Taking together, the NJ phylogenetic tree and cellular locations predictions indicate that *R. communis* sHSP are functionally distributed in different compartments like other land plants (GONG; WANG; LU, 2015; LOPES-CAITAR *et al.*, 2013; MANI; RAMAKRISHNA; SUGUNA, 2015; MU *et al.*, 2013). Additionally, according to the NJ phylogenetic tree, some of the *R. communis* and sHSP subfamily members of other plants were grouped in pairs (Figure 1), suggesting the occurrence of one whole-genome triplication event and two recent doubling events (MAGADUM; BANERJEE; MURUGAN, 2013; MOGHE *et al.*, 2014). For example, *RcsHSP20* (29827.m002646) and *RcsHSP21* (29827.m002650) were clustered together with their closest homolog in *A. thaliana* (At4G10250.1), *S. licopersycum* (Solyc11g020330.1), and *P. trichocarpa* (Potri.013G080200.1) indicating that they resulted from proximal duplication after the divergence among *R. communis*, *A. thaliana*, *S. licopersycum*, and *P. trichocarpa*. Finally, the fact that RcsHSP genes from various species were clustered within the same clades based on their predicted cellular locations, suggest protein evolutionary conservation among these organisms.

3.2 Conserved motifs and gene structure analysis of RcsHSP subfamily members

Conserved motifs were identified by using The Multiple Expectation Maximization for Motif Elicitation (MEME), whereas gene structure was assessed by using Gene Structure Display Server (GSDS). Ten putative motifs were found amongst RcsHSP genes, with lengths ranging from 11–127 aa (Figure 2a, Supplementary Table S4). Most RcsHSP genes contained motifs 3 through 8, but only motifs 4, 6 and 8 were identified as sHSP protein domains (pfam code CL0190). Nevertheless, all 41 sHSP sequences possess at least on of these three motifs. Cytosolic and endoplasmic reticulum proteins show predominantly domains 4 and 6, whereas mitochondrial, chloroplastic, and nuclear proteins show motifs 6 and 8 (Figure 2a, Supplementary table 4).

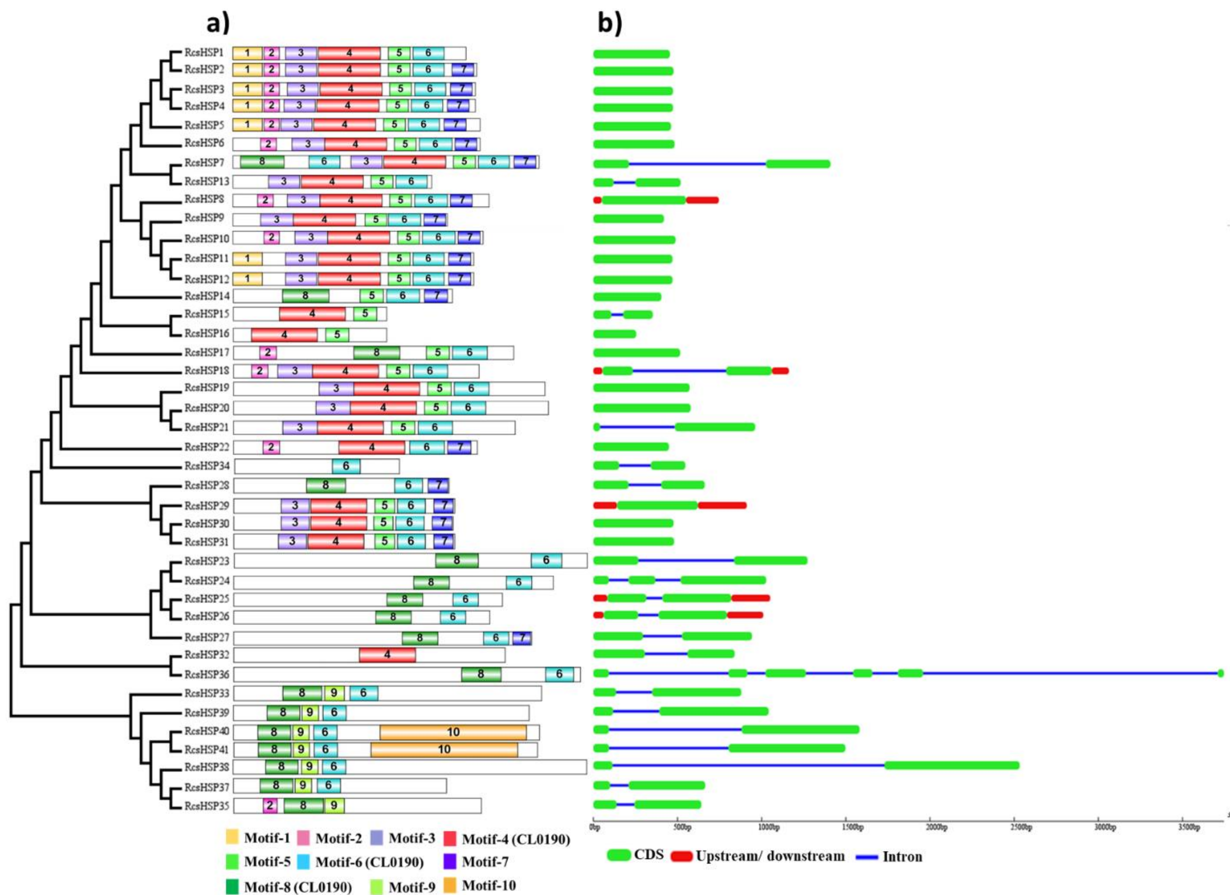

Figure 2. RcsHSP conserved motifs and structure of RcsHSP genes. a) NJ phylogenetic tree and distribution of conserved motifs of 41 RcsHSP proteins. Ten putative motifs are indicated in different colored boxes. b) Exon/intron organization of RcsHsp genes. Green boxes

represent exons (CDS), blue lines represent introns and red boxes represent up- or downstream regions.

To advance our understanding of the structural diversity of RcsHSP genes, we assessed the intron-exon configuration in the coding sequences of RcsHSP genes (Figure 2b). Seventeen out of 41 genes (41.5%) showed no introns, whereas 19 (46.3%) showed one intron and only two (4.9%) genes showed more than one intron (Figure 2b). Closely related sequences in the same subfamily shared similar intron number and exon length. For example, most genes in the cytosolic group C-I had no introns or up- and downstream regions, except for *RcsHSP7*, *RcsHSP13*, *RcsHSP15*, and *RcsHSP18* that showed one intron each of different lengths. Additionally, *RcsHSP8* and *RcsHSP18* showed up- and downstream regions (Figure 2b). All three mithocondrial genes showed one intron each, but only the closely related *RcsHSP25* and *RcsHSP26* showed up- and downstream regions. Most genes in the nuclear group showed one intron and no up- and downstream regions, except for *RcsHSP36* that showed five introns (Figure 2b). These patterns are highly suggestive of a duplication-mediated origin for these genes.

R. communis genome is approximately 320 Mb organized as 10 chromosomes (CHAN *et al.*, 2010). Some models have been proposed to shed some light on the understangin of genome duplication events in dicotyledons. The first model suggests the existence of a common ancestral hexaploidization event Jaillon *et al.*, 2007), whereas the second model suggests that all dicotyledons share one duplication event in their genomes (CHAN *et al.*, 2010; VELASCO, RICCARDO *et al.*, 2007). suggested that *R. communis* genome underwent a hexaploidization event due to the fact that triplications were found in multiple groups of scaffolds. Nevertheless, tandem duplication accounts for most of the diversity acquired by large gene families in plants during evolution (KRSTICEVIC *et al.*, 2016). RcsHSP subfamily presented 19 genes produced by tandem duplications events encompassing only cytosolic and nuclear genes (Supplementary Figure S1). This might have been crucial for the diversification of the RcsHSP subfamily and acquisition of tolerance in *R. communis*. They can be divided into seven groups that might be useful to describe tandem duplication of sHSP genes in *R. communis*. Interestingly, fourteen out of 19 genes are cytosolic, whereas four are

nuclear genes (Supplementary Figure S1). The presence of multiple-copy sHSP genes in *R. communis* may contribute to explain its high tolerance to abiotic stress, opening up uncountable possibilities to exploit phenotypic variation, which could be very useful for breeding programs worldwide.

3.3 Modulation of *RcsHSP* genes during germination and seedling stablishment in response to abiotic stress

Modulation of *RcsHSP* genes in response to abiotic stress was assessed by two different approaches: microarray and qRT-PCR analysis. First, *R. communis* microarray gene expression was mined from unpublished data derived from a genomics approach using microarray analysis during seed germination at 20, 25 and 35 °C (RIBEIRO, PAULO R. *et al.*, 2018). Log-transformed data was used to construct a heatmap representation of *RcsHSP* expression profiles during radicle protusion (RP) and early seedling development (R2) at different temperatures. In general, the expression of *RcsHSP* genes increased gradually with the temperature reaching their maximum expression at 35°C for both RP and R2 stages, suggesting that these genes are involved in temperature-related responses of *R. communis* during seed germination and early seedling development (Suplementary Table S5). However, eleven genes (*RcsHSP8*, *RcsHSP15*, *RcsHSP18*, *RcsHSP27*, *RcsHSP33*, *RcsHSP35*, *RcsHSP37-41*) showed higher expression levels at RP than R2 for all temperatures, suggesting that these genes might play protective function at early seed germination in response to drought stress due to negative matrix potential during seed embebition (RIBEIRO, PAULO R.; WILLEMS; *et al.*, 2015).

We used qRT-PCR to further explore *RcsHSP* response to abiotic stress (Figure 3-5). Ten *RcsHSP* genes were selected for qPCR analysis according to their cellular locations: four cytosolic (*RcsHSP9, RcsHSP10, RcsHSP11* and *RcsHSP12)*, three from the endoplasmic reticulum (*RcsHSP19, RcsHSP20* and *RcsHSP21)* and three mitochondrial (*RcsHSP25, RcsHSP26* and *RcsHSP27)*. Initially, we assessed *RcsHSP* gene expression at radicle protusion (RP) and early seedling development (R2) at three different temperatures (20, 25, and 35°C). In general, high temperatures induced upregulation of *RcsHSP* genes in RP and R2 samples as compared to the lower

temperatures, except for *RcsHSP27* since no difference was observed between samples collected at 35°C as compared with those collected at 20 and 25°C (Figure 3). It is worth noticing, that each gene was upregulated to a different extent. Mithocondrial *RcsHSP26* and cytosolic *RcsHSP10* showed the highest upregulation among the genes if one compares the expression levels at 35°C with those observed for 20 and 25°C within each gene.

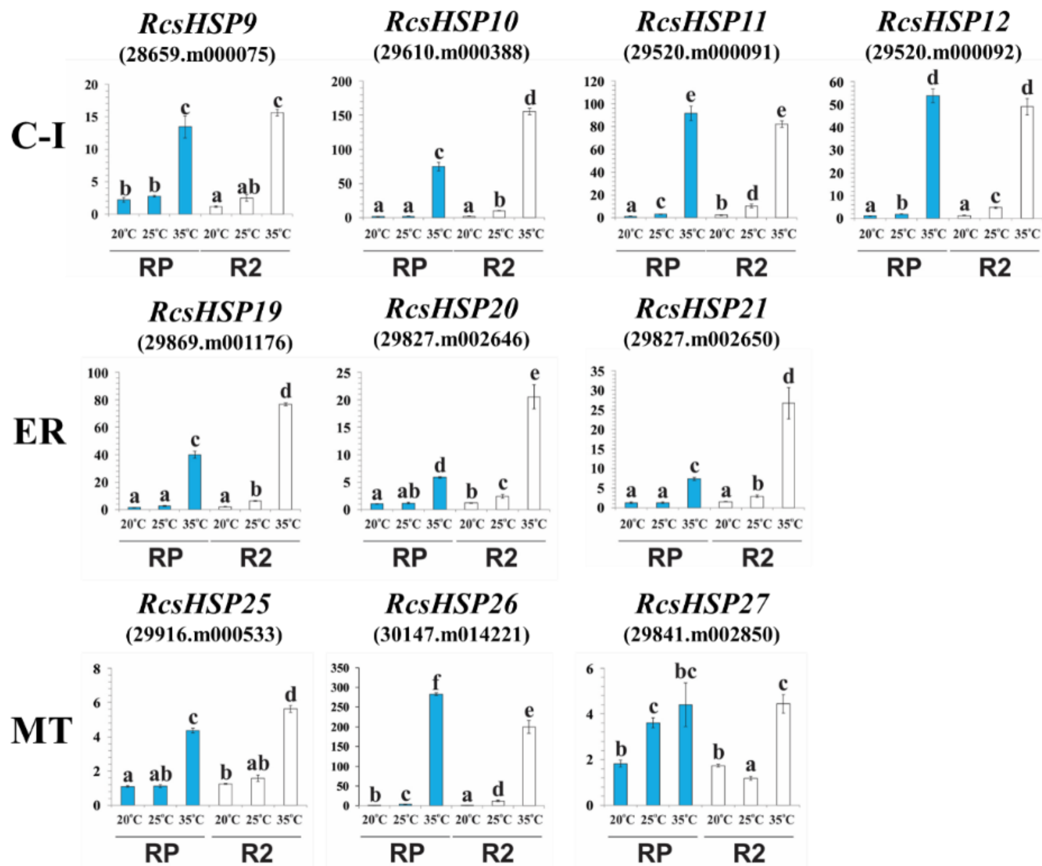

Figure 3. *RcsHSP* gene expression in seeds germinated under differents temperatures. Samples were collected at radicle protusion (RP, blue bars) and early seedling development (R2, white bars) at three different temperatures (20, 25, and 35°C). Means and standard errors of three biological replicates containing 15–20 seeds each are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p < 0.05$).

Additionally, we assessed *RcsHSP* gene expression in roots of seedlings grown at three different temperatures (20, 25, and 35°C) (Figure 4). Among the cytosolic

genes, high temperatures strongly induced upregulation of *RcsHSP10* and *RcsHSP12* as compared to the lower temperatures, whereas for *RcsHSP11* a contrasting pattern was observed: high temperatures downregulated its expression as compared to the lower temperatures. *RcsHSP10* showed hardly no difference in expression levels in response to different temperatures (Figure 4). Among the endoplasmic reticulum genes, only *RcsHSP19* was strongly upregulated in response to high temperatures. *RcsHSP20* and *RcsHSP21* did not show any difference in expression levels in response to temperature.

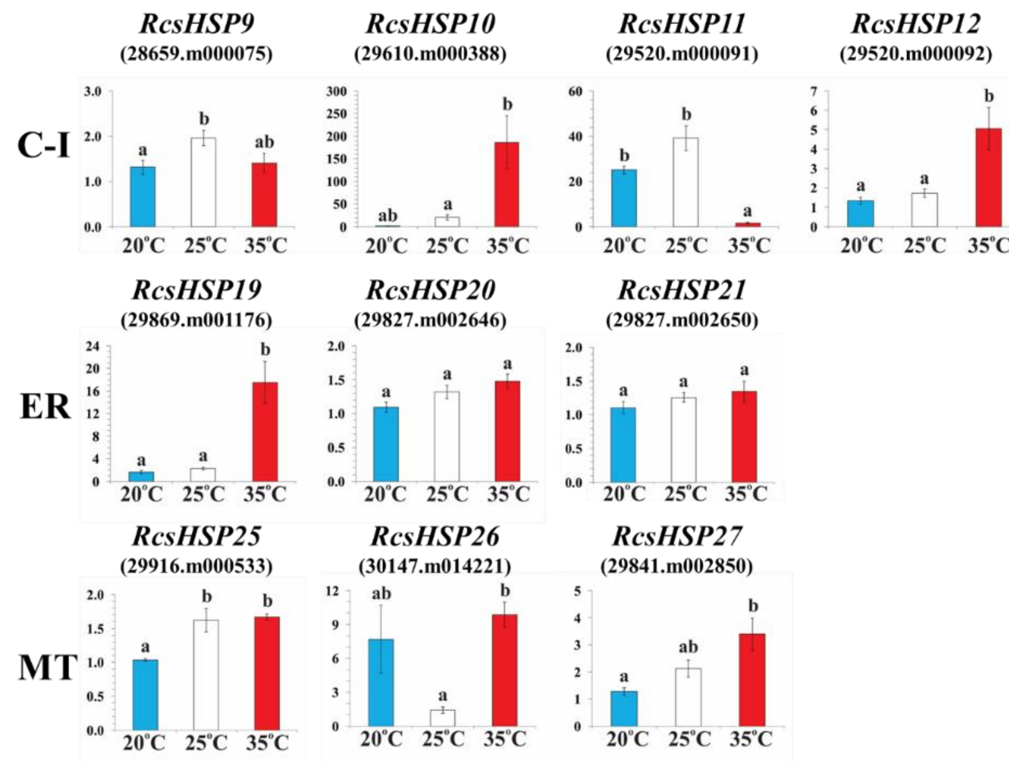

Figure 4. *RcsHSP* gene expression in roots of seedlings grown in different temperatures. Temperatures are depicted in the graphs. Means and standard errors of three biological replicates containing 15–20 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p < 0.05$).

A less clear pattern was observed among the mithocondrial genes, since expression levels of *RcsHSP25* and *RcsHSP27* did not show any difference between

samples collected at 25 and 35°C, whereas expression levels of *RcsHSP26* did not show any difference between samples collected at 20 and 35°C (Figure 4). Thus, suggesting that cytosolic and endoplasmic reticulum *RcsHSP* genes are more likely to be responsive to high temperatures than mithocondrial *RcsHSP* genes in root samples.

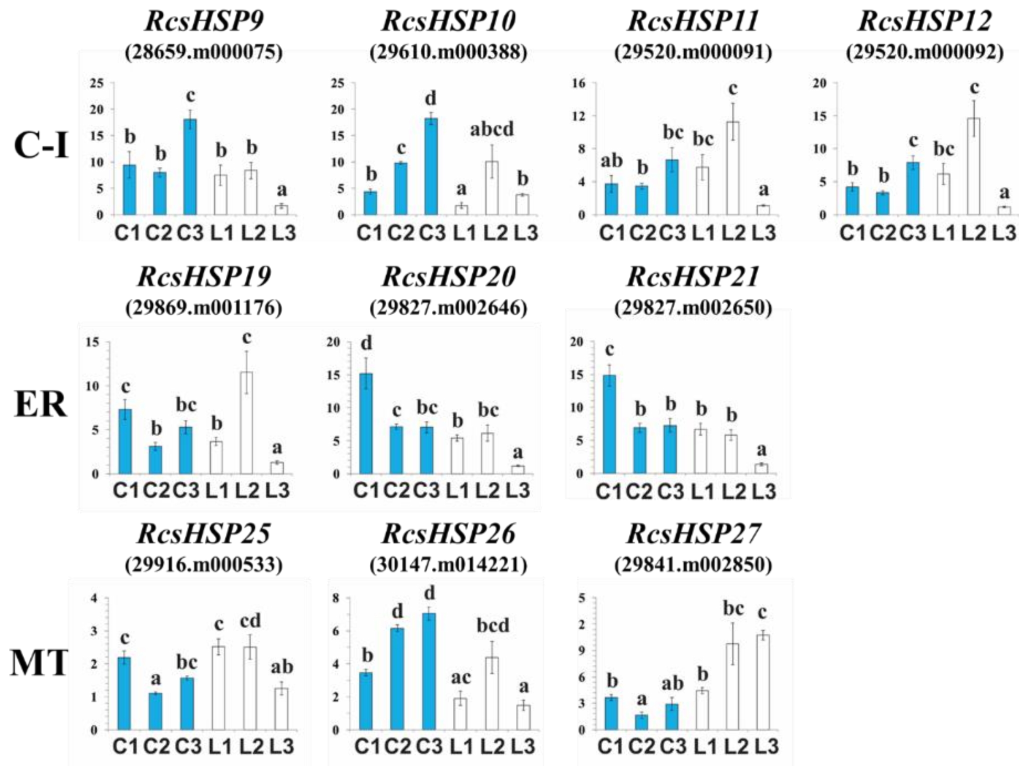

Figure 5. *RcsHSP* gene expression in cotyledons and leaves at different developmental stages. Samples comprised 3-, 6- and 9-day-old cotyledons (C1, C2, and C3) and first real leaves of the different dimensions: L1 (3x2 cm), L2 (7x5 cm)e L3 (11x7 cm). All samples were collected at 25°C Means and standard errors of three biological replicates containing 15–20 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p < 0.05$).

Finally, we assessed *RcsHSP* gene expression in cotyledons and leaves at different developmental stages. This group comprised 3-, 6- and 9-day-old cotyledons and first real leaves of the different dimensions: L1 (3x2 cm), L2 (7x5 cm)e L3 (11x7 cm). In general, expression levels of *RcsHSP* genes were higher in the cotyledons samples as compared to the leaf samples, especially for older leaves (L3) (Figure 5). This is in agreement with the fact that young cotyledons and leaves are more

susceptible to environmental cues than older leaves (IHTISHAM *et al.*, 2018). *RcsHSP27*, however, showed higher levels in the leaf samples as compared to the cotyledons samples, suggesting that this gene might be involved in later responses during seedling development. *R. communis* cotyledons and hypocotyls showed constitutive accumulation of the HSP70 protein regardless the presence of abiotic stresses, fact that was associated with the maintaince of proper functions of the seedlings during development (SCHOBERT *et al.*, 2021). Taking together, these results indicate *RcsHSP subfamily* possesses different regulatory mechanisms in response to various abiotic stress.

sHSP genes are expressed in various tissues and organs, and their expression can be modulated by abiotic stresses, such a high temperature, drought and salt stress. However, not all genes are induced by stress condition in specific tissue. *A. thaliana sHSP18.2* was induced by high temperature stress, but it was no responsive upon drought stress (SAKUMA *et al.*, 2006). Microarray-based expression analysis of tomato under different abiotic stresses showed high variability in the *SlsHSP* expression profile, which included comparison between resistant and susceptible tomato plants under high temperature, salt and drought stress conditions. YU *et al.*, (2016) reported that most of the *SlsHSP* genes were responsive to high temperature, whereas none of the genes were down-regulated. Similarly, nearly all small heat shock protein were upregulated by heat stress in soybean, pepper and watermelon (GONG; WANG; LU, 2015; HE *et al.*, 2019; LOPES-CAITAR *et al.*, 2013). In response to salt and drought stress, however, several *SlHsp20* genes were down-regulated. *OsHSP* displayed tissue-specific expression profile: *OsHSP* genes were strongly upregulated by heat shock stress, whereas none of them were upregulated by cold stress. *OsHSP71.1* was upregulated by ABA, whereas *OsHSP24.1* was downregulated, implying that *OsHSP* genes may play different roles in plant development and abiotic stress responses (ZOU *et al.*, 2009).

3.4 ***Arabidopsis thaliana* overexpressing *RcsHSP* genes showed a specific antioxidant enzyme activity and metabolite signature that led to enhanced abiotic stress tolerance**

To ultimately address the contribution of *RcsHSP* genes in abiotic stress responses, *Arabidopsis thaliana* plants overexpressing two *RcsHSP* genes under the control of the CaMV35S promoter were generated and tested for high temperature, salt stress and water restriction tolerance. Cytosolic *RcsHSP12* and endoplasmic reticulum *RcsHSP19* genes were selected because of their prominent upregulation in response to high temperatures during seed germination and seedling establishment (Figure 3 and 4). Nearly 100% of maximum seed germination was observed in control conditions (seeds imbibed in water at 22°C) for both wild type and transgenic *Arabidopsis thaliana* plants. We chose germination at 35°C as high temperature stress condition based on previously reported germination behaviour of *R. communis* seeds (RIBEIRO *et al.*, 2014; RIBEIRO, PAULO *et al.*, 2015). *R. communis* seeds germinate to a high percentage and faster at 35°C, whereas for *A. thaliana* seeds optimum germination temperature is 22°C (DE ARAÚJO SILVA *et al.*, 2016). At 35°C, wild type *A. thaliana* showed only 7% of germination, whereas *Arabidopsis thaliana* plants overexpressing two *RcsHSP* genes showed up to 40% of maximum germination (Figure 6a), therefore overexpression of *RcsHSP* genes enhanced *A. thaliana* seed germination thermotolerance.

Water restriction was achieved by imbibing seeds in different polyethylene glycol (PEG) potentials (-0.2, -0.4, -0.6, -0.8 and -1.0 MPa) (Figure 6b). No difference whatsoever was observed between wild type and transgenic *Arabidopsis thaliana* plants overexpressing *RcsHSP* genes at -0.2 MPa (Figure 6b). At -0.4 MPa, wild type *A. thaliana* maximum germination dropped to 65%, whereas *Arabidopsis thaliana* plants overexpressing *RcsHSP* genes showed up to 93% of maximum germination (Figure 6c). The effect of water restriction on seed germination became more prominent with the decreasing potentials (-0.6, -0.8 and -1.0 MPa). Even at the lowest potential (-1.0 MPa), *Arabidopsis thaliana* plants overexpressing *RcsHSP* genes showed higher germination than seed from the wild type (Figure 6b).

Salt stress was achieved by imbibing seeds in different NaCl solution concentrations (25, 50, 75 and 100 mM) (Figure 6c). At 25 mM NaCl, wild type *A. thaliana* showed 72% of germination, whereas *Arabidopsis thaliana* plants overexpressing *RcsHSP* genes showed between 83 to 92% of maximum germination (Figure 6b). At 50 mM NaCl, wild type *A. thaliana* maximum germination dropped to 43%, whereas *Arabidopsis thaliana* plants overexpressing *RcsHSP* genes showed between 83 to 92% of maximum germination (Figure 6b). At 75 mM NaCl, wild type *A. thaliana* maximum germination reached 29%, whereas *Arabidopsis thaliana* plants overexpressing *RcsHSP* genes showed between 67 to 85% of maximum germination (Figure 6c). Finally, at 100 mM NaCl nearly no difference was observed between wild type *A. thaliana* and *Arabidopsis thaliana* plants overexpressing *RcsHSP* genes (Figure 6c). Therefore, therefore overexpression of *RcsHSP* genes enhanced *A. thaliana* seed germination under salt stress conditions.

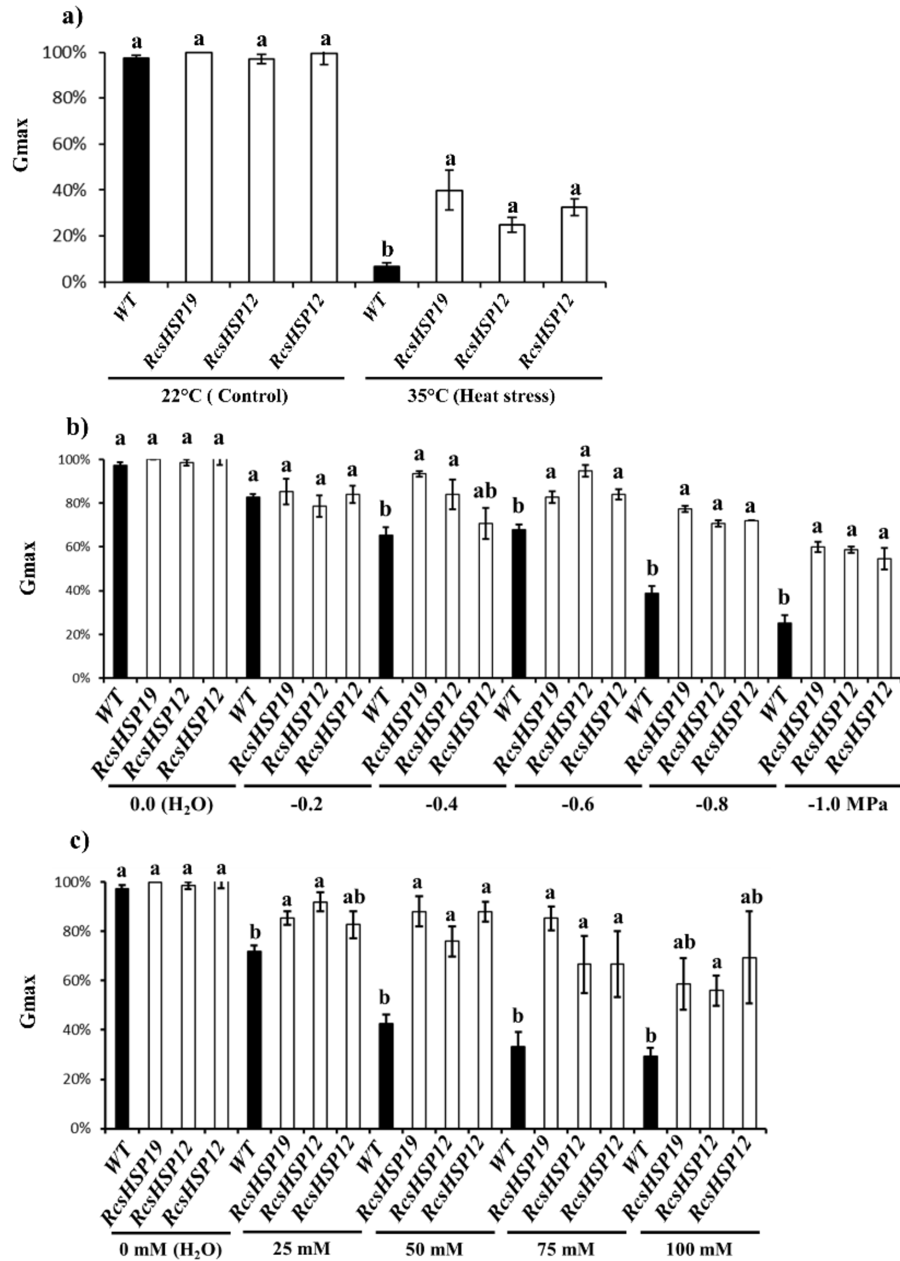

Figure 6. Maximum germination percentage of *A. thaliana* seeds overexpressing *RcsHSP* genes in response to (a) high temperature stress, (b) water restriction, and (c) salt stress. Means and standard errors of three biological replicates are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p < 0.05$).

Overexpression of *AtHSP17.6A* (C-I) increased salt and drought tolerance in Arabidopsis due to its inherent property to function as a molecular chaperone *in vivo*

(SUN, WEINING *et al.*, 2001). Overexpression of *Lilium davidii HSP16.45* (C-II) enhanced tolerance to high temperature, water restriction and salt stress. Transgenic plants presented greater superoxide dismutase and catalase activity as compared to the wild type (MU *et al.*, 2013). Transgenic rice plants overexpression sHSP17.7 showed enhanced drought tolerance (SATO; YOKOYA, 2008). It was hypothesized that sHSP17.7 may contribute drought stress by playing protecting plasma membrane integrity. *RcsHSP* involvement in protective mechanisms might be related to their ubiquitous subcellular distribution. High temperatures induced upregulation of cytosolic *RcsHSP12* and endoplasmic reticulum *RcsHSP19* genes in RP, R2, and root samples as compared to the lower temperatures, which seems to be the most likely explanation to the enhanced tolerance of *Arabidopsis thaliana* plants overexpressing *RcsHSP* genes. Taken together, these results indicate that overexpression of *RcsHSP* genes enhanced *A. thaliana* seed germination under a variety of abiotic stresses, highlighting the fact that *RcsHSP subfamily* might possess different regulatory mechanisms in order to enhance *R. communis* performance under unfavorable conditions.

Total superoxide dismutase (SOD) activity was assessed to investigate its association with the enhanced germination performance of *A. thaliana* plants overexpressing *RcsHSP* genes under a variety of abiotic stresses. For that, we used dry seeds, along with seeds that imbibed in water for 35h at 22°C (control) and 35°C (high temperature stress), as well as seeds that imbibed in -1.0 MPa PEG (water restriction) and 100 mM NaCl (salt stress) solutions. In general, *A. thaliana* plants overexpressing *RcsHSP* genes showed higher total SOD activity than the wild type at all conditions (Figure 7). Nevertheless, the most pronounced differences were observed for water restriction and salt stress conditions.

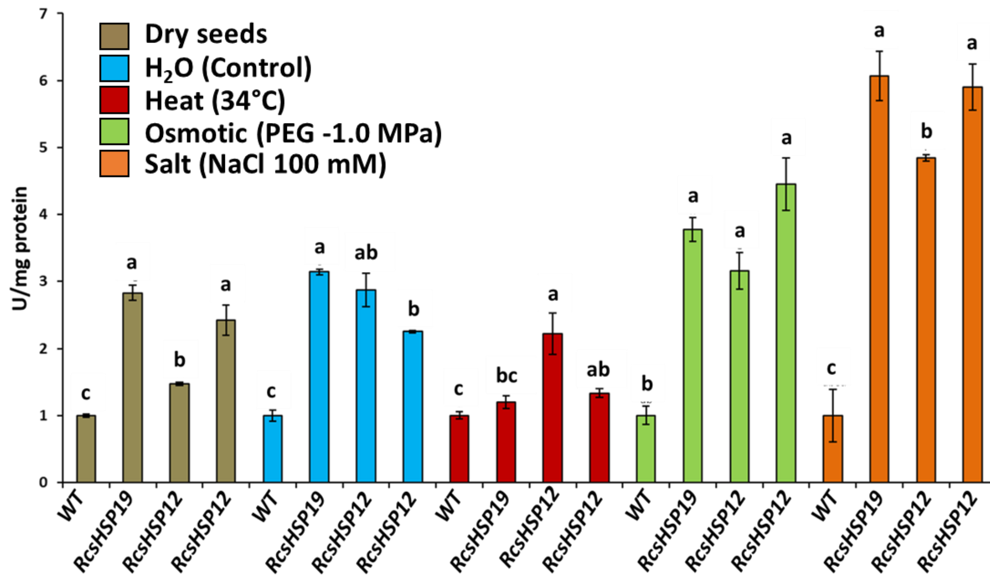

Figure 7. Total superoxide dismutase (SOD) activity of *A. thaliana* seeds overexpressing *RcsHSP* genes in response to high temperature, salt, and water restriction. Means and standard errors of three biological replicates are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p < 0.05$).

Radical oxygen species (ROS) are important for several physiological processes in plant. However, abiotic stresses such as heat, drought, salt, and chilling increase the endogeneous production of ROS, that it is associated to cell damage by oxidative stress (CHOUDHURY *et al.*, 2017). To response to oxidative stress and ROS signaling sugars and antioxidant system as the enzyme superoxide dismutase are modulated to maintain cell homeostase (ELSAYED; RAFUDEEN; GOLLDACK, 2014; GILL *et al.*, 2015). The relationship between sHSP and enzymatic antioxidant system has been described for *Rosa chinensis* and *Lilium davidii* (JIANG *et al.*, 2009; MU *et al.*, 2013). *A. thaliana* plants overexpressing *Lilium davidii HSP16.45* showed enhanced SOD and catalase (CAT) activities in response to high temperature stress, whereas *A. thaliana* plants overexpressing *R. chinensis HSP17.8* showed enhanced SOD and CAT activities in response to high temperature stress (JIANG *et al.*, 2009). Overexpression of *OsHSP18.6* in rice confered increased the tolerance to a variaety of abiotic stresses (heat, drought, salt, and cold). Concomitantly, lower levels of malondialdehyde (MDA) and greater SOD and CAT activities were observed (WANG *et al.*, 2015). Similarly,

Overexpression of *ZmHSP16.9* in tobacco enhanced peroxidase (POD), CAT, and SOD activities (SUN, LIPING *et al.*, 2012). Therefore, higher SOD activity suggest that *A. thaliana* plants overexpressing *RcsHSP* genes possess a peculiar antioxidant program enabling them to efficiently scavenge reactive oxygen species (ROS), which in turn provides the necessary tools to sustain proper germination under abiotic stresses. More importantly, it seems that there is a crosstalk between sHSP overexpression and SOD activity (DRIEDONKS *et al.*, 2015; JACOB; HIRT; BENDAHMANE, 2017).

We used a NMR-based metabolite profiling approach to unravel metabolic signatures associated with the enhanced tolerance of *A. thaliana* plants overexpressing *RcsHSP* genes in response to high temperatures. For that, we used dry seeds, as well as seeds that imbibed in water for 35 h at 22°C and 35°C. We quantified 20 primary metabolites, which included nine amino acids, four carbohydrates, and seven organic acids (Supplementary Table S6). Initially, partial least squares discriminant analysis (PLS-DA) was used to compare the overall variation in metabolite composition associated with temperature, developmental stage, and the overexpression *RcsHSP* genes (Supplementary Figure S2). PLS-DA plot shows that most of the variation in metabolite composition is be attributed to differences in temperature (22°C *versus* 35°C) and developmental stage (dry *versus* imbibed seeds) rather than due to overexpression *RcsHSP* genes. Therefore, in order to dissect high-temperature related effects on the metabolome of overexpressing *RcsHSP* genes, we decided to analyze samples imbibed at 35°C, separately.

Principal component 1 (PC1) explained 32.7% of the variance, whereas principal component 2 (PC2) explained 17.7% (Figure 8a). There is a clear separation along grincipal component 1 between *A. thaliana* wild type (Col-0) and transgenic plants overexpressing *RcsHSP* genes. Based on the PLS-DA analysis, *A. thaliana RcsHSP12* and *RcsHSP19* seeds are closely related related in terms of metabolome, than *A. thaliana* wild type (Col-0) seeds. Nevertheless, just a few metabolites appear to be responsible for this difference (Figure 8b). We set the variable importance in projection (VIP) threshold as 1 to discuss the possible contribution of each individual metabolites to the enhanced tolerance of transgenic plants overexpressing *RcsHSP* genes.

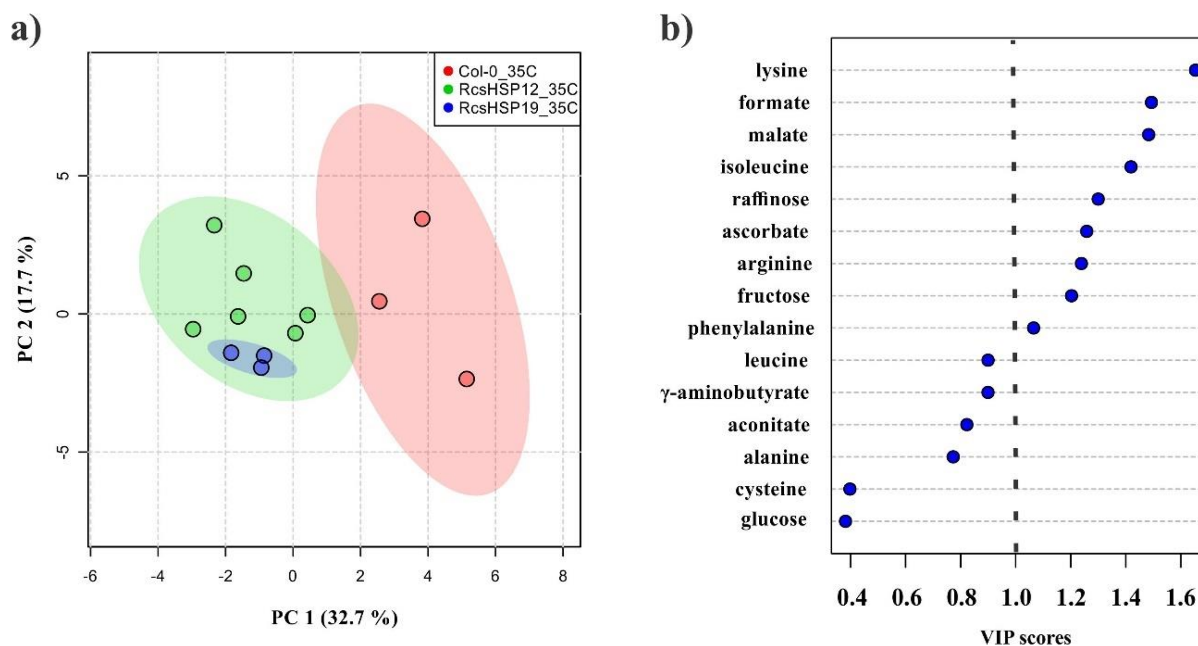

Figure 8. (a) Partial least squares discriminant analysis (PLS-DA) and (b) variable importance in projection (VIP) scores on NMR metabolite profile of seed samples imbibed at 35°C. *A. thaliana* wild type (Col-0) and transgenic plants overexpressing *RcsHSP* genes were used.

On one hand, high temperature led to the accumulation of isoleucine, formate, fructose and raffinose in seeds of the transgenic plants overexpressing *RcsHSP* genes as compared to wild type seeds. On the other hand, high temperature led to the reduction of arginine, lysine, phenylalanine, and malate in seeds of the transgenic plants overexpressing *RcsHSP* genes as compared to wild type seeds (Figure 9). Branched-chain amino acids (BCAAs), such as isoleucine, valine and leucine play important roles in plant to growth and defense (XING; LAST, 2017). BCAAs and amino acids sharing biosynthetic pathways with BCAA, including lysine are generally accumulated upon under abiotic stresses. BCAAs were accumulated upon heat stress during soybean seed development (CHEBROLU *et al.*, 2016), whereas isoleucine levels increased in maize plants upon low- and high-temperature stress (SUN *et al.*, 2016). These amino acids may act either as compatible osmolytes or as an alternative source of electrons for the mitochondrial electron transport chain (JOSHI; ZHANGJUN; JANDER, 2010; OBATA; FERNIE, 2012). Lysine catabolism is responsible for the synthesis of the immune signal pipecolic acid that regulates plant defence mechanisms enhancing systemic acquired

resistance (ZEIER, 2013). Therefore, increased levels of isoleucine along with reduced levels of lysine might support the hypothesis that these amino acids play major role in enhancing abiotic stress tolerance in seeds of transgenic plants overexpressing *RcsHSP* genes.

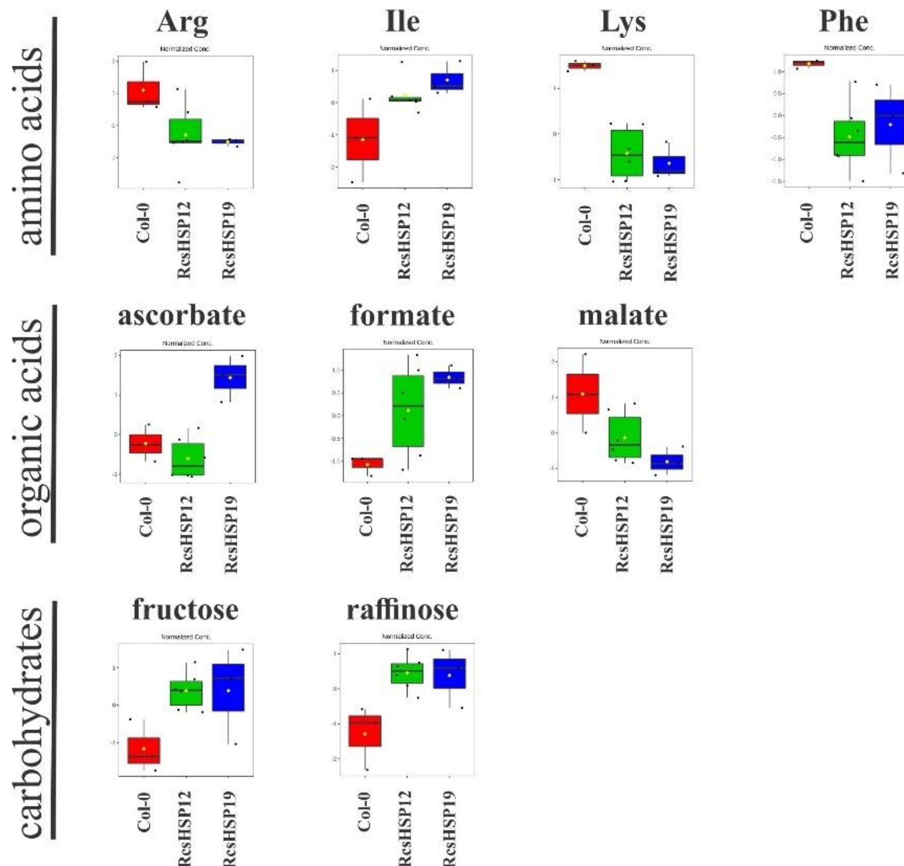

Figure 9. Relative concentration of some metabolites based on NMR metabolite profile of seed samples imbibed at 35°C. *A. thaliana* wild type (Col-0) and transgenic plants overexpressing *RcsHSP* genes were used.

Raffinose family oligosaccharides (RFO) are sucrose galactoside derivatives that act as osmoprotectants in plant cells providing membrane protection and prevent the deleterious effects of ROS (NISHIZAWA-YOKOIA; YABUTAB; SHIGEOKAA, 2008). Raffinose and galactinol were accumulated in *A. thaliana* seedlings upon heat-acclimatation (MUELLER *et al.*, 2015). Raffinose also accumulated in maize leaves subjected to severe heat stress, whereas greater levels of the RFO galactinol were observed in *R. communis* roots grown at 35 ºC than at 20 ºC and 25 ºC (RIBEIRO *et al.*,

2015). Thus, seeds of transgenic plants overexpressing *RcsHSP* genes might prevent oxidative damage by enhancing the biosynthesis of osmoprotectants molecules.

4. CONCLUDING REMARKS

In this study, we have identified 41 sHSP genes from *R. communis* genome. A comprehensive analysis of these genes was performed, including of gene structure, phylogeny, gene duplication, expression profile, and the assessement of abiotic stress tolerance. This work represents the first extensive study of the RcsHSP subfamily. *A. thaliana* plants overexpressing the cytosolic *RcsHSP12* and the endoplasmic reticulum *RcsHSP19* genes showed enhanced germination performance in response to high temperature, water restriction and salt stress then the wild-type plants. *A. thaliana* plants overexpressing *RcsHSP* genes also showed enhanced SOD activity and higher content of osmoprotectants molecules than the wild type at all conditions. Gene expression analysis showed that RcsHSP subfamily possesses different regulatory mechanisms in response to various abiotic stress. Tanken together, our results support that upregulation of sHSP play major role for cellular homeostasis under abiotic stress, including a possible link between sHSP and the regulation of gene expression that are involved in the biosynthesis of primary metabolites and antixiodant system. This will be very helpful for breeding programs aiming at developing high tolerant *R. communis* plants, providing economic and social support for farmers in semiarid areas worldwide.

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest

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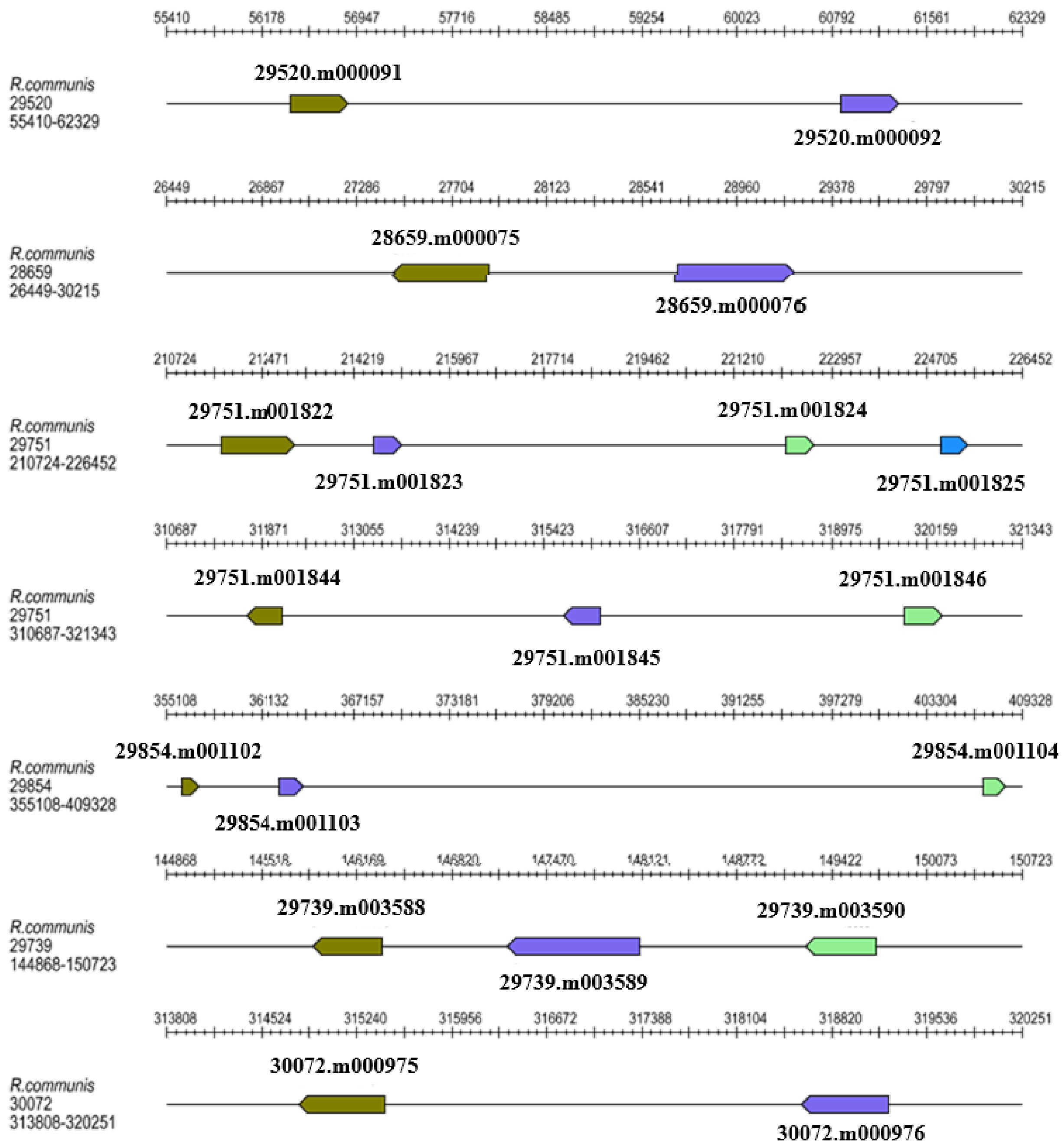
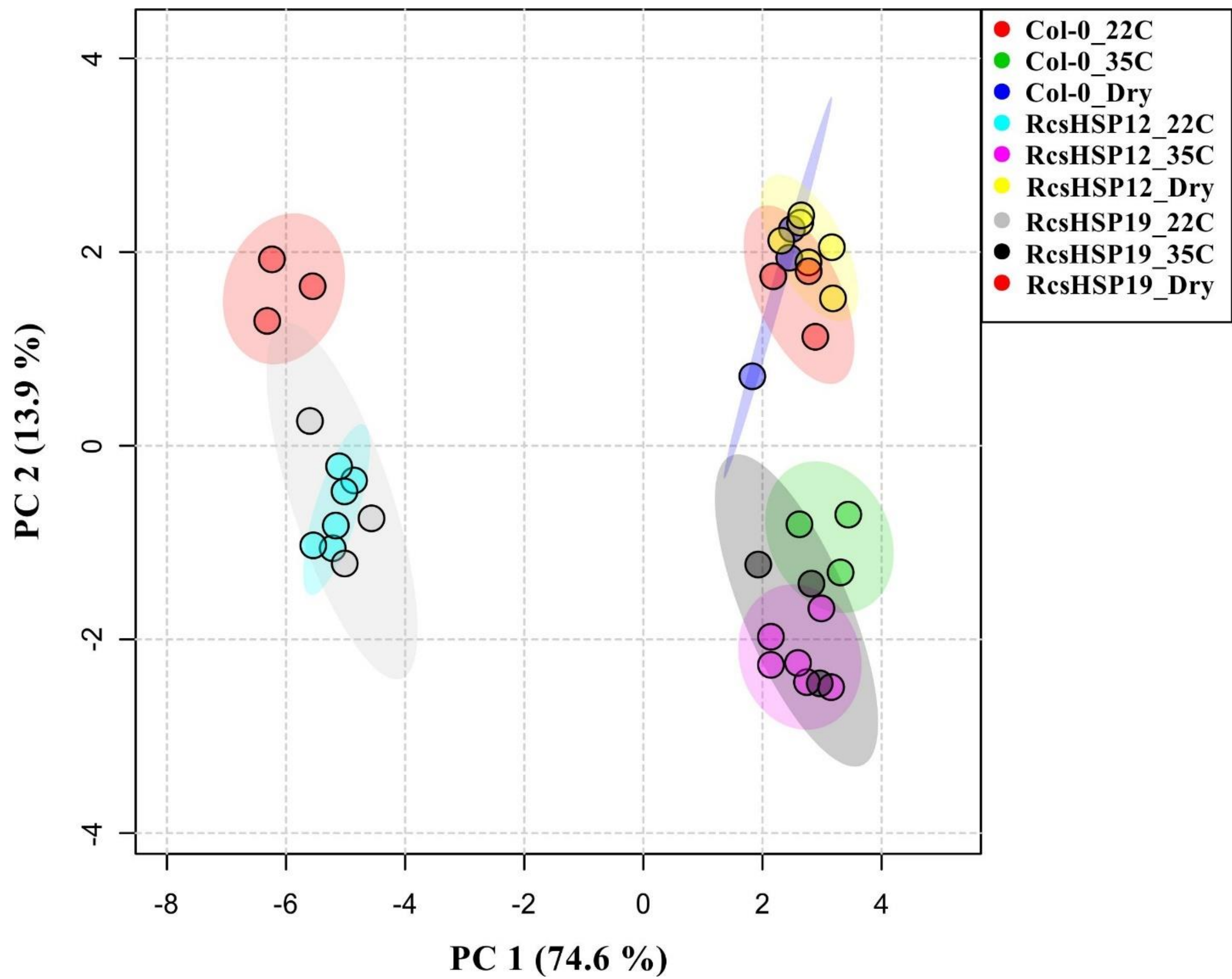
SUPLEMENTARY MATERIAL



Supplementary Figure S1. Tandem duplications of RcsHsp subfamily genes were identified in PTG Base (http://ocri-genomics.org/PTGBase/).



Supplementary Figure S2. Partial least squares discriminant analysis (PLS-DA) based on NMR metabolite profile of seed samples imbibed at different temperatures. *A. thaliana* wild type (Col-0) and transgenic plants overexpressing *RcsHSP* genes were used.

Supplementary Table S1: Primers used for qPCR analysis.

Name	Gene ID	Fw	Rv	Amplicon size
Rc9sHSP	28659.m000075	ATTGTTTGGCAACCCAATGT	CCTGGAAGATCTGCCTTGAA	138
Rc10sHSP	29610.m000388	CCTAGCAACTCCTCGGTCTG	TCTTTAGCCCTGGGAGATCA	119
Rc11sHSP	29520.m000091	TTGACGGCCTCTTCAACTCT	TCCGGTGTTTCTTTCCAGTC	94
Rc12sHSP	29520.m000092	CTCCGCAAATTTACCTGCAT	CATCGGCTTTGAAAATGTGA	101
Rc19sHSP	29869.m001176	GCCATACACAAGGCCATCTT	TTCCACTCCTTTTGGGATTG	100
Rc20sHSP	29827.m002646	TCACCAGCAAGAGTGGACTG	CCTTCTTCAACCCTGGAACA	76
Rc21sHSP	29827.m002650	GAGGCAAATCTGCTGACTGA	CAGTCCACTCTTGCTGGTGA	113
Rc25sHSP	29916.m000533	GATGATCTCGGCACTGTCAA	CCCATCTAGTGGAAAGAAACCA	99
Rc26sHSP	30147.m014221	CCAACAGCCTCACGACTCTT	TCCCAAACGTCTGAGAAGAAA	140
Rc27sHSP	29841.m002850	TCAGACGGGAAAGAAGTTGC	GGGAAGAACTCATAGAGTTGTGGA	134
ACT	30206.m000761	TCCCTCAGTACGTTCCAGCA	CACCTCCATACTCCTCCCT	198
PP2AA1	28883.m000734	GTTGGGTGTTGGGTTCTTTG	ATTCTTCAGCAAGGCGTTT	119

Supplementary Table S2: Primers used for cloning.

Name	Gene ID	Fw	Rv
Rc12sHSP	29520.m000092	ATGGCATCTTCTCTAGCTTT	CTACTCAACCTTAATTTGAACCG
Rc19sHSP	29869.m001176	ATGGCCAGCCCAAGATTC	TCACATCTCAGACTTCACAGTCTT

Supplementary Table S3. Characteristics of the small Heat Shock Protein (sHSP) proteins in *Ricinus communis*.

Group	N°	Gene ID	Cellular locations	Lenght (bp)	Molecular Weight (Da)	Theoretical pI	Arabidopsis ortholog
C- I	1	**29751.m001824**	Cyto	151	17515.81	5.34	AT2G29500/AT1G07400/AT5G59720
	2	**29751.m001844**	Cyto	158	18251.68	5.81	AT2G29500/AT1G07400/AT5G59720
	3	**29751.m001827**	Cyto	157	18119.52	5.54	AT2G29500/AT1G53540/AT5G59720
	4	**29751.m001846**	Cyto	157	18191.68	5.81	AT1G07400/AT2G29500/AT1G53540
	5	**29751.m001845**	Cyto	153	17637.99	6.17	AT2G29500/AT5G59720/AT1G07400
	6	**29751.m001825**	Cyto	160	18421.81	6.32	AT1G53540/AT1G07400/AT2G29500
	7	**29751.m001822**	Cyto/Nucl/Chlo	198	22363.45	8.76	AT2G29500/AT1G07400/AT5G59720
	8	**29610.m000391**	Cyto	166	18980.4	5.74	AT5G59720/AT1G53540/AT3G46230
	9	**28659.m000075**	Cyto	139	15686.94	6.77	AT5G59720/AT2G29500/AT3G46230
	10	**29610.m000388**	Cyto	162	18420.91	6.34	AT5G59720/AT1G53540/AT3G46230
	11	**29520.m000091**	Cyto	156	17789.12	5.85	AT5G59720/AT1G53540/AT3G46230
	12	**29520.m000092**	Cyto	156	17.821.21	7.94	AT5G59720/AT3G46230/AT1G53540
	13	**29751.m001823**	Cyto/Chlo	129	15043.45	6.84	AT1G07400/AT1G59860/AT1G53540
	14	**29633.m000911**	Cyto	134	15364.58	5.81	AT1G53540/AT1G07400/AT1G59860
	15	**27732.m000284**	Mito/Nucl	94	10841.42	9.45	AT2G29500/AT1G07400/AT1G59860
	16	**29751.m001826**	Mito	84	10223.06	10.37	AT2G29500/AT1G59860/AT1G07400
	17	**28659.m000076**	Cyto	171	19486.08	7.93	AT2G29500/AT5G59720/AT3G46230
	18	**28644.m000931**	Cyto	150	17230.11	5.96	AT1G53540/AT5G59720/AT1G07400
ER	19	**29869.m001176**	Cyto/Extr	190	21726.15	6.45	AT4G10250/AT1G07400/AT1G59860
	20	**29827.m002646**	Extr	192	22185.53	6.21	AT4G10250/AT1G07400/AT1G59860
	21	**29827.m002650**	Cyto/Nucl	172	19776.44	5.16	AT4G10250/AT1G07400/AT1G59860
C- VI	22	**30174.m009032**	Cyto	149	16.948.02	5.03	AT1G53540/AT1G07400/AT5G59720
CP	23	**29485.m000142**	Chlo	234	26098.84	8.59	AT4G27670/AT5G51440/AT1G52560
	24	**29929.m004750**	Cyto/Mito/Chlo/nucl	253	29245.34	8.9	AT4G27670/AT1G52560/AT4G25200
MT	25	**29916.m000533**	Chlo	213	23750.62	5.73	AT5G51440/AT4G25200/AT4G27670
	26	**30147.m014221**	Chlo	203	23221.25	5.52	AT4G25200/AT5G51440/AT4G27670
	27	**29841.m002850**	Mito/Nucl	236	26740.18	7.79	AT1G52560/AT1G52560/AT4G27670
C- III	28	**29727.m000463**	Cyto/Nucl	155	17275.74	6.91	AT1G54050/AT5G12030/AT5G12020
C- II	29	**29739.m003589**	Cyto	159	17910.64	6.44	AT5G12020/AT5G12030/AT1G53540

	30	**29739.m003588**	Cyto	158	17762.48	5.93	AT5G12020/AT5G12030/AT1G53540
	31	**29739.m003590**	Cyto	159	17985.58	5.78	AT5G12020/AT5G12030/AT1G53540
C- V	**32**	**28166.m001050**	Cyto	195	22620.5	5.1	AT5G54660/AT1G53540/AT1G59860
	33	**30147.m014075**	Nucl/Cyto	221	25024.04	5.12	AT2G27140/AT5G20970/AT1G54400
C-IV	**34**	**29842.m003614**	Mito	119	13510.24	5.86	AT4G21870/AT2G29500/AT1G59860
NU	**35**	**30072.m000975**	Nucl	178	19941.09	9.31	AT1G54400/AT2G27140/AT5G04890
	36	**29636.m000751**	Nucl	249	27830.62	8.96	AT1G06460/AT1G07400/AT4G27670
	37	**30072.m000976**	Nucl	184	20947.21	9.63	AT1G54400/AT2G27140/AT5G20970
	38	**29852.m002025**	Nucl	305	33560.98	9.12	AT5G04890/AT2G27140/AT5G20970
	39	**29854.m001102**	Nucl	255	29090.72	8.69	AT2G27140/AT5G20970/AT1G54400
	40	**29854.m001103**	Nucl	264	29164.74	9.21	AT2G27140/AT5G20970/AT5G04890
	41	**29854.m001104**	Nucl	262	29105.96	9.62	AT2G27140/AT5G20970/AT5G04890

Supplementary Table S4. Amino acid sequences of ten putative motifs found in 41 *RcsHSP* proteins.

MOTIF	SEQUENCE
1	MAMIPSFFGNPRSNIFDPFS
2	SFDLWDPFKDF
3	PSSLVSRENSALANARVDWKE
4	TPEAHIFKADLPGLKKEEVKVEIEDDRVLQISGERNVEKED
5	WHRVERSSGKFLRRF
6	LPENAKMDQIKASMENGVLTV
7	PKEEIKKPEVKSIZI
8	EAYVLRVDLPGLKKEDVKVQVEBGGLVIK
9	GERPLNDNKWSRFRK
10	HQRDQEVPPKIKTETNDASDRNADNRQEJPDKEKEQKDANGKNSDANSIPGKTLGNEKIEEVDESGKMASIGSKQGLVQ EAGVCCDSKLVHCKQVFGGLLREIKKPRKSTKLVVAAGLLVLVFGLYV

Suplementary Table S5. Heatmap representation of *RcsHSP* expression profiles during radicle protusion (RP) and early seedling development (R2).

Sequence	Gene ID	Samples at different developmental stages and temperatures[a]					
		RP_20	RP_25	RP_35	R2_20	R2_25	R2_35
RcsHSP1	29751.m001824						
RcsHSP2	29751.m001844						
RcsHSP3	29751.m001827						
RcsHSP4	29751.m001846						
RcsHSP5	29751.m001845						
RcsHSP6	29751.m001825						
RcsHSP7	29751.m001822						
RcsHSP8	29610.m000391						
RcsHSP9	28659.m000075						
RcsHSP10	29610.m000388						
RcsHSP11	29520.m000091						
RcsHSP12	29520.m000092						
RcsHSP13	29751.m001823						
RcsHSP14	29633.m000911						
RcsHSP15	27732.m000284						
RcsHSP16	29751.m001826						
RcsHSP17	28659.m000076						
RcsHSP18	28644.m000931						
RcsHSP19	29869.m001176						
RcsHSP20	29827.m002646						
RcsHSP21	29827.m002650						
RcsHSP22	30174.m009032						
RcsHSP23	29485.m000142						
RcsHSP24	29929.m004750						
RcsHSP25	29916.m000533						
RcsHSP26	30147.m014221						
RcsHSP27	29841.m002850						
RcsHSP28	29727.m000463						
RcsHSP29	29739.m003589						
RcsHSP30	29739.m003588						
RcsHSP31	29739.m003590						
RcsHSP32	28166.m001050						
RcsHSP33	30147.m014075						
RcsHSP34	29842.m003614						
RcsHSP35	30072.m000975						
RcsHSP36	29636.m000751						
RcsHSP37	30072.m000976						
RcsHSP38	29852.m002025						
RcsHSP39	29854.m001102						
RcsHSP40	29854.m001103						
RcsHSP41	29854.m001104						

[a]Numbers next to RP and R2 indicates the temperature in which samples were collected

Supplementary Table S6. ^{1}H NMR spectral data of identified metabolites.

Metabolite	δ_H (J in *Hz*)
aconitate	6.40 (s)
Alanine	1.48 (d, 7.2)
arginine	2.75 (t, 7.6)
ascorbate	4.65 (d, 8.0)
asparagine	3.00 (m)
cysteine	3.25 (d, 1.2)
formate	8.35 (s)
fructose	4.43 (d, 5.2)
glucose	5.42 (d, 4.0)
isoleucine	0.99 (d, 7.2)
Lactate	1.34 (d, 6.4)
Leucine	1.04 (t, 7.6)
Lysine	1.75 (q, 7.2)
methionine	2.11 (s)
phenylalanine	7.4 (m)
raffinose	5.44 (d, 4.0)
Serine	3.80 (m)
sucrose	5.24 (d, 3.6)

CAPÍTULO 4: Modulation of NF-YB genes in *Ricinus communis* L. in response to different temperatures and developmental stages and functional characterization of RcNF-YB8 as an important regulator of flowering time in *Arabidopsis thaliana*

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Modulation of NF-YB genes in Ricinus communis L. in response to different temperatures and developmental stages and functional characterization of RcNF-YB8 as an important regulator of flowering time in Arabidopsis thaliana

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Overexpression of *Ricinus communis NF-YB8* transcription factor modulates flowering time in *Arabidopsis thaliana*

ABSTRACT

We have characterized the *NF-YB* gene family in *R. communis* using bioinformatics, ecotopic expression, and transcriptomics. A total of 14 *RcNF-YB* genes were identified in *R. communis* genome using the conserved NF-YB region. This number is similar to what is found in *A. thaliana* (13 genes) and *O. sativa* (11 genes), whereas it is considerably lower to what is found in *P. trichocarpa* (21 genes) and *S. lycopersycum* (29 genes). Several regulatory cis-elements were identified in the promoter region, including low temperature, defense and stress, MIC, MYB, and abscisic acid. *RcNF-YB* is strongly modulated by temperature and it is dependent on the stage of germination. In general, *RcNF-YB* genes showed higher expression levels in dry seeds and early imbibition (EI) samples as compared to later stages of seedling development. Ectopic expression of *RcNF-YB8* reduced flowering time in *Arabidopsis* reducing the time required for the formation of the first visible bud, the time required to open the first flower, and the time required for the formation of the first visible silique. At the end of the life cycle, ectopic expression of *RcNF-YB8* affected plant height (PH), silique length (SL), the total number of silique per plant, 1000-seed weight, and seed size. Our data demonstrated the role of *RcNF-YB8* in flowering time, plant height and seed production, and it shows that it may constitute a key target gene for breeding superior *R. communis* genotypes.

Keywords: Flowering time, Castor bean, phenotyping, *Ricinus communis*

1. INTRODUCTION

Nuclear factor Y (NF-Y) is a highly conserved transcription factor complex from eukaryotes. The NF-Y complex encompasses three different subunits, NF-YA (HAP2-like, CBF-C), NF-YB (HAP3-like, CBF-A), and NF-YC (HAP5-like, CBF-C), which are crucial for the formation and proper functioning of the NF-Y complex since none of the NF-Y subunits are not able to regulate transcription individually. Therefore, they must act coordinately as heterodimer or heterotrimer complexes. They bind to the CCAAT boxes in promoter regions of their target genes and are known to be involved in several physiological processes such as seed dormancy and germination, early seedling development, root growth, flowering, embryogenesis, seed maturation, primary metabolism, and plant responses to abiotic stresses (SWAIN *et al.*, 2017). Each subunit is encoded by families of approximately 10 genes in *Arabidopsis*, allowing the formation of a large number of combinations and diversity of complexes adjusting to many different environmental conditions (SIEFERS *et al.*, 2009).

NF-Y complexes may act by two possible mechanisms. In the first, the heterodimer NF-YB-YC moves towards the nucleus, where it interacts with the NF-YA subunit to form the active NF-YA-YB-YC heterotrimer. Then, the active heterotrimer complex binds to the CCAAT boxes in promoter regions of their target genes, thereby regulating their expression. The second mechanism involves the formation of an NF-YB-YC heterodimer that interacts with a transcription factor (TF) to form an NF-YB-YC-TF heterotrimer that binds to specific regions. It is important to highlight that the NF-YA subunit can competitively inhibit the interaction of TF with the NF-YB/NF-YC heterodimer (ZHAO *et al.*, 2017). *NF-YB* genes have been characterized in *Arabidopsis. thaliana* (SIEFERS *et al.*, 2009), *Popolus trichocarpa* (WANG, RONGKAI *et al.*, 2019), *Solanum lycopersicum* (LI *et al.*, 2016), and *Oriza sativa* (YANG, WENJIE *et al.*, 2017).

Overexpression of *AtNF-YB1* and the *Zea mays* orthologue *ZmNF-YB2* led to increased tolerance to drought stress , through the activation of several drought stress-responsive mechanism, whereas overexpression of *ZmNF-YB2* led to improved grain yield under drought conditions (NELSON *et al.*, 2007). Similar was observed overexpression of ZmNF-YB16 ledding to improved drought resistance and yield

(WANG *et al.*, 2018). *AtNF-YB2* promoted early flowering and root growth, whereas *AtNF-YB3* promoted flowering as a response to high-temperature stress (KUMIMOTO *et al.*, 2008; SATO *et al.*, 2014). Moreover, the *AtNF-YB6* (LEC1) gene plays a role in embryonic development, whereas *At-NF-YB9* is involved in both embryogenesis and hypocotyl elongation. The LEC2 showed to be involved in embryonic resetting of the parental vernalized state (HUANG *et al.*, 2015; JUNKER *et al.*, 2012; KUMAR; JHA; VAN STADEN, 2020; TAO *et al.*, 2019). However, little is known about the function of *Ricinus communis* NF-YB genes. Therefore, we have performed a genome-wide analysis of the *R. communis* NF-YB gene family, as well as the functional characterization of *RcNF-YB8*. RcNF-YB8. Identification of responsive genes for abiotic stresses it is important to use in R. communis breeding.

2. MATERIAL AND METHODS

2.1 Identification of *RcTNF-YB* genes

The conserved region of the NF-YB subfamily (AKETVQECVSEFISFVTGEASDKCQREK RKTINGDDLLWALATLGFEDYY) was used as a query against the *Ricinus communis* genome using the Phytozome database (https://phytozome.jgi.doe.gov/pz/ portal.html#) (MAHESHWARI *et al.*, 2019). Based on homology, the *RcTNF-YB* putative protein sequences were submitted to the Pfam database (https://pfam.xfam.org/) to confirm the presence of the NF-YB conserved domain. The phylogenetic tree of the NF-YB subunit transcription factor proteins was constructed using MEGA 7.0.26 software (https://www.megasoftware.net/). Neighbor-joining (NJ) method with 1000 bootstrap was used to construct a phylogenetic tree with protein sequences from *R. communis* (this work), *A. thaliana* (SIEFERS *et al.*, 2009), *O. sativa* (YANG, WENJIE *et al.*, 2017), *P. trichocarpa* (WANG, RONGKAI *et al.*, 2019) and *S. lycopersicum* (LI *et al.*, 2016).

2.2 Regulatory elements and motif analysis of *RcTFN-YB* genes

Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#) database was used to retrieve the coding sequences (CDS) of all *RcTFN-YB* genes, including 1000bp

upstream of the ATG codon. Promoter analysis was performed on the Plant care database website (http://bioinformatics.psb.ugent.be /webtools/ plantcare/html/). The RcNF-YB conserved motifs were identified by using the Multiple Em for Motif Elicitation (MEME) 5.0.2 online program (https://meme-suite.org/meme/).

2.3 Expression pattern of *RcNF-YB* genes detected by transcriptome data

Gene expression analysis of the *RcNF-Y*B genes was retrieved from a microarray analysis performed on *R. communis* samples collected in different seed germination stages (dry seed, early imbibition - EI, radicle protrusion - RP, and early seedling with 2 cm root - R2) at three different temperatures (20, 25 and 35°C) (RIBEIRO *et al.*, 2018).

2.4 Gene expression of the *RcNF-YB8* gene by qPCR

R. communis samples collected in different developmental stages and temperatures were used to analyze the *RcNF-YB8* genes during germination (RIBEIRO *et al.*, 2015). For that, we used samples collected at different time intervals during germination (dry seed, early imbibition - EI, radicle protrusion - RP and early seedling with 2 cm root - R2), as well as root, cotyledon and leaf samples collected from seedlings grown at three different temperatures (20, 25 and 35°C) (RIBEIRO *et al.*, 2014b). Total RNA was extracted from 5mg of the lyophilized and ground samples using the hot borate method with modifications (WAN; WILKINS, 1994). First-strand cDNA was synthesized with 1 µg of total RNA using the Bio-Rad iScript™ cDNA synthesis kit according to the manufacturer's instructions. qRT-PCR was performed in a total volume of 10 µL containing 2.5 µL of cDNA (20x diluted), 0.5 µL of primers (10 µM), 5 µL of IQ SYBR Green Supermix (Bio-Rad), and 2 µL of ultrapure water. Serine/threonine phosphatase 2A (PP2AA1) gene was used as an internal reference gene. Further details can be found at (GOMES NETO *et al.*, 2018; RIBEIRO *et al.*, 2014a).

2.5 Cloning and *A.thaliana* transformation

The *RcNF-YB8* gene was amplified from *R. communis* cDNA and the *RcNF-YB8* amplicon was recombined into the donor plasmid (pDONr207) to produce pDONr207-*RcNF-YB8* using the Gateway® technology (Ribeiro et al., 2015c). pDONr207-*RcNF-YB8* was recombined with the pGD625 expression vector and the resulting construct

(pGD625-*RcNF-YB8*) was introduced into the AGL0 *Agrobacterium tumefaciens* strain (*CaMV35S::RcNF-YB8*) and subsequently transformed into wild-type (WT) *A. thaliana* plants by the floral dip method (CLOUGH; BENT, 1998; GOMES NETO *et al.*, 2020; ZHANG *et al.*, 2006). The selection of transformants was performed by seed germination in a media supplemented with kanamycin. T4 generation of *A. thaliana* transgenic lines was used in physiological experiments.

2.6 Phenotyping transgenic Arabidopsis lines

Three independent *A. thaliana* overexpressing lines (*RcNF-YB8* (1), *RcNF-YB8* (2), *RcNF-YB8* (3)) were selected for phenotypical characterization, along with wild-type *A. thaliana* (Col-0). Seeds were stratified in half MS medium for 72h at 4°C and germinated at 22°C under constant light. Ten-day-old seedling were transplanted to commercial plant substrate (Carolina Soil) and allowed to grown under 22°C in a 16/8 photoperiod. Three repetitions of six plants were used for phenotypical characterization during the life cycle of the plants.

Plants were phenotyped based on the time required for the formation of the first visible bud, the time required to open the first flower, the time required for the formation of the first visible silique, total leaf number (TLN), number of open flowers (NOF), number of siliques (NS). At the end of the life cycle, plant height (PH), silique length (SL), number of seeds per silique, the total number of silique per plant, 1000-seed weight, and seed size.

For seed size measurements, dried seeds (15 seeds of 3 x technical of 3 x biological) were photographed using a Zeiss AxioCamERc5s microscope and then measured by ImageJ software (https://imagej.nih.gov/ij/). The ‘particle analysis’ feature was used to measure seed area in mm^2. The images were first transformed in binary and to remove background noise the analyze the particles function was performed with the seed size ranging between 0.05 mm^2 and ‘infinity’(REN *et al.*, 2019; XU *et al.*, 2013).

3. RESULTS

3.1 Phylogenetic analysis of *NF-YB* genes in selected angiosperms

A total of 14 RcNF-YB proteins were identified in *R. communis* using the conserved NF-YB region (MAHESHWARI *et al.*, 2019). The putative RcNF-YB proteins were named according to Wang (2018), except for two new genes named subsequently as *RcNF-YB13* and *RcNF-YB14*. The identified *RcNF-YB* proteins were scanned using the Pfam database to confirm the presence of the conserved domain. The 14 *RcNF-YB* proteins of *R. communis* (this work), and all NF-YB proteins from *A. thaliana* (SIEFERS *et al.*, 2009), *O. sativa* (YANG *et al.*, 2017), *P. trichocarpa* (WANG *et al.*, 2019), and *S. lycopersicum* (LI *et al.*, 2016) were used to construct a phylogenetic tree (Figure 1).

The number of *NF-YB* genes in *R. communis* (14 genes) is similar to what is found in *A. thaliana* (13 genes) and *O. sativa* (11 genes), whereas *P. trichocarpa* (21 genes) and *S. lycopersycum* (29 genes) presented considerably higher number of NF-YB genes. A closer relationship between *R. communis* and *P. trichocarpa* genes is observed in a major part of clades (Malpighiales order) despite the difference in the number of genes (14 and 21 respectively). In a few cases, *RcNF-YB* genes showed a close relationship with *A. thaliana* orthologous genes (*RcNF-YB11-AtNF-YB5, RcNF-YB12-AtNF-YB6, RcNF-YB13-AtNF-YB11*) and *O. sativa* gene (*RcNF-YB2-OsNF-YB9*). Groups were classified according to observed clades (a1, a2, b, c, d, e, f, and g). In general, the *RcNF-YB* genes were observed in all groups except group f. Group f was formed only with *SlNF-YB* genes (Figure 1 and Supplementary Table 1). The number of NF-YB genes in each group varied slightly amongst the different species. Group a1 ranged from 2 to 4 members per species, group a2 ranged from 2 to 3 members per species, group b ranged from 0 to 2 members per species, group c only showed 1 member per species, group d ranged from 2 to 6 members per species, group e with 2 members per species, group f ranged from 0 to 11 members per species, and group g ranged from 1 to 3 members per species. *A.thaliana* and *O. sativa* did not present genes in group b (Supplementary Table 2 and Figure 1).

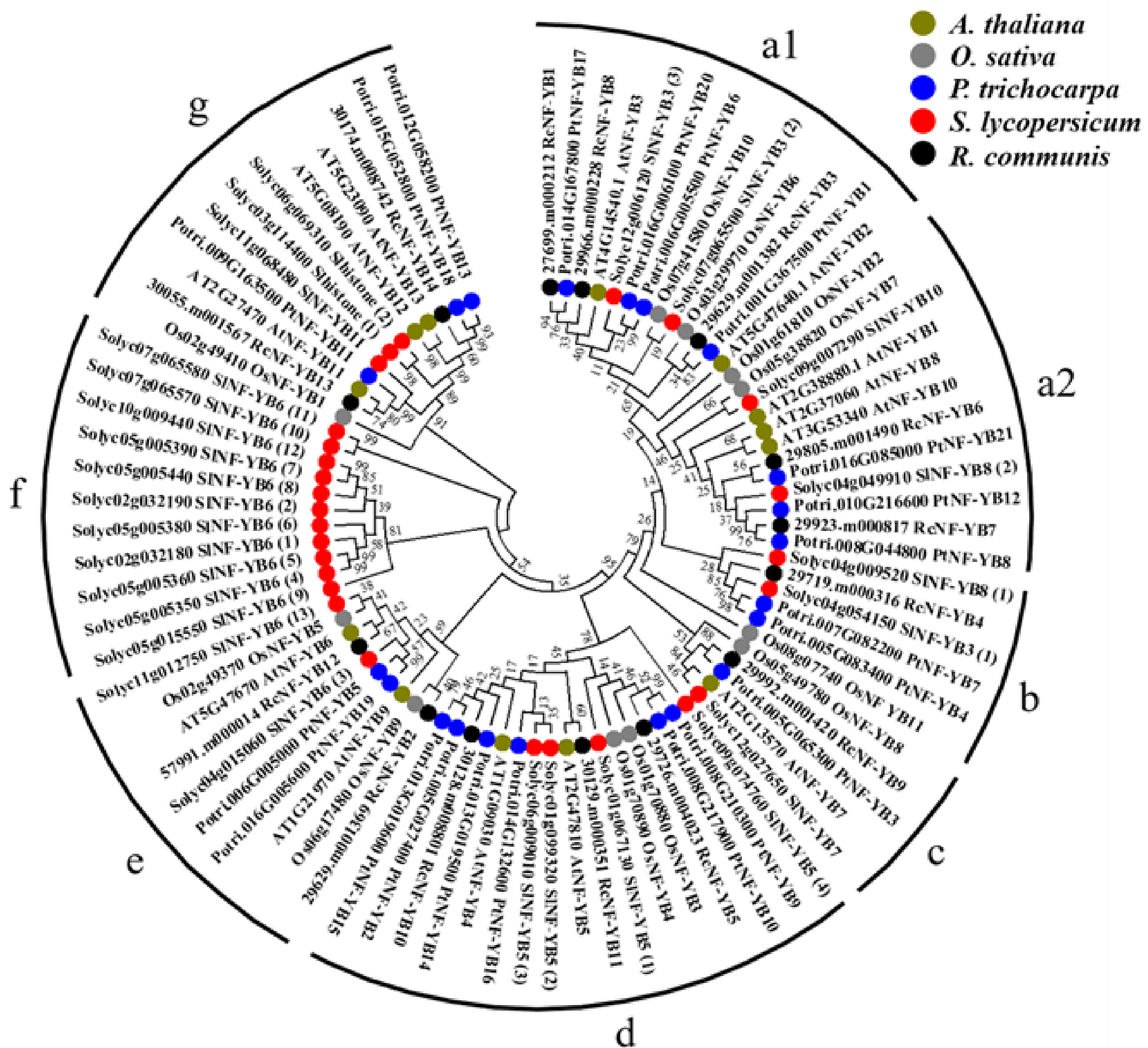


Figure 1. Phylogenetic relationship of *NF-YB* family in selected angiosperms. Genes of different species are shown in different colors. *A. thaliana* (yellow-green), *O. sativa* (gray), *P. trichocarpa* (blue), *S. lycopersicum* (red), and *R.communis* (black). The phylogenetic tree was constructed using MEGA 7.0.26 software (https://www.megasoftware.net/) using the Neighbor-joining (NJ) method with 1000 bootstrap. Groups were classified according to clades.

3.2 *In silico* analysis of *RcNF-YB* regulatory elements

Regulatory elements of the promoter region of *RcNF-YB* genes were analyzed by the Plant care database. For that, we analyzed the 1kb upstream region from the ATG sequence. Cis elements were classified as endosperm expression, auxin response, low temperature, salicylic acid, circadian control, defense and stress, gibberellin, MIC, MYB, meristem expression, light, methyl jasmonate (MeJa), anaerobic induction, and abscisic acid (ABA) (Figure 2). Cis elements related to light responses were found in the promoter region of all *RcNF-YB* genes, the anaerobic induction response was found in 9 genes (*RcNF-YB1*, *RcNF-YB2, RcNF-YB3*, *RcNF-YB4, RcNF-YB5, RcNF-YB6*, *RcNF-YB7, RcNF-YB11,* and *RcNF-YB14*), the low-temperature response was found in 3 genes (*RcNF-YB3*, *RcNF-YB6*, and *RcNF-YB8*), and the defense and stress response was also found in 3 genes (*RcNF-YB2*, *RcNF-YB6*, and *RcNF-YB7*). The MYC transcription factor response was found in 9 genes (*RcNF-YB2, RcNF-YB3, RcNF-YB5, RcNF-YB7, RcNF-YB8, RcNF-YB9, RcNF-YB10, RcNF-YB11,* and *RcNF-YB13*), whereas the MYB transcription factor response was found in 13 genes (all except *RcNF-YB5*). The salicylic acid response was found in 3 genes (*RcNF-YB3, RcNF-YB8, and RcNF-YB11*), the MeJa response was found in 7 genes (*RcNF-YB2, RcNF-YB5, RcNF-YB6*, *RcNF-YB7, RcNF-YB10, RcNF-YB12,* and *RcNF-YB13*), and the auxin response was found in 6 genes (*RcNF-YB1, RcNF-YB3, RcNF-YB6*, *RcNF-YB11,* and *RcNF-YB13*). The abscisic acid (ABA) response was found in 6 genes (*RcNF-YB1*, *RcNF-YB3*, *RcNF-YB6*, *RcNF-YB8*, *RcNF-YB9,* and *RcNF-YB14*), gibberellin response was found in 3 genes (*RcNF-YB1*, *RcNF-YB6*, and *RcNF-YB8*).

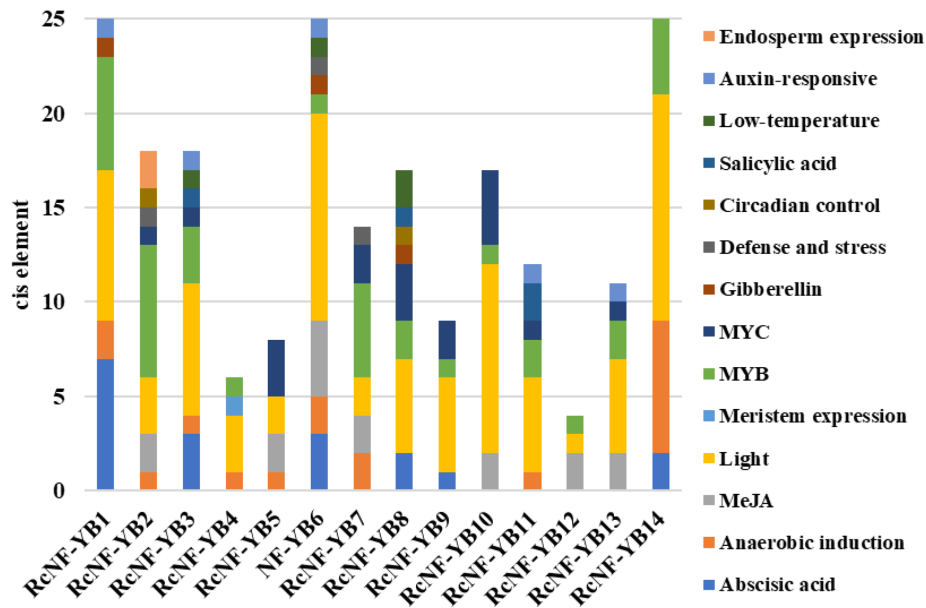


Figure 2. Analysis of the promoter region of Rc*NF-YB* genes. Cis-elements were identified using the Plant care database using 1 kb of the promoter region upstream of the start codon of the *RcNF-YB* genes.

3.3 Conserved motifs analysis of RcNF-YB proteins

Ten putative conserved motifs were identified amongst the *RcNF-YB* proteins, with lengths ranging from 6 to 45 aa (Figure 3, Supplementary Table 3). Motifs 1, 2, and 3 were the most abundant amongst *RcNF-YB* proteins: all *RcNF-YB* proteins contain motif 1, whereas ten *RcNF-YB* proteins contain motif 2 and eleven *RcNF-YB* proteins contain motif 3. Motifs 4 to 10 are only present in a few of the RcNF-YB proteins, with random occurrence throughout *RcNF-YB* proteins. Motif 4 and 9 showed to be exclusive to group a1 (*RcNF-YB1* and *RcNF-YB8*), whereas motif 5 was found in group a1 and group e (*RcNF-YB12*). Motif 6 showed to be exclusive to group a2 (*RcNF-YB6* and *RcNF-YB7*), whereas motif 10 was found in group a2 and group c (*RcNF-YB9*). Motif 7 was found in group b (*RcNF-YB4*) and group e (*RcNF-YB12*). Motif 8 was found in group b and group e (*RcNF-YB2*) (Figure 3).

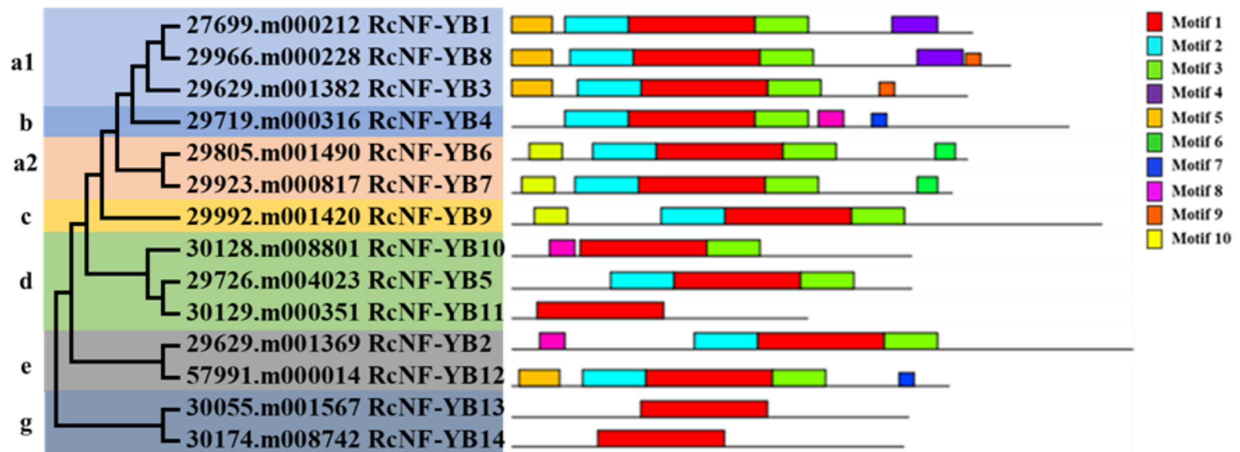


Figure 3: Conserved motifs of RcNF-YB proteins. Ten motifs were identified through the MEME tool search and indicated with different colors.

3.4 Transcriptomic patterns of *RcNF-Y* B subunit genes during germination under heat.

A heatmap plot was constructed using log-transformed transcriptome microarray data to illustrate the expression profile of *RcNF-YB* genes during germination and early seedling development at different temperatures (Figure 4). *RcNF-YB* gene expression seems to be modulated by temperature and it is dependent on the stage of germination. In general, *RcNF-YB* genes showed higher expression levels in dry seeds and early imbibition (EI) samples as compared to radicle protrusion (RP) and seedling of 2cm root (R2) samples.

RcNF-YB2, RcNF-YB3, RcNF-YB4, RcNF-YB5, RcNF-YB6, RcNF-YB7, and *RcNF-YB11* were upregulated upon high-temperature stress during early imbibition (EI), whereas *RcNF-YB1*, *RcNF-YB8, RcNF-YB9, RcNF-YB10, RcNF-YB12,* and *RcNF-YB14* were downregulated. Nevertheless, *RcNF-YB1, RcNF-YB2, RcNF-YB3,* and *RcNF-YB8* were upregulated upon low-temperature stress, whereas *RcNF-YB7, RcNF-YB7, RcNF-YB9, RcNF-YB11,* and *RcNF-YB14* were downregulated.

RcNF-YB4, RcNF-YB5, RcNF-YB9, RcNF-YB10, and *RcNF-YB14* were upregulated upon high-temperature stress during radicle protrusion, whereas *RcNF-*

YB1, RcNF-YB7, RcNF-YB8, RcNF-YB12, and *RcNF-YB13* were downregulated. In contrast, *RcNF-YB2, RcNF-YB3, RcNF-YB4, RcNF-YB5, RcNF-YB8, RcNF-YB10, RcNF-YB11*, and *RcNF-YB14* were upregulated upon low-temperature stress, whereas *RcNF-YB1, RcNF-YB6, RcNF-YB7, RcNF-YB9, RcNF-YB12,* and *RcNF-YB13* were downregulated.

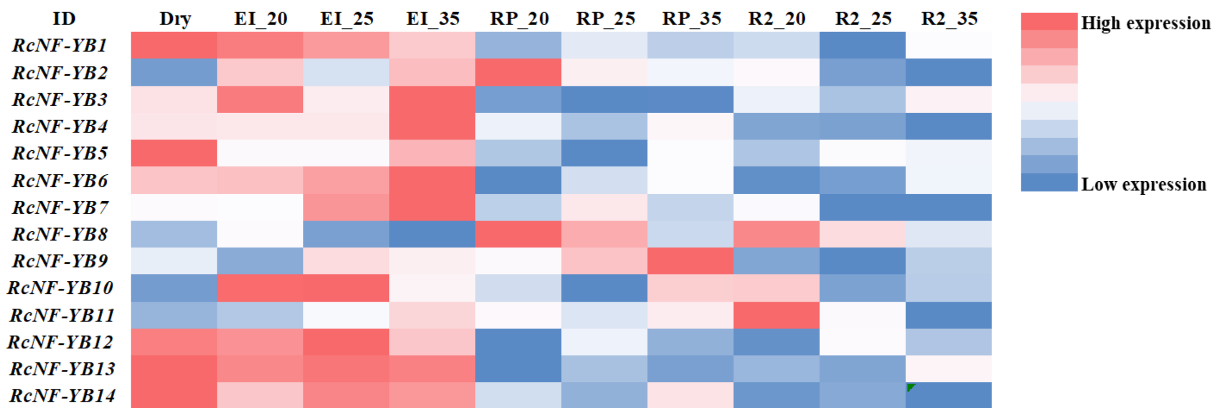


Figure 4. Heatmap of *RcNF-YB* gene expressions during germination at different temperatures. Different colors indicate the difference in gene-level expression (Red-high, white-middle, blue low). EI (early imbibition at 20, 25 and 35 °C), RP (Radical protrusion at 20, 25 and 35 °) and R2 (seedling of 2cm root at 20, 25 and 35 °C).

RcNF-YB1, RcNF-YB3, RcNF-YB6, RcNF-YB9, RcNF-YB10, and RcNF-YB13 were upregulated upon high-temperature stress at seedlings with the root of 2 cm (R2), whereas *RcNF-YB2, RcNF-YB4, RcNF-YB8, RcNF-YB11, RcNF-YB12, and RcNF-YB14* were downregulated. In contrast, *RcNF-YB1, RcNF-YB2, RcNF-YB3, RcNF-YB7, RcNF-YB8, RcNF-YB9, RcNF-YB10, RcNF-YB11,* and *RcNF-YB13* were upregulated upon low-temperature stress, whereas *RcNF-YB4, RcNF-YB5, RcNF-YB6, RcNF-YB12,* and *RcNF-YB14* were downregulated.

3.5 Relative expression patterns of *RcNF-YB8* shows upregulation in leaves

The *RcNF-YB8* gene was selected for further gene expression and cloning experiments because of its interesting expression pattern in the microarray experiment. This gene seems to be responsive to abiotic stress during seed germination, i.e. at radicle protrusion (RP) and seedling development (R2) at different temperatures, which is different from most of the other *RcNF-YB* genes that seem to be responsive to abiotic

stress largely during early seed imbibition (EI). Additionally, this gene was selected based on its position on the phylogenetic tree, forming a small clade with a few other genes from *O. sativa* and *P. trichocarpa*.

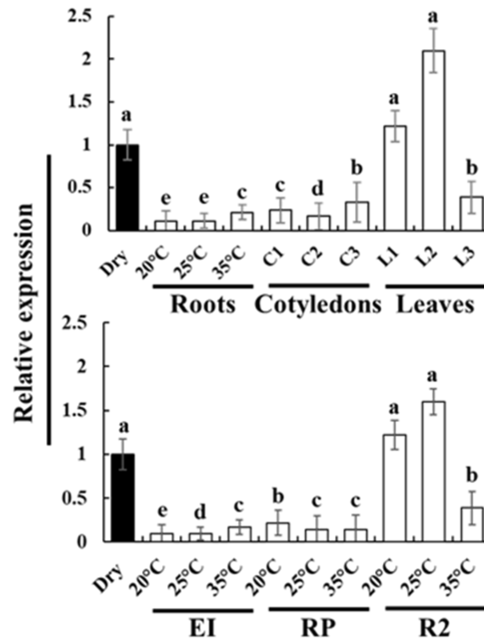


Figure 5. Gene expression of *RcNF-YB8*. a) Relative expression in the roots of seedlings at three different temperatures (20, 25, and 35°C), in cotyledons at three different stages (3, 6 and, 9 days), and leaves at three different dimensions (L1, L2, and L3). **b)** Relative expression in four different stages of germination (dry seed, 6 h of imbibition, the moment of radicle protrusion, and young seedling (2 cm root). The data were analyzed performed with a Student's t-test $P < 0.05$.

The expression levels of *RcNF-YB8* were assessed during germination at three different temperatures (20, 25, and 35°C). *RcNF-YB8* showed almost no difference in expression levels during early imbibition (EI) and moment of radicle protrusion (RP) (Figure 5a), confirming the results observed in the microarray experiment (Figure 4). However, *RcNF-YB8* was downregulated at 35°C in seedlings with a root of 2 cm (R2) as compared to 20 and 25°C. These results suggest that *RcNF-YB8* might not be involved in temperature-related stress responses during germination and early seedling development.

Expression levels of *RcNF-YB8* were also assessed in the root, cotyledon, and leaf samples. Almost no significant differences were observed in *RcNF-YB8* relative expression levels in root samples obtained from young seedlings grown at different temperatures (20, 25, and 35°C) or in cotyledon samples grown at 25°C (3-,6-, and 9-day-old). However, *RcNF-YB8* showed higher expression levels in young leaves (L1 and L2) as compared to older leaves (L3). There is, therefore, an evident tissue-specificity in the expression level which suggests a possible involvement of this gene during the transition from vegetative to flowering stages.

3.6 Multiple alignments of group a1 and interactors of RcNF-YB8

The phylogenetic tree indicated that *RcNF-YB8* is a close orthologue of *AtNF-YB2 and AtNF-YB3*. *AtNF-YB2* and *AtNF-YB3* genes have been associated with flowering time in *A. thaliana* (KUMIMOTO *et al.*, 2008). The amino acid alignment between NF-YB proteins (group a1) showed high similarity amonsgt the sequences and showed all of them contain the conserved histone-fold motif (HFM-PF00808) domain with sites for NF-YA and NF-YC interactions and DNA binding domain (Figure 6).

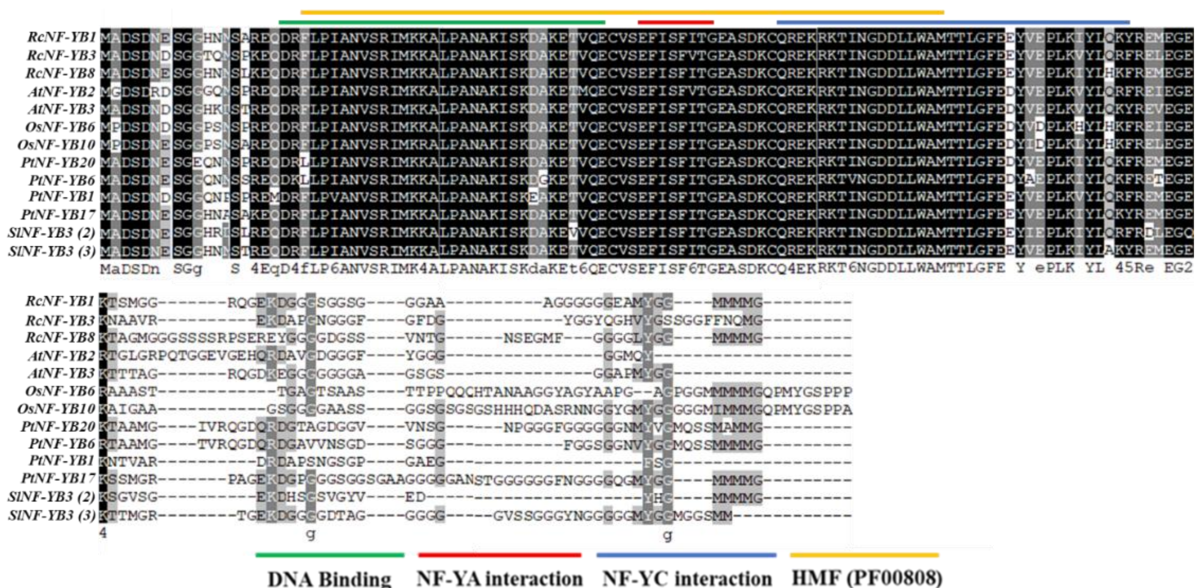


Figure 6. Multiple alignments of the NF-YB proteins of group a1. The green line shows DNA binding. The Redline shows the NF-YA interaction. The blue line shows the NF-YC interaction. The yellow line shows the HMF (PF00808) domain.

Interactors of RcNF-YB8 were predicted by String (Figure 7). The RcNF-YB8 protein showed possible interaction with NF-YA and NF-YC protein subunits. The interaction with NF-YA subunits was predicted for RcNF-YA1, RcNF-YA5 and RcNF-YA6, whereas the interaction with NF-YC subunits was predicted for RcNF-YC4, RcNF-YC5, RcNF-YC6, 29643.m000337, and 29929.m004659. Additionally, the proteins RcCaspase (29806.m000972) and RcKinesin (29912.m005530) seems to be interactor with RcNF-YB8.

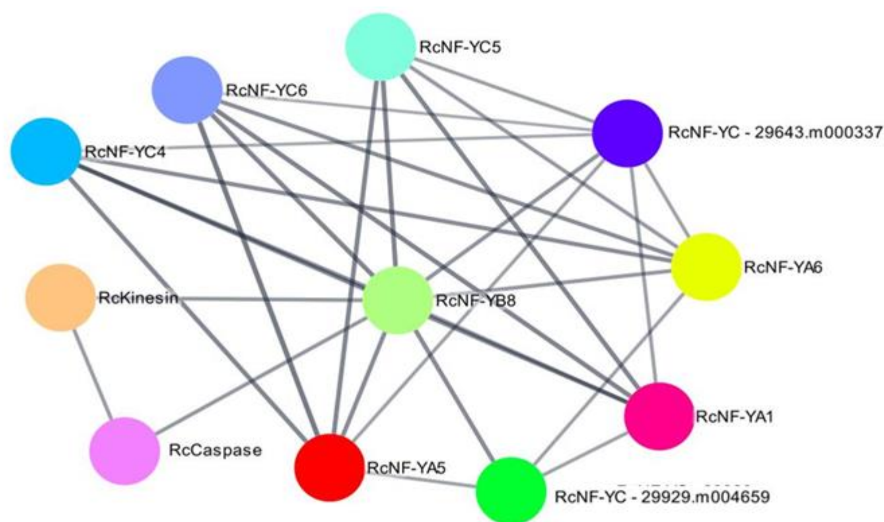


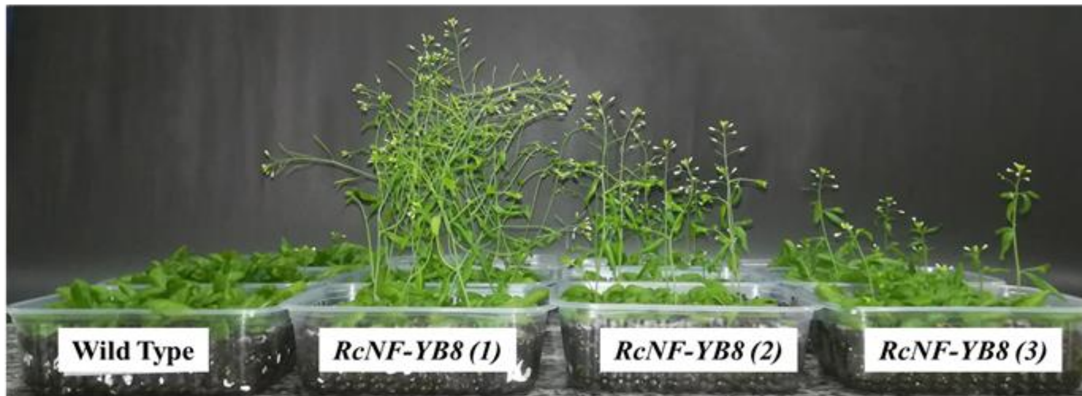
Figure 7. Co-expression network of *RcNF-YB8* in *R. communis*. RcNF-YA and RcNF-YC proteins were named according to Wang et al (2018) and protein domains were checked in the Pfam database. The image was adjusted in Cytoscape v3.8.2.

3.7 Ectopic expression of *RcNF-YB8* induces early flowering in transgenic *Arabidopsis*

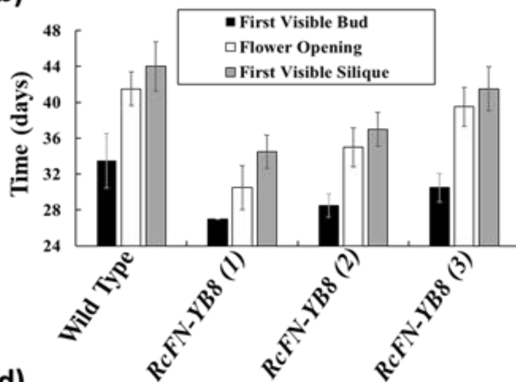
Preliminary observations during propagation of *RcNF-YB8* plants suggested a possible flowering phenotype. Thus, we further investigated the effect of ectopic expression of *RcNF-YB8* on the flowering time of transgenic *A. thaliana* plants. For that, we used three independent overexpressing lines (*RcNF-YB8 (1), RcNF-YB8 (2)*, and *RcNF-YB8 (3)*) and wild-type (Col-0) plants. Plants were phenotyped based on the time required for the formation of the first visible bud, the time required to open the first

flower, the time required for the formation of the first visible silique, total leaf number (TLN), number of open flowers (NOF), number of siliques (NS). At the end of the life cycle, plant height (PH), silique length (SL), number of seeds per silique, the total number of silique per plant, 1000-seed weight, and seed size.

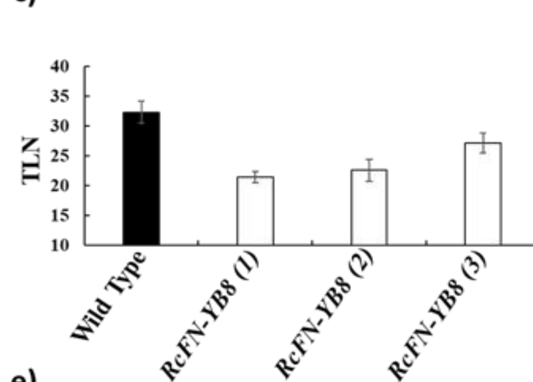
a)



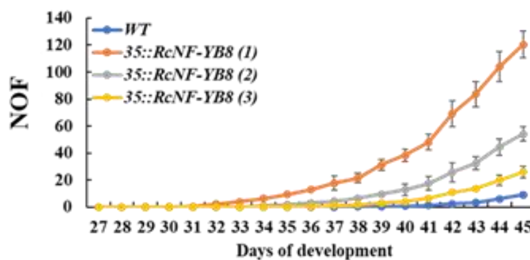
b)



c)



d)



e)

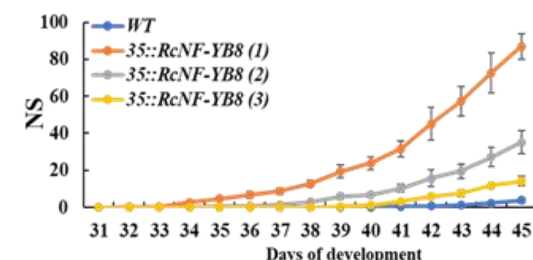


Figure 8. Flowering time of transgenic *RcNF-YB8* and wild type A*. thaliana* plants. **a)** Representative image of 38 days old *35S::RcNF-YB8* transgenic plants showing their flowering phenotype under LD conditions. Three repetitions of six plants were used in this experiment. **b)** Time to first visible bud, open flower, and visible silique. **c)** Total leaf Number of a rosette at the first open flower. **d)** The number of open flowers in transgenic *RcNF-YB8* and wild type *A. thaliana* lines. **e)** The number of visible silique in transgenic *RcNF-YB8* and wild type *A. thaliana* lines.

Ectopic expression of *RcNF-YB8* induced an overall early flowering in transgenic *Arabidopsis* (Figure 8a). Ectopic expression of *RcNF-YB8* reduced the time required for the formation of the first visible bud up to 6.5 days as compared to the wild type, it reduced the time required to open the first flower up to 11 days as compared to the wild type, whereas it reduced the time required for the formation of the first visible silique up to 9.5 days as compared to the wild type (Figure 8b). Ectopic expression of *RcNF-YB8* also induced a reduction in the total leaf number (TLN) (Figure 8c), providing further support to our hypothesis that *RcNF-YB8* is involved in the transition from vegetative to flowering stages. Finally, the number of open flowers (NOF) and the number of siliques (NS) were considerably higher in transgenic *Arabidopsis* as compared to the wild type. After 45 days, ectopic expression of *RcNF-YB8* increased the number of open flowers up to 6.5 days as compared to the wild type (Figure 8d), whereas it increased the number of siliques (NS) up to 11 days as compared to the wild type (Figure 8e).

Ectopic expression of *RcNF-YB8* induced an overall early flowering in transgenic *Arabidopsis*, which in turn had a strong impact on the plant height (PH) and silique length (SL) (Figure 9a). Ectopic expression of *RcNF-YB8* produced smaller plants (up to 29.3%) (Figure 9b) with smaller siliques (up to 9.4%) as compared to the wild type plants (Figure 9c).

Ectopic expression of *RcNF-YB8* had an overall negative impact on productivity and yield parameters, such as a total number of silique per plant, 1000-seed weight, and seed size, but it did not affect the number of seeds per silique (Figure 9 d-g). Ectopic expression of *RcNF-YB8* produced plants with the reduced total number of silique per plant (up to 40.1%), which contained smaller (up to 9.6 %) and lighter seeds (up to 22.9%) within these siliques (Figure 9).

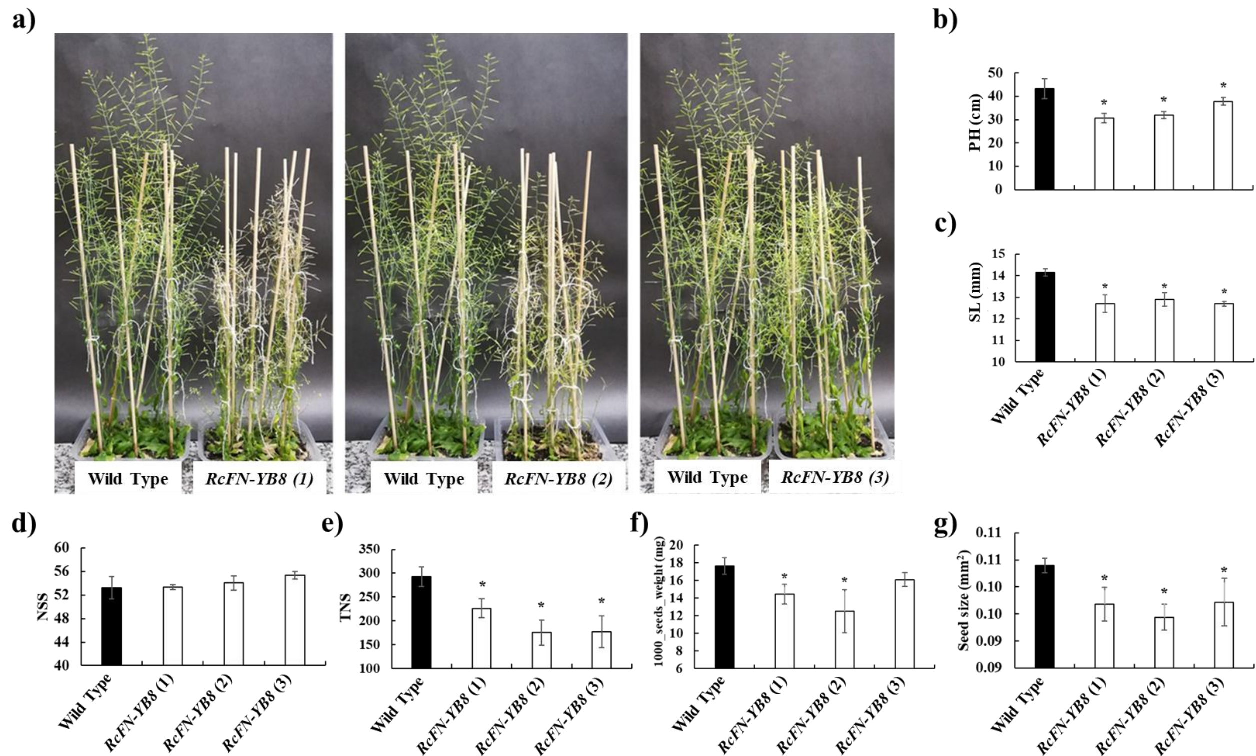


Figure 9: Plant phenotyping of transgenic *RcNF-YB8* and wild type *A. thaliana* plants. **a)** Representative image of *RcNF-YB8* transgenic *A.thaliana* and wild type lines. **b)** Plant height **(PH)** of transgenic *RcNF-YB8* A*. thaliana* plants**.** The PH average of six plants in three repetitions was used. **c)** Silique length **(LS)**. For SL analysis, the average of SL of ten siliques of six plants in three repetitions was used. **d)** Number of silique per plant **(NSS)**. **e)** Total number of silique **(TNS)**. **f)** 1000_seeds weight was obtained from weight 5 mg of dry seeds in 3 technical biology X 3 biological repetition. **g)** Seed size was performed using 3 technical X 3 biological repetitions of 15 seeds. The data were analyzed performed with a Student's t-test $P < 0.05$.

4. DISCUSSION

Phylogenetic relationships amongst genes of different species provide important information about the function, evolution, and conservation of putative genes in different species. Furthermore, in-depth molecular characterization of new genes can be used as important allies in breeding programs that aim at producing high-yield *Ricinus communis* genotypes. It has been demonstrated that the NF-YB genes are related with a large

number of plant events, including also the control of flowering time (CAO *et al.*, 2011; KUMIMOTO *et al.*, 2008; LIANG *et al.*, 2012), besides embryogenesis (KWONG *et al.*, 2003; YAMAMOTO *et al.*, 2009), blue-light (WARPEHA *et al.*, 2007), ABA signaling (WARPEHA *et al.*, 2007; YANG *et al.*, 2017), chloroplast biogenesis (MIYOSHI *et al.*, 2003), fructose and glucose accumulation (MAI *et al.*, 2019) and primary root elongation (BALLIF *et al.*, 2011). The involvment of NF-YB genes in responses to abiotic stresses has also been reported, i.e. the overexpression of NF-YB genes (*AtNF-YB1*, *ZmNF-YB2,* and *PdNF-YB7*) conferred tolerance to drought in Arabidopsis (HAN *et al.*, 2013; NELSON *et al.*, 2007).

The *OsNF-YB1* and *OsNF-YB9* genes demonstrated to be predominantly expressed in the endosperm of rice and interacting with *OsbZIP76* possibly regulating the endosperm cellularization (NIU *et al.*, 2020; ZHIGUO *et al.*, 2018). According to the phylogenetic tree (Figure 1), the *OsNF-YB9* is the closest ortholog of *RcNF-YB2*, whereas the *OsNF-YB1* gene did not present close ortholog in *R. communis*. The close relationship between *OsNF-YB9* and *RcNF-YB2* can indicate that these genes have a similar function and the same interaction with bZIP transcription factors. Besides, *RcNF-YB2* was the only gene of *RcNF-YB* family that presented endosperm expression regulatory elements in the promoter region (Figure 2) and presented high-expression during early imbibition by low and high-temperature compared to other treatments (Figure 4).

The *AtNF-YB2*, *AtNF-YB3*, *PtNF-YB1* genes (group a1) are described to be regulators of flowering time (KUMIMOTO *et al.*, 2008; WANG, RONGKAI *et al.*, 2019). The overexpression of *HvNF-YB1* and *Bd-NF-YB6* genes (ortholog of *AtNF-YB2*, *AtNF-YB3*) in Arabidopsis also produced early flowering timelines (Liang et al., 2012; Cao et al., 2011). According to the phylogenetic tree, the *RcNF-YB1*, *RcNF-YB3,* and *RcNF-YB8* (group a1) are the closest orthologs of these genes showing the possible involvement in regulating the flowering time.

The investigation of possible regulatory elements in the promoter region is an important tool to understand the mechanism by which genes are modulated upon

external stimuli. Phytohormones, abiotic and biotic stresses can induce the activation of genes from specific transcription factors or specific metabolic pathways. Thus, the prediction of regulatory elements in the promoter region of DNA can provide important insights into possible sites that can regulate *RcNF-YB* genes. The analysis of the promoter region implies that *RcNF-YB* genes can be regulated by various abiotic and biotic stimuli during plant development.

MYB and MYC are involved in several abiotic stress responses. The overexpression of *OsMYB6* increased tolerance to drought and salinity stresses (QIN *et al.*, 2012; ROY, 2016; TANG *et al.*, 2019), whereas MYC appears to be involved in *A. thaliana* tolerance cold, osmotic, and salt stresses (FENG *et al.*, 2013; ZUO *et al.*, 2019), with possible regulation of *RcNF-YB* genes in several abiotic stress responses. The circadian control response was found in only 2 genes (*RcNF-YB2* and *RcNF-YB8*). This might suggest that these genes are important for abiotic stress responses, since environmental cues can induce internal signal transduction to coordinate plant development, e.g. the NF-YB subunits have been implicated in ABA/blue light response (WARPEHA *et al.*, 2007; XU *et al.*, 2014).

RcNF-YB genes seem to be involved in a diverse range of physiological processes, including seed germination, seed maturation, seedling development, and response to abiotic stresses. The regulatory *cis*-elements involved in light, phytohormones, and abiotic stress responses were also observed in *NF-YB* genes in *Sorghum bicolor* and *Camellia sinensis* (MAHESHWARI *et al.*, 2019; WANG *et al.*, 2019). Taken together, The distribution of RcNF-YB regulatory *cis*-elements suggests that these genes are potentially involved in abiotic stress tolerance and in the regulation of plant development.

We found conserved motifs and similar arrangements amongst all *RcNF-YB* members, which might indicate potential functional regions of the genes. Motif 1 and 2 correspond to the histone-like transcription factor (CBF/NF-Y) domain (CL0012). Generally, proteins in this clan possess DNA binding properties. The motif 4 (YGGMMMMGQQHQHQGHHQ) and motif 5 (MADSDNESGGHNNNAN) were found exclusively in genes of group a1, suggesting to be conserved in flowering time NF-YB

genes (Figure 6). In general, the *RcNF-YB* genes in the same group shared similar motifs and distribution patterns. The exclusive motifs in each group of *RcNF-YB* genes can be important to the variability of protein interaction and consequently in the expression of the set of genes activated by *RcNF-YB* genes. The differences in genes codified by NF-YB and a slight difference in the presence of motifs sequence in sites of interaction with DNA, NF-YA, and NF-YC subunits might indicate the involvement of NF-YB in a large number of events during plant development and abiotic stresses responses.

Genes can be upregulated or down-regulated by stimulus of abiotic stresses, phytohormones, transcription factors, and microRNA, whereas gene induction by a specific stimulus indicate that this gene can participate in the specific responses towards reestablishment of plant to homeostasis. Several *RcNF-YB* genes showed prompt responses to temperature (Figure 4). *RcNF-YB1, RcNF-YB3,* and *RcNF-YB8* expression was modulated in response to low temperatures (Figures 1 and 4), whereas *RcNF-YB2* and *RcNF-YB3* expression was modulated in response to low and high temperatures. Although *RcNF-YB2* and *RcNF-YB3* are positioned in different clades of the phylogenetic tree (Figure 1), they are located near to each other in the *R. communis* genome at the same direction of transcription, suggesting that low temperature might activate the entire genome locus. *RcNF-YB2* and *RcNF-YB3* share the anaerobic induction, MYB, MYC, and light cis-elements in the promoter region, which can justify the involvement of these genes in temperature-related responses.

RcNF-YB2 was upregulated by drought in two-week-old *R. communis* seedlings, whereas *RcNF-YB8* expression was affected by cold, drought, and heat stresses (WANG, YUE *et al.*, 2018). *RcNF-YB8* response to temperature seems to be dependent on the developmental stage and tissue. In our results, the *RcNF-YB8* was downregulated during germination and early seedling stage (four-day-old) by heat, whereas according Wang et al., (2018) in two-week-old seedlings, *RcNF-YB8* was upregulated by heat. This indicate a high degree of plasticity of *RcNF-YB8* to cope with stress conditions.

Various studies have shown the response of *NF-YB* gene expression with abiotic stresses. In *Glycine max*, five NF-YB genes (*GmNF-YB04, GmNF-YB06, GmNF-YB24, GmNF-YB02,* and *GmNF-YB01*) showed to be induced by drought stress (QUACH *et al.*, 2015). In *Oryza sativa*, the *OsNF-YB3* showed to be induced by drought stress, whereas *OsNF-YB8*, *OsNF-YB9* and *OsNF-YB11* were repressed by drought stress (YANG *et al.*, 2017). In *Brassica napus,* four *BnNF-YB* genes (*BnNF-YB3*, *BnNF-YB7*, *BnNF-YB10*, and *BnNF-YB14*) were induced by salinity, five genes (*BnNF-YB1*, *BnNF-YB8*, *BnNF-YB10*, *BnNF-YB13*, and *BnNF-YB14*) were induced by PEG treatment and seven genes (*BnNF-YB2*, *BnNF-YB3*, *BnNF-YB7*, *BnNF-YB10*, *BnNF-YB11*, *BnNF-YB13*, and *BnNF-YB14*) were induced by ABA treatment (XU, LI *et al.*, 2014). In *Sorghum bicolor*, four *SbNF-YB* genes (*SbNF-YB7*, *SbNF-YB12, SbNF-YB15,* and *SbNF-YB17*) were induced by multiple stress conditions (salt, mannitol, ABA, cold, and high-temperatures). Some *SbNF-YB* genes (*SbNF-YB2, SbNF-YB7, SbNF-YB10, SbNF-YB11, SbNF-YB12, SbNF-YB14, SbNF*-YB16, and *SbNF*-YB17) showed to be modulated by low and high-temperature (MAHESHWARI *et al.*, 2019). Our findings will contribute to the further functional analysis of NF-YB genes in *R. communis* plants.

The nuclear factor Y subunit B can be codified by various genes and interact with subunits Y and A activating different sets of genes. Besides that, some NF-YB protein can interact with other transcription factors as Constans (CO), DREB and BZIP, therefore expanding the arsenal of the set of genes that can be activated and producing a different response to abiotic stresses and plant development. *O. sativa* NF-Y genes (subunits A, B, and C) have contrasting behavior regarding flowering time. *OsNF-YC2* and *OsNF-YC4* genes act as inhibitors of flowering under long-day (LD) conditions, whereas *OsNF-YC6* induces flowering. Physical interactions are observed between OsNF-YC (OsNF-YC2, OsNF-YC4, and OsNF-YC6) and OsNF-YB (OsNF-YB8, OsNF-YB10, and OsNF-YB11) proteins. OsNF-YB10 showed to be the ortholog of RcNF-YB8 suggesting that different interactions by the NF-Y complex can also produce different responses in plant development in *R. communis* (KIM *et al.*, 2016). The *A. thaliana* ortholog (AtNF-YB3) of RcNF-YB8 interacts with AtNF-YA2 protein and DREB2A, an important transcription factor involved in drought and high-temperature stress tolerance, suggesting that this complex can activate different target genes involved in heat and

drought tolerance (SATO *et al.*, 2014). The AtNF-YB2 and AtNF-YB3 proteins showed *in vivo* interaction with At-NF-YC3, At-NF-YC9 (homolog of RcNF-YC4, RcNF-YC2, RcNF-YC3) AtNF-YC4 (homolog of RcNF-YC5) proteins, which are also required for flowering (KUMIMOTO *et al.*, 2010; WANG, YUE *et al.*, 2018). The RcNF-YC4 and RcNF-YC5 show together with the prediction of protein interaction by string the possible interaction with RcNF-YB8.

The overexpression of *AtNF-YB2* and *AtNF-YB3* induced early flowering in Arabidopsis, especially during long-days (LD), by regulating the expression of flowering locus T (FT), a gene involved in the conversion of the vegetative tissue to a floral meristem. Besides, the interaction between AtNF-YB2 and AtCO was demonstrated to regulate FT and promote early flowering (BALLIF *et al.*, 2011; KUMIMOTO *et al.*, 2008). The overexpression of *PtNF-YB1* also induced early flowering in Arabidopsis, but with the possible contribution of PtCO1 and PtCO2 proteins (WANG *et al.*, 2019). In our study, the ecotopic expression of the *RcNF-YB8* gene appeared to be regulated early flowering time in LD conditions. The possible molecular mechanism to regulate early flowering time can be associated with the expression of the Constans (CO), flowering locus T (FT), and Suppressor of overexpression of CONSTANS1 (SOC1) genes. The *RcCO* gene expression during germination in different temperatures follows the same pattern of *RcNF-YB8* gene. Both of them are repressed by high temperature and induced by low-temperature (Supplementary Figure 4). A similar pattern of expression of these genes indicates a possible interaction of *RcCO* and *RcNF-YB8*, similar to what occurs in *P. trichocarpa* (WANG *et al.*, 2019).

In maize, overexpression of *ZmNF-YB2* gene in maize enhances grain yield under drought stress (NELSON *et al.*, 2007). Flowering phenotypes under osmotic stress was analyzed in *At-HAP3b* (*AtNF-YB2*) knockouts and complemented lines showing that wild type and complemented lines flowered, while the knockouts lines did not (CHEN *et al.*, 2007). The overexpression of *OsNF-YB11* (also called *Ghd8* – ortholog of *AtNF-YB2* also called *At-HAP3b*) caused early flowering in Arabidopsis, but in rice (a short-day flowering plant) the Os*NF-YB11* presented the opposite function as it delayed flowering under long-day conditions and promoted flowering under short-day

conditions (YAN *et al.*, 2011). Functional characterization of *RcNF-YB* genes has not been described yet. Herein, *RcNF-YB8* showed to be an important regulator of flowering time. However, the early flowering time showed to affect plant height and seed production. *R. communis* is mainly hand-harvested as green leaves, immature fruits and the large variability in plant height at harvesting time are the major impediment factors for mechanical harvest. Dwarf or growth retardants cultivars may be used to in favor of *R. communis* mechanical harvest (OSWALT *et al.*, 2014). Besides, shorter life cycle can also benefit plants to scape long time exposition to abiotic stresses.

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SUPLEMENTARY MATERIAL

Supplementary Table 1. Predicted *RcNF-YB* orthologs genes in selected angiosperms.

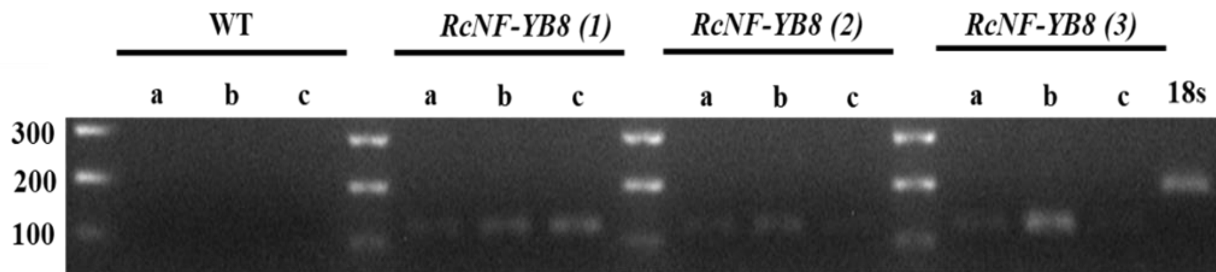
		Putative orthologs			
Name	**Group**	***A. thaliana***	***P. trichocarpa***	***O. sativa***	***S. lycopersicum***
RcNF-YB1	a1	*AtNF-YB2,3*	*PtNF-YB1,6,17,20*	*OsNF-YB6,10*	*SlNF-YB3 (2), (3)*
RcNF-YB2	e	*AtNF-YB6, 9*	*PtNF-YB5,19*	*OsNF-YB5,9*	*SlNF-YB6 (3,13)*
RcNF-YB3	a1	*AtNF-YB2,3*	*PtNF-YB1,6,17,20*	*OsNF-YB6,10*	*SlNF-YB3 (2), (3)*
RcNF-YB4	b	-	*PtNF-YB4,7*	-	*SlNF-YB8(1), 3 (1)*
RcNF-YB5	d	*AtNF-YB4, 5*	*PtNF-YB2,9,10, 14,15,16*	*OsNF-YB3,4*	*SlNF-YB5 (1,2,3,4)*
RcNF-YB6	a2	*AtNF-YB1,8,10*	*PtNF-YB8,12,21*	*OsNF-YB2,7*	*SlNF-YB8(2), 10*
RcNF-YB7	a2	*AtNF-YB1,8,10*	*PtNF-YB8,12,21*	*OsNF-YB2,7*	*SlNF-YB8(2), 10*
RcNF-YB8	a1	*AtNF-YB2,3*	*PtNF-YB1,6,17,20*	*OsNF-YB6, 10*	*SlNF-YB3 (2), (3)*
RcNF-YB9	c	*AtNF-YB7*	*PtNF-YB3*	*OsNF-YB8*	*SlNF-YB7*
RcNF-YB10	d	*AtNF-YB4,5*	*PtNF-YB2,9,10,14,15,16*	*OsNF-YB3,4*	*SlNF-YB5 (1,2,3,4)*
RcNF-YB11	d	*AtNF-YB4,5*	*PtNF-YB2,9,10,14,15,16*	*OsNF-YB3,4*	*SlNF-YB5 (1,2,3,4)*
RcNF-YB12	e	*AtNF-YB6,9*	*PtNF-YB5,19*	*OsNF-YB5,9*	*SlNF-YB6 (3,13)*
RcNF-YB13	g	*AtNF-YB11, 12,13*	*PtNF-YB11,13,18*	*OsNF-YB1*	*SlNF-YB11, Slhis (1,2)*
RcNF-YB14	g	*AtNF-YB11, 12,13*	*PtNF-YB11,13,18*	*OsNF-YB1*	*SlNF-YB11, Slhis (1,2)*

Table 2. Total number of genes of *NF-YB* genes in selected angiosperms.

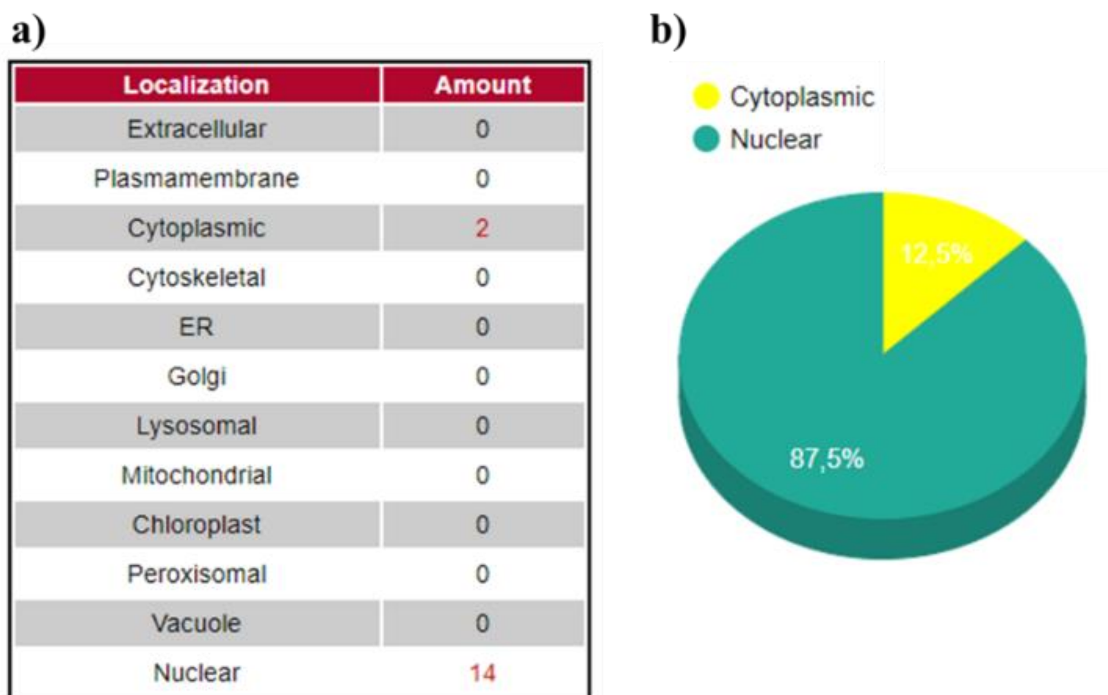
Group	*R. communis*	*A. thaliana*	*P. trichocarpa*	*O. sativa*	*S. lycopersicum*
a1	3	2	4	2	2
a2	2	3	3	2	2
b	1	-	2	-	2
c	1	1	1	1	1
d	3	2	6	2	4
e	2	2	2	2	2
f	-	-	-	-	11
g	2	3	3	1	3

Table 3. Motif sequences found in RcNF-YB genes. The motifs were scanned by the Pfam database to confirm the class of specific NF-YB domains.

Number	Sequence	Pfam
Motif-1	ISKDAKETVQECVSEFISFITGEASDKCQREKRKTINGDDLLWAMTTLGF	**CL0012**
Motif-2	KEQDRFLPIANVSRIMKKALPANAK	**CL0012**
Motif-3	EDYVEPLKVYLHRYREMEGEK	-
Motif-4	YGGMMMMGQQHQHQGHHQ	-
Motif-5	MADSDNESGGHNNNAN	-
Motif-6	QGQHMIVP	-
Motif-7	PMGHPH	-
Motif-8	RHDKQPPPPT	-
Motif-9	QQGHLY	-
Motif-10	PASPAGGSHESGG	-



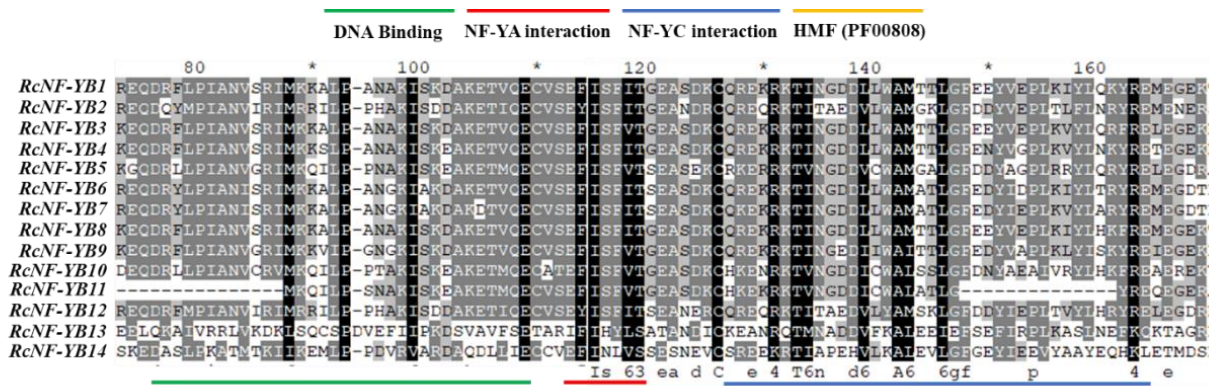
Supplementary Figure 1. Genotyping of the*RcNF-YB8 transgenic* lines. PCR was done with DNA from leaves. To confirm transgenic Arabidopsis lines qPCR primers were used. 18S gene was used for the positive control of WT samples.



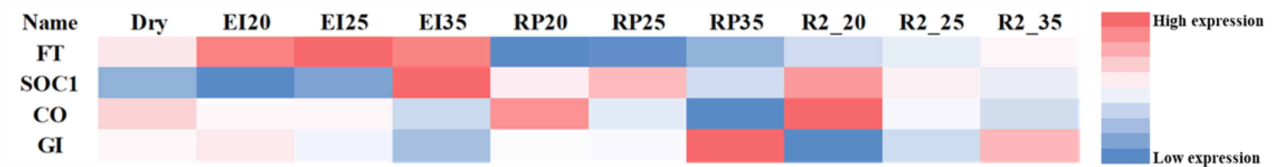
a)

Localization	Amount
Extracellular	0
Plasmamembrane	0
Cytoplasmic	2
Cytoskeletal	0
ER	0
Golgi	0
Lysosomal	0
Mitochondrial	0
Chloroplast	0
Peroxisomal	0
Vacuole	0
Nuclear	14

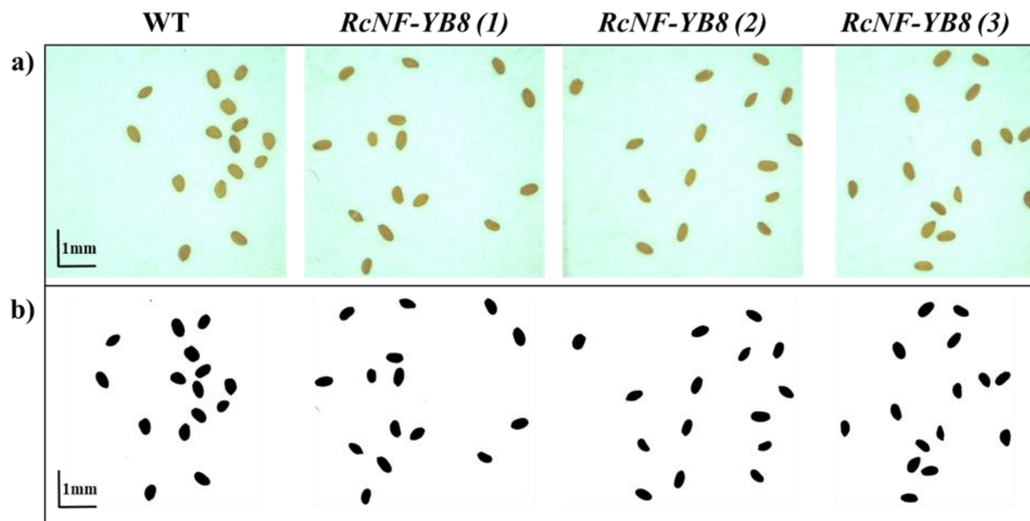
Supplementary Figure 2: Subcellular localization prediction of RcNF-YB gene family. a) Prediction of Localization of 14 RcNF-YB genes. b) Percentage of RcNF-YB cell localization.



Supplementary Figure 3. Alignment of the *RcNF-YB* gene family. The conserved region and specific NF-YB domains are shown.



Supplementary Figure 4: Heatmap of Flowering time *A. thaliana* orthologs genes in *R.communis* during germination at different temperatures. Different colors indicate the difference in gene expression (Red-high, white-middle, blue low).



Supplementary Figure 5. Seed Observation of the transgenic and wild type lines of Arabidopsis Plants. a) Stereo microscope image of mature seeds from transgenic and wild type lines. **b)** Digital image transformed into binary to calculate seed size.

CONSIDERAÇÕES FINAIS

A presente pesquisa de doutoramento teve como foco o entendimento de três famílias de proteínas envolvidas na tolerância a estresses abióticos em mamona (*Ricinus commnuis*), dentre as quais a enzima antioxidante superóxido dismutase (SOD), as proteínas pequenas de choque térmico (sHSP) e a subunidade B do fator de transcrição nuclear Y (NY-B ou TFNY-B).

Os resultados que constam do CAPÍTULO 1 demonstraram que a mamona possui oito genes que codificam para a SOD, dentre os quais houve padrões de indução distintos durante a germinação e crescimento de plântulas, em condições normais e de estresse osmótico. Dentre os quais, os genes *RcCuZnSOD1* e *RcFeSOD8* demonstraram indução por estresse osmótico e melhor perfil fisiológico em resposta ao pré-condicionamento osmótico (osmocondicionamento) em sementes de mamona do genótipo PARAGUAÇU. No CAPÍTULO 2, foi demonstrado novamente um padrão diferente de expressão dos genes da SOD durante a germinação e crescimento de plântulas sob estresse térmico (35ºC), mediante padrão tecido-específico em raizes, cotilédones ou folhas. Os genes *RcCuZnSOD1* e *RcFeSOD8* demonstraram ser responsivos ao estresse térmico no início da germinação, similar ao ocorrido para estresse osmótico. Enquanto que demais genes da SOD foram expressos em estágios mais avançados da embebição das sementes, como momento da protrusão radicular (germinação *per se*) e em plântulas com raízes com 2cm de comprimento. Os genes do grupo Mn demonstraram serem reprimidos com o tempo de embibição e germinação. Os resultados em bioinformática sugerem que os genes da SOD possuem regulação complexa hormônios vegetais como ABA, giberelinas, ácido salicílico e metil jasmonato e coregulação por luz, fatores de transcrição MYB e MYC, fatores relacionados ao desenvolvimento da planta. Ademais, os genes *RcCuZnSOD3* e *RcCCuSOD4* demonstraram uma possível regulação pelo micro RNA (miR398), similarmente ao observado experimentalmente em *A. thaliana*. A fusão dos genes da SOD com GFP em folhas de *N. benthamiana* possibilitou caracterizar a localização subcelular, em que a maior parte dos genes SOD tiverem as mesmas localizações preditas pela análise bioinformática, com excessão do gene *RcMnSOD5* predito como mitocondrial, mas que

demonstrou localização para o citosol (Figura 1). Por fim, concluímos que a família SOD demonstra ter envolvimento na resposta das plantas a estresses abióticos podendo ter papel relevante na tolerância de mamona a estresses abióticos.

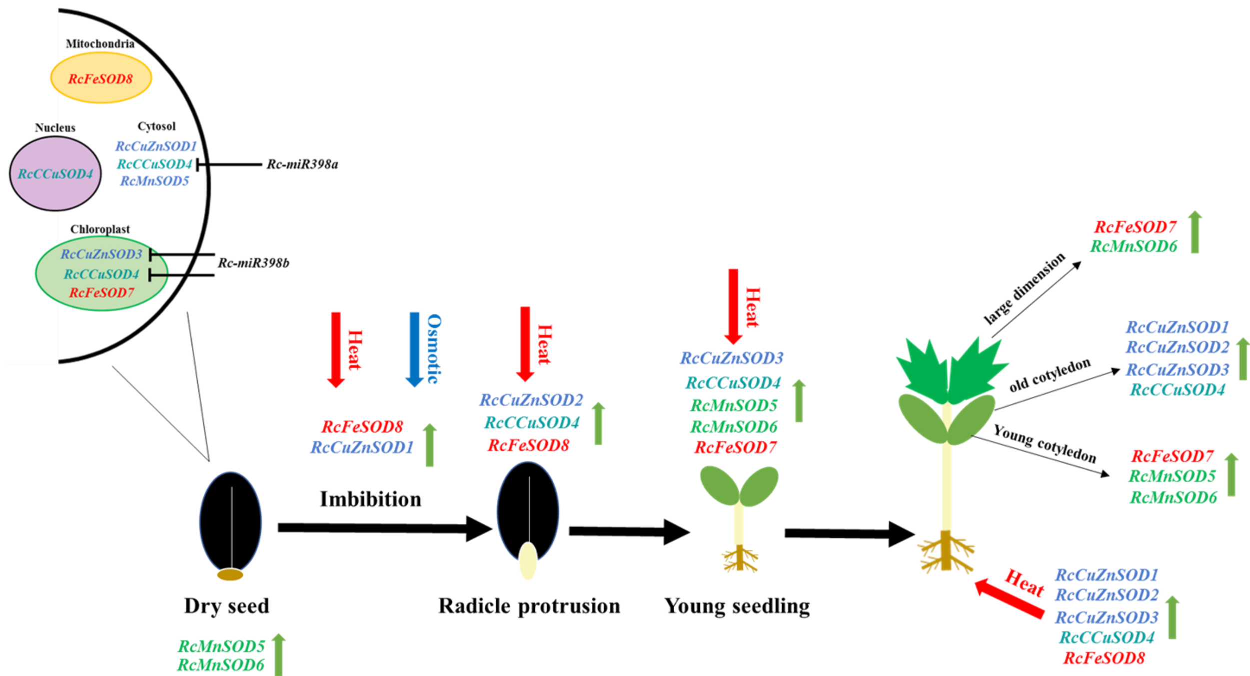


Figura 1: Modelo de indução de genes de SOD em mamona durante a germinação sob estresses abióticos e em diferentes tecidos.

Os resultados no CAPÍTULO 3 demostraram que a mamona possui 41 genes que codificam para as proteínas pequenas de choque térmico (sHSP). Sendo que a maior quantidade de genes sHSP em mamona comparando com *A. thaliana* aparenta resultar de duplicação em tandem. Os genes sHSP preditos para localização subcelular no citosol demonstraram possuir padrões mais simples em suas estruturas, não apresentando íntrons na sua maioria comparado aos que são localizados em compartimentos específicos como mitocôndria e retículo endoplasmático. O perfil de indução dos genes de sHSP demonstram um padrão sob estresse térmico nas sementes no momento da protrusão radicular e plântulas jovens com raiz de 2 cm, mas demonstraram perfis diferentes de indução em raízes, cotilédones e folhas,

demonstrando que genes sHSP possuem diferentes padrões de expressão de acordo com o tecido-específico e estádio de desenvolvimento (Figura 2).

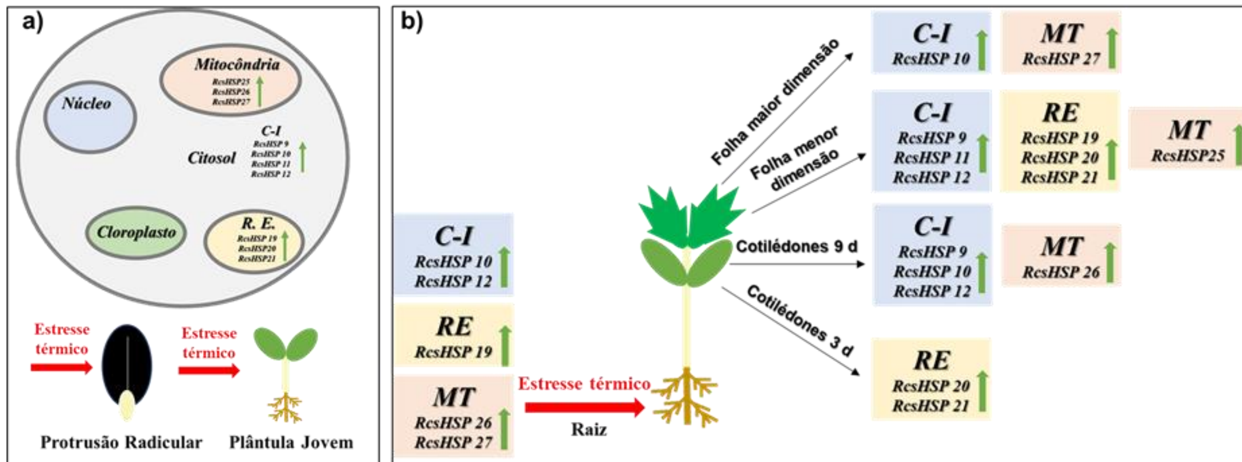


Figura 2: Modelo de indução de genes das sHSP em mamona durante a germinação sob estresses abióticos e em diferentes tecidos. **a)** Perfil de expressão em sementes durante a protrusão radicular. **b)** Perfil de expressão em plântulas jovens com 2 cm de raiz.

A superexpressão dos genes sHSP de mamona (*RcsHSP12* e *RcsHSP19*) em *A. thaliana* resultou em aumento da porcentagem de germinação de sementes sob condições de estresses abióticos. Assim como as sementes transgênicas demonstraram maior potencial antioxidante à partir da maior atividade da SOD observado nas linhagens transgênicas comparado a linhagem selvagem de *A. thaliana* (Col-0), demonstrando que a superexpressão de sHSP é capaz de reforçar o sistema de defesa antioxidante enzimático por meio da maior atividade da SOD. Os dados obtidos convergem com dados da literatura onde sugerem que existem uma possível regulação entre FT e genes de HSP e SOD. Ademais, os resultados demonstraram que alguns metabólitos osmoprotetores e relacionados a resposta da planta a estresses, foram acumulados nas linhagens superexpressoras de sHSP quando comparado com a linhagem selvagem Col-0, tais como rafinose, sacarose, leucina, isoleucina (Figura 3). Podendo-se concluir que as sHSP desenpenham um papel crucial na tolerância de

plantas a estresses abióticos sendo genes igualmente interessantes para possível aplicação no melhoramento genético da mamoneira.

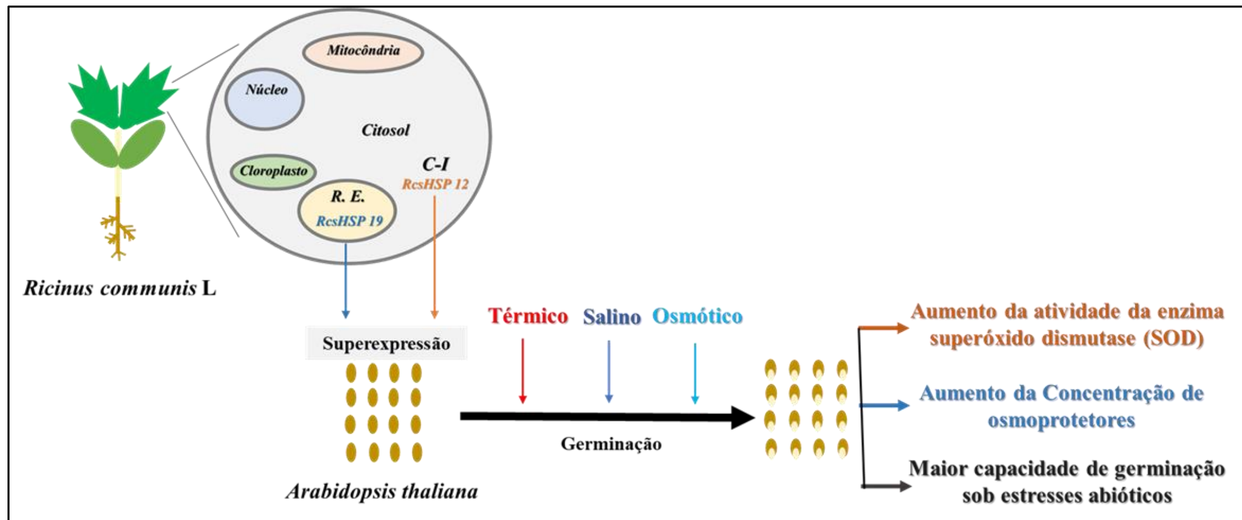


Figura 3: Modelo de caracterização funcional dos genes *RcsHSP12 e RcsHSP19* quanto a resposta da planta a estresses abióticos.

O CAPÍTULO 4 envolveu o estudo dos fatores de transcrição *NF-Y*. Mais especificamente a família de genes que codifica para a subunidade b dos *NF-Y*. Os resultados em bioinformática sugerem que os genes *RcNF-YB* possuem regulação envolvendo hormônios vegetais como ABA, principalmente quando envolve coregulação por luz, fatores de transcrição MYB e MYC, e por baixas temperaturas. Em geral, os genes *RcNF-YB* mostraram serem induzidos ou preexistentes em sementes secas e no início embebição como parte do processo germinativo, quando comparado ao estádio de plântulas jovens. A superexpressão do gene *RcNF-YB8* em *A. thaliana* induziu as plantas ao florescimento precoce, consequentemente também um efeito de redução no tamanho das plantas e frutos, por sua vez impactando na produção média ou rendimento de sementes por planta (Figura 4). Nossos resultados demonstraram que o gene *RcNF-YB8* demonstrou ser um gene importante para mamona, impactado em aspectos relevantes, como o porte da planta, menor ciclo de vida e produtividade, sendo assim um gene também relevante e com potencial para o uso em possíveis programas de melhoramento genético da mamona.

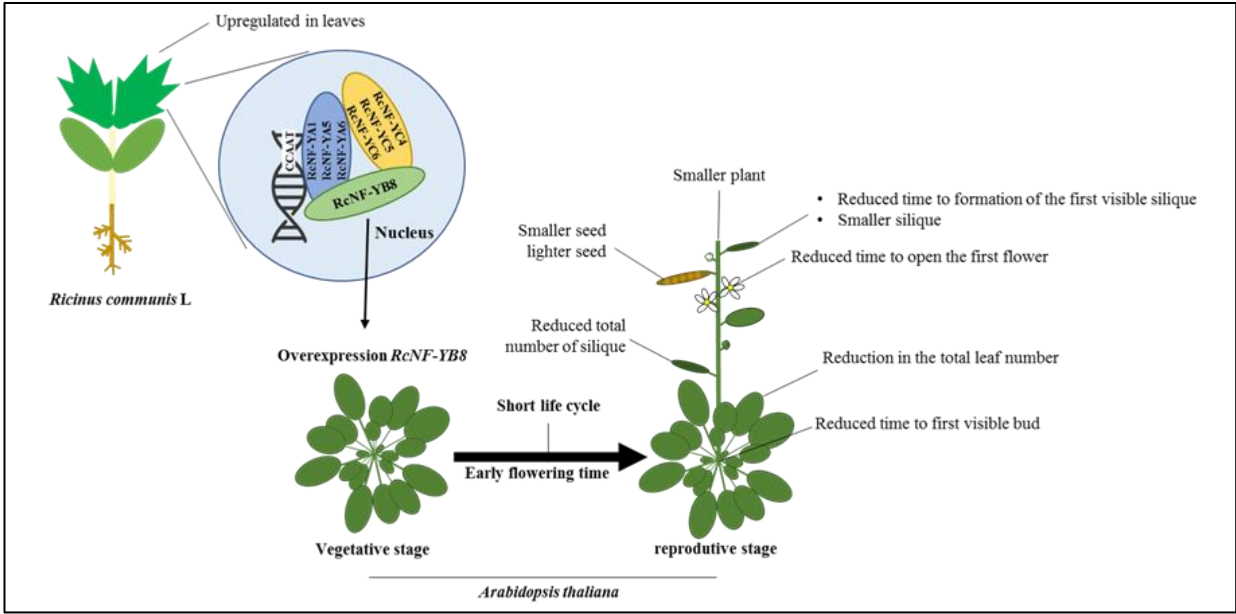


Figura 4: Modelo de caracterização funcional do gene *RcNF-YB8* quanto a indução do florescimento precoce e efeito no estádio reprodutivo da planta.

ANEXOS

Co-autorias em artigos científicos

Artigo 1: Osmopriming-associated genes in Poincianella pyramidalis. https://doi.org/10.1016/j.envexpbot.2020.104345

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Artigo 2: *Stevia Rebaudiana* (Bert.) Bertoni Cultivated under different photoperiod conditions: Improving physiological and Biochemical traits for industrial aplications

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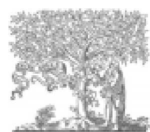

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Stevia rebaudiana (Bert.) Bertoni cultivated under different photoperiod conditions: Improving physiological and biochemical traits for industrial applications

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