FERNANDA FERREIRA SALGADO

PREDIÇÃO E ANOTAÇÃO DE miRNAs E O PAPEL DOS FATORES DE TRANSCRIÇÃO NA RESPOSTA DE *Elaeis guineensis* Jacq. AOS ESTRESSES ABIÓTICOS DE SECA E SALINIDADE

LAVRAS - MG
2023

FERNANDA FERREIRA SALGADO

PREDIÇÃO E ANOTAÇÃO DE miRNAs E O PAPEL DOS FATORES DE TRANSCRIÇÃO NA RESPOSTA DE *Elaeis guineensis* Jacq. AOS ESTRESSES ABIÓTICOS DE SECA E SALINIDADE

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biotecnologia Vegetal, para a obtenção do título de Doutor.

Dr. Manoel Teixeira Souza Júnior
Orientador

Dra. Priscila Grynberg
Coorientadora

LAVRAS - MG
2023

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Salgado, Fernanda Ferreira.
Predição e anotação de miRNAs e o papel dos fatores de transcrição na resposta de Jacq. aos estresses abióticos de seca e salinidade / Fernanda Ferreira Salgado. - 2023.
95 p.

Orientador(a): Manoel Teixeira Souza Junior.
Coorientador(a): Priscila Grynberg .
Tese (doutorado) - Universidade Federal de Lavras, 2023.
Bibliografia.

1. Estresse abiótico. 2. RNA não-codificante. 3. Fator de transcrição. I. Souza Junior, Manoel Teixeira. II. , Priscila Grynberg. III. Título.

FERNANDA FERREIRA SALGADO

PREDIÇÃO E ANOTAÇÃO DE miRNAs E O PAPEL DOS FATORES DE TRANSCRIÇÃO NA RESPOSTA DE *Elaeis guineensis* Jacq. AOS ESTRESSES ABIÓTICOS DE SECA E SALINIDADE

miRNA PREDICTION AND ANNOTATIONS AND THE ROLE OF TRANSCRIPTION FACTORS IN THE RESPONSE OF Elaeis guineensis Jacq. TO THE ABIOTICAL STRESS OF DROUGHT AND SALINITY

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biotecnologia Vegetal, para a obtenção do título de Doutor.

APROVADA em 31 de maio de 2023

Dra. Priscila Grynberg	EMBRAPA Recursos Genéticos e Biotecnologia
Dr. Maurício Antônio Lopes	EMBRAPA Agroenergia
Dr. Jaire Alves Ferreira Filho	EMBRAPA Agroenergia
Dr. Luciano Vilela Paiva	Universidade Federal de Lavras

Dr. Manoel Teixeira Souza Júnior
Orientador

Dra. Priscila Grynberg
Coorientadora

LAVRAS - MG
2023

AGRADECIMENTOS

Primeiramente, agradeço a Deus por me dar forças para superar cada obstáculo que me foi apresentado. Por me amparar e guiar para as melhores escolhas possíveis.

Agradeço aos meus pais Deniara Isabel e Carlos Magno, e a José Lucio por sempre me apoiar e acreditarem que eu seria capaz. Por me ajudarem nos momentos de difíceis tanto emocionalmente quanto financeiramente, pois sabemos que vida de bolsista não é fácil.

À minha irmã, ao Ricardo e a minha filha Ágatha, todos vocês também fazem parte desta conquista. Cada um contribui em pontos fundamentais tornando possível essa vitória, incluindo os mencionados anteriormente.

À Universidade Federal de Lavras por toda a estrutura oferecida e ao programa de pós-graduação em Biotecnologia Vegetal por todo suporte e ensinamento me proporcionando crescimento pessoal e profissional.

Agradeço ao meu orientador Dr. Manoel Teixeira Souza Junior pela excelente orientação, pelos ensinamentos, pela compreensão, pelas palavras de enriquecimento pessoal e profissional. Por ser minha fonte de inspiração e nos mostrar que no tempo certo colhemos os frutos desse nosso plantio.

À minha coorientadora Priscila Grinberg, juntamente com a sua equipe, por todo ensinamento e apoio no desenvolvimento do projeto. Grata por aprender tanto e pelas boas conversas e incentivo.

À meus amigos e colegas de trabalho Thalita Massaro, Thalliton Silva, Ítalo Braga, André Leão e Cleiton Bittencourt pela amizade e colaboração neste trabalho.

Aos funcionários da UFLA e a Embrapa Agroenergia, e a todos aqueles que contribuíram de forma direta ou indiretamente para a realização deste trabalho.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

“*Não há nada a temer na vida, apenas tratar de compreender*.”

Marie Curie

RESUMO GERAL

O dendezeiro (*Elaeis guineensis* Jacq.) é uma oleaginosa de grande importância econômica, podendo render até 10 vezes mais óleo do que outras oleaginosas, como soja e canola. Em 2021/2022, o mundo consumiu aproximadamente 82 milhões de toneladas de óleo de palma e óleo de palmiste e, à medida que a demanda aumenta, é necessário expandir o rendimento. No Brasil, existe uma extensa área propícia ao plantio de dendê; no entanto, essas áreas passam por longos períodos de estiagem, exigindo irrigação artificial para ser sustentável. Cerca de 30% das terras agrícolas irrigadas do mundo são danificadas pela salinidade, afetando negativamente a produtividade das culturas. Assim, para expandir o cultivo de dendê para essas áreas no Brasil, é preciso entender melhor a resposta dessa oleaginosa aos estresses de seca e salinidade. Os microRNAs (miRNAs) estão diretamente relacionados com a regulação pós-transcricional da expressão gênica e são moléculas essenciais na resposta da planta ao estresse abiótico. Nosso grupo realizou um estudo para entender como essa oleaginosa responde a dois estresses abióticos, predizendo e caracterizando miRNAs em dendezeiros jovens expostos à seca e à salinidade e, em seguida, realizando a caracterização de um grupo seleto de fatores de transcrição responsivos a ambos os estresses. Coletamos amostras foliares de dendezeiros jovens - submetidos ou não a estresse salino (12 dias) e privação hídrica (14 dias) - e realizamos RNA-seq e smallRNA-seq. Os dados smallRNA-seq passaram por uma curadoria rigorosa usando mireap versão 0.2, Shortstack versão 3.4 e psRNA-Target e subsequente análise de expressão diferencial e anotação funcional usando software apropriado, enquanto os dados RNA-seq passaram por extensa análise de anotação estrutural e funcional e análise de expressão diferencial usando qPCR. Os dados de sequenciamento gerados permitiram a identificação de genes-alvo de miRNAs putativos no genoma do óleo de palma e entender melhor os papéis desses genes reguladores em resposta ao estresse. Setenta e nove e 81 miRNAs apareceram sob salinidade e estresse hídrico, respectivamente, com 52 miRNAs conhecidos e 29 novos. A anotação funcional dos genes-alvo putativos de miRNAs expressos diferencialmente sob sal e estresse hídrico revelou vários fatores de transcrição. Um grupo de 20 fatores de transcrição - expressos diferencialmente nas folhas de plantas jovens de dendezeiros sob ambos os estresses - foi selecionado e posteriormente caracterizado, mostrando 23 domínios distintos e sete motivos conservados nas regiões codificantes, além de sete elementos de ação cis relacionados a a resposta ao estresse na região promotora. A superexpressão ou nocaute de tais genes resultou em tolerância a esses estresses abióticos em outras espécies de plantas. Os resultados aqui apresentados mostram um padrão de expressão semelhante para ambos os estresses abióticos, indicando que esses genes podem desempenhar papéis essenciais na adaptação das plantas ao estresse. Nossa pesquisa sugere que esses genes devem ser validados in planta como genes candidatos em um programa de melhoramento genético focado na resistência das culturas à seca e ao estresse salino.

Palavras-chave: Estresse abiótico. Fator de transcrição. RNA não-codante. Palma de óleo. Estresse hídrico. Estresse salino.

GENERAL ABSTRACT

The oil palm (*Elaeis guineensis* Jacq.) is an oilseed crop of great economic importance and can yield up to 10 times more oil than other oilseed crops, such as soybean and canola. In 2021/2022, the world consumed approximately 82 million tons of palm oil and palm kernel oil, and as demand increases, expanding the yield is necessary. In Brazil, there is an extensive area suitable for planting oil palm; however, these areas go through long periods of drought, requiring artificial irrigation to be sustainable. Around 30% of the world's irrigated farmland is damaged by salinity, negatively affecting crop productivity. So, to expand oil palm cultivation to such areas in Brazil, one must better understand the response of this oilseed crop to drought and salinity stresses. MicroRNAs (miRNAs) are directly related to the post-transcriptional regulation of gene expression and are essential molecules in the plant response to abiotic stress. Our group performed a study to understand how this oilseed crop responds to two abiotic stresses by predicting and characterizing miRNAs in young oil palms exposed to drought and salinity and then performing the characterization of a select group of transcription factors responsive to both stresses. We collected leaf samples from young oil palm trees - subjected or not to salt stress (12 days) and water deprivation (14 days) - and performed RNA-seq and smallRNA-seq. The smallRNA-seq data underwent rigorous curation using mireap version 0.2, Shortstack version 3.4 and psRNA-Target and subsequent analysis of differential expression and functional annotation using appropriate software, while RNA-seq data underwent extensive analysis of structural and functional annotation and differential expression analysis using qPCR. The sequencing data generated allowed the identification of putative miRNA target genes in the oil palm's genome and to further understand the roles of these regulatory genes in response to stress. Seventy-nine and 81 miRNAs appeared under salinity and drought stress, respectively, with 52 known and 29 new miRNAs. Functional annotation of the putative target genes of differentially expressed miRNAs under salt and water stress revealed several transcription factors. A group of 20 transcription factors - differentially expressed in the leaves of young oil palm plants under both stresses - were selected and further characterized, showing 23 distinct domains and seven conserved motifs in the coding regions, in addition to seven cis-acting elements related to the stress response in the promoter region. Overexpression or knockout of such genes resulted in tolerance to these abiotic stresses in other plant species. The results presented here show a similar expression pattern to both abiotic stresses indicating that these genes may play essential roles in plant adaptation to stress. Our research suggests that these genes should be validated in planta as candidate genes in a genetic improvement program focused on crop resistance to drought and salinity stress.

Keywords: Abiotic stress. Transcription factor. Non-coding RNA. Oil palm. Drought stress. Salt stress.

SUMÁRIO

PRIMEIRA PARTE

1.INTRODUÇÃO GERAL

O *Elaeis guineensis* Jacq, também conhecida como palmeira africana ou dendê, é originário da África ocidental, pertencente à família Arecaceae. O dendê é uma oleaginosa de grande importância econômica e alta produtividade, com uma produção de 85 milhões de toneladas métricas no ano de 2022/2023, superando as demais oleaginosas como soja (60 milhões de toneladas), colza (31 milhões de toneladas) e girassol (18,5 milhões de toneladas) (Barcelos et al., 2015); Shahbandeh, M, 2023; (Statista, 2023; Ritchie & Roser., 2021).

O óleo extraído do fruto do dendê é um produto extremamente versátil, sendo obtido a partir do mesocarpo (óleo de palma) e da amêndoa (óleo de palmiste). 68% da sua produção é destinada ao setor alimentício para produção de margarina, pães, pizzas, chocolates e óleo de cozinha. Sendo também utilizado em aplicações industriais (27%), como produção de detergente e cosméticos, e na produção de bioenergia (5%). Destacando que essa distribuição da porcentagem de óleo destinado a bioenergia varia de pais para pais, onde alguns países chegam a utilizar cerca de 41% da sua produção total para a produção de biocombustível (Ritchie & Roser., 2021).

O dendê é uma planta de clima tropical, que necessita de chuva por um longo período do ano, luz solar adequada e condições úmidas, sendo que as terras adequadas para plantio estão em uma faixa estreita ao redor do equador, tendo sua produção concentrada em países da África, América do Sul e Sudeste Asiático. Dos quais a Indonésia e a Malásia lideram a produção com 84% da oferta global (Ritchie & Roser., 2021; Norrrahim et al., 2022). No Brasil existe uma extensa área apropriada para o plantio do dendê, localizada nas regiões centro-oeste e nordeste, entretanto essas áreas passam por longos períodos de seca não atingindo o nível adequado de água para manter a produtividade do dendê, sendo necessário assim, a utilização de irrigação artificial (Costa et al., 2018; Wei et al., 2021).

O nível de precipitação ideal para que a palma de óleo mantenha sua produtividade e rendimento máximo é de 2.000 mm/ ano, não suportando um período superior a 90 dias de seca. Caso o nível de água esteja abaixo do ideal, o dendê estará exposto ao estresse hídrico, promovendo danos vegetativos em casos de estresse hídrico leve, podendo evoluir a morte em casos mais severos; levando a um impacto negativo nas plantações de dendê (Azzeme et al., 2016) Corley et al., 2018).

A escassez hídrica em regiões áridas e semiáridas, onde a precipitação é insuficiente para atender a demanda hídrica das culturas, lixiviam os sais minerais da zona radicular promovendo assim a salinização do solo (Shahid et al., 2018; Zaman et al., 2018). Além do aumento dos níveis de água subterrânea salina devido a seca, a salinização do solo também é causada por irrigação excessiva e mudanças climáticas, sendo que cerca de 25% a 30% dos solos irrigados são afetados pela salinização (Ma et al., 2022; Zaman et al., 2018), com uma estimativa que em 2050 mais da metade da terra esteja salinizada (Machado & Serralheiro, 2017; Shahid et al., 2018).

O estresse salino nada mais é que o acúmulo excessivo de sal no solo, resultando na inibição do crescimento e desenvolvimento da planta, levando a morte; em escala global, nenhuma outra substancia toxica é tão perigosa quanto o sal para as culturas (Abogadallah, 2010; Ma et al., 2022; Munns & Tester, 2008). As altas concentrações de sal no solo limitam a absorção de água e nutrientes na planta, induzindo estresses primários como o estresse osmótico e o estresse iônico. Sendo que esses estresses primários provocam o estresse oxidativo que levam a uma série de estresses secundários, como alterações de processos metabólicos e fisiológicos (S. Zhao et al., 2021).

Apesar de já existirem diversos estudos de estresse salino e seca em grandes culturas como arroz e milho (Nelson et al., 2007, 2007; B. Zhao et al., 2009), pouco se sabe sobre os mecanismos envolvidos nas respostas do dendê frente ao estresse hídrico e salino sendo necessário melhor compreensão para o desenvolvimento de uma agrícola sustentável, contribuindo assim para promover a segurança alimentar global.

Atualmente diversos estudos vêm sendo realizados para melhor compreensão da reprogramação celular que ocorre nas plantas quando submetidas aos estresses abióticos, sendo que essa reprogramação regula a expressão de diversos genes em níveis transcricionais e pós-transcricionais (Banerjee et al., 2017; Xu et al., 2019). Diante disso os miRNAS, que são reguladores transcricionais e pós-transcricionais, vem sendo frequentemente relacionados com a modulação da resposta e tolerância das plantas aos estresses abióticos (G. Sun, 2012; X. Sun et al., 2019; Xu et al., 2019). Entretanto, quando analisado no contexto do papel dos miRNAs na resposta do dendê ao estresse abiótico, nenhum trabalho foi encontrado.

Simultaneamente, os fatores de transcrição (TFs) desempenham papéis centrais na resposta da planta a diferentes estímulos abióticos, permitindo que ela responda e se adapte rapidamente a determinadas condições, como seca, salinidade, calor, frio, entre outras (Li et al., 2022; Zhang et al., 2022). Os TFs ativam ou reprime a transcrição de genes-alvo, ligando-se a elementos de ação cis dentro das regiões promotoras e participam de vias de transdução de sinal. Cerca de 10% dos genes das plantas codificam fatores de transcrição, sendo classificados em famílias, de acordo com seus domínios de ligação ao DNA (Khaksefidi et al., 2015; Li et al., 2022).

Diante de todo o contexto aqui apresentado sobre a importância do dendê, a necessidade de expansão afim de garantir a segurança alimentar, e os desafios encontrados frente a expansão territorial da palma de óleo; os objetivos desse trabalho foram (I) realizar a previsão e caracterização de miRNAs responsivos ao estresse de seca e salinidade em plantas de dendê, (II) identificar genes-alvo putativos de miRNAs no genoma de dendê, (III) realizar uma comparação in sílico do perfil de expressão dos miRNAs e (IV) caracterização estrutural e funcional de TFs comumente afetados por ambos os estresses.

2.CONSIDERAÇÕES FINAIS

No decorrer desta tese, já destacamos a grande importância do dendê e sua grande expansão comercial. Entretanto, junto com a grande expansão nos esbarramos com uma série de restrições ambientais. Por outro lado, existem áreas que são adequadas para o plantio, mas passam por longos períodos de seca ou que possuem alto teor de salinidade. Diante disso, fez-se necessário estudos para melhor compreender os mecanismos de resposta do dendê aos estresses abióticos afim de desenvolver espécies mais tolerantes às condições adversas.

De acordo com este cenário, já era relatado a participação dos miRNAS na regulação da expressão de genes responsivos ao estresse abiótico, como seca e salinidade, em espécies como arroz, milho e *arabidopsis*. No entanto ainda era escasso os estudos envolvendo a anotação de miRNAs em dendê, sendo que esses estudos já realizados abordavam a participação desses miRNAs no desenvolvimento e crescimento do dendê, mas quanto ao seu envolvimento na resposta ao estresse salino e seca, essa informação era inexistente.

O nosso trabalho possibilitou a identificação de 29 novos miRNAs específicos de dendê, relatados pela primeira vez na literatura, responsivos ao estresse de seca e 27 desses 29 são responsivos ao estresse de sal. Além desse grande avanço, também realizamos a anotação de 81 miRNAs conservados que também estão envolvidos na regulação da resposta das plantas de dênde quando submetidos a ambos os estresses. Com isso, foi possível analisar os genes alvo desses miRNAs, pressupor como esses miRNAS promovem a regulação dos seus genes alvos e por fim, em qual processo esses genes alvos estão envolvidos.

Nossos resultados mostraram que 8 miRNAs foram regulados negativamente em plantas de dendê quando submetidas aos estresses de seca e salinidade, e que seus respectivos genes alvos apresentaram uma regulação positiva, também em ambos os cenários. Através da anotação funcional desses 8 genes alvo, foi mostrado que três deles expressam lncRNAs, sendo que estudos já mostraram o envolvimento dos lncRNAs na resposta adaptativa de plantas ao estresse abiótico. Dos demais, dois codificam proteínas da superfamília facilitadora Major, um para a enzima modificadora de histonas com atividade demetilase, um para uma proteína da família de zíper homeodomínio-leucina de classe III e um para família MIF.

A riqueza dos dados gerados a partir desses dois trabalhos de prospecção e caracterização de miRNAs em dendê, proporciona um grande avanço na busca por seleção de possíveis genes que promovam a resistência aos estresses abióticos. Sendo evidenciado o vasto número de fatores de transcrição que estavam sendo regulados pelos miRNAs, contribuindo na resposta do dendê ao estresse. De acordo com a literatura, os fatores de transcrição exercem uma função essencial na regulação da expressão de genes que auxiliam na adaptação das plantas as condições adversas impostas pelo ambiente.

Ao selecionarmos um grupo de 20 fatores de transcrição responsivos ao estresse de salinidade e seca, conseguidos aprofundar nosso conhecimento a níveis de estrutura, promotores e domínios, na tentativa de encontrar algum padrão entre eles. Ao final, mostramos que dos 20 genes selecionados, 18 apresentam o mesmo comportamento em ambos os estresses, o que nos possibilita o melhoramento genético de ambos os estresses com uma única tecnologia.

A partir dos dados gerados ao final dessa tese, faz-se necessário uma curadoria rigorosa e aprofundada em cada possível gene identificado, para que validações e seleções experimentais sejam realizadas e juntamente com a superexpressão ou repressão desses genes em plantas de ciclo curto próximas há família do dendê, gerando assim informações valiosas para programas de melhoramento genético da palma de óleo.

REFERÊNCIAS

Abogadallah, G. M. (2010). Antioxidative defense under salt stress. *Plant Signaling and Behavior*, *5*(4), 369–374. https://doi.org/10.4161/psb.5.4.10873

Azzeme, A. M., Abdullah, S. N. A., Aziz, M. A., & Wahab, P. E. M. (2016). Oil palm leaves and roots differ in physiological response, antioxidant enzyme activities and expression of stress-responsive genes upon exposure to drought stress. *Acta Physiologiae Plantarum*, *38*(2), 1–12. https://doi.org/10.1007/s11738-016-2073-2

Banerjee, S., Sirohi, A., Ansari, A. A., & Gill, S. S. (2017). Role of small RNAs in abiotic stress responses in plants. *Plant Gene*, *11*, 180–189. https://doi.org/10.1016/j.plgene.2017.04.005

Barcelos, E., De Almeida Rios, S., Cunha, R. N. V., Lopes, R., Motoike, S. Y., Babiychuk, E., Skirycz, A., & Kushnir, S. (2015). Oil palm natural diversity and the potential for yield improvement. *Frontiers in Plant Science*, *6*(MAR), 1–16. https://doi.org/10.3389/fpls.2015.00190

Corley, R. H. V., Rao, V., Palat, T., and Praiwan, T. (2018). Breeding for drought tolerance in oil palm. *J. Oil Palm Res.* 30, 26–35.

Costa, S. J. da, Erasmo, E. A. L., Tavares, T. C. de O., & Silva, J. (2018). Respostas Fisiológicas De Dendê Submetidas Ao Estresse Hídrico Em Condições Do Cerrado. *Revista Brasileira de Agropecuária Sustentável*, *8*(4), 92–99. https://doi.org/10.21206/rbas.v8i4.3055

Khaksefidi, R. E., Mirlohi, S., Khalajin, F., Fakharin, Z., Shiran, B., Fallahi, H., Rafiei, F., Budak, H., & Ebrahimie, E. (2015). Differential expression of seven conserved microRNAs in response to abiotic stress and their regulatory network in Helianthus annuus. *Frontiers in Plant Science*, *6*(September), 1–14. https://doi.org/10.3389/fpls.2015.00741

Li, Y., Yang, Z., Zhang, Y., Guo, J., Liu, L., Wang, C., Wang, B., & Han, G. (2022). *The roles of HD-ZIP proteins in plant abiotic stress tolerance*. *October*, 1–19. https://doi.org/10.3389/fpls.2022.1027071

Ma, L., Liu, X., Lv, W., & Yang, Y. (2022). Molecular Mechanisms of Plant Responses to Salt Stress. *Frontiers in Plant Science*, *13*(June), 1–17. https://doi.org/10.3389/fpls.2022.934877

Machado, R. M. A., & Serralheiro, R. P. (2017). Soil salinity: Effect on vegetable crop growth. Management practices to prevent and mitigate soil salinization. *Horticulturae*, *3*(2). https://doi.org/10.3390/horticulturae3020030

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, *59*, 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Nelson, D. E., Repetti, P. P., Adams, T. R., Creelman, R. A., Wu, J., Warner, D. C., Anstrom, D. C., Bensen, R. J., Castiglioni, P. P., Donnarummo, M. G., Hinchey, B. S., Kumimoto, R. W., Maszle, D. R., Canales, R. D., Krolikowski, K. A., Dotson, S. B., Gutterson, N., Ratcliffe,

O. J., & Heard, J. E. (2007). Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(42), 16450–16455. https://doi.org/10.1073/pnas.0707193104

Norrrahim, M. N. F. et al. Emerging technologies for value-added use of oil palm biomass. Environmental Science: Advances, v. 1, n. 3, p. 259-275, 2022.

Ritchie, H.; Roser, M. Forests and deforestation. Our World in Data, 2021.
Shahid, S. A., Zaman, M., & Heng, L. (2018). Soil Salinity: Historical Perspectives and a World Overview of the Problem. *Guideline for Salinity Assessment, Mitigation and Adaptation Using Nuclear and Related Techniques*, 43–53. https://doi.org/10.1007/978-3-319-96190-3_2

Shahbandeh, M. Production Volume of Palm Oil Worldwide from 2012/13 to 2022/23. Available online: https://www.statista.com/statistics/613471/palm-oil-production-volume-worldwide/#statisticContainer (accessed on 30 Julho 2023).

Sun, G. (2012). MicroRNAs and their diverse functions in plants. *Plant Molecular Biology*, *80*(1), 17–36. https://doi.org/10.1007/s11103-011-9817-6

Sun, X., Lin, L., & Sui, N. (2019). Regulation mechanism of microRNA in plant response to abiotic stress and breeding. *Molecular Biology Reports*, *46*(1), 1447–1457. https://doi.org/10.1007/s11033-018-4511-2

Wei, L., John Martin, J. J., Zhang, H., Zhang, R., & Cao, H. (2021). Problems and prospects of improving abiotic stress tolerance and pathogen resistance of oil palm. *Plants*, *10*(12), 1–16. https://doi.org/10.3390/plants10122622

Xu, J., Hou, Q. M., Khare, T., Verma, S. K., & Kumar, V. (2019). Exploring miRNAs for developing climate-resilient crops: A perspective review. *Science of the Total Environment*, *653*, 91–104. https://doi.org/10.1016/j.scitotenv.2018.10.340

Zaman, M., Shahid, S. A., & Heng, L. (2018). Guideline for Salinity Assessment, Mitigation and Adaptation Using Nuclear and Related Techniques. In *Guideline for Salinity Assessment, Mitigation and Adaptation Using Nuclear and Related Techniques*. https://doi.org/10.1007/978-3-319-96190-3

Zhang, H., Zhu, J., Gong, Z., & Zhu, J. K. (2022). Abiotic stress responses in plants. *Nature Reviews Genetics*, *23*(2), 104–119. https://doi.org/10.1038/s41576-021-00413-0

Zhao, B., Ge, L., Liang, R., Li, W., Ruan, K., Lin, H., & Jin, Y. (2009). Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. *BMC Molecular Biology*, *10*. https://doi.org/10.1186/1471-2199-10-29

Zhao, S., Zhang, Q., Liu, M., Zhou, H., Ma, C., & Wang, P. (2021). Regulation of plant responses to salt stress. *International Journal of Molecular Sciences*, *22*(9), 1–16. https://doi.org/10.3390/ijms22094609

SEGUNDA PARTE – Capítulo de livro

The Role of Noncoding RNAs in the Response of Oil Palm Plants to Abiotic Stresses

Autores: Fernanda Ferreira Salgado, Priscila Grynberg e Manoel Teixeira Souza Junior

Publicado no IntechOpen
https://www.intechopen.com/chapters/82573

Chapter

The Role of Noncoding RNAs in the Response of Oil Palm Plants to Abiotic Stresses

Fernanda Ferreira Salgado, Priscila Grynberg and Manoel Teixeira Souza Junior

Abstract

The genus *Elaeis* comprises two species, *E. guineensis* Jacq. and *E. oleifera* (Kunth) Cortés, which are known as the African and the American oil palms, respectively. The African oil palm originated from West Africa and is the predominant species in commercial plantations. This oilseed crop is the number one source of consumed vegetable oil in the World. Several abiotic stressors affect the plant life cycle interfering with growth and productivity. Salinity and drought are abiotic stresses that affect plantations on all continents, resulting in the loss of billions of dollars annually. MicroRNAs (miRNAs) are small endogenous noncoding RNAs that impact almost all biological processes, affecting either the transcriptional or posttranscriptional regulation of gene expression. Here we describe the R&D initiatives on oil palm miRNAs, highlighting the current knowledge on miRNAs' involvement in oil palm response to abiotic stress and postulating possible miRNA-based strategies for the genetic improvement of oil palm salinity and drought stresses tolerance.

Keywords: abiotic stress, tolerance, transcription factor, transcriptome, noncoding RNA, oil palm, drought stress, salt stress

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is known as the most productive oilseed crop in the World, bearing great economic importance due to its large-scale production and high efficiency of the extraction and refining processes to obtain palm oil and palm kernel oil [1, 2]. In 2021/2022, the World consumed approximately 82 million metric tons of palm oil and palm kernel oil, making this oilseed crop the number one source of consumed vegetable oil [3].

The oil palm industry faces criticism due to a series of unsustainable practices (deforestation and consequent biodiversity loss, increased greenhouse gas emissions, and environmental and aquatic pollution), finding itself under pressure to adopt new and innovative procedures that could help this sector reverse this negative public perception [4]. Darkwah and Ong-Abdullah [5] highlighted some of these procedures, such as choice for intensification over extensification, adoption of precision agriculture technologies, support for smallholder farmers, sustainability certification, and circular economy.

Oil palm plantations are in areas with tropical forests in the equatorial belt, as they need high rainfall throughout the year [6]. In Brazil, for instance, there is an extensive area with favorable conditions for cultivating oil palm outside the Amazon rainforest; however, those areas experience long periods of drought when oil palm does not meet the physiological water requirement to maintain productivity [7], and, consequently, need to be artificially irrigated with proper management to avoid soil salinization. Approximately 30% of the irrigated land area in the World is affected by salt [8], which, to a certain extent, shows a link between drought and salinity stresses.

Studies have shown that the progression of abiotic stresses is limiting global agricultural production, with no sign of reversal shortly [9–11], making it necessary to develop crops that are resilient to different abiotic stresses, such as drought and salinity, ensuring food security. Therefore, it is currently a challenge for plant scientists to develop crops resistant or tolerant to these conditions, capable of withstanding climatic instabilities and environmental stresses, especially combinations of these stresses. So, the research community aiming to develop knowledge and technology to allow oil palm breeding programs in Brazil and elsewhere to breed for superior genotypes must consider that.

Plants respond to environmental stimuli in a complex and highly coordinated manner at biochemical, physiological, and molecular levels [11–13]. When exposed to stress, rapid and effective reprogramming at the molecular level is required to adapt to unfavorable conditions [12, 14, 15]. This reprogramming regulates the expression of stress-responsive genes, especially at transcriptional and posttranscriptional levels [14, 16, 17].

micro RNAs (miRNAs) are posttranscriptional and translational regulators frequently correlated with plant stress tolerance and modulating stress response [15, 18, 19]. miRNAs have enormous potential for crop improvement, being the focus of studies by scientists in recent years [18, 20–22].

Therefore, the main objectives of this review are to describe the R&D initiatives known to date on oil palm (*Elaeis* spp.) miRNAs, to report on their biogenesis and mode of action, to summarize the current knowledge on miRNAs' involvement in oil palm response to abiotic stress, and to postulate possible miRNA-based strategies on the genetic improvement of oil palm's tolerance to abiotic stress.

2. miRNA biogenesis and mode of action

miRNAs are a class of endogenous, noncoding sRNAs transcribed from introns, exons, or intergenic regions [11]. They play a role in posttranscriptional RNA-mediated gene silencing and expression by complementary interaction with their mRNA target site [23, 24]. The biogenesis of plant miRNAs has been reported mainly in *Arabidopsis thaliana* (L.) *Heynh*. (**Figure 1**) [25].

Plant miRNAs range from 21 to 24 nucleotides in length [26]. Similar to protein-coding genes, miRNA genes (MIRs) are commonly transcribed by RNA polymerase II, forming a single-stranded precursor RNA called primary miRNAs (pri-miRNAs) that have imperfect self-complementary folding regions. Still in the nucleus, the 5′ m7G-cap and 3′ polyadenylation are added to promote better

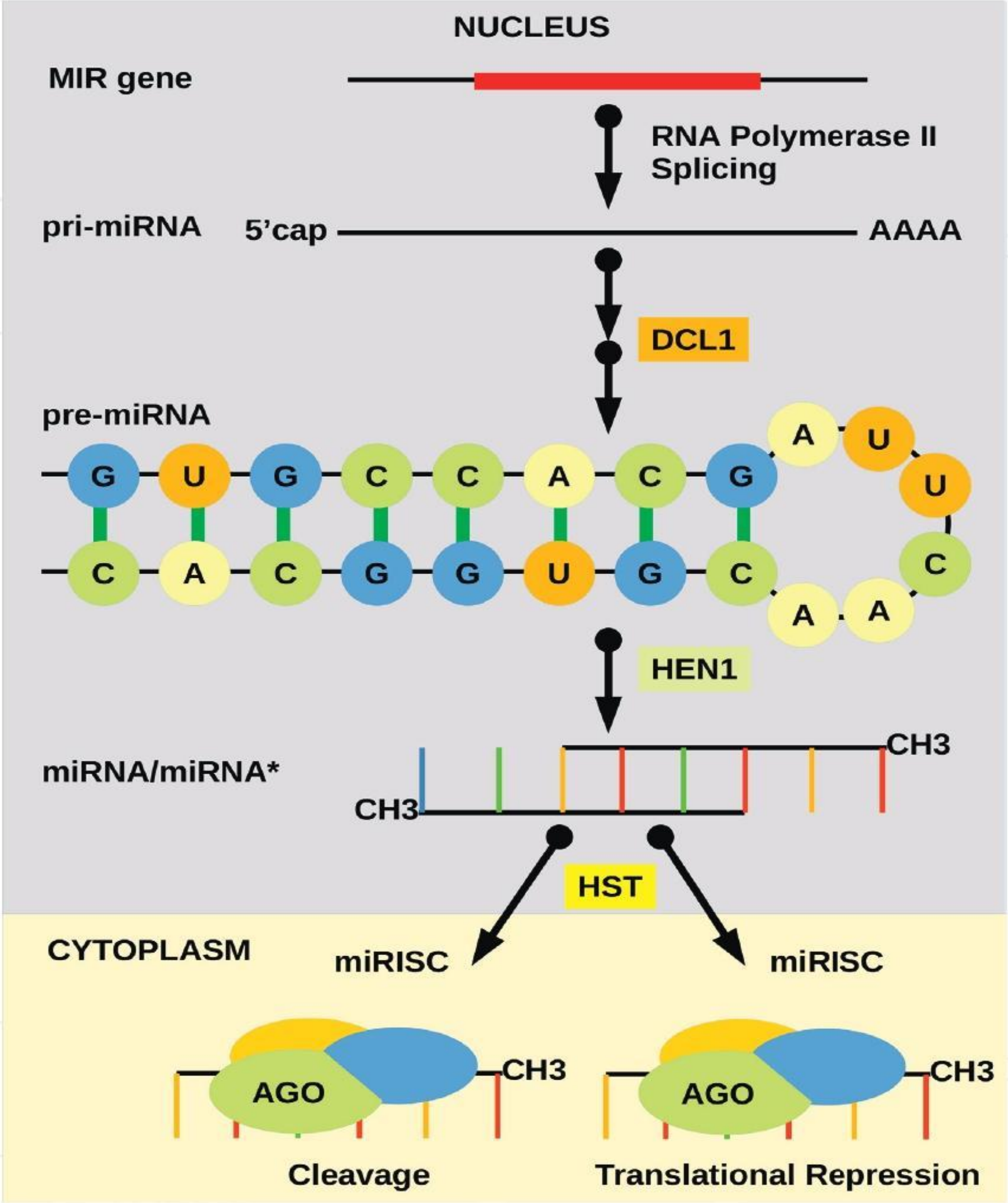

Figure 1.
Biogenesis of plant miRNAs. A miRNA gene is transcribed by RNA polymerase II, giving rise to a primary transcript (pri-miRNA), which is then capped and polyadenylated. DCL1 processes pri-miRNA, perhaps in two or more steps, and HEN1 methylate it to produce the miRNA/miRNA duplex. HST transports the duplex to the cytosol, and the miRNA strand gets incorporated into RISC. The nuclear export of miRNAs may occur before or after RISC assembly. According to the degree of complementarity with the target site, miRISC will either cleave the mRNA or inhibit its translation.*

stability. Then the pri-miRNAs are converted into miRNA precursor sequences (pre-miRNA) by the action of DICER-LIKE 1 (DCL1) along with other associated proteins [15, 27].

The 3′ end of the initial miRNA/miRNA* duplex is methylated by the nuclear protein HUA ENHANCER 1 (HEN1), thus preventing untemplated 3′ polymerization that accelerates miRNA turnover [28]. The mature miRNA is then transported from the nucleus to the cytoplasm by the HASTY protein (HST), then loaded onto AGONAUTE (AGO) proteins and incorporated into an RNA-induced silencing complex (RISC), while often the star miRNA molecules (miRNA *) do undergo degradation. The AGO/miRISC complex search for RNA molecules through sequence complementarity, promoting posttranscriptional gene silencing through endonucleolytic cleavage or translation inhibition [25, 29]. The miRNA mechanisms of biogenesis and function are still unclear; however, studies have shown that they are involved in several cellular processes, including development, differentiation, division, and cell death [11, 26, 30].

An individual miRNA can regulate multiple transcripts, and a single transcript can be acted upon by several distinct miRNAs [11]. The miRNA can regulate the expression of its target gene using three strategies: (a) degradation of a target transcript through almost perfect complementarity; (b) inhibition of translation; or (c) DNA methylation. Regulation via degradation of the target transcript results in the degradation of intron sequences, and these cleaved sequences generate dsRNA molecules with the aid of RDR2 and then produce I-siRNA molecules of 21 and 22 nucleotides. Subsequently, the I-siRNA associated with the AGO-RISC complex directs the cleavage of the target mRNA sequence [31, 32].

Regarding the regulation via translation inhibition, only the roles of AGO1 and AGO10 have so far been understood [33, 34]. On the other hand, the miRNA-mediated inhibition mechanism remains unclear. At last, studies show that miRNAs in *A. thaliana* bind to AGO4, AGO6, and AGO9 to promote transcriptional gene silencing of target genes through RNA-directed DNA methylation (RdDM) [27].

3. miRNAs playing a role in plant response to abiotic-drought and salinity-stress

Abiotic stress, such as deprivation or excess of water, high salinity, low or high temperature, heavy metals, and ultraviolet radiation, is a negative impact caused by nonliving factors on living organisms [35, 36]. They are primary stresses that arise in the plantations limiting seed germination, plant growth, and plant development; and, in some cases, resulting in the death of the plants.

Salinity and drought are two of the most prevalent abiotic stress worldwide, affecting plantations on all continents and resulting in the loss of billions of dollars annually. Plants have developed several mechanisms to combat drought stress, and several genes associated with the response of plants to this stress are known [12, 37]. One of these responses is the positive and/or negative regulation of several transcription factors (TF) related to numerous physiological and cellular functions [38].

The dehydration responsive elements (DREB) are TF related to the activation of genes responsive to drought stress tolerance, and its overexpression can result in tolerance to water deficit in plants. Similarly, the transcription factor MYB regulates negatively in plants during drought stress since it is related to stomata opening [11, 39]. However, the overexpression of other genes associated with drought stress in plants did not result in the expected drought tolerance, demonstrating the complexity of mechanisms responsible for plant tolerance to water stress [40].

Several research groups have identified and reported miRNAs responsive to water stress in several species, such as rice—*Oryza sativa* L. [41], soybean—*Glycine max* (L.) Merr. [42], barley—*Hordeum vulgare* L. [43], and Arabidopsis [44]. Those water stress-responsive miRNAs belong to three classes. The first class includes miRNAs targeting transcription factors that contribute to the gene regulation of the stress response, comprising miR156, miR159, miR165, miR169, miR171, miR172, miR319, and miR396 [21, 44, 45].

In the second class, the miRNAs are involved in the direct response to water stress, some of which are miR167, which is responsible for directing the auxin response factors ARF6 and ARF8 [46]; miR168, which targets the ARGONAUTE 1 (AGO1) mRNA [47]; and miR393 and miR394, which target F-box protein mRNAs that play roles in drought tolerance [45, 48, 49]. The last class contains miRNAs such as miR397 and miR408, which have hydrolase and oxidoreductase genes as their target gene [50, 51].

Zhou and colleagues [41] identified 30 miRNAs differentially expressed in rice under drought stress, 19 were new miRNAs. Regarding their expression, 14 were upregulated (miR159, miR169, miR171, miR319, miR395, miR474, miR845, miR851, miR854, miR896, miR901, miR903, miR1026, and miR1125) and 16 downregulated (miR156, miR159, miR170, miR171, miR172, miR319, miR396, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088 and, miR1126). In soybean, upregulation of miRseq13, miR397ab, miR1513c, miR169-3p, and miR166-5p was observed in sensitive plants, while the same miRNAs were downregulated on tolerant crops [42].

When it comes to salinity stress, several genes are related to the plant's stress response, including those involved in ion channel activation, signal transduction, and modification regulated by plant growth factors, especially the morphological architecture of the root [52]. In *A. thaliana*, studies have shown increased expression of miRNAs miR156, miR158, miR159, miR165, miR167, miR168, miR169, miR171, miR319, miR393, miR394, miR396, and miR397 in response to salt stress, while miR398 was downregulated [21].

According to Ding and colleagues, when analyzing corn variants tolerant and sensitive to salt stress, members of the miR396, miR167, miR164, and miR156 families downregulated, while miRNAs miR474, miR395, miR168, and miR162 upregulated in the tolerant ones [18]. In radish (*Raphanus sativus*), 22 new and 49 already known miRNAs appeared under salt stress [53]. In young oil palm plants under saline stress, 27 new and 52 already known miRNAs appeared [15]. When analyzing the expression profile of these miRNAs, 72 of them showed negative differential expression and the remaining seven had no significant differential expression [15].

Recent studies allowed a better understanding of the mechanisms of action of miRNAs and their relationship in response to different abiotic stresses [38, 54, 55]. It is necessary to highlight that miRNAs are species-specific and present different expression levels when analyzed in distinct plant species or within a specific one under the same stress [15, 24].

4. miRNAs in oil palm (*Elaeis* spp.)

Despite many advancements in the prediction and characterization of miRNAs in plants, there are only a few studies where researchers report the identification and characterization of miRNAs in oil palm, either under abiotic stress conditions or not. Currently, there are 10 published studies reporting miRNAs identification and characterization in oil palm [15, 30, 56–63]. Considering the great economic importance of oil palm, the number of miRNA studies reported on this oilseed crop is relatively low (**Table 1**).

Nasaruddin and colleagues found five potential miRNA encoding sequences in oil palm by a combined homology and structural analysis approach, having roles in regulating the auxin response, floral development, and basal transcription [30]. Mehrpooyan, Othman, and Harikrishna [56] identified in oil palm six paralogs of miR172, a regulator of the APETALA2 (AP2)-like family transcription factors. Their results suggested that the expression of different miR172 precursor paralogs is tissue-specific and showed that each of the two mature miR172 isoforms had different expression patterns during floral development.

Low and colleagues identified 14 miRNAs in contigs assembled from sequences generated from the hypomethylated or gene-rich regions in the genomes of both species from the *Elaeis* genera [57]. Target prediction of these miRNAs identified just one putative target gene, similar to the Rab21-family small GTPase, a small GTP-binding protein of the Ras superfamily. Silva and colleagues identified 57 mature miRNAs in *E. guineensis* and 52 in *E oleifera*, respectively, revealing that majority of them are transcription factors involved in the plant development process [58].

According to Somyong et al. [59], miRNA159 is related to the determination of females in oil palm trees, which is directly related to the higher production of oil palm trees, since palm oil with high number of female inflorescence and of clusters is most favorable. Noting that no expression of EgmiR159a was found in male flowers in their study, confirming its role in female sexual differentiation (**Table 1**). Ho and colleagues identified 15 oil palm-specific miRNA candidates when investigating microRNA expression in female inflorescence at two stages of floral [60].

In Gao et al. [61], the micro RNA EgmiR179 regulates the biosynthesis of metabolites through the negative regulation of its target gene NDT1, increasing oil content in palm oil [61]. Zheng and colleagues identified 452 microRNAs (miRNAs), including 170 conserved miRNAs and 282 new miRNAs, when gaining insights into the oil palm regulatory mechanisms of lipid and fatty acid metabolism. They found 37 fatty acid synthesis-related genes as putative miRNA-target genes and indicated that 22 conserved miRNAs and 14 new ones might be involved in fatty acid metabolism pathways. Tregear et al. [63] reported finding 30 previously unreported oil palm miRNA genes in a molecular study of the process of sexual differentiation in the immature inflorescence of oil palm.

Our research group reported prospecting and characterizing miRNAs in oil palm plants under salinity stress (**Table 1**) [15]. That was the first step in a study prospecting and characterizing miRNAs in oil palm plants under salinity [64] or drought stress and looking for insights on commonalities—miRNAs and putative miRNA-target genes—on the molecular response of young oil palm plants to these two abiotic stresses.

We did find 81 miRNAs—52 known ones and 29 new ones (**Figure 2**)—and 139 differentially expressed putative miRNA-target genes [15, 64]. In Salgado et al. [15], miR166, miR169, miR319, miR396, miR529, and egu-miR24sds showed altered expression profiles in young oil palm plants subjected to salt stress, both targeting TF, indicating a miRNA-dependent posttransitional regulation during the plant's response to the environment.

All miRNAs identification and characterization studies done so far in oil palm allowed the identification of 55 miRNA families or groups of miRNAs that derive from a common ancestor (**Figure 3**).

Technology	Condition	miRNA	Species	Oil palm material	Publication
Small RNA-seq	Normal	miR159a	*E. guineensis*	Female flower	Somyong et al. [59]
3730 sequencing technology/ Blast	Normal	miR2911, miR2916, miR156j, miR2914, miR2910, miR319f, miR167g, miR319e, miR845a, miR845b	*E. guineensis* and *E. oleifera*	Leaf	Low et al. [57]
Blast	Normal	miR156, miR157, miR159, miR160, miR164, miR166, miR167, miR168, miR169, miR171, miR172, miR319, miR390, miR393, miR394, miR395, miR396, miR397, miR398, miR399, miR528, miR529, miR530, miR535, miR815, miR1428, miR1432, miR2118, miR2275, miR5148, miR5179, miR5532, miR5801	*E. guineensis* and *E. oleifera* and *Phoenix dactylifera*	Genome	da Silva et al. [58]
Blast	Normal	miR156, miR157, miR159, miR160, miR854	*E. guineensis*	Genome	Nasaruddin et al. [30]
RNA-seq	Normal	miR5179	*E. guineensis*	Fruit	Gao et al. [61]
Small RNA-seq	Salt stress	miR156, miR391, miR395a, miR536, miR156, miR160, miR166, miR167, miR169, miR171, miR172, miR395, miR396, miR399, miR528, miR156, miR159, miR166, miR319, miR393, miR535, miR156, miR162, miR169, miR529, miR530, miR168, miR319, miR159. egumiR01, egumiR02, egumiR03, egumiR04, egumiR05, egumiR06, egumiR07, egumiR08, egumiR09, egumiR10, egumiR11,egumiR12, egumiR13, egumiR14, egumiR15, egumiR16, egumiR17, egumiR18, egumiR19, egumiR20, egumiR21, egumiR22, egumiR23, egumiR24, egumiR25, egumiR26, egumiR27.	*E. guineensis*	Leaf	Salgado et al. [15]
Small RNA-seq	Normal	miR1432, miR160, miR163, miR166, miR168, miR172, miR1859, miR1873, miR2199, miR2654, miR396, miR4365, miR528, miR535, miR827, miR835, miR858, miR894, egumiR3, egumiR5, egumiR6, egumiR7, egumiR8, egumiR9, egumiR10, egumiR11, egumiR12, egumiR13, egumiR14, egumiR15.	*E. guineensis* *Tenera* hybrid palms (*Dura* × *Pisifera*)	Female Inflorescence	Ho et al. [60]

Technology	Condition	miRNA	Species	Oil palm material	Publication
Small RNA-seq	Normal	miR156, miR160, miR164, miR172, miR444, miR5179, miR528, miR167, egumiR259, egumiR223, egumiR218, egumiR255, egumiR43, egumiR132, egumiR172, egumiR113, egumiR273, egumiR158, egumiR275, egumiR138, egumiR59, egumiR201, egumiR209, egumiR116, egumiR129, egumiR211, egumiR7, egumiR210, egumiR274.	*E. guineensis*	Mesocarp	Zheng et al. [62]
Small RNA-seq	Normal	miR319, miRN1, miR168, miR482, miR2118, miR159, miR171, miR167, miR397, miR160, miR156, miR535, miR396, miR536, miR169, miR319, miR179, miR166, miR394, miR399, miR172, miR164, miRN4, miR390, miR393, miRN5, miRN6, miR162, miR827, miR398, miRN7, miR395, miRN8, miRN9, miRN10, miR408, miR528, miRN11.	*E. guineensis*	Immature Inflorescence	Tregear et al. [63]
Blast	Normal	miR172	*E. guineensis*	Inflorescence	Mehrpooyan et al. [56]

Table 1.
Summary of miRNA studies reported on oil palm.

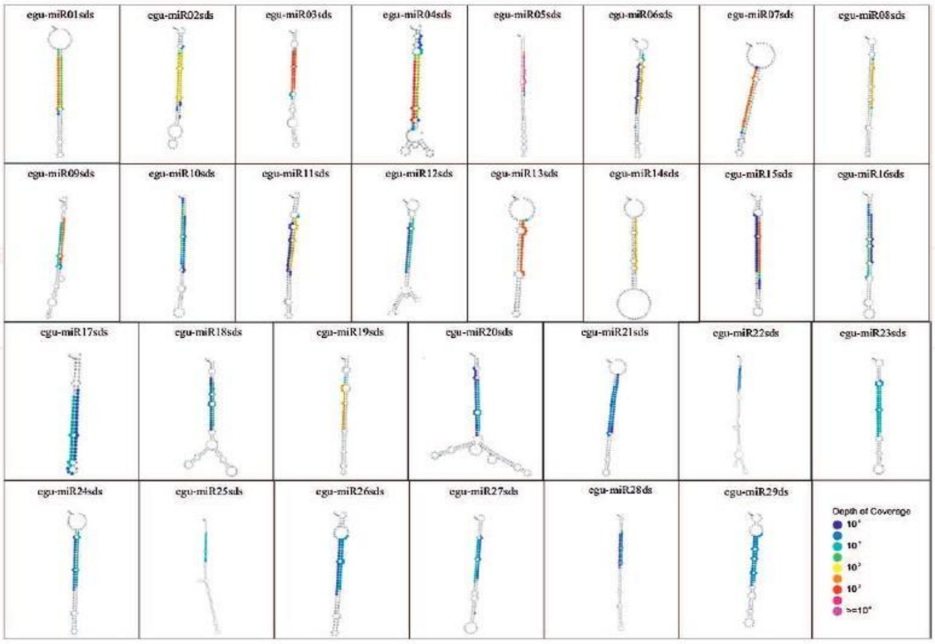

Figure 2.
Structure of the 29 new miRNAs identified in oil palm (Elaeis guineensis) plants exposed to abiotic stresses—salinity and drought (Source: [15, 64]).

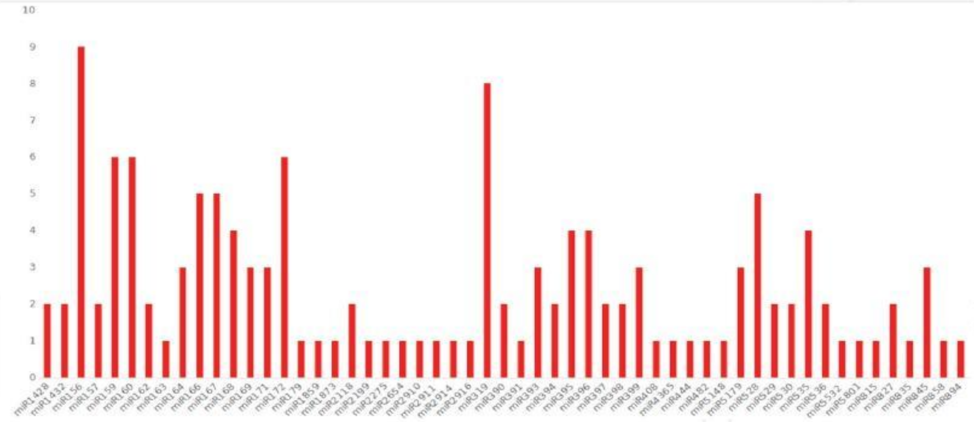

Figure 3.
Families of miRNAs found in oil palm, and the amount of members per family.

5. miRNA-based strategies to improve oil palm tolerance to abiotic stress

In the last decade, due to the recent advances in high-throughput sequencing techniques, vis-à-vis the empowerment of bioinformatics tools, much progress has been made in characterizing and understanding miRNAs mechanism of action, and predicting their target genes [65–68].

The possibility of the involvement of miRNAs in the regulation of genes directly related to the modulation of the impact of abiotic stress on plants has enabled researchers to take the next step by altering the expression levels of miRNAs, either by overexpression or knocking down strategies. This step attempts to gain further insights that could allow the development of distinct ways to generate superior genotypes harboring tolerance to those stresses, either by vertical or horizontal transfer of genes or even by genome editing [8].

According to Hajyzadeh et al. [69], the overexpression of miR408 in chickpea (*Cicer arietinum* L.) resulted in improved drought tolerance. Plants overexpressing the pre-miR408 from *A. thaliana* were without wilting and drying after 8 days of undergoing the drought treatment, unlike the control plants [69]. Arabidopsis plants overexpressing miR408 showed resilience to multi-stress environments—salinity and drought [70]. Through biochemical analysis and fluorescence imaging with measures of the photosystem efficiency, it was reported tolerance of the transformants compared to control ones [70].

Transgenic lines of creeping grass (*Agrostis stolonifer*a L.) overexpressing rice pre-miR393 showed a better tolerance response to saline stress (at 250 mM for 10 days), drought (15 days), and heat (40°C at day, 35°C at night, 13 days), confirming that miR393 is a potent candidate to confer resistance to multiple stresses [71]. According to Baek and colleagues, the overexpression of miR399f in Arabidopsis promoted greater tolerance to salinity and treatment with abscisic acid (ABA) [72]. Arabidopsis' putative miRNA-target genes—ABF3 and CSP41b—had low expression levels in transgenic plants, and these genes are related to stress due to their participation in ABA signaling [72].

Although the strategy of overexpression of miRNAs is dominant among the studies to obtain plants resistant to different stresses, the inhibition approach is also being promoted through the use of the short tandem target mimic (STTM) approach, causing loss of function [11]. Although there has been an increase in studies on the use of miRNAs to obtain plants resistant to multi-stress, there is still a long way to go.

According to Ferdous and colleagues, the overexpression of Hv-miR827 in barley (*H. vulgare* cv. "Golden Promise") influenced the grain weight and allowed the plants to recover after drought treatment, in addition to providing an improvement in the efficiency of the use of water [73]. Such behavior is also in transgenic Arabidopsis plants overexpressing the miRNA Ath-miR827 [74, 75].

miR319 is one of the oldest and most conserved miRNA families in plants, responsive to several stresses, including drought and salinity, based on high-throughput sequencing [76]. Studies have shown that its overexpression in transgenic creeping grass resulted in tolerance to salt and drought stresses; and it was linked to downregulation of at least four putative target genes of miR319 (AsPCF5, AsPCF6, AsPCF8, and AsTCP14) [77].

So far, no studies are known reporting on successfully horizontally transferring (or editing) miRNAs to or from oil palms aiming at achieving tolerance to abiotic stresses. Our research group prospected stress-responsive miRNAs and putative target genes that are good candidates for such endeavor [15, 64]. miRNAs and their putative target genes responsive to both stresses at once are the priority candidates for further studies.

Among putative target genes responsive to both stresses identified by our group in oil palm, there are some lncRNAs. Several studies have shown that lncRNAs play essential roles and different functions in the biological processes of plants. They can play a role as sRNA precursors to produce sRNAs, such as miRNAs and siRNAs. In response to different stresses, lncRNAs also play a role in the RNA-directed DNA methylation (RdDM) pathway [78–80].

6. Conclusions and future perspectives

Due to a series of unsustainable practices used by the oil palm industry, there is criticism pressuring it to adopt new and innovative procedures, including getting away from the tropical rain forests.

Predictably, primary stresses (abiotic and biotic) will arise once the industry establishes itself in those new areas for oil palm cultivation, forcing it to search for tools to deal in an economically sustainable way with them. Salinity and drought are on the top of such a list of primary abiotic stresses. Many studies report successful attempts to increase plants' tolerance to such stresses by altering the expression levels of miRNAs, either by overexpression or knocking down strategies. Despite the great economic importance of oil palm, we found only 10 published studies reporting miRNAs identification and characterization in this oilseed crop. When considering abiotic stresses, the scenario is even worst; only one published study so far. In this sense, it is clear that further studies are necessary on the role of miRNAs - and their putative target genes - in oil palm's response to abiotic stress. Besides that, as candidate genes become available from those studies, it will be necessary to validate their potential as tools to generate superior genotypes harboring tolerance to those stresses, either by vertical or horizontal transfer of genes or even by genome editing.

Acknowledgements

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The grant (01.13.0315.00 - DendêPalm Project) for this study was awarded by the Brazilian Innovation Agency—FINEP.

Conflict of interest

The authors declare no conflict of interest.

Author details

Fernanda Ferreira Salgado[1], Priscila Grynberg[2] and Manoel Teixeira Souza Junior[3]*

1 Universidade Federal de Lavras (UFLA), Lavras, MG, Brazil

2 Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil

3 Embrapa Agroenergia, Brasília, DF, Brazil

*Address all correspondence to: manoel.souza@embrapa.br

References

[1] Priscilla AS, Vanessa SC, Kátia CBR, Kelly SCD, Fábio ML, Roberto LC, et al. Drought tolerance in two oil palm hybrids as related to adjustments in carbon metabolism and vegetative growth. Acta Physiologiae Plantarum. 2017;**39**(58):1-12

[2] Le W, May L, Baoqing Y, Gen HY. Genes, pathways and networks responding to drought stress in oil palm roots. Scientific Reports. 2020;**10**(1):1-13. DOI: doi.org/10.1038/s41598-020-78297-z

[3] Statista. 2022. The Statistics Portal. Available from: www.statista.com/statistics/263937/vegetable-oils-global-consumption [Accessed: May 10, 2019]

[4] Matin Q, Kibrom TS, Hermanto S, Ingo G. Environmental, economic, and social consequences of the oil palm boom. Annual Review of Resource Economics. 2020;**12**:321-344. DOI: 10.1146/annurev-resource-110119-024922

[5] Dickson OD, Meilina OA. Sustainability of the oil palm industry. In: *Elaeis guineensis*. London: IntechOpen; 2021. DOI: 10.5772/intechopen.100156

[6] Furumo PR, Aide TM. Characterizing commercial oil palm expansion in Latin America: Land use change and trade. Environmental Research Letters. 2017;**12**(2):024008. DOI: 10.1088/1748-9326/aa5892

[7] Sérgio JC, Eduardo ALE, Taynar COT, Joedna S. Respostas fisiológicas de dendê submetidas ao estresse hídrico em condições do Cerrado. Revista Brasileira de Agropecuária Sustentável. 2018;**8**(4):92-99. https://doi.org/10.21206/rbas.v8i4.3055

[8] Vinay K, Tushar K, Varsha S, Shabir HW. Plant small RNAs: The essential epigenetic regulators of gene expression for salt-stress responses and tolerance. Plant Cell Reports. 2018;**37**(1):61-75. DOI: 10.1007/s00299-017-2210-4

[9] Petronia C, Maria GA, Giovanni P, Amodio F, Pasqualina W. Salinity Stress and Salt Tolerance. London, UK: Intech; 2012. p. 13. DOI: 10.1039/C7RA00172J%

[10] Khaled MH, Jonathan MF, David N, Alain L, Khaled M, Khaled MAA. Prospects for the study and improvement of abiotic stress tolerance in date palms in the post-genomics era. Frontiers in Plant Science. 2020;**11**(March):1-13. DOI: 10.3389/fpls.2020.00293

[11] Xu J et al. Exploring MiRNAs for developing climate-resilient crops: A perspective review. Science of the Total Environment. 2019;**653**:91-104. DOI: 10.1016/j.scitotenv.2018.10.340

[12] Banerjee S, Sirohi A, Ansari AA, Gill SS. Role of small RNAs in abiotic stress responses in plants. Plant Gene. 2017;**11**:180-189. DOI: 10.1016/j.plgene.2017.04.005

[13] Woodrow P et al. Ttd1a promoter is involved in DNA-protein binding by salt and light stresses. Molecular Biology Reports. 2011;**38**(6):3787-3794

[14] Djami-Tchatchou AT, Sanan-Mishra N, Ntushelo K, Dubery IA. Functional roles of microRNAs in agronomically important plants-potential as targets for crop improvement and protection. Frontiers in Plant Science. 2017;**8**(March)

[15] Salgado FF et al. Expression analysis of MiRNAs and their putative target genes confirm a preponderant role of transcription factors in the early response of oil palm plants to salinity stress. BMC Plant Biology. 2021;**21**(1):1-17. DOI: 10.1186/s12870-021-03296-9

[16] Sun G. MicroRNAs and their diverse functions in plants. Plant Molecular Biology. 2012;**80**(1):17-36

[17] Sunkar R. MicroRNAs with macro-effects on plant stress responses. Seminars in Cell and Developmental Biology. 2010;**21**(8):805-811. DOI: 10.1016/j.semcdb.2010.04.001

[18] Ding D et al. Differential expression of MiRNAs in response to salt stress in maize roots. Annals of Botany. 2009;**103**(1):29-38

[19] Sunkar R, Chinnusamy V, Zhu J, Zhu JK. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. Trends in Plant Science. 2007;**12**(7):301-309

[20] Denver JB, Ullah H. MiR393s regulate salt stress response pathway in arabidopsis thaliana through scaffold protein RACK1A mediated ABA signaling pathways. Plant Signaling and Behavior. 2019;**14**(6):1-7. DOI: 10.1080/15592324.2019.1600394

[21] Liu HH et al. Microarray-based analysis of stress-regulated MicroRNAs in arabidopsis thaliana. RNA. 2008;**14**(5):836-843

[22] Yu Y et al. Overexpression of soybean MiR169c confers increased drought stress sensitivity in transgenic arabidopsis thaliana. Plant Science. 2019;**285**(February):68-78. DOI: 10.1016/j.plantsci.2019.05.003

[23] Basso MF et al. MicroRNAs and new biotechnological tools for its modulation and improving stress tolerance in plants. Plant Biotechnology Journal. 2019;**17**(8):1482-1500

[24] Wang J et al. Non-coding RNAs and their roles in stress response in plants. Genomics, Proteomics and Bioinformatics. 2017a;**15**(5):301-312. DOI: 10.1016/j.gpb.2017.01.007

[25] Ding J, Zhou S, Guan J. Finding MicroRNA targets in plants: Current status and perspectives. Genomics, Proteomics and Bioinformatics. 2012;**10**(5):264-275. DOI: 10.1016/j.gpb.2012.09.003

[26] Sun X, Lin L, Sui N. Regulation mechanism of MicroRNA in plant response to abiotic stress and breeding. Molecular Biology Reports. 2019;**46**(1):1447-1457. DOI: 10.1007/s11033-018-4511-2

[27] Borges F, Martienssen RA. The expanding world of small RNAs in plants filipe. Nature reviews. Molecular Cell Biology. 2015;**16**(12):727-741

[28] Li J et al. Methylation protects MiRNAs and SiRNAs from a 3′-End Uridylation activity in arabidopsis. Current Biology. 2005;**15**(16):1501-1507

[29] Wang et al. Non-coding RNAs and their roles in stress response in plants Wang J et Al/MiRNA and LncRNA in plant stress response. Genomics, Proteomics and Bioinformatics. 2017b;**15**(5):301-312. DOI: 10.1016/j.gpb.2017.01.007

[30] Nasaruddin M, Nadirah, et al. Computational prediction of MicroRNAs from oil palm (Elaeis Guineensis Jacq.) expressed sequence tags. Asia-Pacific Journal of Molecular Biology and Biotechnology. 2007;**15**(3):107-113

[31] Arribas-Hernández L et al. The slicer activity of ARGONAUTE1 is

required specifically for the phasing, not production, of trans-acting short interfering RNAs in arabidopsis. Plant Cell. 2016;**28**(7):1563-1580

[32] Meng Y, Shao C, Ma X, Wang H. Introns targeted by plant MicroRNAs: A possible novel mechanism of gene regulation. Rice. 2013;**6**(1):1-10

[33] Li S et al. MicroRNAs inhibit the translation of target MRNAs on the endoplasmic reticulum in arabidopsis. Cell. 2013;**153**(3):562-574

[34] Shan Q et al. Multiplex and homologous recombination–mediated genome editing in arabidopsis and nicotiana benthamiana using guide RNA and Cas9. Nature Biotechnology. 2013;**31**(8):686-688

[35] Gull A, Lone AA, Wani NUI. Biotic and Abiotic Stresses in Plants. In: de Oliveira A, editor. Abiotic and Biotic Stress in Plants. London: IntechOpen; 2019. 6p. https://doi.org/10.5772/intechopen.85832

[36] Zhang H, Zhu J, Gong Z, et al. Abiotic stress responses in plants. Nature Reviews. Genetics. 2022;**23**:104-119. DOI: 10.1038/s41576-021-00413-0

[37] Shinozaki K, Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance. Journal of Experimental Botany. 2007;**58**(2): 221-227

[38] Ayubov MS et al. Role of MicroRNAs and small RNAs in regulation of developmental processes and agronomic traits in gossypium species. Genomics. 2019;**111**(5):1018-1025. DOI: 10.1016/j.ygeno.2018.07.012

[39] Kumar R. Role of MicroRNAs in biotic and abiotic stress responses in crop plants. Applied Biochemistry and Biotechnology. 2014;**174**(1):93-115

[40] Ferdous J, Hussain SS, Shi BJ. Role of MicroRNAs in plant drought tolerance. Plant Biotechnology Journal. 2015;**13**(3):293-305

[41] Zhou L et al. Genome-wide identification and analysis of drought-responsive MicroRNAs in oryza sativa. Journal of Experimental Botany. 2010;**61**(15):4157-4168

[42] Kulcheski FR, de Oliveira LF, Molina LG, Almerão MP, Rodrigues FA, Marcolino J, et. al. Identification of novel soybean microRNAs involved in abiotic and biotic stresses. BMC genomics. 2011;**12**(307):1-17. https://doi.org/10.1186/1471-2164-12-307

[43] Deng P et al. Global identification of MicroRNAs and their targets in barley under salinity stress. PLoS One. 2015;**10**(9):1-20

[44] Sunkar R, Zhu J-K. Novel and stress-regulated MicroRNAs and other small RNAs from arabidopsis. 2004;**18**(August):957

[45] Jones-Rhoades MW, Bartel DP. Computational identification of plant MicroRNAs and their targets, including a stress-induced MiRNA. Molecular Cell. 2004;**14**(6):787-799

[46] Mahdavi-Darvari F, Noor NM, Ismanizan I. Epigenetic regulation and gene markers as signals of early somatic embryogenesis. Plant Cell, Tissue and Organ Culture. 2014;**120**(2):407-422. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-84921809786&doi=10.1007%2Fs11240-014-0615-0&partnerID=40&md5=9558f1d988ad53c4638fdf1142f417d2

[47] Vaucheret H, Vazquez F, Crété P, Bartel DP. The action of ARGONAUTE1 in the MiRNA pathway and its regulation by the MiRNA pathway are crucial

for plant development. Genes and Development. 2004;**18**(10):1187-1197

[48] Jain M et al. F-Box proteins in rice. Genome-wide analysis, classification, temporal and spatial gene expression during panicle and seed development, and regulation by light and abiotic stress. Plant Physiology. 2007;**143**(4): 1467-1483

[49] Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, et al. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science. New York, N.Y. 2006;**312**(5772):436-439. https://doi.org/10.1126/science.1126088

[50] Apel K, Hirt H. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology. 2004;**55**:373-399

[51] Kimura M et al. Analysis of hydrogen peroxide–independent expression of the high-light–inducible ELIP2 gene with the aid of the ELIP2 promoter–luciferase fusion. Photochemistry and Photobiology. 2003;**77**(6):668

[52] Ku YS et al. Small RNAs in plant responses to abiotic stresses: Regulatory roles and study methods. International Journal of Molecular Sciences. 2015;**16**(10):24532-24554

[53] Sun X et al. Preparation and luminescence properties of Sr2EuxLa1-XAlO5 phosphor. Journal of Luminescence. 2015;**157**:197-200

[54] Kord H, Fakheri B, Ghabooli M, et al. Salinity-associated microRNAs and their potential roles in mediating salt tolerance in rice colonized by the endophytic root fungus Piriformospora indica. Funct Integr Genomics. 2019;**19**:659-672. https://doi.org/10.1007/s10142-019-00671-6

[55] Xu J, Chen Q, Liu P, Jia W, Chen Z, Xu Z. Integration of mRNA and miRNA Analysis Reveals the Molecular Mechanism Underlying Salt and Alkali Stress Tolerance in Tobacco. International journal of molecular sciences. 2019;**20**(10):1-18, 2391. https://doi.org/10.3390/ijms20102391

[56] Mehrpooyan F, Othman RY, Harikrishna JA. Tissue and temporal expression of MiR172 paralogs and the AP2-like target in oil palm (Elaeis Guineensis Jacq.). Tree Genetics and Genomes. 2012;**8**(6):1331-1343

[57] Low ET, Rosli R, Jayanthi N, Mohd-Amin AH, Azizi N, Chan KL, et al. Analyses of hypomethylated oil palm gene space. PloS one. 2014;**9**(1):1-15, e86728. https://doi.org/10.1371/journal.pone.0086728

[58] da Silva A, Cunha, et al. Computational identification and comparative analysis of MiRNA precursors in three palm species. Planta. 2016;**243**(5):1265-1277

[59] Somyong S et al. ACC oxidase and MiRNA 159a, and their involvement in fresh fruit bunch yield (FFB) via sex ratio determination in oil palm. Molecular Genetics and Genomics. 2016;**291**(3):1243-1257

[60] HuiliH, GudimellaR, Ong-AbdullahM, Harikrishna JA. Expression of microRNAs during female inflorescence development in African oil palm (Elaeis guineensis Jacq.). Tree Genetics and Genomes. 2017;**35**(13):1-17. https://doi.org/10.1007/s11295-017-1120-5

[61] Gao LC et al. EgmiR5179 from the mesocarp of oil palm (Elaeis Guineensis Jacq.) regulates oil accumulation by targeting NAD transporter 1. Industrial Crops and Products. 2019;**137**(October 2018):126-136. DOI: 10.1016/j.indcrop.2019.05.013

[62] Zheng Y, Chen C, Liang Y, Sun R, Gao L, Liu T, et al. Genome-wide association analysis of the lipid and fatty acid metabolism regulatory network in the mesocarp of oil palm (*Elaeis guineensis* Jacq.) based on small noncoding RNA sequencing. Tree Physiology. 2019;**39**(3):356-371. DOI: doi.org/10.1093/treephys/tpy091

[63] Tregear JW, Richaud F, Collin M, Esbelin J, Parrinello H, Cochard B, et al. Micro-RNA-regulated *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL)* gene expression and cytokinin accumulation distinguish early-developing male and female inflorescences in oil palm (*Elaeis guineensis*). Plants (Basel, Switzerland). 2022;**11**(5):1-16, 685. https://doi.org/10.3390/plants11050685

[64] Salgado et al. The early response of oil palm (*Elaeis guineensis* Jacq.) plants to water deprivation: Expression analysis of miRNAs and their putative target genes, and similarities with the response to salinity stress. Unpublished

[65] Chuammitri P, Vannamahaxay S, Sornpet B, Pringproa K, Patchanee P. Detection and characterization of microRNA expression profiling and its target genes in response to canine parvovirus in Crandell Reese Feline Kidney cells. PeerJ. 2020;**8**:1-25, e8522. https://doi.org/10.7717/peerj.8522

[66] Khaksefidi, Reyhaneh Ebrahimi et al. 2015. "Differential expression of seven conserved MicroRNAs in response to abiotic stress and their regulatory network in helianthus annuus." Frontiers in Plant Science 6(September): 1-14.

[67] Zhang B, Wang Q. MicroRNA, a new target for engineering new crop cultivars. Bioengineered. 2016;**7**(1):7-10. DOI: 10.1080/21655979.2016.1141838

[68] Zhu QH, Wang MB. Molecular functions of long non-coding RNAs in plants. Genes. 2012;**3**(1):176-190

[69] Hajyzadeh M, Turktas M, Khawar KM, Unver T. MiR408 overexpression causes increased drought tolerance in chickpea. Gene. 2015;**555**(2):186-193. DOI: 10.1016/j.gene.2014.11.002

[70] Ma C, Burd S, Lers A. MiR408 is involved in abiotic stress responses in arabidopsis. Plant Journal. 2015;**84**(1):169-187

[71] Zhao J et al. Transgenic creeping bentgrass overexpressing osa-MiR393a exhibits altered plant development and improved multiple stress tolerance. Plant Biotechnology Journal. 2019;**17**(1):233-251

[72] Baek D et al. A role for arabidopsis MiR399f in salt, drought, and ABA signaling. Molecules and Cells. 2016;**39**(2):111-118

[73] Ferdous J et al. Drought-inducible expression of Hv-MiR827 enhances drought tolerance in transgenic barley. Functional and Integrative Genomics. 2017;**17**(2-3):279-292

[74] Aukerman M. Drought tolerant plants and related constructs and methods involving genes encooing MIR827. 2013;**1**(61)

[75] Aukerman M, Park W. Drought tolerant plants and related constructs and methods involving genes encoding MiR827. 2008;**1**(19). Available from: https://patents.google.com/patent/US20090165168A1/en

[76] Sunkar R, Zhu JK. Novel and stress-regulated MicroRNAs and other small RNAs from. Arabidopsis Plant Cell. 2004;**16**(August 2004):2019

[77] Man Z, Hong L. Role of microRNA319 in creeping bentgrass salinity and drought stress response. Plant Signaling and Behavior. 2014;**9**(4):e28700, DOI:10.4161/psb.28700

[78] Raj KJ, Swati M, Urmila B, Muhammad HR, Nat NVK. Genome wide identification and functional prediction of long non-coding RNAs responsive to sclerotinia sclerotiorum infection in brassica napus. PLoS One. 2016;**11**(7):1-19. DOI: 10.1371/journal.pone.0158784

[79] Matzke MA, Rebecca AM. RNA-directed DNA methylation: An epigenetic pathway of increasing complexity. Nature Reviews Genetics. 2014;**15**(6):394-408. DOI: 10.1038/nrg3683

[80] Wierzbicki AT. The role of long non-coding RNA in transcriptional gene silencing. Current Opinion in Plant Biology. 2012;**15**(5):517-522. DOI: 10.1016/j.pbi.2012.08.008

TERCEIRA PARTE – ARTIGOS

ARTIGO I

Expression analysis of miRNAs and their putative target genes confirm a preponderant role of transcription factors in the early response of oil palm plants to salinity stress

Autores: Fernanda Ferreira Salgado, Letícia Rios Vieira, Vivianny Nayse Belo Silva, André Pereira Leão, Priscila Grynberg, Marcos Mota do Carmo Costa, Roberto Coiti Togawa, Carlos Antônio Ferreira de Sousa e Manoel Teixeira Souza Júnior

Publicado no periódico BMC Plant Biology
https://bmcplantbiol.biomedcentral.com/articles/10.1186/s12870-021-03296-9

Salgado *et al. BMC Plant Biol* (2021) 21:518
https://doi.org/10.1186/s12870-021-03296-9

BMC Plant Biology

RESEARCH

Open Access

Expression analysis of miRNAs and their putative target genes confirm a preponderant role of transcription factors in the early response of oil palm plants to salinity stress

Fernanda Ferreira Salgado[1], Letícia Rios Vieira[1], Vivianny Nayse Belo Silva[1], André Pereira Leão[2], Priscila Grynberg[3], Marcos Mota do Carmo Costa[3], Roberto Coiti Togawa[3], Carlos Antônio Ferreira de Sousa[4] and Manoel Teixeira Souza Júnior[1,2*]

Abstract

Background: Several mechanisms regulating gene expression contribute to restore and reestablish cellular homeostasis so that plants can adapt and survive in adverse situations. MicroRNAs (miRNAs) play roles important in the transcriptional and post-transcriptional regulation of gene expression, emerging as a regulatory molecule key in the responses to plant stress, such as cold, heat, drought, and salt. This work is a comprehensive and large-scale miRNA analysis performed to characterize the miRNA population present in oil palm (*Elaeis guineensis Jacq.*) exposed to a high level of salt stress, to identify miRNA-putative target genes in the oil palm genome, and to perform an in silico comparison of the expression profile of the miRNAs and their putative target genes.

Results: A group of 79 miRNAs was found in oil palm, been 52 known miRNAs and 27 new ones. The known miRNAs found belonged to 28 families. Those miRNAs led to 229 distinct miRNA-putative target genes identified in the genome of oil palm. miRNAs and putative target genes differentially expressed under salinity stress were then selected for functional annotation analysis. The regulation of transcription, DNA-templated, and the oxidation-reduction process were the biological processes with the highest number of hits to the putative target genes, while protein binding and DNA binding were the molecular functions with the highest number of hits. Finally, the nucleus was the cellular component with the highest number of hits. The functional annotation of the putative target genes differentially expressed under salinity stress showed several ones coding for transcription factors which have already proven able to result in tolerance to salinity stress by overexpression or knockout in other plant species.

Conclusions: Our findings provide new insights into the early response of young oil palm plants to salinity stress and confirm an expected preponderant role of transcription factors - such as NF-YA3, HOX32, and GRF1 - in this response. Besides, it points out potential salt-responsive miRNAs and miRNA-putative target genes that one can utilize to develop oil palm plants tolerant to salinity stress.

Keywords: Abiotic stress, *Elaeis guineensis*, Transcription factor, Transcriptome, Non-coding RNA

Background

MicroRNAs (miRNAs) are small endogenous non-coding RNAs, usually 21 nucleotides long, known to impact almost all biological processes [1]. miRNAs play roles

---

*Correspondence: manoel.souza@embrapa.br
[2] Embrapa Agroenergia, CEP 70770-901, Brasília, DF, Brazil
Full list of author information is available at the end of the article

important in the transcriptional and post-transcriptional regulation of gene expression, emerging as a regulatory molecule key in the responses to plant stress, and the main components of miRNA show high conservation between species [2, 3].

The synthesis of plant miRNAs happens in the nucleus, where the encoded plant miRNA genes are processed by polymerase II to form miRNAs [4]. Long sequences of miRNAs are folded into a hairpin structure, known as primary miRNAs or pri-miRNAs, and are then cleaved by DCL1 to form short and incomplete double-stranded structures called pre-miRNAs [5]. Pre-miRNAs are cleaved by DCL1 or DCL4 to form a double-stranded miRNA known as miRNA dimer. Subsequently, the methyltransferase HEN1 carries out methylation of the 3′ end, and the miRNA is then transported to the cytoplasm by the plant homologous protein exportin-5 (HASTY, HST). Then the miRNA single-chain and the AGO protein form the RISC complex, binding to the complementary target mRNA to cleave or inhibit translation, obtaining negative regulation of the target gene [6, 7].

Several abiotic stressors, such as cold, heat, drought, and salt, affect the plant life cycle interfering with growth and productivity [8]. Several mechanisms regulating gene expression contribute to restore and reestablish cellular homeostasis so that plants can adapt and survive in adverse situations. miRNAs play a role important in regulating gene expression in response to stress conditions [1, 9].

Abiotic stresses upregulate some genes and downregulate others, depending on the role played by the gene. miRNAs responsive to water stress are present in *Oryza sativa* [10, 11], *Arabidopsis thaliana* [12], and *Medicago truncatula* [13, 14]. Sunkar and Zhu [15] showed that miR319c is positively regulated in Arabidopsis when subjected to cold stress but did not change when subjected to dehydration, salt, or ABA. Several miRNAs, such as miR156, miR159, miR167, miR171, miR319, and miR396, showed differential expression levels during the response to salt stress in *Arabidopsis* sp. [12] and *Zea mays* [16]. Using state-of-the-art sequencing technology (NGS) to identify miRNAs responsive to salt, Dong et al. [17] identified 104 differentially expressed miRNAs in soybean nodules under salt stress.

Some studies have reported miRNAs in oil palm. Nasaruddin et al. [18] found five new potential miRNA encoding sequences in a collection of 7284 oil palm EST sequences by a combined homology and structural analysis approach, having roles in regulating the auxin response, floral development, and basal transcription. Low et al. [19] applied a homology approach to identify 14 miRNAs in contigs assembled from sequences generated from the hypomethylated or gene-rich regions of *Elaeis guineensis* and *E. oleifera* genomes. Silva et al. [20] identified 57 mature miRNA in *E. guineensis* and 52 in *E oleifera*, respectively, and miRNA-target prediction revealed that most of these miRNA-putative target genes are transcription factors involved in the plant development process, particularly the regulation of root development. Ho et al. [21] investigated microRNA expression in oil palm female inflorescences at two stages of floral development corresponding to the emergence of floral meristems and the formation of floral organs, identifying 15 oil palm-specific miRNA candidates. Zheng et al. [22] identified 52 miRNAs in a study aiming to gain insights into the regulatory mechanisms of lipid and fatty acid (FA) metabolism in oil palm.

Oil palm (*Elaeis guineensis* Jacq.) is a source of vegetable oil that has great importance in many economic sectors. In Brazil, over 95% of the oil palm plantations are in the Amazon region. Due to environmental restrictions imposed on the use of the Amazon rainforest, and the logistical difficulties to flow the production to the main industrial centers in the country, there is a crescent demand of growers for the cultivation of oil palm in other geographic regions in the country. One must use irrigation in oil palm plantations outside the Amazon region in Brazil, mainly due to long periods of drought observed in these alternative regions with potential for oil palm cultivation [23, 24]. Between 25 and 30% of the irrigated land area in the World is affected by salt and is essentially commercially unproductive [25]. Because of that, Embrapa started working on a multi-omics approach to characterize morphophysiological and molecular responses of oil palm (*E. guineensis*) to drought and salinity stresses [26].

The current study is a follow-up to the characterization of the morphophysiological responses of oil palm plants to drought and salinity stress [24, 26]. We carried out a comprehensive, large-scale miRNA analysis to characterize the miRNA population present in oil palm exposed to a high level of salt stress, to identify miRNA-putative target genes in the oil palm genome, and to perform an in silico comparison of the expression profile of the miRNAs and their putative target genes.

Results

As shown previously in Vieira et al. [24], the electrical conductivity (EC) of the saturation extract increased, and the water potential (Ψw) decreased in a NaCl dose-dependent manner. At the 12th day after imposing the stress (DAT), the EC values ranged from ±2 dS m^{-1} (control plants) to ±45 dS m^{-1} (stressed plants in substrate treated with 2.0g of NaCl per 100g of the substrate), while the Ψw values varied from zero to -1.42 MPa, respectively (data not shown). There were no differences in the average evapotranspiration between the groups on day zero; however, when subjected to stress, the plants started to show differences in the evapotranspiration rates, remaining until the end of the experiment (data not shown).

At 12 DAT, it is visible a reduction in the rates of CO_2 assimilation (*A*), stomatal conductance to water vapor (*gs*), and transpiration (*E*), which correlated with the amount of NaCl used (Fig. 1A, B, and D). On the other hand, the increase in intercellular CO_2 concentration (*Ci*) also correlated with the amount of salt used (Fig. 1C). Stressed plants at the highest NaCl dose were already showing senescence of the leaves at 12 DAT (Fig. 2). Based on the morphophysiological responses of young oil palm plants to salinity stress (Figs. 1 and 2, and Vieira et al. [24]), both the control and the 2.0 g of NaCl per 100 g of the substrate treatments - which will be from now on referred as control and stressed treatments – were selected to the characterization of the microRNA and mRNA profiles.

Identification of known and novel miRNAs, and differential expression analysis of miRNAs

The small RNA raw sequence data (9 fastq files) used in this study have been uploaded in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information under *Elaeis guineensis* microRNA_Drought and Salinity Stresses - BioProject

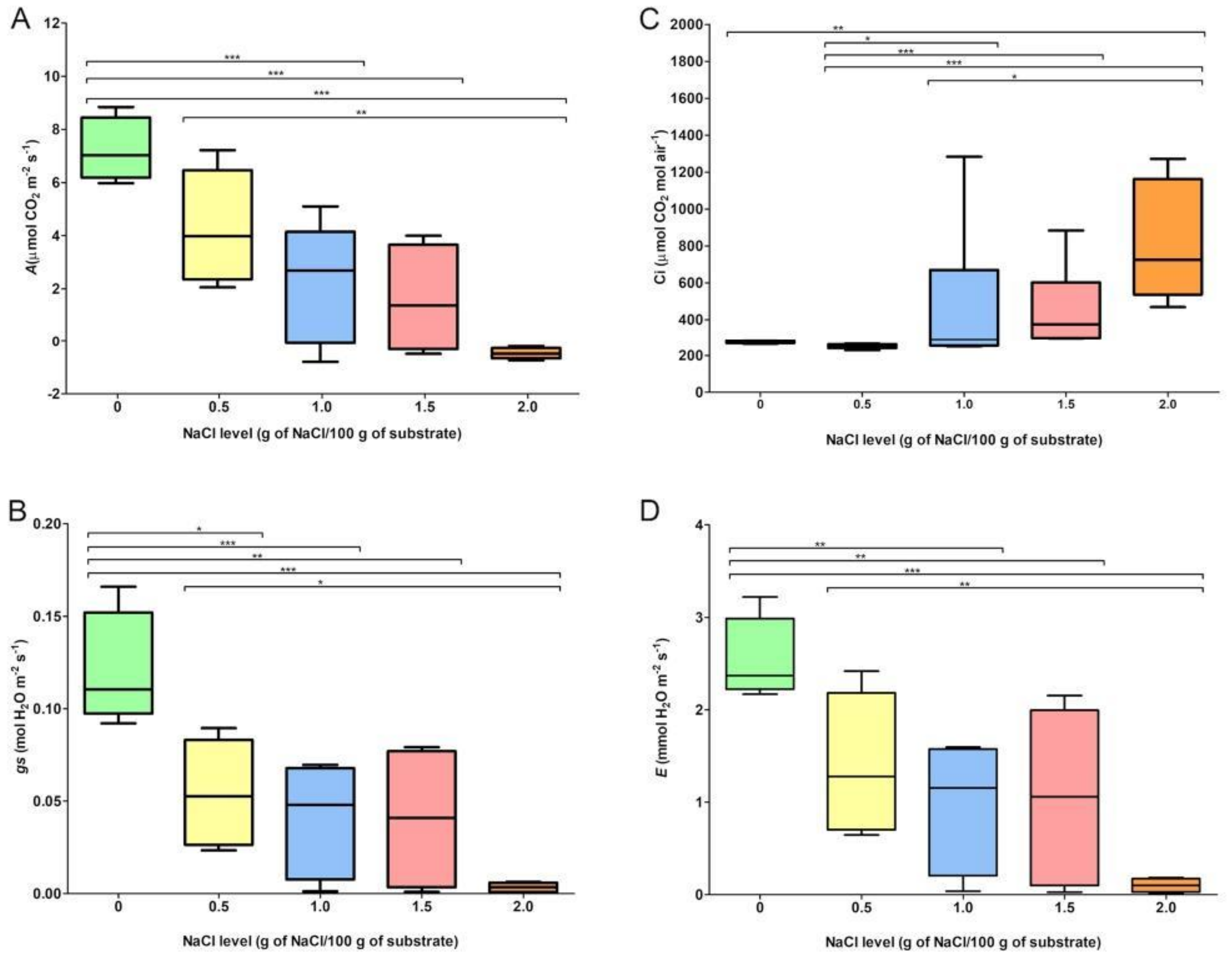

Fig. 1 Box plots of the changes in gas exchange parameters in oil palm plants grown under increasing concentrations of NaCl in the cultivation substrate. **A** Net CO_2 assimilation rate (*A*); (**B**) stomatal conductance rate to water vapor (*gs*); (**C**) intercellular CO_2 concentration (*Ci*); and (**D**) transpiration rate (*E*). Medians and interquartile ranges are shown. The values represent the average of four replicates. The significance of differences was calculated using the Kruskal-Wallis test with *post-hoc* Dunn ($p < 0.05$). nsP > 0.05, non-significant comparisons were not shown in the graph; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$

Fig. 2 Young oil palm (*Elaeis guineensis*) plants at the bifid-sapling growth stage, after 12 days under salt stress. The value below each pot represents the amount of NaCl added to each 100 g of substrate, oven-dried at 105 °C for 24 h

number of PRJNA646488, BioSample SAMN12799239. All adapter-free small RNA sequences (from all nine fastq files) were concatenated into a single file and submitted for miRNA prediction using mireap version 0.2 and Shortstack version 3.4, generating 96 positive hits for potential miRNAs (data not shown). Concomitantly, all adapter-free small RNA sequences from control and salt-stressed samples (three replicates) were submitted to assemble and then mapped against the oil palm reference genome [27], generating 3384 positive hits (data not shown).

A search in the database with the 3384 hits to the oil palm genome, using the database with the potential miRNAs, led to a total of 79 miRNAs, being 52 known miRNAs and 27 new ones (Fig. 3). The length of the 27 new ones ranges from 21 (24 miRNAs) to 22 (3 miRNAs) nucleotides (Supplementary Table 1). The genes of the 79 miRNAs identified in this study ranged from 68 to 285 bp in length and spread throughout all 16 chromosomes of the *E. guineensis* genome (Supplementary Table 2). Several miRNAs are present in more than one place in the genome, in different chromosomes, or at different positions in the same chromosome. Chromosomes 01, 04, and 08 had the highest miRNAs amount, 12, 11, and 11, respectively. Twenty-eight miRNAs got mapped to 28 unplaced scaffolds. The highest number of miRNAs in one unplaced scaffold was three, in scaffold NW_011551039.1 (Supplementary Table 2).

The new putative miRNA genes are between 68 and 267 bp in length (Supplementary Table 2). Regarding the location of these genes in the genome of *E. guineensis*, 20 of them were in intragenic and nine in intergenic regions (Table 1). Of the genes present inside genes, only the egu-miR16sds, egu-miR17sds, egu-miR22sds, and egu-miR26sds genes are in non-characterized ones (Table 1).

The 52 known miRNAs found in this study belonged to 28 families. Among these families, the largest were miR156 and miR169 (5 members each), followed by miR166 and miR396 (4 members), miR159 and miR171 (3 members), miR168, miR319, miR393, miR395, miR399 and miR529 (2 members), and miR160, miR162, miR167, miR172, miR390, miR391, miR397, miR4 82, miR528, miR530, miR535, miR536, miR827, miR828, and miR2637 (one member).

Among the 79 known and novel miRNAs, 72 showed a significant (probability $\geq$0.95) different level of expression under saline stress; however, all were downregulated (Supplementary Table 2). These differentially expressed (DE) miRNAs had their expression level reduced in the range from 39.75 to 99.82%. In general, those DE miRNAs with their genes located in different regions in the genome did not present very distinct Log_2(FC) values; the only exception was ppe-miR397 (Supplementary Table 2).

Prediction and differential expression analysis of miRNA-putative target genes

The psRNA-Target online program, version 2, led to 425 positive hits as miRNA-putative target genes. When analyzing the mode of inhibition of these positive hits, the vast majority, 398, presented an mRNA cleavage mode and the remaining 27 a translation inhibition (Supplementary Table 3). It usually occurs because of some

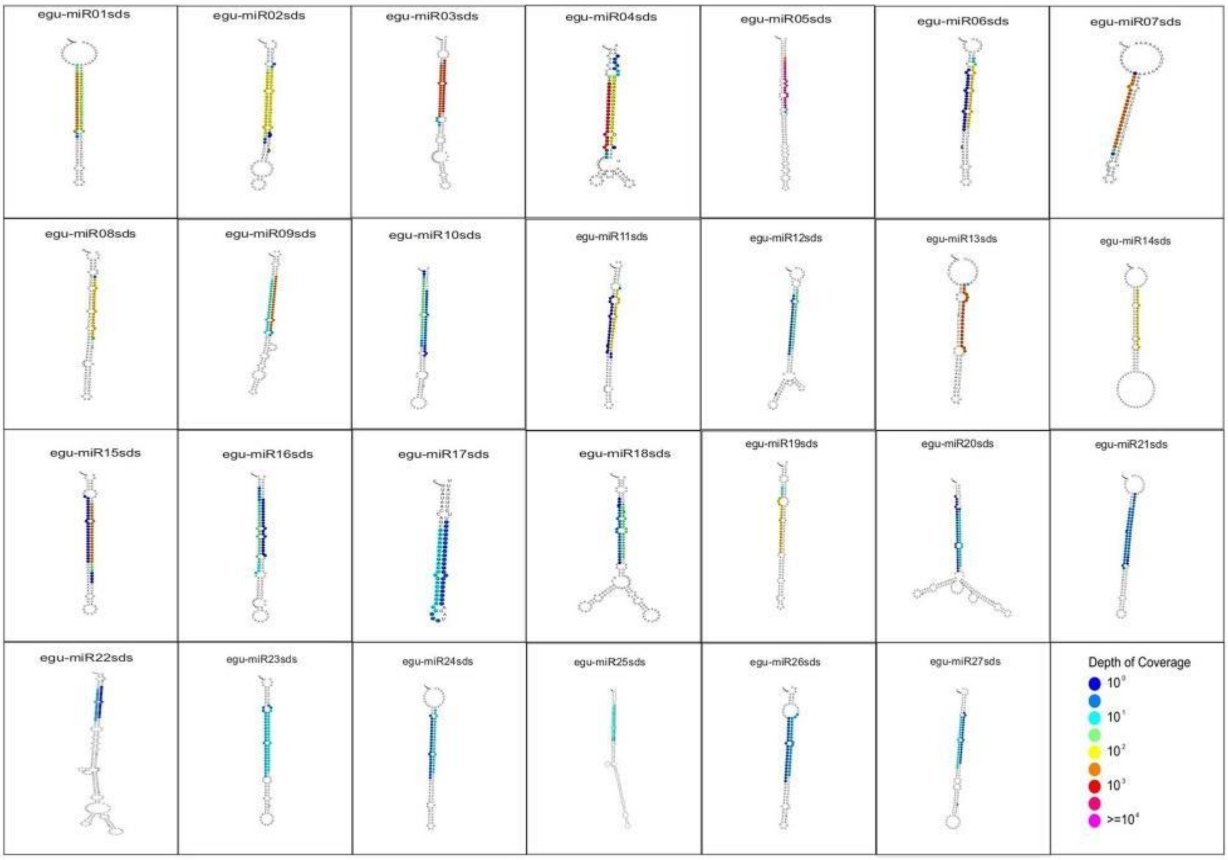

Fig. 3 Structure of the 27 new miRNAs identified in oil palm (*Elaeis guineensis*) plants exposed to a high level of salt stress

incompatibility around the center of the complementary region, as the central area is essential for cleavage [27].

Out of the 425 positive hits, there were 229 distinct putative target genes; based on the LOC Ids from the oil palm reference genome. Among these putative target genes, 150 were target to just one miRNA, and 79 were target to more than one miRNA - ranging from two to 36 miRNA per target gene (Supplementary Table 3).

The RNA-seq fastq files used in this study - from control and stressed plant samples, three replicates/treatment - are part of a group of 18 fastq files that have been uploaded in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information under *Elaeis guineensis* Transcriptome_Drought and Salinity Stresses - BioProject PRJNA573093, BioSample SAMN12799239. The transcriptome analysis showed that over 90% of the raw read pairs survived the preprocessing stage requiring a minimum average quality of reads $\geq$30 and the minimum length of reads $\geq$75 nucleotides. Over 95% of the high-quality read pairs mapped to the reference genome available at NCBI [28]. The reference genome has 29,567 genomic features of type 'gene' retrieved from 2781 ref. sequences in GCF_000442705.1_EG5_genomic.fna file; however, 4213 of these features had no aligned reads detected in any of the samples (Table 2).

When comparing control against stressed plants, the pairwise differential expression analysis revealed that out of the 29,567 features from the *E. guineensis* genome [28], 5366 were DE at False Discovery Rate (FDR) < 0.05 (data not shown); being 2380 upregulated ($\mathrm{Log}_2(\mathrm{FC}) > 0$) and 2986 downregulated ($\mathrm{Log}_2(\mathrm{FC}) < 0$). By applying the same criteria for the differential expression analysis of the 229 distinct miRNA-putative target genes previously prospected (Supplementary Table 3), a group of 24 upregulated and 27 downregulated genes were identified (data not shown). These 51 DE putative target genes were integratively and functionally annotated (Table 3, Supplementary Table 4).

Table 1 New miRNAs identified in the oil palm (*Elaeis guineensis*) genome

miRNA Name	Shortstack cord	Location	Gene ID[a]	Gene description
egu-miR01sds	NC_025994.1:56908176–56,908,276	intragenic	LOC105039763	26S proteasome non-ATPase regulatory subunit 8 homolog A
egu-miR01sds	NW_011550933.1:850430–850,534	intragenic	LOC105061524	ubiquitin carboxyl-terminal hydrolase 3
egu-miR02sds	NC_025996.1:22888879–22,888,988	intragenic	LOC105042830	E3 ubiquitin-protein ligase HAKAI homolog
egu-miR03sds	NC_025997.1:33290362–33,290,493	intragenic	LOC105045464	hydroxymethylglutaryl-CoA lyase, mitochondrial
egu-miR04sds	NC_025998.1:32639960–32,640,067	intergenic	NA	NA
egu-miR05sds	NC_026000.1:8768033–8,768,169	intergenic	NA	NA
egu-miR06sds	NC_026001.1:23573097–23,573,199	intragenic	LOC105051421	plant UBX domain-containing protein 11
egu-miR07sds	NW_011552138.1:10926–10,993	intragenic	LOC105035654	probable peptide/nitrate transporter At3g43790
egu-miR07sds	NC_026007.1:3222601–3,222,690	intergenic	NA	NA
egu-miR08sds	NW_011551761.1:8894–9002	intragenic	LOC105035396	probable DNA helicase MCM8
egu-miR09sds	NW_011553407.1:22959–23,079	intragenic	LOC105036110	vesicle-associated membrane protein 721
egu-miR10sds	NW_011552437.1:17925–18,039	intergenic	NA	NA
egu-miR11sds	NC_025995.1:12779980–12,780,080	intragenic	LOC105040786	serine/threonine-protein phosphatase PP1
egu-miR12sds	NC_026007.1:23368340–23,368,461	intergenic	NA	NA
egu-miR13sds	NC_025996.1:46674015–46,674,106	intergenic	NA	NA
egu-miR14sds	NC_026002.1:30893315–30,893,427	intragenic	LOC105053431	E3 ubiquitin protein ligase DRIP2
egu-miR14sds	NC_026001.1:19506680–19,506,793	intergenic	NA	NA
egu-miR14sds	NC_025997.1:18353407–18,353,516	intergenic	NA	NA
egu-miR14sds	NW_011550939.1:17612–17,724	intragenic	LOC105031979	probable GDP-L-fucose synthase 1
egu-miR14sds	NW_011551049.1:134162–134,274	intragenic	LOC105033684	mevalonate kinase
egu-miR15sds	NC_026003.1:10876077–10,876,178	intragenic	LOC105053747	26S proteasome non-ATPase regulatory subunit 2 homolog A
egu-miR16sds	NC_025993.1:8597526–8,597,635	intragenic	LOC105039415	uncharacterized LOC105039415 – lncRNA
egu-miR17sds	NW_011551539.1:60051–60,120	intragenic	LOC105035181	uncharacterized LOC105035181 – lncRNA
egu-miR18sds	NC_025997.1:42070048–42,070,195	intergenic	NA	NA
egu-miR19sds	NC_026006.1:8032758–8,032,862	intragenic	LOC105057555	UPF0496 protein At5g66675-like
egu-miR20sds	NW_011551034.1:756120–756,299	intragenic	LOC105033525	histone-lysine N-methyltransferase ATXR2
egu-miR21sds	NC_025994.1:23652674–23,652,776	intragenic	LOC105038330	transcription factor VIP1
egu-miR22sds	NC_025996.1:56111754–56,112,020	intragenic	LOC105044280	uncharacterized LOC105044280 - protein coding
egu-miR23sds	NC_026002.1:13066673–13,066,777	intergenic	NA	NA
egu-miR24sds	NC_025996.1:46873993–46,874,094	intragenic	LOC105043881	ATP-dependent DNA helicase SRS2-like protein At4g25120
egu-miR25sds	NW_011551090.1:713220–713,417	intragenic	LOC105034003	ubiquitin receptor RAD23d
egu-miR26sds	NC_025993.1:37595103–37,595,199	intragenic	LOC105054928	uncharacterized LOC105054928 – protein coding
egu-miR27sds	NC_025996.1:40372686–40,372,807	intragenic	LOC105043419	shaggy-related protein kinase epsilon

[a] BioProject PRJNA192219 and BioSample SAMN02981535, available at NCBI

Integrating the expression profiles from DE miRNA-putative target genes and their respective DE miRNAs

By integrating the expression profiles of the upregulated miRNA-putative target genes and their respective miRNAs, twenty-one of them showed just one DE miRNA, one showed two (LOC105047586), and one showed four (LOC105046708) linked to the miRNA-target gene, while one had a non-differentially expressed miRNA linked to it (LOC105059776) (Table 3). On the other hand, by integrating the expression profiles of the downregulated miRNA-putative target genes and their respective miRNAs, twenty-five of them showed just one, and two showed two (LOC105054987, LOC105059511) DE miRNA linked to the miRNA-target gene, while one had a non-differentially expressed miRNA linked to it (LOC105058639) (Table 3).

Among the 40 DE miRNAs with DE miRNA-putative target genes, 28 had only one target gene, nine had three, and three had three (Table 3). DE miRNAs vvi-miR171j, gu-miR18sds, and egu-miR03sds had three distinct DE putative target genes presenting different profiles when submitted to salinity stress. Vvi-miR171j downregulated to 15% of its expression level in the control plants, while its putative target genes downregulated to 38 (LOC105060969), 41 (LOC105059511), and 50%

Table 2 Statistics of RNA-Seq data from six samples of oil palm plants submitted to two treatments (0 and 2 g of NaCl per 100 g of the substrate); three replicates per treatment. Mapping to reference genome EG5 (BioProject PRJNA192219 and BioSample SAMN02981535) available at NCBI

Sample	Control_R1	Control_R2	Control_R3	Stressed_R1	Stressed_R2	Stressed_R3
Input Read Pairs	30,974,342	32,078,783	21,419,898	21,159,877	22,423,080	23,655,116
Both Surviving Reads	28,199,684 / 91.04%	28,906,033 / 90.11%	19,458,233 / 90.84%	19,244,188 / 90.95%	20,264,942 / 90.38%	21,601,656 / 91.32%
Uniquely Mapped Reads	26,504,692 / 93.989%	27,293,855 / 94.423%	17,156,499 / 88.171%	17,076,951 / 88.738%	17,363,794 / 85.684%	19,753,290 / 91.443%
Average Mapped Length	294.02	294.58	295.41	295.44	295.79	295.68
Reads Mapped to Multiple Loci	730,067 / 2.589%	654,325 / 2.264%	1,775,953 / 9.127%	1,621,318 / 8.425%	2,403,469 / 11.86%	1,270,546 / 5.882%
Reads Aligned to Feature of Type 'gene'[a]	24,370,328 / 86.72%	25,185,822 / 87.68%	15,143,708 / 72.45%	14,791,141 / 72.16%	13,979,922 / 62.26%	17,597,354 / 78.43%
Reads Not Aligned to Feature	1,701,727 / 6.06%	1,665,256 / 5.80%	1,758,058 / 8.41%	2,081,861 / 10.16%	3,185,020 / 14.18%	1,930,248 / 8.60%
Reads Aligned to More Than One Feature	1,599,065 / 5.69%	1,432,045 / 4.99%	3,746,405 / 17.92%	3,421,162 / 16.69%	5,091,435 / 22.67%	2,683,016 / 11.96%

[a] 29,567 genomic features of type 'gene', retrieved from 2781 ref. sequences in GCF_000442705.1_EG5_genomic.fna & 4213 features (14.25%) for with no aligned reads was detected in any of the samples

(LOC105054987). DE miRNA egu-miR18sds also downregulated to 15% of its expression level in the control plants, while one of its putative target genes upregulated to 189% (LOC105055689) and two downregulated to 39 (LOC105031985) and 67% (LOC105061136). On the other hand, while egu-miR03sds downregulated to 21% of its expression level in the control plants, one of the putative target genes downregulated to 74% (LOC105048718) and the other two upregulated to 137 (LOC105040914) and 143% (LOC105059001) of their initial expression level (Table 3).

LOC109505530 was the miRNA-target gene that experienced the highest expression level increase in the leaf of oil palm plants due to saline stress. This gene is one of two found as targetted by aof-miR536, but the only one to upregulate due to this stress. The saline stress led aorf-miR536 to downregulate to less than 10% the level found in the control plants, while LOC109505530 upregulated to almost eight times its initial expression level (Fig. 4A). LOC105046708 is target of three distinct miRNAs (ata-miR166d-3p, sly-miR166c-3p, and osa-miR166i-3p) in the genome of oil palm. This gene experienced an increase of approximately 80% due to saline stress, while the miRNAs targetting it downregulated to between 13 and 18% the level found in the control plants (Fig. 4A).

All oil palm new miRNAs identified in this study downregulate due to saline stress. However, their differentially expressed putative target genes belonged to two groups according to their response to saline stress, seven upregulated and 13 downregulated (Fig. 4B). In the case of egu-miR13sds and egu-miR18sds, the fate of their respective putative target genes – each one had 3 - were completely distinct, with some upregulating and some downregulating due to saline stress (Fig. 4B).

Functional annotation of the differencially expressed putative target genes

Among the 51 DE miRNA-putative target genes selected for functional annotation analysis, twenty had positive hits for biological process, 33 for molecular function, and eight for cellular component (Supplementary Table 4). The regulation of transcription, DNA-templated (GO:0006355), was the biological process with the highest number of hits, six, followed by the oxidation-reduction process (GO:0055114) with five. Protein binding (GO:0005515) and DNA binding (GO:0003677) were the molecular functions with the highest number of hits, six, five, respectively. The cellular component with the highest number of hits was the nucleus (GO:0005634), four, followed by the membrane (GO:0016021). Four genes had hits for domains from the GRAS family, a player important in gibberellin signaling [29].

The functional annotation analysis led to 18 known proteins, besides three lncRNA genes and three uncharacterized/unknown proteins. Among the 18 proteins there are six different kinds of transcription factors (LOC105054175, LOC105056468, LOC105046708, LOC105039459, LOC105048659, and LOC105043768). The remaining protein are: SPX-MFS proteins (LOC105043377,

Table 3 Profile of differentially expressed miRNA and their differentially expressed target genes from oil palm. False discovery rate (FDR), counts per milllion (CPM), and fold change (FC)

Target Gene					miRNA			
ID[a]	Expression profile	FDR	log$_2$FC	log$_2$CPM	Name	Expression profile	Probability	log$_2$FC
LOC105032827	UP	0.017	0.639	7.657	osa-miR159a.2	DOWN	0.96	−1.73
LOC105032890	UP	0.005	0.527	6.145	egu-miR12sds	DOWN	1.00	−5.49
LOC105034273	UP	0.007	0.710	5.881	ssp-miR827	DOWN	1.00	−2.93
LOC105035262	UP	0.020	0.500	6.502	atr-miR393	DOWN	0.98	−0.92
LOC105039459	UP	0.029	1.704	−0.968	bdi-miR529-5p	DOWN	1.00	−2.30
LOC105040914	UP	0.047	0.451	4.664	egu-miR03sds	DOWN	1.00	−2.23
LOC105043377	UP	0.000	1.670	5.767	ssp-miR827	DOWN	1.00	−2.93
LOC105043768	UP	0.008	0.530	7.110	atr-miR319e	DOWN	0.99	−1.93
LOC105043777	UP	0.001	0.591	6.996	vvi-miR828a	DOWN	1.00	−1.87
LOC105046708	UP	0.047	0.823	1.782	ata-miR166d-3p	DOWN	1.00	−2.51
LOC105046708	UP	0.047	0.823	1.782	atr-miR166b	DOWN	1.00	−2.76
LOC105046708	UP	0.047	0.823	1.782	osa-miR166i-3p	DOWN	1.00	−2.92
LOC105046708	UP	0.047	0.823	1.782	sly-miR166c-3p	DOWN	1.00	−1.76
LOC105047586	UP	0.000	1.863	0.439	bra-miR168a-5p	DOWN	1.00	−2.32
LOC105047586	UP	0.000	1.863	0.439	bra-miR168c-5p	DOWN	1.00	−1.83
LOC105048659	UP	0.013	0.705	3.253	egu-miR24sds	DOWN	0.95	−1.51
LOC105050858	UP	0.001	0.699	5.484	egu-miR11sds	DOWN	1.00	−4.26
LOC105051200	UP	0.001	1.303	2.815	atr-miR535	DOWN	1.00	−1.09
LOC105052568	UP	0.002	0.836	5.573	mtr-miR2673b	DOWN	1.00	−6.43
LOC105054175	UP	0.047	1.208	0.522	ata-miR396b-5p	DOWN	1.00	−2.53
LOC105054413	UP	0.000	1.031	5.014	osa-miR2118p	DOWN	1.00	−0.81
LOC105055689	UP	0.000	0.921	4.005	egu-miR18sds	DOWN	0.99	−2.72
LOC105056468	UP	0.000	1.366	4.716	ata-miR169d-5p	DOWN	0.98	−2.57
LOC105056609	UP	0.008	1.037	2.980	ata-miR167d-5p	DOWN	1.00	−2.55
LOC105059001	UP	0.016	0.514	4.698	egu-miR03sds	DOWN	1.00	−2.23
LOC105059776	UP	0.009	0.654	4.620	egu-miR27sds	NDE[b]	NDE	NDE
LOC105059810	UP	0.045	0.419	5.282	egu-miR09sds	DOWN	1.00	−3.22
LOC109505530	UP	0.000	2.950	−0.450	aof-miR536	DOWN	1.00	−3.55
LOC105031985	DOWN	0.000	−1.367	3.216	egu-miR18sds	DOWN	0.99	−2.72
LOC105032107	DOWN	0.006	−0.512	5.732	mes-miR393d	DOWN	1.00	−1.48
LOC105033129	DOWN	0.002	−0.622	4.658	egu-miR17sds	DOWN	0.99	−4.15
LOC105034164	DOWN	0.000	−1.053	5.714	ata-miR172b-3p	DOWN	0.98	−1.27
LOC105035561	DOWN	0.000	−1.006	3.118	ata-miR399a-3p	DOWN	1.00	−8.83
LOC105038401	DOWN	0.000	−0.902	3.311	egu-miR10sds	DOWN	1.00	−2.95
LOC105039220	DOWN	0.004	−0.617	4.451	aof-miR391	DOWN	1.00	−6.71
LOC105041147	DOWN	0.000	−0.834	3.888	egu-miR09sds	DOWN	1.00	−3.22
LOC105043694	DOWN	0.000	−1.320	4.891	aof-miR536	DOWN	1.00	−3.55
LOC105046087	DOWN	0.035	−0.596	7.237	egu-miR07sds	DOWN	1.00	−2.05
LOC105046096	DOWN	0.037	−0.493	4.096	egu-miR24sds	DOWN	0.95	−1.51
LOC105048141	DOWN	0.000	−1.356	2.377	egu-miR21sds	DOWN	0.98	−2.78
LOC105048606	DOWN	0.000	−2.036	2.656	ata-miR395c-3p	DOWN	1.00	−2.65
LOC105048718	DOWN	0.022	−0.434	5.325	egu-miR03sds	DOWN	1.00	−2.23
LOC105048722	DOWN	0.000	−0.948	3.893	egu-miR08sds	DOWN	1.00	−2.50
LOC105048783	DOWN	0.038	−1.133	2.064	ata-miR171a-3p	DOWN	0.99	−3.64
LOC105049211	DOWN	0.005	−0.757	3.512	ata-miR167d-5p	DOWN	1.00	−2.55
LOC105052116	DOWN	0.006	−0.515	5.757	egu-miR04sds	DOWN	1.00	−9.11
LOC105054869	DOWN	0.011	−0.624	3.628	egu-miR23sds	DOWN	0.96	−1.65

Table 3 (continued)

Target Gene					miRNA			
ID[a]	Expression profile	FDR	log_2FC	log_2CPM	Name	Expression profile	Probability	log_2FC
LOC105054987	DOWN	0.000	−0.990	5.245	mdm-miR171b	DOWN	1.00	−1.94
LOC105054987	DOWN	0.000	−0.990	5.245	vvi-miR171j	DOWN	0.99	−2.74
LOC105057798	DOWN	0.018	−1.098	2.516	osa-miR159a.2	DOWN	0.96	−1.73
LOC105058639	DOWN	0.001	−0.688	4.776	gma-miR482a-3p	NDE	NDE	NDE
LOC105059511	DOWN	0.000	−1.281	3.868	mdm-miR171b	DOWN	1.00	−1.94
LOC105059511	DOWN	0.000	−1.281	3.868	vvi-miR171j	DOWN	0.99	−2.74
LOC105060969	DOWN	0.000	−1.382	1.982	vvi-miR171j	DOWN	0.99	−2.74
LOC105061136	DOWN	0.007	−0.578	3.982	egu-miR18sds	DOWN	0.99	−2.72
LOC105061318	DOWN	0.002	−1.663	0.209	ata-miR396b-5p	DOWN	1.00	−2.53
LOC105061572	DOWN	0.038	−0.470	4.505	egu-miR07sds	DOWN	1.00	−2.05

[a] BioProject PRJNA192219 and BioSample SAMN02981535, available at NCBI; [b] *NDE* non differentially expressed

LOC105034273), JMJ16 protein (LOC105055689), TIR-1 like protein (LOC105035262), Delta4-sphingolipid-FADS-like protein (LOC105059001), PPR repeat-containing protein (LOC105032890), TPR repeat-containing protein (LOC105059776), Exportin 1-like protein (LOC105040914), 4-Oxalocrotonate Tautomerase protein (LOC105050858), Glycosyltransferase protein (LOC105056609), protein ORANGE (LOC105043777), and Actin protein (LOC105032827).

Discussion

Among the few studies reporting miRNA in oil palm [18–22], none of them has studied the role of this type of macromolecule in the response of this species to salinity stress. So, to the best of our knowledge, this is the first report on the expression profile of oil palm miRNAs and their putative target genes when subjected to saline stress.

The 52 orthologous miRNAs identified in this study belong to 28 different families previously reported in oil palm as expressed in floral meristems - miR156, miR160, miR166, miR167, miR168, miR172, miR396, miR528, and miR535, by Ho et al. [21]; in the development of the mesocarp – miR156, miR395, and miR528, by Fang et al. [30]; and in shoot apical meristem, immature and mature flowers - miR156, miR159, and miR160, by Nasaruddin et al. [18] using ESTs from a study by Ho et al. [31].

The expression profile of the 79 miRNAs found in this study revealed that 72 of them downregulate in the salt-stressed plants, and the remaining had no significant differential expression (Supplementary Table 2). The genotypic specificity of the miRNAs behavior is usually evident since different genotypes express the same miRNA but at different levels [8, 32]. According to Sunkar et al. [8], a miRNA that presents negative regulation during stress probably targets positive regulators of stress tolerance, generating an accumulation of gene products.

Dong et al. [17] reported that miR159, miR169, and miR319 showed highly significant negative regulation in soybean nodules when subjected to salt stress. Other studies showed that miR393, miR394, miR396, and miR156 were responsive to this stress in *Arabidopsis thaliana*, *Zea mays*, *Populus tremula*, *Populus trichocarpa*, *Oryza sativa*, and *Glycine max* [15, 16, 33–35]. According to Ding et al. [16], miR159, miR160, miR162, miR164, miR166, miR167, miR168, miR171, miR319, miR395, miR396, and miR399 were responsive to the salt stress in the roots of corn when subjected to a 200 mM NaCl concentration. In *P. trichocarpa*, miR530 downregulate during salt stress, the same behavior observed in *Arabidopsis* spp. to miR396 [36]. These results corroborate with the miRNAs found in oil palm when subjected to salt stress.

Some studies show that a single miRNA can selectively regulate its targets in a non-linear dose-dependent manner, so preferred mRNA targets may vary over the developmental stages, depending on the level of expression of regulatory miRNAs [21, 37]. In Ho et al. [21], validation using RNA degradome data supports that a single miRNA may regulate multiple targets, and an mRNA may be regulated by more than one miRNA, suggesting a complex and fine-tuned interaction network between miRNAs and their targets at the post-transcriptional level. Ho and colleagues' results corroborate with ours in oil palm subjected to salt stress, as miR171, egu-miR03sds and egu-miR18sds showed differential expression, and each one of them had three putative target genes also differentially expressed; the same is true to a putative target gene regulated by multiple miRNAs.

Many miRNAs play a role in the response of plants to abiotic stresses, such as salinity, through post-transcriptional regulation, which has been the focus

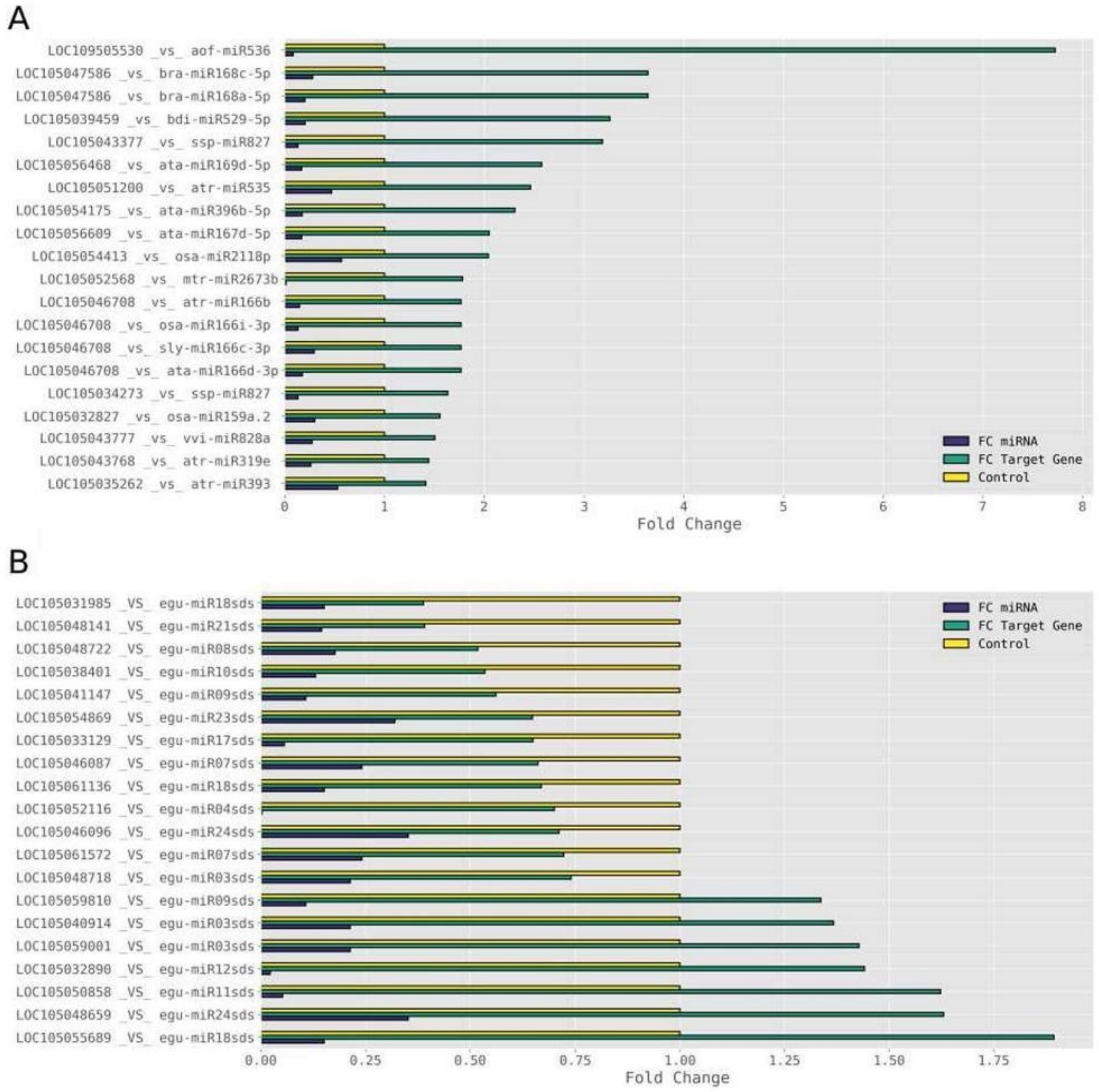

Fig. 4 Expression profiles in fold change (FC) of the differentially expressed (DE) miRNAs and their respective DE target gene(s), resulted from submission of young oil palm plants to salinity stress, and in comparisson with the control treatment (FC = 1). A – DE known miRNAs and their upregulated putative target genes; and B - DE new miRNAs and their DE putative target genes

of several studies [1, 8, 22, 38]. Different reports demonstrate that several miRNA-target genes are transcription factor mRNAs (TFs), indicating miRNA-dependent post-transitional regulation during the development and response to the environment [39, 40]. In plants, approximately 7–10% of the genes code for TFs at distinct moments, and dozen plant *TF* gene families precisely coordinate the spatial and temporal expression of downstream genes associated with abiotic stress [41, 42]. The present study showed that miRNAs miR166, miR169, miR319, miR396, miR529, and egu-miR24sds showed altered expression profiles in young oil palm plants subjected to salt stress; and, through functional analysis, that they regulate TFs transcript levels, which in turn affect the protein levels of the TFs.

The squamosa promoter-binding-like protein 17 homolog gene in oil palm (LOC105039459) is the putative target gene to miR529. While this miRNA underwent an 80% decrease in expression, its target gene showed a 70% increase in the apical leaf of young oil palm plants under very high salt stress. Squamosa promoter-binding (SBP) and SBP-Like (SPL) proteins are putative transcription factors having a plant-specific SBP domain consisting of 76 amino acids in length that regulates several biological processes, including salinity stress. Hou et al. [43] overexpressed the *VpSBP16* gene from grape (*Vitis vinifera*) in *A. thaliana* and observed an enhancement in the tolerance to salt and drought stress during seed germination, as well in seedlings and mature plants, by regulating SOS and ROS signaling cascades. On the other hand, the *OsSPL10* and *CaSBP12* genes negatively control salt tolerance in rice and pepper, respectively [29, 44].

The transcription factor bHLH143 homolog gene in oil palm (LOC105048659) is the putative target gene of egu-miR24sds. While this miRNA underwent a 65% decrease in expression, its putative target gene showed a 63% increase in the apical leaf of young oil palm plants under very high salt stress. The basic helix-loop-helix (bHLH) TFs are a large gene family in the plant genome, and some of these TFs regulate plant responses to abiotic stresses, including salt stress. The overexpression of *the SlbHLH22* gene in tomato plants increased the tolerance to drought and salinity stress by improving the ROS scavenging system, increasing osmotic potential, and enhanced the accumulation of secondary metabolites [45]. Qiu et al. [46] overexpressed the *MfbHLH38* gene - from the resurrection plant *Myrothamnus flabellifolia* - in Arabidopsis and observed enhanced tolerance to both drought and salinity stresses through increasing water retention ability, regulating osmotic balance, decreasing stress-induced oxidation damage, and possibly participated in ABA-dependent stress-responding pathway.

The GATA transcription factor 27 homolog gene in oil palm (LOC105043768) is the putative target gene to miR319. While this miRNA underwent a 74% decrease in expression, its target gene showed a 44% increase under very high salt stress. GATA TFs belong to one of the most conserved families of zinc-finger TFs [47]. Transcript abundance analysis using salt-sensitive and salt-tolerant rice genotypes indicated differential expression of GATA TF genes in response to various abiotic stresses such as salinity, drought, and exogenous ABA, suggesting inherent roles of diverse GATA factors in abiotic stress signaling [48]. Nutan et al. [49] have shown that the overexpression of the *OsGATA8* gene results in salinity tolerance in rice seedlings, as it maintains ion homeostasis and restricts membrane damage.

The homeobox-leucine zipper protein HOX32 homolog gene in oil palm (LOC105046708) is the putative target gene to four distinct miRNAs from the miR166 family. While these miRNAs underwent a 70–87% decrease in expression, its target gene showed a 78% increase in the apical leaf of young oil palm plants under very high salt stress. *Arabidopsis thaliana* has four distinct classes of homeodomain leucine zipper (HD-ZIP) transcription factors – HD-ZIPI to HD-ZIPIV – organized in multi-genes families [50]. Bhattacharjee et al. [51] carried out a functional analysis of two candidates stress-responsive HD-ZIP I class homeobox genes from rice, *OsHOX22*, and *OsHOX24*, and showed that these genes were highly upregulated under various abiotic stress conditions, including salinity stress, at different stages of development, including seedling, mature and reproductive stages. Besides that, Bhattacharjee and colleagues also overexpressed the *OsHOX24* gene in Arabidopsis plants showing that its overexpression does not result in a detectable difference in the phenotype and various growth parameters compared to the wild type under normal growth conditions; however, it does result in higher sensitivity to salinity stress.

The growth-regulating factor 10 homolog gene in oil palm (LOC105054175) is the putative target gene to miR396. While this miRNA underwent an 83% decrease in expression, its target gene showed a 131% increase in the apical leaf of young oil palm plants under very high salt stress. microRNA miR396 controls the expression of several growth-regulating factors (GRFs), and the GRF-miRNA396 regulatory module appears to be central to several developmental processes, including flower and seed formation, root development, and the coordination of growth processes under adverse environmental conditions, including salt stress [52–54]. Genetically modified creeping bentgrass (*Agrostis stolonifera*) overexpressing *Osa-miR396c*, a rice miRNA396 gene, showed enhanced salt tolerance associated with improved water retention, increased chlorophyll content, cell membrane integrity, and Na^+ exclusion during high salinity exposure; however, they exhibited altered development [53]. RNA-sequencing analysis revealed that GRF1 and GRF3 regulate the expression of many clock core genes and genes with stress- and defense-related functions [55]. *AtGRF7* - a repressor of stress-responsive genes under non-stress conditions – suppresses *DREB2A* expression to preserve plant growth rate [56]. DREB2A is a TF whose transcriptional and post-translational activation increases osmotic stress tolerance in *Arabidopsis* [55]. *atgrf7* lost function mutants are more tolerant to drought and salinity stresses [52].

Nuclear factor Y (NF-Y) proteins are widespread in plants, animals, and other eukaryotes and are also known as *CCAAT* Binding Factor (CBF) or Heme Activator Protein (HAP); and they modulate the expression of downstream putative target genes via two main mechanisms [57]. The heterotrimer – NF-YA-YB-YC – binds to the CCAAT box present in the promoter region of the downstream putative target genes through NF-YA and regulates the expression of the putative target genes. The idea of NF-YA competing with TFs, and suppressing the formation of the NF-YB-YC-TF complex, was postulated [58]; however, according to Zhao et al. [57], there is still no direct molecular evidence to support it.

Different members of the NFY gene family, including NF-YA, are targets of the miR169 family, and studies have shown that overexpression of NF-YA in Arabidopsis increased the plant's tolerance to salt stress, increasing the expression of abscisic acid [59]. A hypothetical model presented by Leyva-González et al. [58] proposes that in plants growing under non-stress conditions, NF-YA expression is low due to high levels of miR169 but sufficient to activate the transcription of genes which promoters contain the CCAAT box. In plants exposed to abiotic stress, NF-YA levels increase due to their transcriptional activation and to the reduction in the miR169 levels. Increased NF-YA levels repress early abiotic stress response genes probably by sequestering NF-YB-YC, creating a regulatory loop to arrest early responses that represent high energy and carbon costs, and participating in the activation of a late one.

Our results showed that the NF-YA3 homolog gene (LOC105056468) expression level in the apical leaf of salt-stressed young oil palm increased 158%, while miR169 had its expression level decreased to 17%. The gene that expresses the miR169 targetting the NF-YA3 homolog gene in oil palm is at two places in the oil palm genome, chromosomes 08 and 13, but only the one in the former chromosome differentially expressed under salinity stress (Supplementary Table 2). As the salinity stress reduces the amount of miR169 in the leaves of young oil palm plants, we postulate that more NF-YA3 would be available to compete with any NF-YB-YC-TF complex, resulting in more of the NF-YA-YB-YC complex, which could restore some of the main biological functions of this complex, such as drought tolerance.

A high concentration of soluble salts in the soil can directly affect plant growth in two distinct phases – osmotic and ionic -, whose duration and intensity vary according to the plant species and salt levels [60]. There is a rapid reduction in the osmotic potential in the osmotic stress phase that restricts water absorption and, therefore, reduces transpiration rates [60, 61]. Salinity in its first phase of salt stress is much similar to that of drought stress, and many common responses between salinity and drought stresses are also expected [62]. In the present study, the stressed plants showed a rate of evapotranspiration about half of the one in the control ones, which shows that the young oil palm plants were experiencing the osmotic stress at 12 DAT [24]. The ionic phase, on the contrary, occurs more slowly and depends not only on the saline concentration but also on the exposure time and on the plant's capacity to accumulate or expel toxic ions [60, 63].

The young oil palm plants used in this study had been for 12 days under salinity stress when collecting leaves for the transcriptome characterization [24], which can be considered a short period when dealing with a perennial crop. Those plants had already shown - in the highest level of NaCl used - premature senescence, chlorosis, and necrosis of adult leaves, and consequently a reduction in the photosynthetic area available to support continued growth (Fig. 2). Such symptoms result from a high Na^+ level in the plant that disrupts protein synthesis and interferes with enzyme activity [25]. In the case of these young oil palm plants, one can see an increase of almost 4X in Na^+ and 2X in Cl^- in the absorption roots in the highest level of NaCl used, but not in the apical and basal leaves; showing that these plants were already starting to experience ionic stress [24].

Conclusion

This comprehensive and large-scale miRNA analysis characterized the miRNA population present in the leaves of young oil palm plants exposed to a high level of salt stress, to identify miRNA-putative target genes in the oil palm genome, and to perform an in silico comparison of the expression profile of the miRNAs and their putative target genes, resulting in:

a) The identification of 79 miRNAs, 52 known miRNAs, and 27 new ones; 72 of them differentially expressed under salinity stress. The new ones received the names egu-miR(01to27)sds, where egu is the abbreviation of *Elaeis guineensis* and sds stands for salinity and drought stress;

b) The prediction of 229 distinct genes as the targets to these 79 miRNAs in the oil palm genome; 150 of them were target to just one miRNA and the remaining 79 to two or more. Fifty-one miRNA-putative target genes differentially expressed under salinity stress;

c) The functional annotation of 24 putative target genes upregulated under salinity stress. Among these genes, there were six that code for transcription factors and three for lncRNA; and.

d) The identification of potential targets genes – based upon evidence of a target gene-miRNA interaction under salinity stress - that can be tested as candidate genes to develop salinity stress tolerant oil palm plants. The development of salt-tolerant oil palm genotypes can come from overexpression or knock out of some of these miRNA or their respective putative target genes, either by a CRISPR/Cas genome editing strategy or by employing classic *Agrobacterium*- or biolistic-mediated genetic modification.

Methods

Plant material and growth conditions

The oil palm plants used in this study were clones regenerated in our lab out of embryogenic calluses obtained from leaves of an adult plant belonging to the *E. guineensis* genotype AM33, a Deli x Ghana from ASD Costa Rica (http://www.asd-cr.com). The protocols and procedures implemented to regenerate the plants are described in Corrêa et al. [64]. Plants were kept in black plastic pots (5 L), containing 1700 g of a mix of vermiculite, soil, and a commercial substrate (Bioplant®), in a 1:1:1 ratio on a dry basis, and fertilized using 2.5 g/L of the formula 20–20-20. Before starting the experiments, plants were standardized accordingly to the developmental stage, size, and number of leaves. The experiment was performed in a greenhouse at Embrapa Agroenergy (www.embrapa.br/en/agroenergia) in Brasília, DF, Brazil (S-15.732°, W-47.900°). The main environmental variables (temperature, humidity, and radiation) measured at a nearby meteorological station (S-15.789°, W-47.925°) fluctuated according to the weather conditions. The oil palm plants used in this study were in the growth stage known as "bifid saplings" when subjected to salt stress.

Experimental design and saline stress

The experiment was carried out in March 2018 and consisted of five treatments (0.0, 0.5, 1.0, 1.5, and 2.0 g of NaCl per 100 g of substrate), with four replicates in a completely randomized design. For details regarding moisture content, field capacity, and electric conductivity in the substrate, determined preliminarily, see Vieira et al. [24].

To salinize the substrate, the amount of NaCl corresponding to the level to be applied to each treatment was dissolved in an amount of tap water standardized and calculated by the difference between the amount of water previously present in the fresh substrate and the amount of water retained for the substrate to reach field capacity. Applying the right amount of water to get the substrate field capacity was a means of ensuring that there was no extravasation of the solution and loss of Na^+ or Cl^-. Thus, the amount of salt added would remain in the substrate.

Plants were under stress for 12 days, with daily water maintenance by replacing the lost volume with tap water. The difference between total weight (TW) (container, soil, water added to reach field capacity, and plant weights, altogether) and the daily weight (DW) is equal to the amount of water necessary to replace daily water losses due to evapotranspiration. Such a procedure was essential to allow the same level of electric conductivity and water potential accordingly to the dose of salt added to the substrate.

Gas exchange measurements

Gas exchange was measured on the middle third of the apical leaf, in a previously marked area, between 9:00 and 11:00 a.m. [24]. The parameters of leaf gas exchange [net CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance to water vapor (gs), and intercellular CO_2 concentration (Ci)] were measured by a portable infrared gas analyzer LI-COR Mod. 6400XT (LI-COR, Lincoln, NE, USA) equipped with a measuring chamber (2 × 3 cm) with artificial light system LI-COR Mod. 6400-02B. The extracted data was provided by the OPEN software version 6.3. The block temperature was 25 °C, PAR was 1500 μmol/m^2/s, the relative humidity of the air inside the measuring chamber was between 50 and 60%, the airflow index was 400 μmol/s, and the CO_2 concentration was 400 ppm in the reference cell, using the model 6400–01 CO_2 mixer with cylinder CO_2 (7.5 g). After submitting the gas exchange data to the Kruskal-Wallis test, we applied the Dunn's test ($p < 0.05$) to those data with significant differences between treatments.

Transcriptomics

Apical leaves from three control and stressed plants (0.0 and 2.0 g of NaCl per 100 g of substrate), collected 12 days after imposition of the treatments (DAT), were immediately immersed in liquid nitrogen and then stored at − 80 °C until RNA extraction, library preparation, and sequencing.

Total RNA extraction and quality analysis, library preparation and sequencing

Total RNA was isolated from oil palm leaves using the Qiagen RNeasy® Plant Mini kit (QIAGEN, CA, USA) following the manufacturer's protocol. RNA quantity and quality were measured using a Nanodrop Qubit 2.0 Fluorometer (Life Technologies, CA, USA) and an Agilent Bioanalyzer Model 2100 (Agilent Technologies, Palo Alto, CA). The GenOne Company (Rio de Janeiro, RJ, Brazil) performed the RNA-Seq using an Illumina HiSeq platform and the paired-end strategy. The Functional Genomics Center / ESALQ-USP (Piracicaba, SP, Brazil) performed the small RNAs sequencing using an Illumina HiSeq platform.

RNA-Seq data analysis

The OmicsBox version 1.3 [65] was employed to perform all RNA-Seq analyses. We used FastQC [66] and Trimmomatic [67] for quality control, filter reads, and remove low-quality bases. The oil palm reference genome [27] – files downloaded from NCBI (BioProject PRJNA192219; BioSample SAMN02981535) on October 2020 - was used to align the RNA-Seq data using default parameters from OmicsBox version 1.3 through software STAR [68]. The default parameters from OmicsBox version 1.3 through HTSeq version 0.9.0 were employed to quantify expression at the gene or transcript level [69]. The pairwise differential expression analysis between experimental conditions (Control vs. Stressed) was performed through edgeR version 3.28.0 [70], applying a simple design and an exact statistical test without a filter for low counts genes.

miRNAs data analysis

The small RNA raw data was submitted to the cutadapt software version 2.7 [71], generating adapter-free small RNA reads 20–24 nucleotides long. The Rfam version 12.0 database was used to remove contaminants, followed by mapping to the oil palm reference genome [27] using Bowtie2 [72].

All adapter-free small RNA sequences (stressed and control) were concatenated into a single file for miRNA prediction. The prediction was then made using mireap version 0.2 (https://sourceforge.net/projects/mireap) and Shortstack version 3.4 (https://github.com/MikeAxtell/ShortStack), independently or in an association. Both programs generate clusters of sequences lined up in genomic regions. Ideally, these clusters indicate the genomic location and the miRNA precursor, mature miRNA, and miRNA* sequences. Shortstack also analyzes precursor and hairpin metrics formed according to parameters established by Axtell and Meyers and classifies them in Y (confirmed miRNA) or N1-N15, where N15 means that the candidate has all the correct metrics, but the miRNA* is absent [73]. The clusters formed by the mireap were analyzed by Shortack to obtain the classifications of each miRNA. StrucVis (https://github.com/MikeAxtell/strucVis) was used in sequences classified as Y or N15 by ShortStack and/or ShortStack-mireap for structural evaluation of miRNA. Finally, manual curation was made of all miRNAs classified as Y and N15. The length of the strings, the predicted structure of the hairpin, and the annotation by homology were evaluated (miRBase - http://www.mirbase.org/search.shtml).

The prediction of miRNA-putative target genes was performed using the psRNA-Target online program, version 2 (https://bio.tools/psrnatarget), with the following parameters: 5 of top targets, 5 expectation, 1 Penalty for other mismatches. For the analysis of differential expression of miRNAs, we used the NOISeq R package [74]. For this, the individual counts of each sample were used as input. The genes that showed *p* values ≥ 0.95 were designated as differentially expressed.

To functionally annotate the differentially expressed miRNA-putative target genes we used the LOC id to get to the protein sequence at NCBI, and then submitted it to the InterProScan search at InterPro (http://www.ebi.ac.uk/interpro/) [75].

Abbreviations

Embrapa: Brazilian agricultural research corporation; PGBV: Graduate program in plant biotechnology; UFLA: Federal university of Lavras; TF: Transcription factors; DE: Differentially expressed; FDR: False discovery rate; DEG: Differentially expressed gene; ROS: Reactive oxygen species; CRISPR: Clustered regularly interspaced short palindromic repeats.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-03296-9.

Additional file 1: Supplementary Table 1. Name, sequence and size of oil palm miRNAs predicted using mireap version 0.2 and Shortstack version 3.4, independently or in association, from the smallRNA raw data obtained from six samples of oil palm plants submitted to two treatments (0 and 2 g of NaCl per 100 g of the substrate); three replicates per treatment.

Additional file 2: Supplementary Table 2. Name of the miRNA, localization and size of oil palm miRNA gene, and differential expression profile of the putative miRNA genes in oil palm plants under salinity stress (2 g of NaCl per 100 g of the substrate), in comparison to control plants. FC – Fold change.

Additional file 3: Supplementary Table 3. Prediction of oil palm miRNAs-putative target genes using psRNA-Target online program, version 2, and the 79 miRNAs predicted in this study.

Additional file 4: Supplementary Table 4. Gene ontology (GO) classification of the oil palm gene targets for orthologous and new miRNAs prospected under salinity stress. The mRNA-putative target genes were classified into biological process, molecular function, and cellular component at the second level of GO classification.

Acknowledgements

We would like to express sincere gratitude to Prof. Sergio Motoike, from the Universidade Federal de Viçosa - UFV, located in the state of Minas Gerais, Brazil, for supplying the embryogenic calluses from *E. guineensis* genotype AM33.

Authors' contributions

M.T.S.Jr. and C.A.F.S. conceived the experiment(s), F.F.S., L.R.V., and A.P.L. conducted the experiment(s), M.T.S.Jr., F.F.S., V.N.B.S., P.G., M.M.C.C., and R.C.T. analyzed the results. M.T.S.Jr. and F.F.S. wrote the manuscript. All authors have read and approved the manuscript.

Funding

This work was funded by the Brazilian Innovation Agency - FINEP (01.13.0315.00 DendêPalm Project), and the Coordination for the Improvement of Higher Education Personnel – CAPES (Scholarships for F.F.S. and L.R.V.). The funding bodies covered all student and project costs but were not involved in the design, collection, analysis and interpretation of the data or the preparation of the manuscript.

Availability of data and materials

The smallRNA raw sequence data used in this study have been uploaded in the SRA database of the NCBI under *Elaeis guineensis* microRNA_Drought and Salinity Stresses - BioProject PRJNA646488 (SUB7775347), BioSample SAMN12799239 (SUB6325749), SRA submission SUB7897143 (accessions from SRR12424937 to SRR12424945), and will be available after publication of this study. All RNA-seq fastq files used in this study have been uploaded in the SRA database of the NCBI under *Elaeis guineensis* Transcriptome_Drought and Salinity Stresses - BioProject PRJNA573093 (SUB6324604), BioSample SAMN12799239 (SUB6325749), SRA submission SUB6335775 (accessions from SRR10219424 to SRR10219441), and will be available after publication of this study. The data-sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors declare receiving consent from the Publication Committee at Embrapa Agroenergia.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]PGBV - Universidade Federal de Lavras – UFLA, CEP 37200-000, Lavras, MG, Brazil. [2]Embrapa Agroenergia, CEP 70770-901, Brasília, DF, Brazil. [3]Embrapa Recursos Genéticos e Biotecnologia, CEP 70770-917, Brasília, DF, Brazil. [4]Embrapa Meio Norte, Av. Duque de Caxias, 5650, CEP 64.006-245, Teresina, PI, Brazil.

Received: 14 June 2021 Accepted: 26 October 2021

References

1. Wang, J., Meng, X., Dobrovolskaya, O. B., Orlov, Y. L., & Chen, M. Non-coding RNAs and their roles in stress response in plants. Genomics Proteomics Bioinformatics 2017; 15(5), 301–312. https://doi.org/https://doi.org/10.1016/j.gpb.2017.01.007.
2. Ayubov, M. S., Mirzakhmedov, M. H., Sripathi, V. R., Buriev, Z. T., Ubaydullaeva, K. A., Usmonov, D. E., Norboboyeva, R. B., Emani, C., Kumpatla, S. P., & Abdurakhmonov, I. Y. Role of MicroRNAs and small RNAs in regulation of developmental processes and agronomic traits in Gossypium species. Genomics. 2019; 111(5), 1018–1025. https://doi.org/https://doi.org/10.1016/j.ygeno.2018.07.012.
3. Xu, X.W., Zhou, X.H., Wang, R.R. et al. Functional analysis of long intergenic non-coding RNAs in phosphate-starved rice using competing endogenous RNA network. 2016; Sci Rep 6, 20715. https://doi.org/https://doi.org/10.1038/srep20715.
4. Budak, H., & Akpinar, B. A. Plant miRNAs: biogenesis, organization and origins. Funct Integr Genomics 2015; 15(5), 523–531. https://doi.org/https://doi.org/10.1007/s10142-015-0451-2.
5. Kim, Young Kook, Boseon Kim, and V. Narry Kim. "Re-Evaluation of the Roles of DROSHA, Exportin 5, and DICER in microRNA Biogenesis." Proc Natl Acad Sci U S A 2016; 113(13): E1881–E1889. https://doi.org/10.1073/pnas.1602532113.
6. Chuammitri, P., Vannamahaxay, S., Sornpet, B., Pringproa, K., & Patchanee, P. Detection and characterization of microRNA expression profiling and its target genes in response to canine parvovirus in Crandell Reese Feline Kidney cells. 2020; PeerJ, *8*, e8522. https://doi.org/https://doi.org/10.7717/peerj.8522.
7. Sun, X., Lin, L., & Sui, N. Regulation mechanism of microRNA in plant response to abiotic stress and breeding. Mol Biol Rep 2019; 46(1), 1447–1457. https://doi.org/https://doi.org/10.1007/s11033-018-4511-2.
8. Sunkar, R., Chinnusamy, V., Zhu, J., & Zhu, J. K. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. Trends Plant Sci 2007; 12(7), 301–309. https://doi.org/https://doi.org/10.1016/j.tplants.2007.05.001.
9. Xu, J., Hou, Q. M., Khare, T., Verma, S. K., & Kumar, V. Exploring miRNAs for developing climate-resilient crops: a perspective review. Sci Total Environ 2019; 653, 91–104. https://doi.org/https://doi.org/10.1016/j.scitotenv.2018.10.340.
10. Zhao, B., Liang, R., Ge, L., et al. Identification of drought-induced microRNAs in rice. Biochem Biophys Res Commun. 2007; 354(2), 585–590. DOI: https://doi.org/https://doi.org/10.1016/j.bbrc.2007.01.022.
11. Zhou, Liguo et al. "Genome-wide identification and analysis of drought-responsive microRNAs in Oryza Sativa." J Exp Bot 2010; 61(15): 4157–4168. https://doi.org/https://doi.org/10.1093/jxb/erq237.
12. Liu, H. H., Tian, X., Li, Y. J., Wu, C. A., & Zheng, C. C. Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. RNA. *2008; 14*(5), 836–843. https://doi.org/https://doi.org/10.1261/rna.895308.
13. Wang, T., Chen, L., Zhao, M., Tian, Q., & Zhang, W. H. Identification of drought-responsive microRNAs in Medicago truncatula by genome-wide high-throughput sequencing. BMC Genomics 2011; 12, 367. https://doi.org/https://doi.org/10.1186/1471-2164-12-367.
14. Li, C., Li, Y., Bai, L., Chaoxing, H.E., Xianchang, Y.U. Dynamic expression of miRNAs and their targets in the response to drought stress of grafted cucumber seedlings. Hortic Plant J 2016; 2, 41–49. http://dx.doi.org/https://doi.org/10.1016/j.hpj.2016.02.002
15. Sunkar, R., and Zhu, J.K. Novel and stress regulated microRNAs and other small RNAs from Arabidopsis w inside box sign. Plant Cell 2004; 16(8), 2001–2019. https://doi.org/https://doi.org/10.1105/tpc.104.022830.
16. Ding, D., Zhang, L., Wang, H., Liu, Z., Zhang, Z., & Zheng, Y. Differential expression of miRNAs in response to salt stress in maize roots. Ann Bot 2009; 103(1), 29–38. https://doi.org/https://doi.org/10.1093/aob/mcn205.
17. Dong, Z., Shi, L., Wang, Y., Chen, L., Cai, Z., Wang, Y., Jin, J., & Li, X. Identification and dynamic regulation of microRNAs involved in salt stress responses in functional soybean nodules by high-throughput sequencing. Int J Mol Sci 2013; 14(2), 2717–2738. https://doi.org/https://doi.org/10.3390/ijms14022717.
18. Md Nasaruddin N, Harikrishna K, Othman R, Hoon L, Ann Harikrishna J. Computational prediction of microRNAs from oil palm (Elaeis guineensis Jacq.) expressed sequence tags. Asia Pac J Mol Biol Biotechnol. 2007;15(3):107–13.
19. Low, E. T., Rosli, R., Jayanthi, N., Mohd-Amin, A. H., Azizi, N., Chan, K. L., Maqbool, N. J., Maclean, P., Brauning, R., McCulloch, A., Moraga, R., Ong-Abdullah, M., & Singh, R. Analyses of hypomethylated oil palm gene space. PLoS One 2014; 9(1), e86728. https://doi.org/https://doi.org/10.1371/journal.pone.0086728.
20. da Silva, A. C., Grativol, C., Thiebaut, F., Hemerly, A. S., & Ferreira, P. C. Computational identification and comparative analysis of miRNA precursors in three palm species. Planta. 2016; 243(5), 1265–1277. https://doi.org/https://doi.org/10.1007/s00425-016-2486-6.
21. Ho, H., Gudimella, R., Ong-Abdullah, M. et al. Expression of microRNAs during female inflorescence development in African oil palm (*Elaeis guineensis* Jacq.). Tree Genet Genomes. 2017; 13, 35. https://doi.org/https://doi.org/10.1007/s11295-017-1120-5.
22. Zheng, Y., Chen, C., Liang, Y., Sun, R., Gao, L., Liu, T., & Li, D. Genome-wide association analysis of the lipid and fatty acid metabolism regulatory network in the mesocarp of oil palm (Elaeis guineensis Jacq.) based on small noncoding RNA sequencing. Tree Physiol 2019; 39(3), 356–371. https://doi.org/https://doi.org/10.1093/treephys/tpy091.
23. Abrapalma. Diagnóstico da Produção Sustentável da Palma de Óleo. 2018; http://www.abrapalma.org/pt/wpcontent/uploads/2018/06/DIAGNOSTICO_PALMA.pdf
24. Vieira, L. R., et al. "Morphophysiological responses of young oil palm plants to salinity stress". Pesq Agrop Brasileira 2020; v.55, e01835. https://doi.org/https://doi.org/10.1590/S1678-3921.pab2020.v55.01835.
25. Carillo, P., Annunziata, M. G., Pontecorvo, G., Fuggi, A., Woodrow, P. Salinity Stress and Salt Tolerance, Abiotic Stress in Plants - Mechanisms and Adaptations, Prof. Arun Shanker (Ed.), ISBN: 978–953–307-394-1, InTech, 2011. Available from: http://www.intechopen.com/books/abiotic-stress-in-plants-mechanisms-and-adaptations/salinity-stress-and-salt-tolerance
26. Vieira, L. R. Morphophysiological, Metabolomic and transcrytomic responses of oil palm (Elaeis guineensis) to drought and salinity stresses. 2019. 158 p. d.Sc. Thesis – Universidade federal de Lavras, Lavras, MG, Brazil. http://repositorio.ufla.br/jspui/handle/1/46074

27. Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y. Y., Sieburth, L., & Voinnet, O. Widespread translational inhibition by plant miRNAs and siRNAs. Science 2008; *320*(5880), 1185–1190. https://doi.org/https://doi.org/10.1126/science.1159151.
28. Singh, R., Ong-Abdullah, M., Low, E. T., Manaf, M. A., Rosli, R., Nookiah, R., Ooi, L. C., Ooi, S. E., Chan, K. L., Halim, M. A., Azizi, N., Nagappan, J., Bacher, B., Lakey, N., Smith, S. W., He, D., Hogan, M., Budiman, M. A., Lee, E. K., DeSalle, R., Sambanthamurthi, R. Oil palm genome sequence reveals divergence of interfertile species in old and new worlds. Nature. 2013; 500(7462), 335–339. https://doi.org/https://doi.org/10.1038/nature12309.
29. Zhang, H. X., Zhu, W. C., Feng, X. H., Jin, J. H., Wei, A. M., & Gong, Z. H. Transcription factor *CaSBP12* negatively regulates salt stress tolerance in pepper (*Capsicum annuum* L.). Int J Mol Sci. 2020; *21*(2), 444. https://doi.org/https://doi.org/10.3390/ijms21020444.
30. Fang L, Liang Y, Li D, Cao X, Zheng Y. Dynamic expression analysis of miRNAs during the development process of oil palm mesocarp. Plant Sci J. 2013;31:304–12.
31. Ho, C. L., Kwan, Y. Y., Choi, M. C., Tee, S. S., Ng, W. H., Lim, K. A., Lee, Y. P., Ooi, S. E., Lee, W. W., Tee, J. M., Tan, S. H., Kulaveerasingam, H., Alwee, S. S., & Abdullah, M. O. Analysis and functional annotation of expressed sequence tags (ESTs) from multiple tissues of oil palm (*Elaeis guineensis* Jacq.). BMC Genomics. 2007; 8, 381. https://doi.org/https://doi.org/10.1186/1471-2164-8-381.
32. Mica, E., Gianfranceschi, L., & Pè, M. E. Characterization of five microRNA families in maize. J Exp Bot 2006; 57(11), 2601–2612. https://doi.org/https://doi.org/10.1093/jxb/erl013.
33. Gao, P., Bai, X., Yang, L., Lv, D., Pan, X., Li, Y., Cai, H., Ji, W., Chen, Q., & Zhu, Y. Osa-MIR393: a salinity- and alkaline stress-related microRNA gene. Mol Biol Rep 2011; 38(1), 237–242. https://doi.org/https://doi.org/10.1007/s11033-010-0100-8.
34. Guo Q, Li L, Zhao K, Yao W, Cheng Z, Zhou B, Jiang T. Genome-wide analysis of poplar SQUAMOSA-promoter-binding protein (SBP) family under salt stress. Forests. 2021; 12(4):413. https://doi.org/https://doi.org/10.3390/f12040413.
35. Lu, S., Sun, Y. H., & Chiang, V. L. Stress-responsive microRNAs in Populus. Plant J 2008; 55(1), 131–151. https://doi.org/https://doi.org/10.1111/j.1365-313X.2008.03497.x.
36. Sunkar R. MicroRNAs with macro-effects on plant stress responses. Semin Cell Dev Biol 2010; 21(8), 805–811. https://doi.org/https://doi.org/10.1016/j.semcdb.2010.04.001.
37. Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P., Linsley, P. S., & Johnson, J. M. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 2005; 433(7027), 769–773. https://doi.org/https://doi.org/10.1038/nature03315.
38. Xu, J., Chen, Q., Liu, P., Jia, W., Chen, Z., & Xu, Z. Integration of mRNA and miRNA analysis reveals the molecular mechanism underlying salt and alkali stress tolerance in tobacco. Int J Mol Sci 2019; 20(10), 2391. https://doi.org/https://doi.org/10.3390/ijms20102391.
39. Cheng, Y., & Long, M. A cytosolic NADP-malic enzyme gene from rice (Oryza sativa L.) confers salt tolerance in transgenic Arabidopsis. Biotechnol Lett 2007; 29(7), 1129–1134. https://doi.org/https://doi.org/10.1007/s10529-007-9347-0.
40. Prashanth, S. R., Sadhasivam, V., & Parida, A. Over expression of cytosolic copper/zinc superoxide dismutase from a mangrove plant Avicennia marina in indica rice var Pusa Basmati-1 confers abiotic stress tolerance. Transgenic Res 2008; 17(2), 281–291. https://doi.org/https://doi.org/10.1007/s11248-007-9099-6.
41. Javed, T., Shabbir, R., Ali, A., Afzal, I., Zaheer, U., & Gao, S. J. Transcription factors in plant stress responses: challenges and potential for sugarcane improvement. Plants 2020; *9*(4), 491. https://doi.org/https://doi.org/10.3390/plants9040491.
42. Wang, J., Ye, Y., Xu, M. et al. Roles of the SPL gene family and miR156 in the salt stress responses of tamarisk (*Tamarix chinensis*). BMC Plant Biol 2019; 19, 370. https://doi.org/https://doi.org/10.1186/s12870-019-1977-6.
43. Hou, H., Jia, H., Yan, Q., & Wang, X. Overexpression of a SBP-box gene (VpSBP16) from Chinese wild Vitis species in Arabidopsis improves salinity and drought stress tolerance. Int J Mol Sci 2018; 19(4), 940. https://doi.org/https://doi.org/10.3390/ijms19040940.
44. Lan, T., Zheng, Y., Su, Z., Yu, S., Song, H., Zheng, X., Lin, G., & Wu, W. *OsSPL10*, a SBP-box gene, plays a dual role in salt tolerance and trichome formation in rice (*Oryza sativa* L.). G3 2019; 9(12), 4107–4114. https://doi.org/https://doi.org/10.1534/g3.119.400700.
45. Waseem, M., Rong, X., & Li, Z. Dissecting the role of a basic Helix-loop-Helix transcription factor, *SlbHLH22*, under salt and drought stresses in transgenic *Solanum lycopersicum* L. Front Plant Sci 2019; 10, 734. https://doi.org/https://doi.org/10.3389/fpls.2019.00734.
46. Qiu, J. R., Huang, Z., Xiang, X. Y., Xu, W. X., Wang, J. T., Chen, J., Song, L., Xiao, Y., Li, X., Ma, J., Cai, S. Z., Sun, L. X., & Jiang, C. Z. MfbHLH38, a Myrothamnus flabellifolia bHLH transcription factor, confers tolerance to drought and salinity stresses in Arabidopsis. BMC Plant Biol 2020; 20(1), 542. https://doi.org/https://doi.org/10.1186/s12870-020-02732-6.
47. Reyes, J. C., Muro-Pastor, M. I., & Florencio, F. J. The GATA family of transcription factors in Arabidopsis and rice. Plant Physiol 2004; 134(4), 1718–1732. https://doi.org/https://doi.org/10.1104/pp.103.037788.
48. Gupta, P., Nutan, K. K., Singla-Pareek, S. L., & Pareek, A. Abiotic stresses cause differential regulation of alternative splice forms of GATA transcription factor in Rice. Front Plant Sci 2017; 8, 1944. https://doi.org/https://doi.org/10.3389/fpls.2017.01944.
49. Nutan, K.K., Singla-Pareek, S.L., Pareek, A. The Saltol QTL-localized transcription factor OsGATA8 plays an important role in stress tolerance and seed development in Arabidopsis and rice, J Exp Bot. 2020; V. 71, Issue 2, 7 January 2020, Pages 684–698, https://doi.org/https://doi.org/10.1093/jxb/erz368.
50. Brandt R, Cabedo M, Xie Y, Wenkel S. Homeodomain leucine-zipper proteins and their role in synchronizing growth and development with the environment. J Integr Plant Biol. 2014;56(6):518–26. https://doi.org/10.1111/jipb.12185 Epub 2014 Mar 13. PMID: 24528801. https://doi.org/10.1111/jipb.12185.
51. Bhattacharjee, A., Khurana, J. P., & Jain, M. Characterization of Rice Homeobox genes, OsHOX22 and OsHOX24, and over-expression of OsHOX24 in transgenic Arabidopsis suggest their role in abiotic stress response. Front Plant Sci 2016; 7, 627. https://doi.org/https://doi.org/10.3389/fpls.2016.00627.
52. Omidbakhshfard, M. A., Proost, S., Fujikura, U., & Mueller-Roeber, B. Growth-regulating factors (GRFs): a small transcription factor family with important functions in plant biology. Mol Plant 2015; 8(7), 998–1010. https://doi.org/https://doi.org/10.1016/j.molp.2015.01.013.
53. Yuan, S., Zhao, J., Li, Z., Hu, Q., Yuan, N., Zhou, M., Xia, X., Noorai, R., Saski, C., Li, S., & Luo, H. MicroRNA396-mediated alteration in plant development and salinity stress response in creeping bentgrass. Horticulture Res 2019; 6, 48. https://doi.org/https://doi.org/10.1038/s41438-019-0130-x.
54. Rossmann, S., Richter, R., Sun, H., Schneeberger, K., Töpfer, R., Zyprian, E., & Theres, K. Mutations in the miR396 binding site of the growth-regulating factor gene VvGRF4 modulate inflorescence architecture in grapevine. Plant J 2020; 101(5), 1234–1248. https://doi.org/https://doi.org/10.1111/tpj.14588.
55. Piya, S., Liu, J., Burch-Smith, T., Baum, T. J., & Hewezi, T. A role for Arabidopsis growth-regulating factors 1 and 3 in growth-stress antagonism. J Exp Bot 2020; 71(4), 1402–1417. https://doi.org/https://doi.org/10.1093/jxb/erz502.
56. Kim, J. S., Mizoi, J., Kidokoro, S., Maruyama, K., Nakajima, J., Nakashima, K., Mitsuda, N., Takiguchi, Y., Ohme-Takagi, M., Kondou, Y., Yoshizumi, T., Matsui, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. Arabidopsis growth-regulating factor7 functions as a transcriptional repressor of abscisic acid- and osmotic stress-responsive genes, including DREB2A. Plant Cell 2012; 24(8), 3393–3405. https://doi.org/https://doi.org/10.1105/tpc.112.100933.
57. Zhao H, Wu D, Kong F, Lin K, Zhang H, Li G. The *Arabidopsis thaliana* nuclear factor Y transcription factors. Front Plant Sci 2017;7:2045. https://doi.org/https://doi.org/10.3389/fpls.2016.02045.
58. Leyva-González, M. A., Ibarra-Laclette, E., Cruz-Ramírez, A., & Herrera-Estrella, L. Functional and transcriptome analysis reveals an acclimatization strategy for abiotic stress tolerance mediated by Arabidopsis NF-YA family members. PLoS One 2012; 7(10), e48138. https://doi.org/https://doi.org/10.1371/journal.pone.0048138.
59. Li, Y. J., Fang, Y., Fu, Y. R., Huang, J. G., Wu, C. A., & Zheng, C. C. NFYA1 is involved in regulation of postgermination growth arrest under salt stress in Arabidopsis. PLoS One 2013; 8(4), e61289. https://doi.org/https://doi.org/10.1371/journal.pone.0061289.
60. Munns, Rana, and Mark Tester. "Mechanisms of salinity tolerance." Annu Rev Plant Biol 2008; 59: 651–681. https://doi.org/https://doi.org/10.1146/annurev-arplant-050718-100005.

61. Parihar, P., Singh, S., Singh, R., Singh, V. P., & Prasad, S. M. Effect of salinity stress on plants and its tolerance strategies: a review. Environ Sci Pollut Res Int 2015; 22(6), 4056–4075. https://doi.org/https://doi.org/10.1007/s11356-014-3739-1.
62. Uddin, M. N., Hossain, M. A., and Burritt, D. "Salinity and drought stress: similarities and differences in oxidative responses and cellular redox regulation," in Water Stress and Crop Plants: A Sustainable Approach, ed. P. Ahmad (Hoboken, NJ: Wiley). 2016; 86–101. https://doi.org/https://doi.org/10.1002/9781119054450.ch7.
63. Kumari, A., Das, P., Parida, A. K., & Agarwal, P. K. Proteomics, metabolomics, and ionomics perspectives of salinity tolerance in halophytes. Front Plant Sci 2015; 6,537. https://doi.org/https://doi.org/10.3389/fpls.2015.00537.
64. Corrêa TR, Motoike SY, Coser SM, DA Silveira G, De Resende MDV, Chia GS. estimation of genetic parameters for *in vitro* oil palm characteristics (*Elaeis guineensis* Jacq.) and selection of genotypes for cloning capacity and oil yield. Ind Crop Prod. 2015;77:1033–8.
65. OmicsBox – Bioinformatics Made Easy, BioBam Bioinformatics, March 3, 2019. https://www.biobam.com/omicsbox
66. Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013; 29(1), 15–21. https://doi.org/https://doi.org/10.1093/bioinformatics/bts635.
67. Andrews S. FastQC: a quality control tool for high Thoughput sequence 2010. Data. Retrieved 2018, from https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
68. Bolger, A. M., Lohse, M., & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014; 30(15), 2114–2120. https://doi.org/https://doi.org/10.1093/bioinformatics/btu170.
69. Anders, S., Pyl, P. T., & Huber, W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics 2015; 31(2), 166–169. https://doi.org/https://doi.org/10.1093/bioinformatics/btu638.
70. Robinson, M. D., McCarthy, D. J., & Smyth, G. K. EdgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010; *26*(1), 139–140. https://doi.org/https://doi.org/10.1093/bioinformatics/btp616.
71. Martin, Marcel. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal, [S.l.], v. 17, n. 1, p. pp. 10–12, may 2011. ISSN 2226–6089. Available at: <http://journal.embnet.org/index.php/embnetjournal/article/view/200>. Date accessed: 15 july 2020. doi:https://doi.org/https://doi.org/10.14806/ej.17.1.200
72. Langmead, B., Trapnell, C., Pop, M., et al. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009 10, R25. https://doi.org/https://doi.org/10.1186/gb-2009-10-3-r25.
73. Axtell, M. J., & Meyers, B. C. Revisiting criteria for plant MicroRNA annotation in the era of big data. Plant Cell 2018, 30(2), 272–284. https://doi.org/https://doi.org/10.1105/tpc.17.00851.
74. Tarazona, S., Furió-Tarí, P., Turrà, D., Pietro, A. D., Nueda, M. J., Ferrer, A., & Conesa, A. Data quality aware analysis of differential expression in RNA-seq with NOISeq R/bioc package. Nucleic Acids Res 2015, 43(21), e140. https://doi.org/https://doi.org/10.1093/nar/gkv711.
75. Blum, M., Chang, H, et al. The InterPro protein families and domains database: 20 years on. Nucleic Acids Research, Nov 2020, https://doi.org/https://doi.org/10.1093/nar/gkaa977.

Publisher's Note

ARTIGO II

The early response of oil palm (Elaeis guineensis Jacq.) plants to water deprivation: Expression analysis of miRNAs and their putative target genes, and similarities with the response to salinity stress

Autores: Fernanda Ferreira Salgado, Thalliton Luiz Carvalho da Silva, Letícia Rios Vieira, Vivianny Nayse Belo Silva, André Pereira Leão, Marcos Mota do Carmo Costa, Roberto Coiti Togawa, Carlos Antônio Ferreira de Sousa, Priscila Grynberg e Manoel Teixeira Souza Jr

Publicado no periódico Frontiers in Plant Science
https://www.frontiersin.org/articles/10.3389/fpls.2022.970113/full

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 23 September 2022
DOI 10.3389/fpls.2022.970113

OPEN ACCESS

EDITED BY
Douglas S. Domingues,
University of São Paulo, Brazil

REVIEWED BY
Boon Chin Tan,
University of Malaya,
Malaysia
Muhammad Zeeshan,
Guangxi University, China

*CORRESPONDENCE
Manoel Teixeira Souza Jr
manoel.souza@embrapa.br

SPECIALTY SECTION
This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

RECEIVED 16 June 2022
ACCEPTED 25 August 2022
PUBLISHED 23 September 2022

CITATION
Salgado FF, da Silva TLC, Vieira LR, Silva VNB, Leão AP, Costa MMDC, Togawa RC, de Sousa CAF, Grynberg P and Souza MT Jr (2022) The early response of oil palm (*Elaeis guineensis* Jacq.) plants to water deprivation: Expression analysis of miRNAs and their putative target genes, and similarities with the response to salinity stress.
Front. Plant Sci. 13:970113.
doi: 10.3389/fpls.2022.970113

The early response of oil palm (*Elaeis guineensis* Jacq.) plants to water deprivation: Expression analysis of miRNAs and their putative target genes, and similarities with the response to salinity stress

Fernanda Ferreira Salgado[1], Thalliton Luiz Carvalho da Silva[1], Letícia Rios Vieira[1], Vivianny Nayse Belo Silva[1], André Pereira Leão[2], Marcos Mota do Carmo Costa[3], Roberto Coiti Togawa[3], Carlos Antônio Ferreira de Sousa[4], Priscila Grynberg[3] and Manoel Teixeira Souza Jr[1,2]*

[1]Graduate Program of Plant Biotechnology, Federal University of Lavras, Lavras, MG, Brazil, [2]The Brazilian Agricultural Research Corporation, Embrapa Agroenergy, Brasília, DF, Brazil, [3]The Brazilian Agricultural Research Corporation, Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil, [4]The Brazilian Agricultural Research Corporation, Embrapa Mid-North, Teresina, PI, Brazil

Oil palm (*Elaeis guineensis* Jacq.) is a oilseed crop of great economic importance drastically affected by abiotic stresses. MicroRNAs (miRNAs) play crucial roles in transcription and post-transcription regulation of gene expression, being essential molecules in the response of plants to abiotic stress. To better understand the molecular mechanisms behind the response of young oil palm plants to drought stress, this study reports on the prediction and characterization of miRNAs and their putative target genes in the apical leaf of plants subjected to 14 days of water deprivation. Then, the data from this study were compared to the data from a similar study that focused on salinity stress. Both, the drought-and salt-responsive miRNAs and their putative target genes underwent correlation analysis to identify similarities and dissimilarities among them. Among the 81 identified miRNAs, 29 are specific for oil palm, including two (egu-miR28ds and egu-miR29ds) new ones – described for the first time. As for the expression profile, 62 miRNAs were significantly differentially expressed under drought stress, being five up-regulated (miR396e, miR159b, miR529b, egu-miR19sds, and egu-miR29ds) and 57 down-regulated. Transcription factors, such as MYBs, HOXs, and NF-Ys, were predicted as putative miRNA-target genes in oil palm under water deprivation; making them the most predominant group of such genes. Finally, the correlation analysis study revealed a group of putative target genes with similar behavior under salt and drought stresses. Those genes that are upregulated by these two abiotic stresses encode lncRNAs and proteins linked to stress tolerance, stress memory, modulation of ROS signaling, and defense response regulation to abiotic and biotic stresses. In summary, this study provides molecular evidence for the possible involvement of miRNAs in the drought stress response in oil palm. Besides, it shows that, at the molecular level, there are many similarities in the response of young oil palm plants to these two abiotic stresses.

KEYWORDS

abiotic stress, tolerance, transcriptome, transcription factor, non-coding RNA, lncRNA

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a palm tree from the Arecaceae family, classified as one of the most productive oil seed crops (Wang, L. et al., 2020). Originally from West Africa, it has been successfully introduced and exploited commercially in Asia, Africa, and Latin America. Indonesia and Malaysia are the largest palm oil-producing nations (EPOA, 2020). This species has great economic importance due to the high fruit production and oil efficiency extraction, with refining processes that render both palm oil and palm kernel oil (Corley, 2009; Silva et al., 2016). Palm oil is the raw material for cosmetics, medicines, candles, soaps, biofuels, and lubricating greases, and its demand is increasing (Abrapalma, 2018).

Oil palm does not withstand long periods of severe or moderate drought, and its fruit yield decreases considerably under water scarcity (Azzeme et al., 2016). It requires ~2,000 mm/year of water and does not tolerate drought for more than 90 days (Corley et al., 2018). According to Silva et al. (2016, 2017), water stress from seven to 21 days induced physiological changes and affected the growth of oil palm seedlings; while repetitive water deficit events induced photosynthetic acclimation in young oil palm plants (Lopes Filho et al., 2021). However, there is not much information about the molecular mechanisms behind the responses of oil palm plants to drought stress.

MicroRNAs (miRNAs) are small molecules of non-protein-coding RNAs, 20 to 24 nucleotides (nts) in length, derived from single-stranded precursors, which form a secondary stem-loop structure (Xin et al., 2015). They are involved in gene expression regulation at a post-transcription level in plants, animals, fungi, and viruses (Denli et al., 2004; Lytle et al., 2007; Ventura et al., 2008; Xu et al., 2019). miRNAs molecules are highly conserved in plants (da Silva et al., 2016).

Several studies have shown that miRNAs are involved in many biological and metabolic processes (Comai and Zhang, 2012; Sun, 2012). They play crucial roles in plant growth regulation (Mallory et al., 2004), flower development (Chen, 2004; Zhu et al., 2009), and responses to abiotic stresses, such as drought and salinity (Liu et al., 2008; Ding et al., 2009; Lv et al., 2010; Chen et al., 2018; Qiu et al., 2020; Salgado et al., 2021; Zeeshan et al., 2021).

Water stress-responsive miRNAs are present in *Oryza sativa* (Zhou et al., 2010), *Medicago truncatula* (Wang et al., 2011), and *Arabidopsis thaliana* (Liu et al., 2008). Zhou et al. (2010) described 11 down-and eight up-regulated miRNAs in rice plants under drought stress. Li et al. (2008) reported that miR169a and miR169c are significantly down-regulated by drought, promoting increased drought resistance in arabidopsis. They postulated that it was because one of the miR169 targets, NFYA5 (Nuclear Factor YA5), is a crucial transcription factor that regulates the expression of numerous drought stress-responsive genes (Li et al., 2008).

Studies have shown that miR398 and miR408 are positively regulated by water deficit in *M. truncatula*, leading to the negative regulation of its target genes (COX5b, CSD1, and plantacyanin; Trindade et al., 2010). In *Zea mays*, miRNAs modulate the expression of MAPK (mitogen-activated protein kinase), PLD (phospholipase D), PHD (proline dehydrogenase), and POD (peroxidase; Wei et al., 2009); which are known to be involved in plant response to environmental stresses as part of signaling pathways (MAPK and PLD) or as having role in the ROS-scavenging system (PHD and POD).

Although some studies identified miRNAs in oil palm (Md Nasaruddin et al., 2007; Xiao et al., 2013; Low et al., 2014; da Silva et al., 2016; Salgado et al., 2021), only a few miRNAs from *E. guineensis* are in miRBase (version 2.1). To the best of our knowledge, there are no reports on prospecting and characterizing drought-responsive miRNAs in oil palm. This study reports on the prediction and characterization of miRNAs responsive to drought stress in oil palm plants and their target genes. Besides, the drought- (from this study) and salt-responsive (from Salgado et al., 2021) miRNAs and their putative target genes underwent correlation analysis to identify similarities and dissimilarities among them.

Materials and methods

Plant material, growth conditions, experimental design, and drought stress

The oil palm plants used in this study were clones regenerated out of embryogenic calluses (Corrêa et al., 2015) obtained from leaves of an adult plant belonging to the *E. guineensis* genotype AM33, a Deli x Ghana from ASD Costa Rica.[1] The plants used in this study were different of the one used in the Salgado et al. (2021) study; although, all plants – from both studies – came from the same embryogenic calluses; consequently, they are all clones of the same plant.

The embryogenic calluses were transferred to a regeneration medium on January 2016 and kept in a BOD chamber at 30°C and a 16/8-h light/dark photoperiod. On July and December 2016, the plants regenerated *in vitro* were put in 200-mL plastic cups containing vermiculite and a commercial substrate (Bioplant® – Bioplant Agrícola Ltda., Nova Ponte, MG, Brazil), in a 1:1 ratio, on a dry basis; transferred to the PGW40 growth chamber (Conviron, Winnipeg, Canada), with air temperature at 25 ± 2°C, relative humidity at 60 ± 10%, and light intensity at $500 \pm 50\,\mu\text{mol m}^{-2}\,\text{s}^{-1}$, for acclimation; and then transferred to a greenhouse. Once acclimated, they were transferred to black plastic pots (3.5 L) containing 1,200 g of vermiculite, soil, and a commercial substrate (Bioplant®) mix, in a 1:1:1 ratio – on a dry basis; and fertilized using 2.5 g/L of the formula nitrogen (N), phosphorus (P), and potassium (K) fertilizer 20-20-20.

Before starting the experiments, plants were screened accordingly to their development stage, size, and number of leaves, to use the most uniform group of plants possible. The experiment was performed in a greenhouse in Brasília, DF, Brazil (S-15.732°, W-47.900°). Main environmental variables (temperature, humidity, and radiation), measured at a nearby meteorological station (S-15.789°, W-47.925°), fluctuated according to the weather conditions. Plants underwent drought stress in the growth stage known as bifid saplings.

Two experiments were carried out in November 2017 (Experiment 1) and March 2018 (Experiment 2), and both consisted of two treatments: a control one, with four replicates, and a drought stress one, with six replicates. The experimental design was completely randomized blocks. All plants in the control treatment were maintained at field capacity throughout the entire experiment, while plants in the drought stress treatment were initially at field capacity, and then they were deprived of water addition for 14 days.

Evapotranspiration rate and gas exchange measurements

Plant weight was measured daily and individually to determine the water lost by evapotranspiration. The weight of the vessels containing control plants was measured, and the soil received water to field capacity, with no water added in the remaining treatment. The daily evapotranspiration average measured before the onset of experiments 1 and 2 was considered 100%. The gathered data was then calculated from this initial value, as follows: evapotranspiration (%) = day evapotranspiration (mL) × 100 (%)/mean evapotranspiration before stress (mL). After submitting the data for normality analyses using the Shapiro–Wilk test, an statistical analysis was performed by the Student's *t*-test at 5% probability using GraphPad.

The parameters of leaf gas exchange [net CO_2 assimilation rate (*A*), transpiration rate (*E*), stomatal conductance to water vapor (*gs*), and intercellular CO_2 concentration (*Ci*)] were measured using a portable infrared gas analyzer LI-COR Mod. 6400XT (LI-COR, Lincoln, NE, United States) equipped with a measuring chamber (2 × 3 cm) with artificial light system LI-COR Mod. 6400-02B. We used the OPEN software version 6.3 to extract the data. The block temperature was 25°C, PAR was $1,500\,\mu\text{mol/m}^2\text{/s}$, the relative humidity of the air inside the measuring chamber was between 50 and 60%, the airflow index was 400 µmol/s, and the CO_2 concentration was 400 ppm in the reference cell, using the model 6400-01 CO2 mixer with cylinder CO_2 (7.5 g). After submitting the gas exchange data to the Kruskal-Wallis test, we applied the Dunn's test ($p < 0.05$) to those data with significant differences between treatments. The gas exchange measurements were on the middle third of the apical leaf, in a previously marked area, between 9:00 and 11:00 a.m., only in the first experiment.

Transcriptomics analysis

Apical leaves from three control and three drought-stressed plants, collected 14 days after setting up the treatments (DAT), were immediately immersed in liquid nitrogen and stored at −80°C until RNA extraction, library preparation, and sequencing. Total RNA isolation, as well as the RNA quantity and quality analysis, were performed as described in Salgado et al. (2021). The GenOne Company (Rio de Janeiro, RJ, Brazil) performed the RNA-Seq using an Illumina HiSeq platform and the paired-end strategy. The Functional Genomics Center / ESALQ-USP (Piracicaba, SP, Brazil) performed the small RNAs sequencing using an Illumina HiSeq platform.

The OmicsBox version 1.3 (OmicsBox, 2019) was employed to perform all RNA-Seq analyses, using the same pipeline of analysis described previously in Salgado et al. (2021). Here, we also used FastQC (Andrews, 2018) and Trimmomatic (Bolger et al., 2014) for quality control, STAR (Dobin et al., 2013) to align the high-quality reads to the oil palm genome (Singh et al., 2013), and HTseq to quantify expression at the gene or transcript level (Anders et al., 2015). The small RNA raw data was also submitted to the same pipeline of analysis described previously in Salgado et al. (2021); and here we generated adapter-free small RNA reads using the Cutadapt software (Martin, 2011), mapped them to the reference genome using Bowtie2 (Langmead et al., 2009). The following parameters were used to run bowtie: -a (report all alignments per read) and -V 0 (no mismatches were allowed). The oil palm genome (Singh et al., 2013) – files downloaded from NCBI (BioProject PRJNA192219; BioSample SAMN02981535) on October 2020 – was again used as reference genome.

---

[1] www.asd-cr.com

All adapter-free small RNA sequences (stressed and control) were concatenated into a single file for miRNA prediction. The miRNA prediction was made using mireap version 0.2[2] and Shortstack version 3.4,[3] independently or in an association. Both programs generate clusters of sequences lined up in genomic regions. Ideally, these clusters indicate the genomic location and the miRNA precursor, mature miRNA, and miRNA* sequences. StrucVis[4] was used in sequences classified as Y or N15 by ShortStack and/or ShortStack-mireap for structural evaluation of miRNA. At last, manual curation was made of all miRNAs classified as Y (confirmed miRNA) or N1-N15, where N15 means that the candidate has all the correct metrics, but the miRNA* is absent (Axtell and Meyers, 2018). The length of the strings, the predicted structure of the hairpin, and the annotation by homology were evaluated in miRBase using the default criteria.[5]

The prediction of miRNA-putative target genes was performed using the psRNA-Target online program, version 2,[6] with the same parameters used in Salgado et al. (2021). The NOISeq R package (Tarazona et al., 2015) was used for the analysis of differential expression of miRNAs, having the individual counts of each sample as input. The genes that showed p-values ≥ 0.95 were designated as differentially expressed (DE).

Determination of biomass and soil water potential

Shoots and roots from three control and three drought-stressed plants, collected 14 days after setting up the treatments (DAP), were taken apart and weighed for fresh biomass determination and then dried in a forced-air oven at 65°C to constant weight to determine dry biomass. The leaf relative water content (RWC) was measured after 14 DAP in the oil palm plants; as well as the water potential (Ψw) of the substrate.

After harvesting the plants and homogenizing the soil, three samples were collected per pot to analyze soil water potential, using the WP4C equipment (Dew Point PotentiaMeter, METER Group, Inc.). Soil samples were placed in the equipment in the accurate reading mode, as indicated by the manufacturer. The results allow the comparison between the different treatments concerning the energy state of the soil water, which refers to the soil water content. After submitting the data for normality analyses using the Shapiro–Wilk test, an statistical analysis was performed by Student's t-test at 5% probability, using GraphPad.

Correlation analysis of differentially expressed miRNAs and mRNAs under two distinct scenarios – salinity and drought stresses

To perform correlation analysis of differentially expressed (DE) miRNAs and their respective putative target genes under two distinct scenarios, we used sets of data having the DE miRNAs and mRNAs from this present study and re-used the respective ones from Salgado et al. (2021). First, to check the data distribution, we used the Data Overview module of the Omics Fusion (Brink et al., 2016), the web platform for integrative analysis of Omics data,[7] and then the Scatter Plot one for the correlation analysis between the sets of data—a pairwise combination of the different molecules and scenarios evaluated. The input data used was the Log_2 (FC) data of the DE miRNAs and the DE target genes obtained from the single-omics analysis.

Results

Morphophysiological responses of young oil palm plants to drought stress

The evapotranspiration rate of the control plants remained high and constant during experiments 1 and 2. Meanwhile, the water deficit caused a gradual reduction in the evapotranspiration rates in the drought-stressed plants, in both experiments (Figures 1A,B). Oil palm plants kept for 14 days under drought stress started to show morphological changes in the leaves, such as yellowing and necrosis of the edge and tip (Figure 1C). The leaf relative water content (RWC) in the oil palm plants subjected to drought stress for 14 days dropped ~50% compared to the control plants. The water potential (Ψw) of the substrate at the end of the experiment averaged 0.19 MPa in the control treatment and −13.60 in the drought-stressed one.

The stressed plants showed a reduction in shoots and roots fresh weight, averaging 87.19 and 61.59 g, respectively. Meanwhile, the control plants averaged 122.18 and 220.34 g, respectively. Such a significant reduction did not happen in either shoot or root dry weight, where stressed plants averaged 36.37 and 40.23 g, compared to an average of 36.85 and 55.44 g, respectively, for the control plants.

The reduction in the Ψw led to a reduction in most of the gas exchange parameters assessed. The net CO_2 assimilation rate (A), the stomatal conductance rates (gs), and the transpiration rate (E), showed a significant reduction of 81.03%, 87.74%, and 86.17%, respectively, in comparison with their respective control plants (Figures 2A–C). The intracellular concentration of CO_2 (Ci) in the drought stressed plants showed a low percentage of reduction

---

[2] https://sourceforge.net/projects/mireap

[3] https://github.com/MikeAxtell/ShortStack

[4] https://github.com/MikeAxtell/strucVis

[5] http://www.mirbase.org/search.shtml

[6] https://bio.tools/psrnatarget

[7] https://fusion.cebitec.uni-bielefeld.de

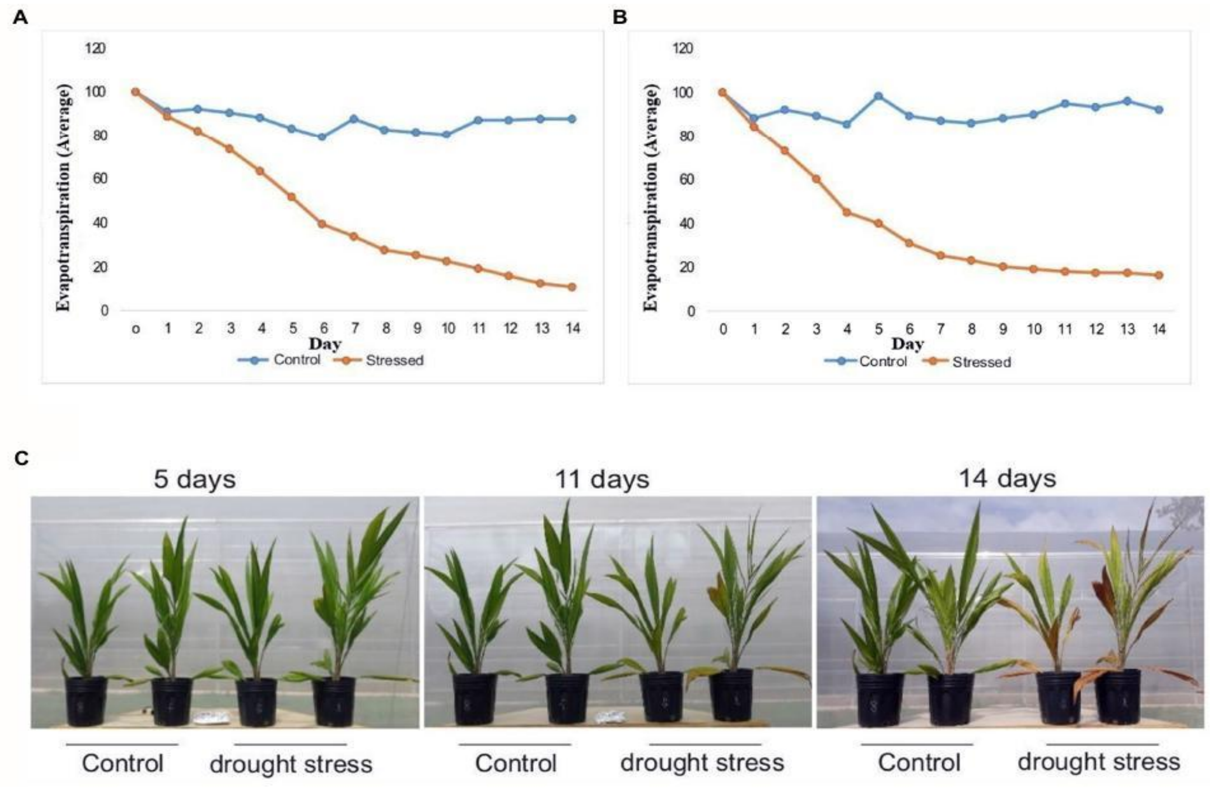

FIGURE 1
Morphophysiological responses of young oil palm plants to drought stress: **(A)** average daily rate of evapotranspiration of irrigated and non-irrigated oil palm plants from experiment 1, **(B)** average daily rate of evapotranspiration of irrigated and non-irrigated oil palm plants from experiment 2, **(C)** morphological changes of oil palm at 5, 11, and 14 days of drought stress. Evapotranspiration (Average): Percentage of the initial value measured in day 0.

(19.28%) when compared to the control, with no statistical difference (Figure 2D).

Identification of known and novel miRNAs and differential expression analysis of miRNAs

The raw reads generated ranged from 14.2 and 57.6 million per sample, presenting an extensive resource for discovering miRNAs (Table 1). After filtering out low-quality reads and removing adapters, the number of remaining clean reads ranged from three and 15 million. A total of 85,871,550 sequences, ranging from 20 to 24 nts in length, was then submitted to further analysis in the Rfam version 12.0 database, in order to remove non-coding RNAs – rRNA, tRNA, snRNA and snoRNA. The miRNA prediction was performed after mapping the remaining 61,666,402 small RNA sequences (Table 1) against the oil palm reference genome and concatenating them into a single file, generating 5,701 positive hits, from which 163 were Y and 5,538 were N15. The miRNA prediction was performed after mapping the remaining 61,666,402 small RNA sequences (Table 1) against the oil palm reference genome and concatenating them into a single file, generating 5,701 positive hits, from which 163 present all necessary characteristics, including the exact miRNA-star, to be annotate as a miRNA (code Y), and 5,538 were classified as a "maybe" (N15), in accordance with miRNA analysis codes from Shortstack, requiring a manual curation.

A total of 81 miRNAs resulted from a manual curatorship evaluating the length of the strings (20–22 nts), predicting the structure of the hairpin by strucVis version 0.4,[8] and annotation by homology in the miRBase database,[9] being 52 conserved miRNAs already reported in other species and 29 oil palm-specific miRNAs (Table 2). It is a fact that plant miRNAs are generally 20 to 22 nt in size, with 23 and 24 being rare (Axtell and Meyers, 2018). The 24 nt sequences represent siRNAs, and none of them was pointed out as a possible miRNA by the Shortstack and mireap programs after the curation process employed in this

---

[8] www.github.com/MikeAxtell/strucVis
[9] www.mirbase.org

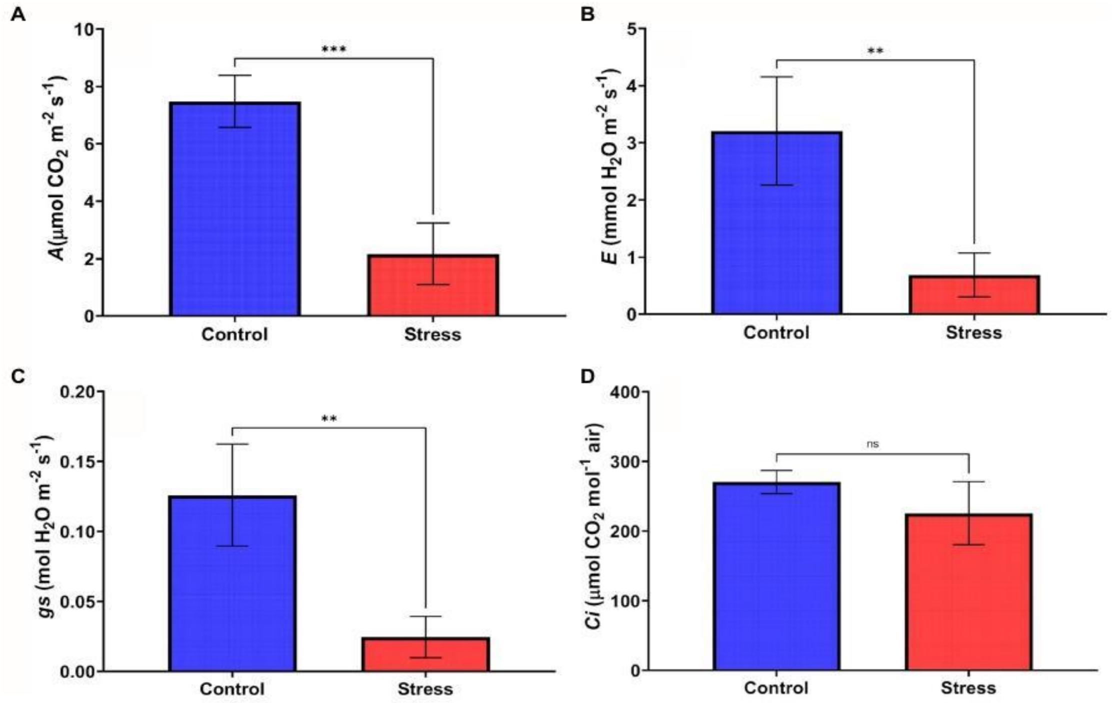

FIGURE 2
Gas exchange rates in leaves of young oil palm plants subjected to 14 days of drought stress in greenhouse conditions: **(A)** net CO_2 assimilation rate – *A*; **(B)** transpiration rate – *E*; **(C)** stomatal conductance – *gs*; and **(D)** intercellular CO_2 concentration – *Ci*. After submitting the gas exchange data to the Kruskal-Wallis test, Dunn's test ($p<0.05$) was applied to data with significant differences between treatments. ns - not significantly different, and ** or *** - significantly different.

TABLE 1 Oil palm sRNA statistics and clean read length distribution.

Samples	Raw reads (%)	After Cutadapt (20–24 nt)	After Rfam (20–24 nt)
Control_R1_Lane1	17,359,260 (100%)	5,701,540 (32.84%)	4,405,869 (25.38%)
Control_R1_Lane2	19,137,482 (100%)	6,283,384 (32.83%)	4,809,227 (25.13%)
Control_R2_Lane1	19,766,560 (100%)	6,304,031 (31.89%)	5,179,368 (26.20%)
Control_R2_Lane2	21,788,523 (100%)	6,965,496 (31.97%)	5,682,583 (26.08%)
Control_R3_Lane1	25,695,279 (100%)	5,769,021 (22.45%)	3,307,121 (12.87%)
Control_R3_Lane2	28,288,139 (100%)	6,370,094 (22.52%)	3,560,220 (12.59%)
Drought_R1_Lane1	23,959,300 (100%)	6,471,468 (27.01%)	4,410,743 (18.41%)
Drought_R1_Lane2	26,514,939 (100%)	7,182,696 (27.09%)	4,824,005 (18.19%)
Drought_R2_Lane1	14,201,378 (100%)	3,063,342 (21.57%)	1,825,359 (12.85%)
Drought_R2_Lane2	15,714,905 (100%)	3,063,342 (19.49%)	1,978,270 (12.59%)
Drought_R3_Lane1	52,289,340 (100%)	13,652,218 (26.11%)	10,366,551 (19.83%)
Drought_R3_Lane2	57,646,162 (100%)	15,044,918 (26.10%)	11,317,086 (19.63%)

study, demonstrating the robustness of the prediction, annotation, and curation process used. Among the 29 new miRNAs, 27 were similar to those already reported by Salgado et al. (2021) in oil palm under salinity stress – egu-miR01sds to egu-miR27sds. The two new miRNAs identified in this study, egu-miR28ds, and egu-miR29ds, have a length of 21 nts and are located in intragenic regions of the *E. guineensis* genome (Table 2). The former is the target GTPase-activating protein GYP7 gene (LOC105043478) and has a length of 144 nts, while the latter is in an uncharacterized protein (LOC105044755) and has 101 nts. The GTPase-activating protein GYP7 gene is 9,498 nts long, has five exons, and code for two protein isoforms in the *E. guineensis* genome. The egu-miR28ds gene is located in the third and longest exon. The uncharacterized LOC105044755, on the other hand, is 2,165 nts long and has no intron, and the egu-miR29ds gene is located pretty much in the middle of it.

TABLE 2 The two new oil palm-specific miRNAs predicted in this study, and their putative target genes predicted using psRNA-Target online program, version 2.

miRNA	**miRNA size**	**Mireap**	**Shortstack**	**miRNA gene size**
egu-miR28ds	21 nt	o	–	144 nt
Location	**Gene ID***	**Gene description**	**miRNA_Aligned Fragment**	
intragenic	LOC105043478	GTPase-activating protein GYP7	UAGUAGUCUCCAAAUCACAUG	
Alignment	**Target Gene_Aligned Fragment**	**Target Gene ID**	**Target Gene Description**	
.:::::::::::::::.::.	5′ UAUGUGAUUUGAAGACUGCUG 3′	LOC105039667	Probable protein-S-isoprenylcysteine O-methyltransferase	
.::::::::::::.::::::	5′ UGUGAAAUUUGGAGGCUACUA 3′	LOC105050641	GBF-interacting protein 1-like	
.::::::::::::.::::::	5′ UGUGAAAUUUGGAGGCUACUA 3′			
.::::::::::::.::::::	5′ UGUGAAAUUUGGAGGCUACUA 3′			
.::::::::::::.::::::	5′ UGUGAAAUUUGGAGGCUACUA 3′			
miRNA	**miRNA size**	**Mireap**	**Shortstack**	**miRNA gene size**
egu-miR29ds	21 nt	o	o	101 nt
Location	**Gene ID***	**Gene description**	**miRNA_Aligned Fragment**	
intragenic	LOC105044755	Uncharacterized – protein coding	UCUCGGGCGGCGACCUCCUCC	
Alignment	**Target Gene_Aligned Fragment**	**Target Gene ID**	**Target Gene Description**	
:::::::::::::::::::::	5′GGAGGAAGUCGCCGCCCGAGA 3′	LOC105054028	Probable xyloglucan galactosyltransferase GT19	
::::::::::::::::::::	5′GGAAGAGGUCGCCGCCGGAGC 3′	LOC109506582	Probable protein phosphatase 2C 8	
::::::::::::::::::::	5′UGAGGCGG-CGCCGCCCGAGA 3′	LOC105041954	Protein FATTY ACID EXPORT 2	
::::::::::::::::::::	5′UGAGGCGG-CGCCGCCCGAGA 3′	LOC105053017	Protein FATTY ACID EXPORT 7	
::::::.:::::::::::.	5′GGAUGCGGUUGCCGCCCGAGG 3′	LOC105041155	EF1A lysine methyltransferase 2	

To avoid the annotation of false-positive miRNAs, we followed the curation criteria suggested by Axtell and Meyers (2018), evaluating the predicted structure of the hairpin of the new miRNAs (Figure 3), both show precise excision of the initial stalk duplex of a precursor RNA, without secondary stalks or large internal loops (greater than five nts), with a processing precision above 94%.

Sixty-two out of the 81 known and novel miRNAs showed a significant (probability $\geq$0.95) different level of expression under drought stress. While miR396e, miR159b, miR529b, egu-miR19sds, and egu-miR29ds were up-regulated, the remaining 57 miRNAs had their expression level reduced, compared to the level in the control treatment (Supplementary Table 1). The ppe-miR397 miRNA appeared in different positions of the genome, with distinct differential expression levels, and, at position NC_025997.1:28868274-28868388, it was down-regulated ($p < 0.97$), while at position NC_025993.1:61023997-61024130 it did not show significant ($p < 0.77$) differential expression (Supplementary Table 1).

Prediction and differential expression analysis of miRNAs-putative target genes

The psRNA-Target online program – version 2[10] – led to 357 positive hits as DE miRNA-putative target genes. Based on the LOC Ids from the oil palm reference genome (Singh et al., 2013), there were 185 distinct putative target genes out of the 357 positive hits. All 62 DE miRNA had two or more putative target genes.

Almost 100% of the high-quality read pairs in all samples mapped to the oil palm reference genome, which had 29,567 genomic features of type "gene" retrieved from 2,781 ref. sequences in GCF_000442705.1_EG5_genomic.fna file; however, 4,115 of these features had no aligned reads detected in any of the samples (Table 3).

When comparing drought-stressed against control plants, the pairwise differential expression analysis revealed that out of the 185 distinct miRNA-putative target genes, 88 differentially expressed at False Discovery Rate (FDR) $\leq$ 0.05; being 44 positively regulated ($\text{Log}_2(\text{FC}) > 0$) and 44 negatively regulated ($\text{Log}_2(\text{FC}) < 0$). The down-regulated miRNA-putative target genes experienced a reduction in the expression level, ranging from 28% to almost 100%. On the other hand, the up-regulated miRNA-putative target genes increased from 1.28X to 166.24X (Table 4).

Integrating the expression profiles from DE miRNA-putative target genes and their respective DE miRNAs

The interaction between DE miRNAs and their putative target genes was investigated using Cytoscape – version 3.8.2,[11] which led to the identification of a total of 102 miRNA-mRNA

---

[10] www.bio.tools/psrnatarget

[11] https://cytoscape.org

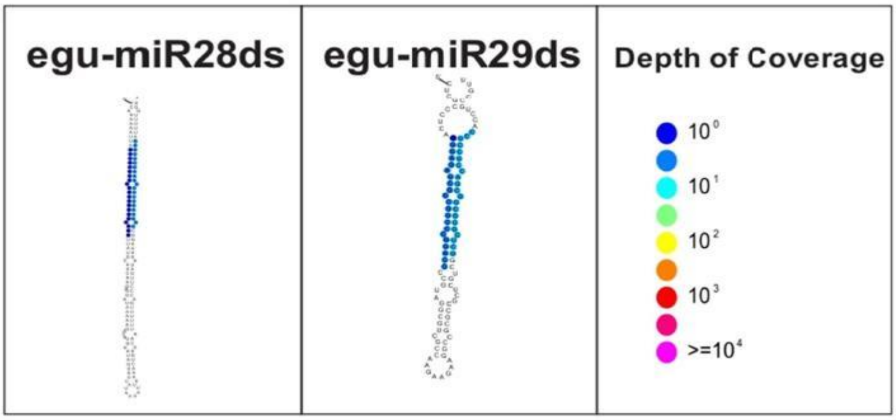

FIGURE 3
Structure of the new miRNAs identified in oil palm under water deficit.

TABLE 3 Statistics of RNA-Seq data from six samples of oil palm plants subjected to two treatments (control and drought stress); three replicates per treatment.

Sample*	Control treatment			Stressed treatment		
	R1	R2	R3	R1	R2	R3
Input high quality read pairs	28,506,037	27,758,299	27,482,363	29,085,207	29,056,879	30,875,128
Aligned reads to feature	15,462,284	23,380,536	14,294,486	17,136,348	20,031,805	25,942,349
Aligned reads to no feature	4,422,784	2,283,704	4,201,816	5,803,528	4,325,704	2,977,275
Ambiguous	230.22	401.78	225.45	199.44	228.05	315.77
Alignment not unique	8,390,751	1,692,282	8,760,612	5,945,893	4,471,325	1,639,731
Low alignment quality	0	0	0	0	0	0
Not aligned	2	2	0	0	0	0

Mapping to reference genome EG5 (BioProject PRJNA192219 and BioSample SAMN02981535) available at NCBI.
*29,567 genomic features of type "gene", retrieved from 2,781 ref sequences in GCF_000442705.1_EG5_genomic.fna & 4,115 features (13.92%) for with no aligned reads was detected in any of the samples.

interactions, involving 62 DE miRNAs and 88 DE mRNAs. The analysis showed that a single miRNA can regulate multiple mRNAs and that a single mRNA can correlate with more than one miRNA, suggesting that the miRNA-mRNA interaction network involved in water stress is highly complex.

By integrating the expression profiles of the miRNA-putative target genes and their related miRNAs, 26 DE miRNA had just one DE putative target gene, 15 had two, eight had three, five had four, and eight had none. On the other hand, among the 88 DE putative target genes, 79 of them had just one DE miRNA, seven had two, one had three, and one had four (Table 4).

miR396e and egu-miR29ds, with a respective increment of 116% and 361%, were up-regulated due to drought stress, while their putative target genes had their expression level reduced to 25% and 71%, respectively (Table 4; Figure 4). LOC105053992 was the putative target gene that experienced the highest increase in expression, 166.24X higher than in the control plant. It experienced regulation by two miRNAs (ama-miR156 and ata-miR156e-5p) that were down-regulated to about 50% of the level in the control plant (Figure 4).

Functional annotation of the differentially expressed target genes

The target genes underwent functional annotation using the InterProScan database. Among the 88 DE target genes selected for functional annotation, 32 had positive hits for biological processes, 50 for molecular function, and 19 for cellular components. Transmembrane transport (GO:0055085) and regulation of transcription by DNA (GO:0006355) had the highest number of biological processes present, five; followed by hormone response processes (GO: 0009725), protein phosphorylation (GO: 0006468), pigment biosynthetic process (GO: 0046148), histone lysine methylation (GO: 0034968), lipid metabolic process (GO: 0006629), oxidation–reduction process (GO: 0055114) and

TABLE 4 Profile of differential expressed miRNA and their differential expressed putative target genes from oil palm plants.

miRNA					Putative target gene				
Name	**EP**	**Probability**	**FC**	**Log_2FC**	**ID**	**EP**	**FC**	**Log_2FC**	**FDR**
ama-miR156	Down	1.00	0.54	−0.89	LOC105053992	Up	166.24	7.38	0.00
ata-miR156e-5p	Down	1.00	0.57	−0.81	LOC105053992	Up	166.24	7.38	0.00
bra-miR168a-5p	Down	1.00	0.25	−2.02	LOC105047586	Up	44.07	5.46	0.00
bra-miR168c-5p	Down	1.00	0.20	−2.33	LOC105047586	Up	44.07	5.46	0.00
ppe-miR397	Down	0.97	0.38	−1.40	LOC105046309	Up	30.64	4.94	0.00
egu-miR02sds	Down	1.00	0.01	−7.42	LOC105042156	Up	28.34	4.82	0.01
ppe-miR397	Down	0.97	0.38	−1.40	LOC105036954	Up	22.40	4.49	0.00
ata-miR528-5p	Down	0.99	0.24	−2.05	LOC105036904	Up	19.12	4.26	0.00
egu-miR20sds	Down	0.97	0.07	−3.91	LOC105046327	Up	14.61	3.87	0.01
ssp-miR827	Down	1.00	0.12	−3.04	LOC105043377	Up	12.69	3.67	0.00
ata-miR167d-5p	Down	1.00	0.31	−1.67	LOC105053405	Up	8.21	3.04	0.00
gma-miR482a-3p	Down	0.99	0.77	−0.38	LOC105043410	Up	7.96	2.99	0.00
egu-miR25sds	Down	0.97	0.23	−2.10	LOC105036224	Up	7.73	2.95	0.00
ata-miR166d-3p	Down	1.00	0.15	−2.78	LOC105046708	Up	6.30	2.66	0.00
atr-miR166b	Down	1.00	0.27	−1.88	LOC105046708	Up	6.30	2.66	0.00
osa-miR166i-3p	Down	1.00	0.13	−2.99	LOC105046708	Up	6.30	2.66	0.00
sly-miR166c-3p	Down	0.99	0.32	−1.63	LOC105046708	Up	6.30	2.66	0.00
ata-miR396e-5p	Down	0.97	0.49	−1.03	LOC105033560	Up	4.72	2.24	0.03
ata-miR160c-5p	Down	1.00	0.13	−2.99	LOC105044300	Up	4.66	2.22	0.00
egu-miR07sds	Down	1.00	0.23	−2.09	LOC105044919	Up	4.34	2.12	0.00
ata-miR160c-5p	Down	1.00	0.13	−2.99	LOC105038384	Up	3.49	1.80	0.00
aof-miR395a	Down	1.00	0.09	−3.45	LOC105041393	Up	3.45	1.79	0.00
atr-miR535	Down	0.99	0.57	−0.82	LOC105051200	Up	3.44	1.78	0.00
mtr-miR2673b	Down	0.99	0.03	−5.21	LOC105060921	Up	3.44	1.78	0.00
mdm-miR171b	Down	1.00	0.34	−1.57	LOC105043623	Up	3.12	1.64	0.00
osa-miR2118p	Down	1.00	0.58	−0.78	LOC105054413	Up	3.05	1.61	0.00
egu-miR04sds	Down	1.00	0.00	−7.79	LOC105040907	Up	2.86	1.51	0.00
bra-miR319-3p	Down	1.00	0.27	−1.89	LOC105032078	Up	2.71	1.44	0.00
atr-miR156c	Down	1.00	0.55	−0.87	LOC105061255	Up	2.67	1.42	0.03
bdi-miR529-5p	Down	1.00	0.24	−2.08	LOC105061255	Up	2.67	1.42	0.03
egu-miR07sds	Down	1.00	0.23	−2.09	LOC105061572	Up	2.59	1.37	0.00
osa-miR2118p	Down	1.00	0.58	−0.78	LOC105054674	Up	2.50	1.32	0.00
egu-miR18sds	Down	0.97	0.32	−1.63	LOC105055689	Up	2.45	1.29	0.00
egu-miR15sds	Down	0.99	0.32	−1.64	LOC105044088	Up	2.41	1.27	0.00
egu-miR05sds	Down	1.00	0.12	−3.09	LOC105045735	Up	2.40	1.26	0.00
egu-miR10sds	Down	1.00	0.15	−2.70	LOC105043130	Up	1.78	0.83	0.00
ata-miR528-5p	Down	0.99	0.24	−2.05	LOC105055547	Up	1.77	0.82	0.00
aof-miR395a	Down	1.00	0.09	−3.45	LOC105038678	Up	1.67	0.74	0.00
aof-miR391	Down	0.99	0.09	−3.53	LOC105045520	Up	1.63	0.71	0.00
egu-miR11sds	Down	0.99	0.08	−3.62	LOC105050858	Up	1.63	0.70	0.00
egu-miR10sds	Down	1.00	0.15	−2.70	LOC105034850	Up	1.62	0.69	0.00
ssp-miR827	Down	1.00	0.12	−3.04	LOC105034273	Up	1.60	0.68	0.00
egu-miR02sds	Down	1.00	0.01	−7.42	LOC105047587	Up	1.60	0.68	0.01
ata-miR169i-3p	Down	1.00	0.07	−3.92	LOC105043671	Up	1.60	0.68	0.00
ata-miR528-5p	Down	0.99	0.24	−2.05	LOC105034839	Up	1.52	0.61	0.00
ata-miR169i-3p	Down	1.00	0.07	−3.92	LOC105060868	Up	1.47	0.55	0.00
egu-miR08sds	Down	1.00	0.11	−3.22	LOC105042656	Up	1.45	0.53	0.00
bra-miR168a-5p	Down	1.00	0.25	−2.02	LOC105051551	Up	1.39	0.48	0.01
bra-miR168a-5p	Down	1.00	0.25	−2.02	LOC105042722	Up	1.28	0.36	0.04

(Continued)

TABLE 4 (Continued)

miRNA					**Putative target gene**				
Name	**EP**	**Probability**	**FC**	**Log_2FC**	**ID**	**EP**	**FC**	**Log_2FC**	**FDR**
ssp-miR827	Down	1.00	0.12	−3.04	LOC105054157	Down	0.00	−7.75	0.00
ata-miR169i-3p	Down	1.00	0.07	−3.92	LOC105051537	Down	0.03	−5.26	0.00
ata-miR399a-3p	Down	1.00	0.01	−6.21	LOC105035561	Down	0.03	−5.13	0.00
ata-miR395c-3p	Down	1.00	0.08	−3.64	LOC105048606	Down	0.04	−4.60	0.00
egu-miR20sds	Down	0.97	0.07	−3.91	LOC105032247	Down	0.06	−4.18	0.00
egu-miR08sds	Down	1.00	0.11	−3.22	LOC105048722	Down	0.14	−2.82	0.00
atr-miR159	Down	1.00	0.23	−2.13	LOC105043705	Down	0.16	−2.65	0.00
egu-miR05sds	Down	1.00	0.12	−3.09	LOC105041381	Down	0.16	−2.61	0.00
egu-miR03sds	Down	0.99	0.48	−1.05	LOC105059001	Down	0.17	−2.58	0.00
aof-miR391	Down	0.99	0.09	−3.53	LOC105039220	Down	0.17	−2.57	0.00
mes-miR399f	Down	1.00	0.01	−6.62	LOC105052027	Down	0.20	−2.29	0.00
egu-miR09sds	Down	1.00	0.21	−2.22	LOC105041147	Down	0.22	−2.21	0.00
ata-miR396e-5p	Down	0.97	0.49	−1.03	LOC105033558	Down	0.25	−2.02	0.00
ata-miR528-5p	Down	0.99	0.24	−2.05	LOC105048962	Down	0.29	−1.77	0.00
ata-miR169i-3p	Down	1.00	0.07	−3.92	LOC105047549	Down	0.31	−1.71	0.03
ata-miR390-5p	Down	0.98	0.22	−2.20	LOC105053593	Down	0.31	−1.70	0.00
bdi-miR169c-3p	Down	1.00	0.08	−3.67	LOC105046060	Down	0.33	−1.61	0.03
gma-miR482a-3p	Down	0.99	0.77	−0.38	LOC105058639	Down	0.33	−1.58	0.00
egu-miR01sds	Down	1.00	0.16	−2.60	LOC105055591	Down	0.34	−1.56	0.00
egu-miR04sds	Down	1.00	0.00	−7.79	LOC105052116	Down	0.36	−1.47	0.00
ata-miR167d-5p	Down	1.00	0.31	−1.67	LOC105049211	Down	0.38	−1.41	0.00
aof-miR395a	Down	1.00	0.09	−3.45	LOC105042288	Down	0.38	−1.41	0.00
vvi-miR828a	Down	0.96	0.58	−0.78	LOC105051514	Down	0.41	−1.28	0.00
ata-miR396e-5p	Down	0.97	0.49	−1.03	LOC105052992	Down	0.44	−1.20	0.00
ata-miR396e-5p	Down	0.97	0.49	−1.03	LOC105042167	Down	0.48	−1.05	0.00
egu-miR11sds	Down	0.99	0.08	−3.62	LOC105056373	Down	0.49	−1.02	0.00
egu-miR26sds	Down	0.97	0.13	−2.91	LOC105056373	Down	0.49	−1.02	0.00
mdm-miR171b	Down	1.00	0.34	−1.57	LOC105059511	Down	0.49	−1.02	0.00
egu-miR18sds	Down	0.97	0.32	−1.63	LOC105031985	Down	0.50	−1.01	0.00
bdi-miR530b	Down	0.99	0.21	−2.23	LOC105055997	Down	0.50	−1.00	0.00
mdm-miR171b	Down	1.00	0.34	−1.57	LOC105054013	Down	0.53	−0.92	0.00
mdm-miR171b	Down	1.00	0.34	−1.57	LOC105054987	Down	0.58	−0.79	0.00
egu-miR15sds	Down	0.99	0.32	−1.64	LOC105050166	Down	0.58	−0.78	0.01
vvi-miR828a	Down	0.96	0.58	−0.78	LOC105043777	Down	0.59	−0.77	0.00
vvi-miR828a	Down	0.96	0.58	−0.78	LOC105039801	Down	0.61	−0.70	0.00
egu-miR11sds	Down	0.99	0.08	−3.62	LOC105049000	Down	0.62	−0.69	0.01
egu-miR03sds	Down	0.99	0.48	−1.05	LOC105048718	Down	0.63	−0.66	0.00
egu-miR08sds	Down	1.00	0.11	−3.22	LOC105049925	Down	0.63	−0.66	0.00
egu-miR03sds	Down	0.99	0.48	−1.05	LOC105060476	Down	0.67	−0.58	0.00
egu-miR07sds	Down	1.00	0.23	−2.09	LOC105058953	Down	0.68	−0.56	0.02
egu-miR22sds	Down	0.99	0.06	−4.18	LOC105060538	Down	0.68	−0.56	0.02
ata-miR399a-3p	Down	1.00	0.01	−6.21	LOC105047412	Down	0.68	−0.56	0.00
mes-miR399f	Down	1.00	0.01	−6.62	LOC105047412	Down	0.68	−0.56	0.00
aof-miR395a	Down	1.00	0.09	−3.45	LOC105041383	Down	0.71	−0.50	0.00
osa-miR2118p	Down	1.00	0.58	−0.78	LOC105044139	Down	0.72	−0.48	0.02
cme-miR396e	Up	0.97	2.16	1.11	LOC105033558	Down	0.25	−2.02	0.00
egu-miR29ds	Up	0.97	4.61	2.20	LOC105053017	Down	0.71	−0.49	0.01
cme-miR396e	Up	0.97	2.16	1.11	LOC105033560	Up	4.72	2.24	0.03

(Continued)

TABLE 4 (Continued)

miRNA					**Putative target gene**				
Name	**EP**	**Probability**	**FC**	**Log_2FC**	**ID**	**EP**	**FC**	**Log_2FC**	**FDR**
egu-miR19sds	Up	0.95	1.45	0.54	LOC105049219	Up	3.62	1.86	0.00
cpa-miR159b	Up	0.97	2.32	1.21	LOC105032078	Up	2.71	1.44	0.00
osa-miR529b	Up	0.98	3.28	1.71	LOC105061255	Up	2.67	1.42	0.03

False discovery rate (FDR), Identity in the reference genome EG5 at NCBI (ID), and fold change (FC). EP, Expression profile; FC, Fold Change (Drought/Control).

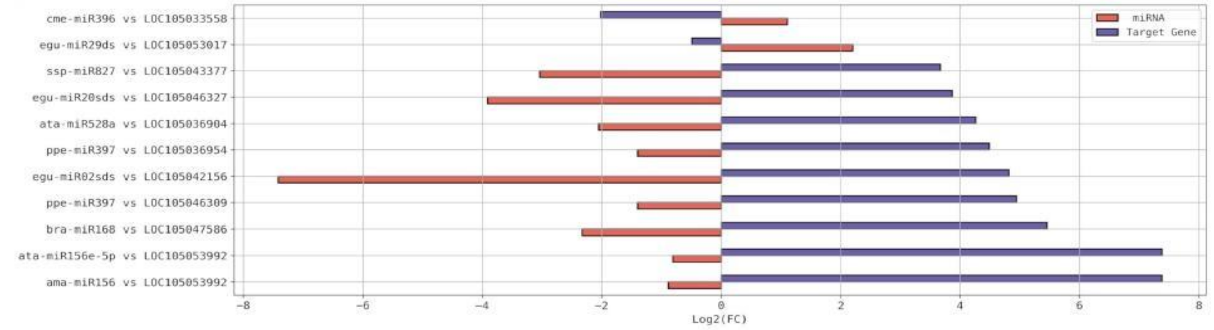

FIGURE 4
Expression profiles in Log_2(FC) of the differentially expressed (DE) miRNAs and their respective DE putative target gene(s), resulted from submission of young oil palm plants to drought stress. FC, Fold Change.

histone methylation (GO: 0016571), with two each (Supplementary Table 2).

For molecular functions, the GO terms that appeared the most were protein binding (GO: 0005515), with nine occurrences, followed by DNA binding (GO: 0003677), with eight, and ATP binding (GO: 0005524), with seven. The cellular component most frequently present was the nucleus (GO: 0005634), with seven occurrences, followed by the membrane (GO: 0016020), with six, and the integral membrane component (GO: 0016021), with four (Supplementary Table 2).

When analyzing the most expressed domains, TF_GRAS (IPR005202) came first with 4, followed by SPX domain (IPR004331) with three, and SET_dom (IPR001214), Tyrosinase_Cu-bd (IPR002227), Protein kinase domain (IPR000719), SANT/Myb (IPR001005), and SBP_dom (IPR004333) with two hits each. MFS family, with three members, was the largest one, followed by the ABC, Polyphenol_oxidase, and Hist-Lys_N-MeTrfase_plant families with two members each (Supplementary Table 2).

To further characterize the putative target genes, the LOC numbers were used to identify the ID of the protein coded by them. Nine LOC numbers were from genes coding for ncRNAs, and the remaining 79 led to the identification of 138 proteins (distinct XP id). These 138 proteins were submitted to analysis in the GhostKOALA platform (Kanehisa et al., 2016), and in the EggNOG v5.0 platfom (Huerta-Cepas et al., 2019; Cantalapiedra et al., 2021). The results of the hierarchical, functional and phylogenetic annotation using the EggNOG platform, having the Liliopsida class as taxonomic scope, is presented in Supplementary Table 3; only 132 out of the 138 proteins were annotated. Only 44 proteins were annotated in the GhostKOALA platform, being 11 transcription factors from three families – MYB, HOX, and NF-Y.

Correlation analyses of DE miRNA-putative target genes and their respective DE miRNAs

First, 72 miRNAs differentially expressed in young oil palm plants under salinity stress (Salgado et al., 2021) were submitted to correlation analysis against 62 from the present study (Figure 5A). Then, 51 DE miRNA-putative target genes from Salgado et al. (2021) underwent correlation analysis against the 88 from the present study (Figure 5B). These results revealed eight miRNA-putative target genes upregulated in both scenarios, salinity and drought stress (Figure 5B), that also had the miRNAs targeting them down-regulating in those scenarios (Figure 5A). Among them, there were three genes expressing lncRNAs, two coding for proteins from the Major facilitator superfamily, one for a putative histone-modifying enzyme harboring a demethylase

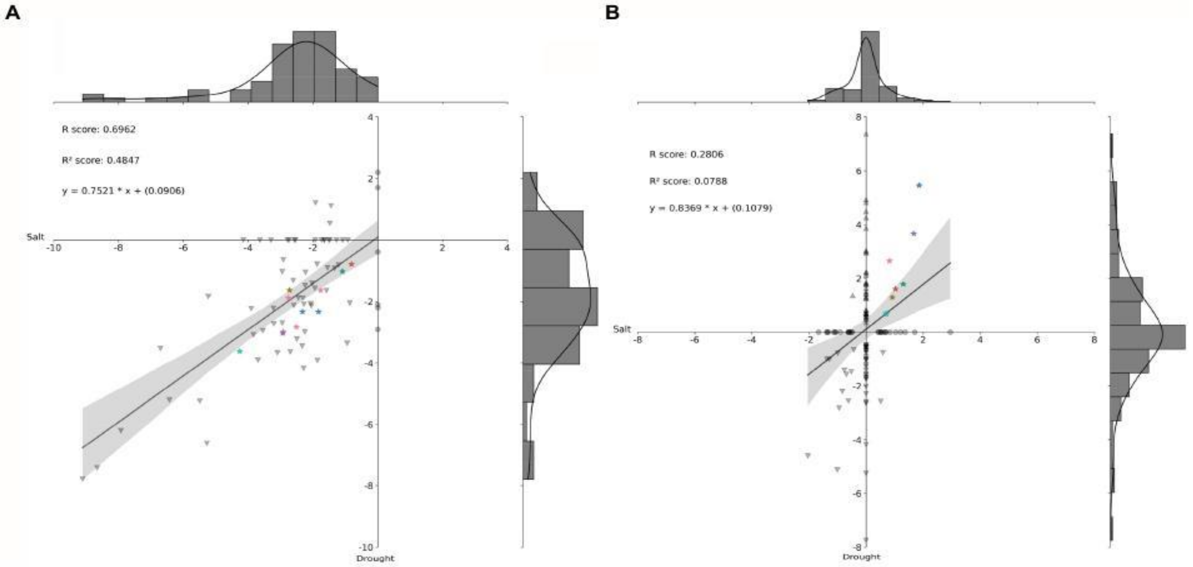

FIGURE 5
Histogram and correlation analysis of the Log_2 (FC) of differentially expressed (DE) miRNAs **(A)** and their respective DE putative target genes **(B)**, by pairwise comparison of two scenarios: Salt stress and Drought stress. Stars of same color identify miRNAs (in A) and their respective putative target genes upregulated in both stresses (in B). FC, Fold Change.

activity, one for a protein from the class III homeodomain-leucine zipper family, and one for a protein from the Macrophage migration inhibitory factor (MIF) family.

Discussion

Palms are considered the third most important economic group of plants, second only to grasses and legumes (Meerow et al., 2012). miRNAs have been described as master regulators of gene expression and involved in controlling growth and development and plant responses to different stresses (Liu et al., 2008; Ding et al., 2009; Djami-Tchatchou et al., 2017). Still, little information on palm miRNA is available (Md Nasaruddin et al., 2007; Low et al., 2014; da Silva et al., 2016; Ho et al., 2017; Zheng et al., 2019; Salgado et al., 2021), and none of them studied the miRNAs involved in the response of oil palm plants to drought stress. Thus, this is the first report of prospecting oil palm miRNAs and analyzing their expression profile together with their target genes under water deprivation.

The reduction of *gs* and Ψw seen in the present study, inducing an abrupt stomatal closure, is a shred of evidence that oil palm has a high sensitivity to drought. It suggests that mechanisms for water control mediate the decrease in *gs*; in other words, that water deprivation is sensed by the roots, triggering the production of abscisic acid (ABA), which results in stomatal closure mediated by a transduction cascade (Chaves et al., 2009; Cutler et al., 2010; Silva et al., 2016). The effects of *A* reduction during water deficit (Figure 2A) may be related to the increase in diffusive (stomatal and mesophilic restrictions) and biochemical limitations (Chaves et al., 2009; Flexas et al., 2012).

This study identified 81 miRNAs, where 52 miRNAs are deeply conserved among various plant species, such as *A. thaliana* (Liu et al., 2008), *O. sativa* (Zhou et al., 2010), *Populus trichocarpa* (Lu et al., 2008), and *M. truncatula* (Wang et al., 2011). Some of them, miR156, miR160, miR166, miR167, miR168, miR172, miR396, miR528, and miR535, have already been found in oil palm by Ho et al. (2017) in floral meristems, while Fang et al. (2013) identified miR156, miR395, and miR528 in the mesocarp of oil palm fruits. Also, Md Nasaruddin et al. (2007) identified miR156, miR159, and miR160 in oil palm's apical meristem and immature and mature flowers. The other 29 miRNAs are specific for oil palm (Table 3), from which 27 have been already reported in our previous study (Salgado et al., 2021) – regarding oil palm responses to salinity stress, and two (egu-miR28ds and egu-miR29ds) reported for the first time.

Sixty-two out of the 81 miRNAs showed significant differential expression. While five of them up-regulated under drought stress, the remaining 57 were down-regulated (Table 4). That is a behavior similar to the one seen usually in genes encoding proteins – meaning that a miRNA expression is also up-or down-regulated in response to stress (Fang et al., 2013), as also shown for *A. thaliana* (Liu et al., 2008), *O. sativa* (Zhou et al., 2010), *P. trichocarpa* (Lu et al., 2008), *M. truncatula* (Wang et al., 2011), among others.

A series of transcription factor (TFs) putative genes were also targeted by miRNAs in such plants when submitted to drought stress, as already seen in young oil palm plants under salinity stress (Salgado et al., 2021). TFs, such as auxin response factors (ARFs), squamosa promoter binding proteins, ethylene response transcription factors, MYB transcription factor, homeobox-leucine protein zipper HOX, transcription factor GAMYB, and nuclear transcription factor Y, which are responsible for regulating plant growth and development (Wu and Poethig, 2006; Gandikota et al., 2007), were predicted as putative miRNA-target genes in oil palm under water deficit in this study.

Most of these miRNAs targeting TFs were down-regulated (miR156, miR160c-5p, miR166d-3p, miR169i-3p, miR156c, miR166b, miR529-5p, miR319-3p, egu-miR02sds, egu-miR07sds, egu-miR11sds, egu-miR26sds, miR171b, miR166i-3p, miR166c-3p), indicating that the decrease in these miRNAs will increase the expression of some corresponding transcription factors, thus promoting the activation of a set of encoding genes to play defensive roles against abiotic stresses. This behavior is present in other species under water deficit (Liu et al., 2008; Zhou et al., 2010).

The plant response to drought stress is very similar to the initial plant response to salinity stress, and many common occurrences between these two primary stresses are also expected (Uddin et al., 2016). Salgado et al. (2021) observed that miRNAs miR166, miR169, miR319, miR396, and miR529 shows reduced expression profiles in young oil palm plants subjected to salt stress, a similar behavior to what happened in oil palm plants under drought stress. It is proved, through functional analysis, that these miRNAs regulate the levels of transcription of TFs, thus affecting the levels of TF proteins.

miR529 in the leaves of oil palm plants under salinity (Salgado et al., 2021) and drought stress showed a reduction of 80% and 76% in expression levels, respectively. Both showed up-regulation of their putative target gene – SBP (squamosa promoter-binding-like protein) – by ~70%. Squamosa promoter binding proteins are putative transcription factors with a plant-specific SBP domain consisting of 76 amino acids in length responsible for regulating various biological processes, including drought and saline stress. According to studies carried out by Hou et al. (2018), the overexpression of the homologous SBP16 gene from grapes (*Vitis vinifera*) in *A. thaliana* promoted an increase in tolerance to drought and salinity stresses during seed germination, as well as in seedlings and mature plants, regulating the signaling cascades of SOS and ROS.

egu-miR02sds showed a 99% reduction in its expression level in plants under drought stress, promoting a 60% increase in the expression of its putative target gene NF-YB (LOC105047587 – nuclear transcription factor Y subunit B-4-like), a similar behavior shown when under salt stress (Salgado et al., 2021). The Nuclear factor Y (NF-Y) is a ubiquitous transcription factor with high affinity and sequence specificity for the CCAAT box, a cis-element present in about 25% of eukaryotic gene promoters. NF-Y is a heterotrimeric complex composed of three distinct subunits (NF-YA, NF-YB, and NF-YC; Mantovani, 1999). Studies shows that NF-Y is responsible for activating a transcriptional cascade critical for drought resistance, where its overexpression improves resistance and promotes drought tolerance in arabidopsis and maize (Nelson et al., 2007; Liu et al., 2008).

According to Li et al. (2008), overexpression of NF-YA5 in arabidopsis reduced water loss in leaves and resulted in a resistance to water stress higher than the wild type. Nelson et al. (2007) showed that NF-YB overexpression promoted drought tolerance in maize, based on the responses of several stress-related parameters, including chlorophyll content, stomatal conductance, leaf temperature, reduced wilting, and maintenance of photosynthesis. Thus, NF-Y is crucial for expressing a series of genes responsive to drought stress, and its induction takes place at both transcriptional and post-transcriptional levels (Li et al., 2008; Liu et al., 2008).

The miRNAs miR168, miR395, miR396, miR397 were also down-regulated and are directly involved in the stress response process or stress tolerance (Sunkar et al., 2005; Gray-Mitsumune and Matton, 2006). miR168 regulates the *Argonaute 1* (AGO1) gene in *A. thaliana* and *P. trichocarpa* (Lu et al., 2005). AGO1 proteins can be directly associated with siRNAs / miRNAs before and after recognizing their mRNA targets and are required for normal plant development (Vaucheret et al., 2004). In the present study, miR168 expression level experienced a 75% reduction in drought-stressed plants, while its putative target gene (LOC105042722 – protein argonaute 1A-like) up-regulated 28%.

The present study led us to identify two new oil palm-specific miRNAs and enabled a comparative analysis of expression levels with miRNAs in oil palm reported under salt stress (Salgado et al., 2021). Comparative studies of miRNAs can help to increase our understanding, at a regulatory level, of the events that give rise to new species or the emergence of specific characteristics. The correlation analysis done in this present study showed that lncRNAs might play some role in the response of young oil palm plants to both drought and salinity stresses. Long non-coding RNAs (lncRNAs) are >200 nucleotides-long RNAs that are not translated into functional proteins but have cellular functions of a structural and/or regulatory nature (Statello et al., 2021). Accordingly to Jha and colleagues, lncRNAs play an essential role in plant adaptation to various abiotic stresses, such as drought, heat, cold, heavy metal toxicity, and nutrient deficiency (Jha et al., 2020).

The Major Facilitator Superfamily (MFS) is the largest group of secondary active membrane transporters that shows a much larger number of genes in plant genomes than in bacteria, yeast, or animals (Niño-González et al., 2019). Again, the correlation analysis done in this present study showed that an MFS gene plays a role in the response of young oil palm plants to both abiotic stresses under consideration. Genes from the MFS are involved in plant response to abiotic stress in arabidopsis, mediating drought and salt tolerance (Remy et al., 2013; Wang, D. et al., 2020).

Finally, regarding the remaining three proteins shown to be potentially playing a role in the response of young oil palm plant to drought and salinity stresses – a putative histone-modifying enzyme harboring a demethylase activity, a protein from the class III homeodomain-leucine zipper family, and a protein from the MIF family, reports are there linking them to stress memory (Bhadouriya et al., 2021), regulation of defense response to abiotic and biotic stresses (Sharif et al., 2021), and modulation of ROS signaling (Zhao et al., 2021).

Many studies are available where the main objective is to compare the effects of those or more two types of stresses on plant growth and development (Chaves et al., 2009; de Oliveira et al., 2013; Uddin et al., 2016; Ma et al., 2020). This present study allowed us to visualize some of the similarities and dissimilarities – regarding expression analysis of miRNAs and their putative target genes – young oil palm plants have with the response to two highly important environmental stresses, drought and salinity. As far as we know, this is the first time such type of study is done in oil palm.

The present study and our previous one (Salgado et al., 2021) used plants with the same genetic background (clones from the same plant), the same age (young oil palm plants), almost similar duration of stress (12 and 14 days, respectively for salinity and drought stress), same omics platform (transcriptome of mRNAs and smallRNAs), and the same group of analytical tools. All these commonalities between these two studies allowed us analyze the similarities and dissimilarities regarding expression analysis of miRNAs and their putative target genes, what it a valuable set of information to help us in the search for genes that can be used to promote future attempts to horizontally transfer tolerance at once to both stresses; not only to oil palm, but also other plant species (Patel et al., 2019; Chaudhary et al., 2021).

Conclusion

This study characterized the miRNA population and their miRNA-target genes present in the leaves of young oil palm plants exposed to drought stress, besides it performed correlation analysis of the miRNAs and their target genes differentially expressed under drought and salinity stresses (from Salgado et al., 2021). Together, those activities resulted in:

(a) The identification of two new miRNAs that received the names egu-miR28ds and egu-miR29ds, where egu is the abbreviation of *Elaeis guineensis* and ds stands for drought stress;
(b) The prediction of 185 distinct genes as the targets to the 81 miRNAs in the genome of oil palm and a total of 102 miRNA-mRNA interactions involving 62 DE miRNAs and 88 DE mRNAs;
(c) Among the 88 DE target genes selected for functional annotation, 32 had positive hits for biological processes, 50 for molecular function, and 19 for cellular components; and.
(d) Eight miRNA-putative target genes – upregulated under salinity as well as drought stress – that code for lncRNAs, proteins from the Major facilitator superfamily, a putative histone-modifying enzyme harboring a demethylase activity, a protein from the class III homeodomain-leucine zipper family, and a protein from the Macrophage migration inhibitory factor (MIF) family.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, The smallRNA raw sequence data used in this study have been uploaded in the SRA database of the NCBI under Elaeis guineensis microRNA_Drought and Salinity Stresses – BioProject PRJNA646488 (SUB7775347), BioSample SAMN12799239 (SUB6325749), SRA submission SUB7897143 (accessions from SRR12424937 to SRR12424945). All RNA-seq fastq files used in this study have been uploaded in the SRA database of the NCBI under Elaeis guineensis Transcriptome_Drought and Salinity Stresses – BioProject PRJNA573093 (SUB6324604), BioSample SAMN12799239 (SUB6325749), SRA submission SUB6335775 (accessions from SRR10219424 to SRR10219441). The data-sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

CS and MS designed the study. FS, TS, LV, VS, and AL performed the experiments and generated the data. FS, PG, MC, RT, CS, and MS analyzed the data. FS, PG, CS, and MS wrote first draft of the manuscript, which was extensively edited and approved the submitted version by all authors. MS was responsible for the funding acquisition, project administration, and group supervision. All authors contributed to the article and approved the submitted version.

Funding

The grant (01.13.0315.00—DendePalm Project) for this study was awarded by the Brazilian Ministry of Science, Technology, and Innovation (MCTI) *via* the Brazilian Research and Innovation Agency (FINEP). The authors confirm that the funder had no influence over the study design, the content of the article, or the selection of this journal.

Acknowledgments

The authors acknowledge funding to FS, TS, LV, and VS by the Coordination for the Improvement of Higher Education Personnel (CAPES), *via* the Graduate Program in Plant Biotechnology at the Federal University of Lavras (UFLA).

Conflict of interest

AL, MC, RT, CS, PG, and MS were employed by The Brazilian Agricultural Research Corporation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.970113/full#supplementary-material

References

Abrapalma (2018). Retrospecto e Projeções da Palma de Óleo No Brasil. Available at: http://www.abrapalma.org/pt/retrospecto-e-projecoes-da-palma-de-oleo-no-brasil-2018-2019/.

Anders, S., Pyl, P. T., and Huber, W. (2015). HTSeq–a python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166–169. doi: 10.1093/bioinformatics/btu638

Andrews, S. (2018). FastQC: A quality control tool for high Thoughput sequence 2010. Data. Available at: from https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Retrieved 2018).

Axtell, M. J., and Meyers, B. C. (2018). Revisiting criteria for plant micro RNA annotation in the era of big data. *Plant Cell* 30, 272–284. doi: 10.1105/tpc.17.00851

Azzeme, A. M., Abdullah, S. N. A., Aziz, M. A., and Wahab, P. E. M. (2016). Oil palm leaves and roots differ in physiological response, antioxidant enzyme activities and expression of stress-responsive genes upon exposure to drought stress. *Acta Physiol. Plant.* 38:52. doi: 10.1007/s11738-016-2073-2

Bhadouriya, S. L., Mehrotra, S., Basantani, M. K., Loake, G. J., and Mehrotra, R. (2021). Role of chromatin architecture in plant stress responses: an update. *Front. Plant Sci.* 11:603380. doi: 10.3389/fpls.2020.603380

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Brink, B. G., Seidel, A., Kleinbölting, N., Nattkemper, T. W., and Albaum, S. P. (2016). Omics fusion – a platform for integrative analysis of omics data. *J. Integr. Bioinform.* 13, 43–46. doi: 10.1515/jib-2016-296

Cantalapiedra, C. P., Hernández-Plaza, A., Letunic, I., Bork, P., and Huerta-Cepas, J. (2021). eggNOG-mapper v2: functional annotation, Orthology assignments, and domain prediction at the metagenomic scale. *Mol. Biol. Evol.* 38, 5825–5829. doi: 10.1093/molbev/msab293

Chaudhary, S., Grover, A., and Sharma, P. C. (2021). MicroRNAs: potential targets for developing stress-tolerant crops. *Life (Basel, Switzerland)* 11:289. doi: 10.3390/life11040289

Chaves, M. M., Flexas, J., and Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann. Bot.* 103, 551–560. doi: 10.1093/aob/mcn125

Chen, X. (2004). A MicroRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science* 303, 2022–2025. doi: 10.1126/science.1088060

Chen, G., Zou, Y., Hu, J., and Ding, Y. (2018). Genome-wide analysis of the Rice PPR gene family and their expression profiles under different stress treatments. *BMC Genomics* 19:720. doi: 10.1186/s12864-018-5088-9

Comai, L., and Zhang, B. (2012). MicroRNAs: key gene regulators with versatile functions. *Plant Mol. Biol.* 80:1. doi: 10.1007/s11103-012-9947-5

Corley, R. H. V. (2009). How much palm oil do we need? *Environ. Sci. Pol.* 12, 134–139. doi: 10.1016/j.envsci.2008.10.011

Corley, R. H. V., Rao, V., Palat, T., and Praiwan, T. (2018). Breeding for drought tolerance in oil palm. *J. Oil Palm Res.* 30, 26–35.

Corrêa, T. R., Motoike, S. Y., Coser, S. M., DA Silveira, G., De Resende, M. D. V., and Chia, G. S. (2015). Estimation of genetic parameters for *in vitro* oil palm characteristics (*Elaeis guineensis* Jacq.) and selection of genotypes for cloning capacity and oil yield. *Ind. Crop. Prod.* 77, 1033–1038. doi: 10.1016/j.indcrop.2015.09.066

Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R., and Abrams, S. R. (2010). Abscisic acid: emergence of a Core signaling network. *Annu. Ver. Plant Biol* 61, 651–679. doi: 10.1146/annurev-arplant-042809-112122

da Silva, A. C., Grativol, C., Thiebaut, F., Hemerly, A. S., and Ferreira, P. C. (2016). Computational identification and comparative analysis of miRNA precursors in three palm species. *Planta* 243, 1265–1277. doi: 10.1007/s00425-016-2486-6

de Oliveira, A. B., Alencar, N. L. M., and Gomes-Filho, E. (2013). "Comparison between the water and salt stress effects on plant growth and development," in *Responses of Organisms to Water Stress* (IntechOpen)

Denli, A. M., Tops, B. B. J., Plasterk, R. H. A., Ketting, R. F., and Hannon, G. J. (2004). Processing of primary microRNAs by the microprocessor complex. *Nature* 432, 231–235. doi: 10.1038/nature03049

Ding, D., Zhang, L., Wang, H., Liu, Z., Zhang, Z., and Zheng, Y. (2009). Differential expression of miRNAs in response to salt stress in maize roots. *Ann. Bot.* 103, 29–38. doi: 10.1093/aob/mcn205

Djami-Tchatchou, A. T., Sanan-Mishra, N., Ntushelo, K., and Dubery, I. A. (2017). Functional roles of microRNAs in Agronomically important plants-potential as targets for crop improvement and protection. *Front. Plant Sci.* 8:378. doi: 10.3389/fpls.2017.00378

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635

EPOA (2020). Palm oil production. Available at: https://www.palmoilandfood.eu/en/palm-oil-production

Fang, L., Liang, Y., Li, D., Cao, X., and Zheng, Y. (2013). Dynamic expression analysis of miRNAs during the development process of oil palm mesocarp. *Plant Sci.* 31, 304–312. doi: 10.3724/SP.J.1142.2013.30304

Flexas, J., Barbour, M. M., Brendel, O., Cabrera, H. M., Carriquí, M., Díaz-Espejo, A., et al. (2012). Corrigendum to 'mesophyll diffusion conductance to CO2: an unappreciated central player in photosynthesis. *Plant Sci.* 196:31. doi: 10.1016/j.plantsci.2012.08.001

Gandikota, M., Birkenbihl, R. P., Hohmann, S., Cardon, G. H., Saedler, H., and Huijser, P. (2007). The miRNA156/157 recognition element in the 3′ UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings. *Plant J.* 49, 683–693. doi: 10.1111/j.1365-313X.2006.02983.x

Gray-Mitsumune, M., and Matton, D. P. (2006). The egg apparatus 1 gene from maize is a member of a large gene family found in both monocots and dicots. *Planta* 223, 618–625. doi: 10.1007/s00425-005-0174-z

Ho, H., Gudimella, R., Ong-Abdullah, M., and Harikrishna, J. A. (2017). Expression of microRNAs during female inflorescence development in African oil palm (*Elaeis guineensis* Jacq.). *Tree Genet. Genomes* 13:35. doi: 10.1007/s11295-017-1120-5

Hou, H., Jia, H., Yan, Q., and Wang, X. (2018). Overexpression of a SBP-box gene (VpSBP16) from Chinese wild Vitis species in Arabidopsis improves salinity and drought stress tolerance. *Int. J. Mol. Sci.* 19:940. doi: 10.3390/ijms19040940

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S. K., Cook, H., et al. (2019). eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res.* 47, D309–D314. doi: 10.1093/nar/gky1085

Jha, U. C., Nayyar, H., Jha, R., Khurshid, M., Zhou, M., Mantri, N., et al. (2020). Long non-coding RNAs: emerging players regulating plant abiotic stress response and adaptation. *BMC Plant Biol.* 20:466. doi: 10.1186/s12870-020-02595-x

Kanehisa, M., Sato, Y., and Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and Metagenome sequences. *J. Mol. Biol.* 428, 726–731. doi: 10.1016/j.jmb.2015.11.006

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25. doi: 10.1186/gb-2009-10-3-r25

Li, W. X., Oono, Y., Zhu, J., He, X. J., Wu, J. M., Lida, K., et al. (2008). The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and Posttranscriptionally to promote drought resistance. *Plant Cell* 20, 2238–2251. doi: 10.1105/tpc.108.059444

Liu, H. H., Tian, X., Li, Y. J., Wu, C. A., and Zheng, C. C. (2008). Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA* 14, 836–843. doi: 10.1261/rna.895308

Lopes Filho, W. R. L., Rodrigues, F. H. S., Ferreira, I. V. L., Correa, L. O., Cunha, R. L., and Pinheiro, H. A. (2021). Physiological responses of young oil palm (*Elaeis guineensis* Jacq.) plants to repetitive water deficit events. *Ind. Crop. Prod.* 172:114052. doi: 10.1016/j.indcrop.2021.114052

Low, E. T., Rosli, R., Jayanthi, N., Mohd-Amin, A. H., Azizi, N., Chan, K. L., et al. (2014). Analyses of hypomethylated oil palm gene space. *PLoS One* 9:e86728. doi: 10.1371/journal.pone.0086728

Lu, S., Sun, Y. H., and Chiang, V. L. (2008). Stress-responsive microRNAs in Populus. *Plant J.* 55, 131–151. doi: 10.1111/j.1365-313X.2008.03497.x

Lu, S., Sun, Y. H., Clark, C., Li, L., and Chiang, V. L. (2005). Novel and mechanical stress-responsive MicroRNAs in *Populus trichocarpa* that are absent from Arabidopsis. *Plant Cell* 17, 2186–2203. doi: 10.1105/tpc.105.033456

Lv, D. K., Bai, X., Li, Y., Ding, X. D., Ge, Y., Cai, H., et al. (2010). Profiling of cold-stress-responsive miRNAs in Rice by microarrays. *Gene* 459, 39–47. doi: 10.1016/j.gene.2010.03.011

Lytle, J. R., Yario, T. A., and Steitz, J. A. (2007). Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. *Proc. Natl. Acad. Sci. U. S. A.* 104, 9667–9672. doi: 10.1073/pnas.0703820104

Ma, Y., Dias, M. C., and Freitas, H. (2020). Drought and salinity stress responses and microbe-induced tolerance in plants. *Front. Plant Sci.* 11:591911. doi: 10.3389/fpls.2020.591911

Mallory, A. C., Reinhart, B. J., Jones-Rhoades, M. W., Tang, G., Zamore, P. D., Barton, M. K., et al. (2004). MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5′ region. *EMBO J.* 23, 3356–3364. doi: 10.1038/sj.emboj.7600340

Mantovani, R. (1999). The molecular biology of the CCAAT-binding factor NF-Y. *Gene* 239, 15–27. doi: 10.1016/s0378-1119(99)00368-6

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J*, 17, 10–12. ISSN 2226-6089. Available at: http://journal.embnet.org/index.php/embnetjournal/article/view/200

Md Nasaruddin, N., Harikrishna, K., Othman, R., Hoon, L., and Ann Harikrishna, J. (2007). Computational prediction of microRNAs from oil palm (*Elaeis guineensis Jacq.*) expressed sequence tags. *Asia Pac. J. Mol. Biol. Biotechnol.* 15, 107–113.

Meerow, A. W., Krueger, R. R., Singh, R., Low, E. T. L., Ithnin, M., and Ooi, L. C. L. (2012). "Coconut, date, and oil palm genomics," in *Genomics of Tree Crops*. eds. R. Schnell and P. Priyadarshan (New York, NY: Springer).

Nelson, D. E., Repetti, P. P., Adams, T. R., Creelman, R. A., Wu, J., Warner, D. C., et al. (2007). Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and Lead to improved corn yields on water-limited acres. *Proc. Natl. Acad. Sci. U. S. A.* 104, 16450–16455. doi: 10.1073/pnas.0707193104

Niño-González, M., Novo-Uzal, E., Richardson, D. N., Barros, P. M., and Duque, P. (2019). More transporters, more substrates: the Arabidopsis Major facilitator superfamily revisited. *Mol. Plant* 12, 1182–1202. doi: 10.1016/j.molp.2019.07.003

OmicsBox (2019). Bioinformatics made easy, BioBam bioinformatics, March 3. Available at: https://www.biobam.com/omicsbox

Patel, P., Yadav, K., Ganapathi, T. R., and Penna, S. (2019). "Plant miRNAome: cross talk in abiotic stressful times," in *Genetic enhancement of crops for tolerance to abiotic stress: Mechanisms and approaches, Vol. I. Sustainable Development and Biodiversity*. eds. *vol. 20* V. Rajpal, D. Sehgal, A. Kumar and S. Raina, (Cham: Springer).

Qiu, C.-W., Liu, L., Feng, X., Hao, P.-F., He, X., Cao, F., et al. (2020). Genome-wide identification and characterization of drought stress responsive microRNAs in Tibetan wild barley. *Int. J. Mol. Sci.* 21:2795. doi: 10.3390/ijms21082795

Remy, E., Cabrito, T. R., Baster, P., Batista, R. A., Teixeira, M. C., Friml, J., et al. (2013). A major facilitator superfamily transporter plays a dual role in polar auxin transport and drought stress tolerance in Arabidopsis. *The Plant cell vol.* 25, 901–926. doi: 10.1105/tpc.113.110353

Salgado, F. F., Vieira, L. R., Silva, V., Leão, A. P., Grynberg, P., do Carmo Costa, M. M., et al. (2021). Expression analysis of miRNAs and their putative target genes confirm a preponderant role of transcription factors in the early response of oil palm plants to salinity stress. *BMC Plant Biol.* 21:518. doi: 10.1186/s12870-021-03296-9

Sharif, R., Raza, A., Chen, P., Li, Y., el-Ballat, E. M., Rauf, A., et al. (2021). HD-ZIP gene family: potential roles in improving plant growth and regulating stress-responsive mechanisms in plants. *Genes* 12:1256. doi: 10.3390/genes12081256

Silva, P. A., Cosme, V. S., Rodrigues, K. C. B., Detmann, K. S. C., Leão, F. M., Cunha, R. L., et al. (2017). Drought tolerance in two oil palm hybrids as related to adjustments in carbon metabolism and vegetative growth. *Acta Physiol. Plant.* 39:58. doi: 10.1007/s11738-017-2354-4

Silva, P. A., Oliveira, I. V., Rodrigues, K. C. B., Cosme, V. S., Bastos, A. J. R., Detmann, K. S. C., et al. (2016). Leaf gas exchange and multiple enzymatic and non-enzymatic antioxidant strategies related to drought tolerance in two oil palm hybrids. *Trees – Struct. Function* 30, 203–214. doi: 10.1007/s00468-015-1289-x

Singh, R., Ong-Abdullah, M., Low, E. T., Manaf, M. A., Rosli, R., Nookiah, R., et al. (2013). Oil palm genome sequence reveals divergence of interfertile species in old and new worlds. *Nature* 500, 335–339. doi: 10.1038/nature12309

Statello, L., Guo, C. J., Chen, L. L., and Huarte, M. (2021). Gene regulation by long non-coding RNAs and its biological functions. *Nat. Rev. Mol. Cell Biol.* 22, 96–118. doi: 10.1038/s41580-020-00315-9

Sun, G. (2012). MicroRNAs and their diverse functions in plants. *Plant Mol. Biol.* 80, 17–36. doi: 10.1007/s11103-011-9817-6

Sunkar, R., Girke, T., Jain, P. K., and Zhu, J. K. (2005). Cloning and characterization of microRNAs from Rice. *Plant Cell* 17, 1397–1411. doi: 10.1105/tpc.105.031682

Tarazona, S., Furió-Tarí, P., Turrà, D., Pietro, A. D., Nueda, M. J., Ferrer, A., et al. (2015). Data quality aware analysis of differential expression in RNA-seq with NOISeq R/bioc package. *Nucleic Acids Res.* 43:e140. doi: 10.1093/nar/gkv711

Trindade, I., Capitão, C., Dalmay, T., Fevereiro, M. P., and dos Santos, D. M. (2010). miR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*. *Planta* 231, 705–716. doi: 10.1007/s00425-009-1078-0

Uddin, M. N., Hossain, M. A., and Burritt, D. (2016). "Salinity and drought stress: similarities and differences in oxidative responses and cellular redox regulation" in *Water Stress and Crop Plants: A Sustainable Approach*. ed. P. Ahmad (Hoboken, NJ: Wiley), 86–101.

Vaucheret, H., Vazquez, F., Crété, P., and Bartel, D. P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev.* 18, 1187–1197. doi: 10.1101/gad.1201404

Ventura, A., Young, A. G., Winslow, M. M., Lintault, L., Meissner, A., Erkeland, S. J., et al. (2008). Targeted deletion reveals essential and overlapping functions of the miR-17~92 family of miRNA clusters. *Cells* 132, 875–886. doi: 10.1016/j.cell.2008.02.019

Wang, T., Chen, L., Zhao, M., Tian, Q., and Zhang, W. H. (2011). Identification of drought-responsive microRNAs in *Medicago truncatula* by genome-wide high-throughput sequencing. *BMC Genomics* 12:367. doi: 10.1186/1471-2164-12-367

Wang, L., Lee, M., Ye, B., and Yue, G. H. (2020). Genes, pathways and networks responding to drought stress in oil palm roots. *Sci. Rep.* 10, 21303–21313. doi: 10.1038/s41598-020-78297-z

Wang, D., Liu, H., Wang, H., Zhang, P., and Shi, C. (2020). A novel sucrose transporter gene *IbSUT4* involves in plant growth and response to abiotic stress through the ABF-dependent ABA signaling pathway in Sweetpotato. *BMC Plant Biol.* 20:157. doi: 10.1186/s12870-020-02382-8

Wei, L., Zhang, D., Xiang, F., and Zhang, Z. (2009). Differentially expressed miRNAs potentially involved in the regulation of defense mechanism to drought stress in maize seedlings. *Int. J. Plant Sci.* 170, 979–989. doi: 10.1086/605122

Wu, G., and Poethig, R. S. (2006). Temporal regulation of shoot development in *Arabidopsis thaliana* by miRr156 and its target SPL3. *Development* 133, 3539–3547. doi: 10.1242/dev.02521

Xiao, Y., Xia, W., Yang, Y., Mason, A. S., Lei, X., and Ma, Z. (2013). Characterization and evolution of conserved MicroRNA through duplication events in date palm (Phoenix Dactylifera). *PLoS One* 8:71435. doi: 10.1371/journal.pone.0071435

Xin, C., Liu, W., Lin, Q., Zhang, X., Cui, P., Li, F., et al. (2015). Profiling microRNA expression during multi-staged date palm (*Phoenix dactylifera* L.) fruit development. *Genomics* 105, 242–251. doi: 10.1016/j.ygeno.2015.01.004

Xu, J., Chen, Q., Liu, P., Jia, W., Chen, Z., and Xu, Z. (2019). Integration of mRNA and miRNA analysis reveals the molecular mechanism underlying salt and alkali stress tolerance in tobacco. *Int. J. Mol. Sci.* 20:2391. doi: 10.3390/ijms20102391

Zeeshan, M., Qiu, C.-W., Naz, S., Cao, F., and Wu, F. (2021). Genome-wide discovery of miRNAs with differential expression patterns in responses to salinity in the two contrasting wheat cultivars. *Int. J. Mol. Sci.* 22:12556. doi: 10.3390/ijms222212556

Zhao, M., Chang, Q., Liu, Y., Sang, P., Kang, Z., and Wang, X. (2021). Functional characterization of the wheat macrophage migration inhibitory factor TaMIF1 in wheat-stripe rust (*Puccinia striiformis*) interaction. *Biology* 10:878. doi: 10.3390/biology10090878

Zheng, Y., Chen, C., Liang, Y., Sun, R., Gao, L., Liu, T., et al. (2019). Genome-wide association analysis of the lipid and fatty acid metabolism regulatory network in the mesocarp of oil palm (*Elaeis guineensis* Jacq.) based on small noncoding RNA sequencing. *Tree Physiol.* 39, 356–371. doi: 10.1093/treephys/tpy091

Zhou, L., Liu, Y., Liu, Z., Kong, D., Duan, M., and Luo, L. (2010). Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza Sativa*. *J. Exp. Bot.* 61, 4157–4168. doi: 10.1093/jxb/erq237

Zhu, Q. H., Upadhyaya, N. M., Gubler, F., and Helliwell, C. A. (2009). Over-expression of miR172 causes loss of spikelet determinacy and floral organ abnormalities in Rice (Oryza Sativa). *BMC Plant Biol.* 9:149. doi: 10.1186/1471-2229-9-149

ARTIGO III

Similarities in the behavior of transcription factors in young oil palm (*Elaeis guineensis* Jacq.) plants under salt or water stress provide strategies to develop stress-tolerant oil palm plants at once

Autores: Fernanda Ferreira Salgado, Thalliton Luiz Carvalho da Silva, André Pereira Leão, Roberto Coiti Togawa, Carlos Antônio Ferreira de Sousa, Priscila Grynberg e Manoel Teixeira Souza Jr

Aceito para publicação no periódico International Journal of *Molecular Sciences*

International Journal of
Molecular Sciences

MDPI

Article

Similarities in the behavior of transcription factors in young oil palm (*Elaeis guineensis* Jacq.) plants under salt or water stress provide strategies to develop stress-tolerant oil palm plants at once

Fernanda Ferreira Salgado [1], Thalliton Luiz Carvalho da Silva [1], André Pereira Leão [2], Roberto Coiti Togawa [3], Carlos Antônio Ferreira de Sousa [4], Priscila Grynberg [3] and Manoel Teixeira Souza Júnior [1,2,*]

[1] Graduate Program of Plant Biotechnology, Federal University of Lavras, Lavras, MG, Brazil; fernandasalgadopbv@gmail.com, ThallitonS@gmail.com

[2] The Brazilian Agricultural Research Corporation, Embrapa Agroenergy, Brasília, DF, Brazil; andre.leao@embrapa.br, manoel.souza@embrapa.br

[3] The Brazilian Agricultural Research Corporation, Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil; roberto.togawa@embrapa.br, priscila.grynberg@embrapa.br

[4] The Brazilian Agricultural Research Corporation, Embrapa Mid-North, Teresina, PI, Brazil; carlos.antonio@embrapa.br

* Correspondence: manoel.souza@embrapa.br

Citation: Salgado, F.F.; Carvalho da Silva, T.L.; Leão, A.P.; et al. Similarities in the behavior of transcription factors in young oil palm (*Elaeis guineensis* Jacq.) plants under salt or water stress provide strategies to develop stress-tolerant oil palm plants at once. *Int. J. Mol. Sci.* **2023**, *24*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date

Accepted: date

Published: date

Abstract: In recent years, many studies have reported that several distinct families of transcription factor (TF) genes play crucial roles in the response of plants to abiotic stress. Although some of these families got systematically studied in many species, little knowledge exists about these genes in oil palm (Elaeis guineensis Jacq.). In this study, 20 genes differentially expressed in the leaves of oil palm plants subjected to salinity or drought stress and encoding TFs belonging to four families of TFs - MYB, HD-ZIP, NF-Y, and HSFF - got selected for further characterization. The genes underwent a structural and functional annotation, besides having their RNA-Seq expression profile validated by the qPCR technique, and a correlation analysis of their response under both stresses got performed. The annotation analysis of the promoter region showed the presence of seven cis-acting elements, all already reported to be involved in plants' response to abiotic stress. The structural and functional characterization of the proteins encoded by the selected genes showed that some groups present patterns of conserved regions and are orthologs to genes already linked to salinity or drought resistance in other plant species. It was possible to confirm the RNA-Seq expression profile of 19 genes, only gene 4 showed the opposite response when using qPCR. Through correlation analysis, it was possible to observe a high level of similarity in the behavior of the 20 genes in response to drought or salinity stress, a behavior already reported in other species. The results presented here shed light on the role of 20 TFs in the leaves of young oil palm plants under severe levels of soil salinity or water deprivation. The high level of similarity in the molecular response to both stresses allowed the identification of genes that might grant strategies aiming to develop oil palm plants with a tolerance to both salinity and drought at once.

Keywords: Abiotic stress, tolerance, salinity, drought, transcriptome, MYB, NFY, HD-ZIP, HSFF.

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is the number one oilseed crop in the world, considering the amount of vegetal oil consumed yearly. The consumption of palm oil and palm kernel oil in the 2022/23 season was about 85 million metric tons, ahead of soybean oil (60 million metric tons), rapeseed oil (31), and sunflower-seed oil (18.5) [1].

Over 95% of the oil palm plantations in Brazil are in the Legal Amazon Area, specifically in the Northeast region of the Pará State. Although Brazil has millions of hectares with an aptitude for oil palm production, it currently has only about 200,000 hectares destined for this purpose [2]. Any initiative aiming to increase the area cultivated with oil palm in Brazil faces a dilemma, stay in the Amazon Biome, where this crop finds a natural environment of high rainfall throughout the year, or go outside this area. Both options have its pro and cons.

One of the main cons of going somewhere outside the Amazon Biome, more specifically to the Mid-West and Northeast regions in Brazil, is that there oil palm will face long periods of drought throughout the year when it will not receive the physiological water level required to maintain productivity and will demand the use of artificial irrigation. Accordingly to Corley et al. [3], oil palm requires ~2,000 mm/year of water and does not tolerate water deprivation for more than 90 days.

The climate in the Cerrado Biome of the Mid-West region of Brazil has two seasons, rainy summers and dry winters. Some areas in that region experience a period of about four months without rain. The Northeast region is a semi-arid region where precipitation is insufficient to meet the water demand of crops. Water scarcity in places like those leaches mineral salts from the root zone, promoting soil salinization, a global problem due to its negative impact on agricultural productivity and sustainability [4]. About 30% of the world's irrigated agricultural land suffers from secondary salinity. That is a problem experiencing constant growth and expecting to reach more than half of the land by 2050 [5-7].

Plant response to drought and salinity stresses is a complex and coordinated process that rapidly and effectively works to restore homeostasis. It consequently enables the plant to adapt to unfavorable conditions [8]. Such a process regulates the expression of several stress-responsive gene groups [9], especially at transcriptional and post-transcriptional levels, with attention given to transcription factors (TFs). There are plenty of studies available showing that transcription factors (TFs) play central roles in the plant's response to different abiotic stimuli, allowing them to respond and adapt promptly to certain conditions, such as heat, drought, salinity, cold, and others [10]. Approximately 7–10% of all plant genes code for TFs at distinct moments, and several TF gene families are known for precisely coordinating the spatial and temporal expression of downstream genes associated with abiotic stress [11-12]. So far, 60 distinct TF families got identified in plants, including heat shock factors (HSPs), which are a class of great importance that play crucial roles in protecting plants against the effects caused by various abiotic stresses through the regulation of heat shock proteins [9, 13].

Studies show that overexpression of certain stress-inducible transcription factors, such as NF-Y, MYB, HD-ZIP, and DREB, can increase tolerance to salinity, drought, or extreme temperatures and that they play central roles in plant response to different abiotic stimuli [14, 15]. The homeodomain leucine zipper (HD-Zip) family of transcription factors is one of the largest plant-specific superfamilies. The TFs from this family got linked to plant growth modulation and responses to environmental stresses [16-19], and studies show that their overexpression does promote tolerance to drought [18, 20, 21], and to drought and salt stresses [22].

In two previous studies, our research group reported comprehensive, large-scale miRNA and RNA-Seq analyses applied to characterize young oil palm responses to a high level of salinity [23] and water deprivation [24]. In those studies, we showed that TFs play a preponderant role in the early response of this oilseed crop to those two abiotic stresses. Here we describe an additional step toward analyzing those datasets, aiming at getting more insights into the TF families and their TF genes commonly affected by those two stresses. To do so, all TF genes differentially expressed got considered, not only those identified as putative miRNA-target genes. A group of 20 TF genes (and their promoter sequences) from four distinct TF families underwent structural and functional annotation, with subsequent qPCR analysis of their behavior in both stress conditions.

2. Results

2.1. Selection of transcription factors responsive to drought and salinity stress

The results from the GhostKOALA annotation tool, specifically the ones from the KO0300 – Transcription Factor group, revealed 19 and 36 groups of different families of transcription factors responsive to salinity and drought stresses, respectively. There were 18 families of differentially expressed TFs present in both sets of results (Supplementary Table 1). Based on information gathered from previous studies [23-26], 20 differentially expressed genes got selected, and they belong to five of those 18 TFs families. Among them, there were 13 genes from the MYB family, three genes from the homeobox-leucine zipper protein (HD-ZIP) family, two genes from the heat shock transcription factor (HSFF) family, one gene from the nuclear transcription family Y subunit beta (NFY-B), and one of the nuclear transcription factor Y gamma (NFY-C) family (Table 1).

The RNASeq analysis of those 20 genes selected showed that eleven expressed differentially under saline stress, with fold change values ranging from 1.8 to 10.4 times the expression level seen in the non-stressed plants. Among the nine genes negatively regulated under salinity, gene 17 (XP_010936941.1) had a 17.2 times reduction in the expression level found in the non-stressed plants. Under drought stress, nine out of the 20 TFs genes expressed positively; gene 1 (XP_010904805.1) led the rank with a fold change of 50.2. The remaining eleven genes negatively regulated showed reductions ranging from -1.9 to -21.2, compared to the control (Table 1).

2.2. Selection of transcription factors responsive to drought and salinity stress

The phylogenetic tree obtained using the stretch of 1,432 nucleotides upstream of the start codon of each gene separated the 20 TFs genes into four groups; however, when comparing the expression profiles of the genes under the two stress tested, there was no particular pattern at all (Figure 1A, Table 1). The MEME software revealed three conserved promoter motifs in all 20 genes, and, again, no particular pattern appeared within the four groups found in the phylogenetic tree (Figure 1B).

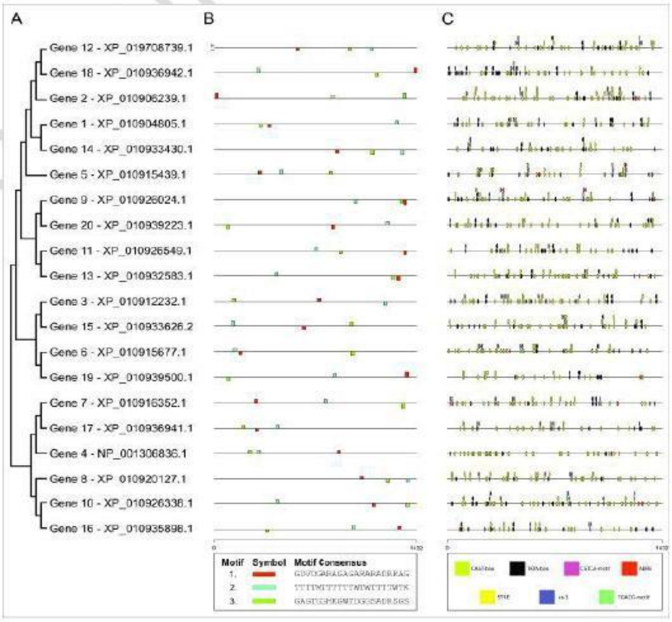

Figure 1. Structural annotation performed on the 1432 bp long sequence upstream of the initiation codon of selected salt and drought stress-responsive genes. A: phylogenetic tree performed using the MEGA11 software. B: Analysis of conserved motifs and their position in the nucleotide sequence. C: Localized cis action elements, their location and frequency.

The use of the PlantCare database revealed seven cis-acting elements related to stress response present in the promoter sequences of those 20 genes, which were: ABRE (ABA-responsive element), STRE (activated by heat shock, osmotic stress, low pH and nutrient deprivation), CGTCA-motif (responsive to methyl jasmonate (MeJA)), TGACG-motif (involved in MeJA responsiveness), TATA-box, CAAT-box, and the salicylic acid and auxin-responsive element as-1 (Figure 1C).

The CAAT- and TATA- box elements were present in large amounts in all 20 promoter sequences, being directly related to the initiation of gene transcription and enhancing activity, improving the ability of genes to react to adversity to respond to stress abiotic process more efficiently and quickly [27]. Two genes - 3 and 5 - from the HSFF family presented the elements ABRE, as-1, CAAT-box, CGTCA – motif, TATA-box, TGACG – motif, while genes 2, 6, 7, 8, 9, and 10 showed all seven cis-acting elements (Figure 1C).

2.3. Structural and functional annotation of the coding region

Regarding the size of the genes and the numbers and disposition of introns and exons, there was considerable diversity in the coding sequence of the 20 TFs genes analyzed. The longest gene was gene 4, almost 7,500 nucleotides long. The number of introns varied from zero (gene 20) to nine (gene 12). When analyzed by family, one can observe that egMYB presented two introns in most genes, while egHSFF presented one, egNFY zero or one intron, and in egHD-ZIP ranging from two to nine introns (Figure 2).

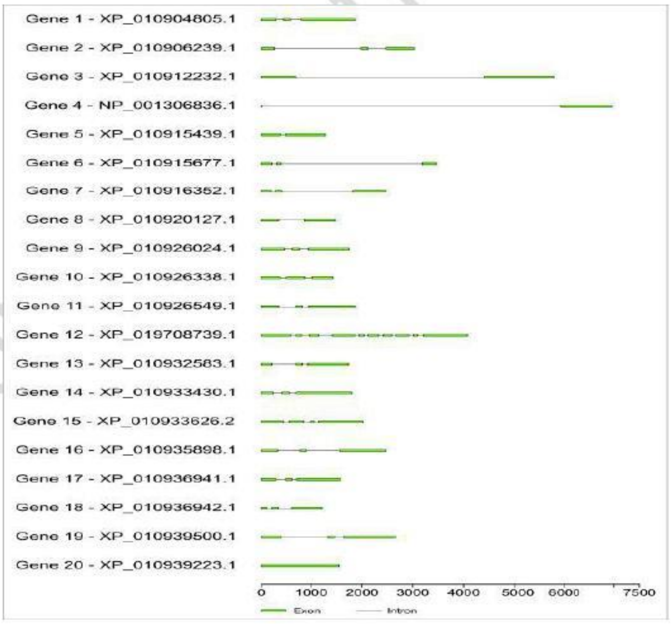

Figure 2. Number and location of intron and exon of selected salt and drought stress-responsive genes in the oil palm genome. The green boxes represent the number of exons and their location in the gene.

A phylogenetic tree resulted from analyzing the protein sequences of the 20 genes to explore the evolutionary relationship between transcription factor families, besides characterizing the features of conserved motifs and domains (Figure 3). The 20 TFs did group into three clades, where one clade had only the protein from gene 9 - an MYB family individual, and the second one had eight proteins - from genes also belonging to the MYB family. At last, the third clade includes the proteins of the remaining four genes of the MYB family, three genes of the HD-ZIP family, two genes of the HSFF family, and the two genes of the NFY family; both being side by side with their respective groups.

As for the conserved domains, only the protein coded by gene six had no conserved domain. The remaining 19 proteins showed 23 distinct conserved domains. The PLN03091 and PLN03212 domains appeared in the proteins coded by genes 1, 2, 7-9, 11, 13, 14, 16-19. Those proteins belong to the MYB family of transcription factors; and had the REB1, Myb, and SANT domains randomly distributed in them (Figure 3b, Supplementary Table 2).

In the coding regions of genes 3 and 5 (both of the HSFF family), the domains HSF, HSF1, HSF_DNA-bind, and HSF_DNA-bind superfamily were found. While in the protein coded by genes 4, 10, 12, 15, and 20, the domains HOX, homeodomain, homeodomain superfamily, Homeobox, HHT1, HALZ, H4, Histone superfamily, START, HAP5, HD-ZIP_N, START_ArGLABRA2_like, CBFD_NFYB_HMF were also found and SRPBCC (Figure 3b, Supplementary Table 2).

The MEME program predicted seven conserved motifs in 18 of the 20 proteins analyzed (Figure 3c). The numbers of conserved motifs vary from one to five per protein, with the proteins coded by genes 11 and 19 having the highest number of motifs, and the length of the motifs ranged from eight to 50 amino acids. Furthermore, in domains PLN03091 and PLN03212, one can see motifs 1, 2, and 4. Motif 5 was present in the proteins coded by genes 3 and 5, while motif 6 was present in the proteins coded by genes 10, 12, and 15. Motif 3 was present in nine proteins, while motif 7 was in three (genes 10, 11, and 19) (Figure 3c).

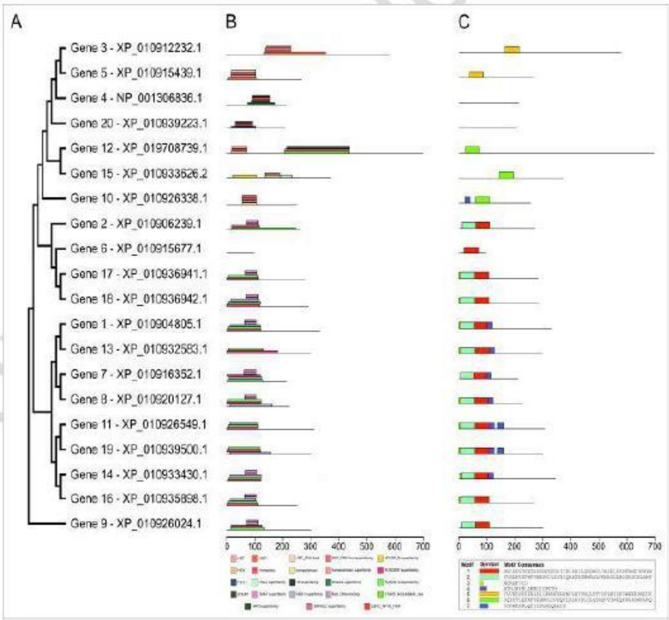

Figure 3. Structural annotation of the protein sequence of selected salt and drought stress-responsive genes. A: phylogenetic tree performed using the MEGA11 software. B: Domains of the 19 proteins that were positive on the NCBI platform, where each color represents a different domain. C: Conserved motifs of the 18 proteins that were positive and their position in the amino acid sequence, where each color represents a different motif.

From the functional annotation of the TFs coded by the 20 genes responsive to drought and salinity stress, 87 activities for biological processes, nine for molecular functions, and four for cell components were identified (Figure 4, Supplementary Table 3). One should notice that the protein coded by gene 14 had the highest number of GO terms annotated, with 29 GO terms, followed by those coded by genes 9, 11, and 19, with 23 GO terms for each one (Supplementary Table 3).

2.4. Differential expression and correlation analyses

To further characterize the 20 genes selected, their expression levels got determined using qPCR (Figure 5, Supplementary Table 4). In water deprivation, genes 1, 5, 9, and 11 were significantly positively regulated, while the remaining genes were negatively regulated. Under salt stress, genes 1, 5, 9, 11-16, and 19 were positively regulated, while genes 2-4, 6-8, 10, 17, 18, and 20 were negatively regulated.

The differential expression of the 20 genes under salt and drought stress underwent a correlation analysis. Interestingly, almost all genes showed the same behavior when analyzed under drought and salinity stress conditions, with an R2 score of 0.7317. Only genes 12 and 13 showed opposite behavior when subjected to different stress conditions, being positively regulated under salt stress and negatively under water deprivation (Figure 6).

The expression levels of all genes, measured by RNA-Seq and qPCR, were compared under both abiotic stresses (Figure 7). Among the 20 genes tested, only gene 4 showed a different expression pattern, showing a positive regulation response to both conditions when using the RNA-Seq and a negative regulation response when using qPCR.

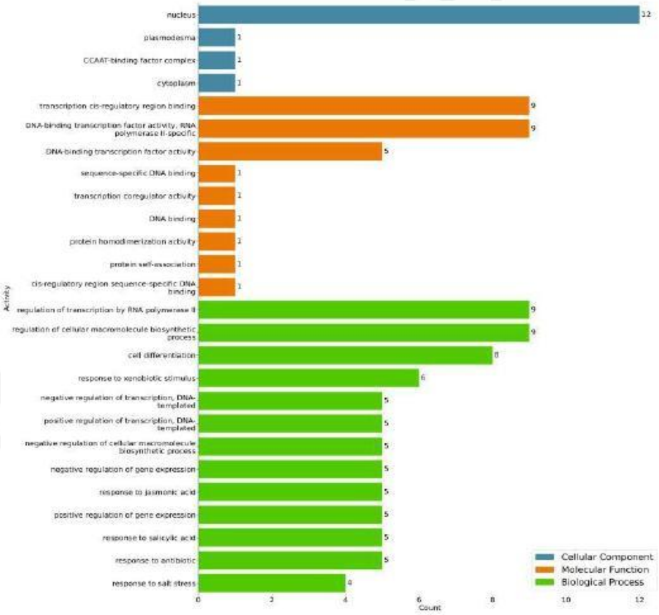

Figure 4. Gene Ontology (GO) plot of annotation rank statistics of selected salt and drought stress-responsive genes in the oil palm genome; classified according to cellular component (blue), molecular function (orange) and biological process (green). The numbers represent the number of positive hits.

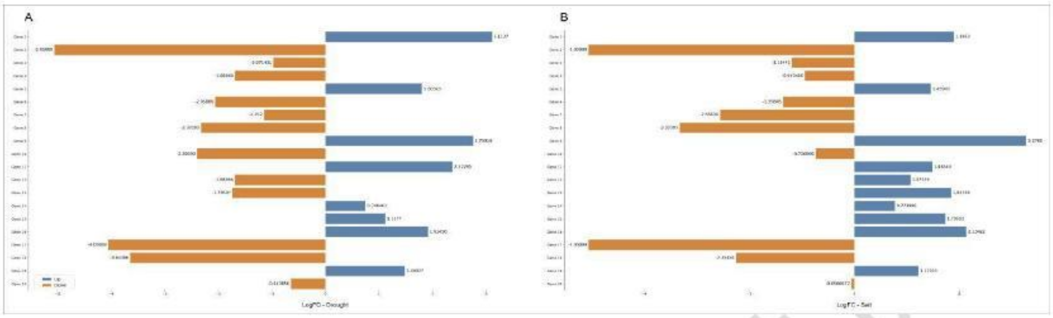

Figure 5. Expression profiles in $Log_2(FC)$ of the 20 genes selected using qPCR, A: resulting from the submission of young oil palm plants to drought stress, in comparison with the control treatment; and B - resulting from the submission of young oil palm plants to saline stress, in comparison with the control treatment. FC - fold change.

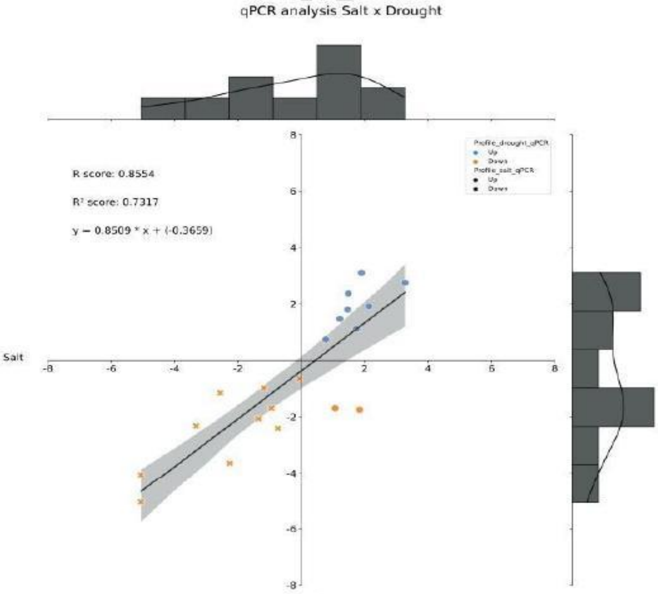

Figure 6. $Log_2(FC)$ correlation analysis of the 20 selected genes; resulting from the qPCR analysis, by pairwise comparison of two scenarios: saline stress and water stress. FC - fold change.

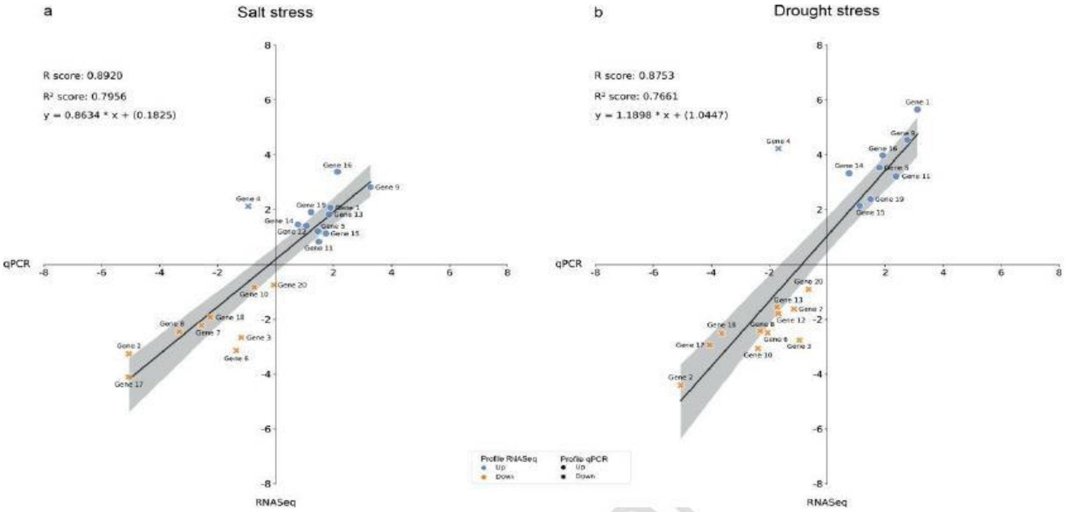

Figure 7. Correlation analysis of the Log$_2$(FC) of the 20 selected genes. A: Differential gene expression under saline stress, pairwise comparison of two scenarios: qPCR and RNASeq; and B: Differential expression of genes under drought stress, pairwise comparison of two scenarios: qPCR and RNASeq. FC - fold change.

3. Discussion

Soil salinity and water deficit are two of the most severe abiotic stress factors, restricting the growth and development of many plant species and generating significant losses in crop productivity [28]. Plants respond to unfavorable conditions through two pathways, namely the abscisic acid (ABA)-dependent pathway and the ABA-independent pathway [29]. Transcription factors (TFs) play vital roles in mediating stress tolerance in both of them, stimulating osmotic homeostasis, and ionic balance, among other elements, in an attempt to reduce the damage caused by stress in plants [30, 31].

There is a lack of knowledge about the functioning of transcription factors (TFs) in regulating gene expression in oil palm plants under drought and salt stresses. Recently, our group reported several transcription factors been affected by those two stresses in connection with miRNAs [23, 24]. The goal of the present study was to characterize TFs' found as responsive to both stresses in young oil palm plants, independently of been or not a miRNA-target gene. To do so, a group of 20 TF genes got selected for further analysis. Those TFs belong to four distinct families - MYB, HD-ZIP, HSFF, and NF-Y - previously reported as directly involved in salt and drought resistance in some plant species.

The first step in the characterization process focused on identifying features commonly found in the promoter sequences of those genes. Gene promoters contain essential regulatory cis-elements for regulating gene expression [32], and, in the present study, as expected, many important cis-elements were present in all 20 promoter regions. Numerous CAAT-box and TATA-box sequences appeared, presenting themselves possibly as the most relevant cis-elements of the ones identified. CAAT-box is a cis-acting element commonly found in promoter sequences with enhancing activity that may contribute to increased activity of promoters in response to salinity and drought stress [32]. Meanwhile, the genomic distribution of the TATA box tends to favor function-specific genes. Such as, TATA-box is rich in promoters of stress-related genes and is absent in promoters of essential genes, in addition to being associated with rapid and variable regulation under stress [27, 33].

Besides CAAT – box and TATA – box, other cis-acting elements also reported as having links with response to abiotic stresses appeared, such as ABRE (response to ABA), STRE (activated by heat and osmotic stresses), as-1 (Oxidative Stress-Responsive Element activated by salicylic acid), and CGTCA – motif and TGACG – motif, both responsive to methyl jasmonate (MEJA). Although 18 out of the 20 genes presented similar qualitative profiles in the two scenarios - salt and drought stresses - evaluated, which got validated by RNA-Seq and qPCR analysis, not one specific pattern in terms of the number and position of those above cited cis-acting elements was evident.

The second step in the characterization process focused on identifying features in the sequence of the proteins coded by those genes. As expected, regarding the motifs and domains present in the proteins coded by the selected genes, it was possible to identify equivalent patterns among the representatives of each specific TFs family, corroborating the results from the GhostKOALA annotation tool, specifically the ones from the KO0300 – Transcription Factor group.

Thirteen of the selected genes belong to the MYB transcription factors family. Several studies have shown that MYB proteins perform multiple functions in response to stresses such as salt, drought, and cold [34-36]. MYB20 (gene 13) showed a positive regulation in the leaves of young oil palm plants under saline stress and a negative under drought. This gene is known for acting as a negative regulator of ABA-mediated stomatal closure under conditions of water stress, and its overexpression results in higher susceptibility to desiccation [37]. According to Wang et al. [36], it positively regulates plant salt tolerance by repressing key ABA repressor, PP2Cs. MYB30 (gene 16) had its expression increased in the leaves of young oil palm plants in response to both stresses. This FT is known for modulating plants' tolerance to salt through the regulation of mitochondrial alternative oxidase AOX1s; and the AOX1a overexpression rescued salt-sensitive phenotypes [36, 38]. On the other hand, MYB30 positively regulates wax synthesis genes that have a direct link with the biosynthesis of the cuticle, one of the most important plant structures protecting plant leaves from desiccation [36, 39].

Two of the selected genes belong to the NF-Y transcription factors family. Nuclear factor Y (NF-Y) proteins, also known as CCAAT Binding Factor (CBF) or Heme Activator Protein (HAP), modulate the expression of downstream genes via a heterotrimer – NF-YA-YB-YC – that binds through NF-YA to the CCAAT box present in the promoter region of those genes. Several members of the NF-Y family and their subunits are regulators of abiotic stress responses in plants [40-44. NF-YA is a target of the miR169 family, and the overexpression of NF-YA in Arabidopsis increases its tolerance to salt stress [42].

Our previous studies have shown that members of the NF-Y family are regulated by microRNAs (miR169 and egu-miR02sds) in the leaves of young oil palm plants under salinity and drought stress [23, 24]. Even though the miR169 gene got negatively regulated in oil palm plants under salinity and drought stress, the levels of NF-YA increased only under salt stress [23]. In the present study, NF-YB3 (gene 20) and NF-YC2 (gene 4) got differentially expressed in the leaves of oil palm plants under both abiotic stresses, with the former negatively regulated under salinity and drought stress and the latter positively regulated.

A hypothetical model presented by Leyva-González et al. [41] proposes that in plants exposed to abiotic stress, NF-YA levels increase due to their transcriptional activation and to the reduction in the miR169 levels, corroborating the results from Salgado et al. [23]. The higher amount of NF-YA represses early abiotic stress response genes probably by sequestering NF-YB-YC, creating a regulatory loop to arrest early responses that represent high energy and carbon costs. If that is the case, that might happen in the leaves of young oil palm plants only under salinity stress, considering that the reduction in the levels of miR169 due to drought stress does not show to affect the level of NF-YA, as seen in Salgado et al. [24].

Previous studies have reported that AtNF-YB1 improves plant performance in drought conditions, and its ortholog in maize, ZmNF-YB2, conferred resistance to water stress in the field [40]. That suggests that this would be a starting point for identifying possible markers for genetic improvement. Gene 4, an NF-YC2, was overexpressed in the leaves of oil palm plants under drought and salinity stress; this behavior was reported in conifers (Picea wilsonii) as well, where overexpression of PwHAP5 (Arabidopsis homolog

NF-YC2) partially rescued the increased sensitivity of NF-YC2 to salt, drought, and ABA treatments, through its positive regulation [42].

Three of the selected genes belong to the HD-Zip transcription factors family, one positively (gene 15) and one negatively (gene 10) regulated in both scenarios, and one positively regulated under salinity stress and negatively under drought stress (gene 12). The homeodomain leucine zipper (HD-Zip) transcription factor family is one of the largest plant-specific superfamilies and includes genes with roles in modulating plant growth and response to environmental stresses, and categorized into four subfamilies - HD-Zip I, HD-Zip II, HD-Zip III, and HD-Zip IV [16, 18, 19].

The overexpression in Arabidopsis of HaHB4, a drought/ABA-inducible HD-Zip family I gene from sunflower, upregulated transcript levels of several genes involved in JA biosynthesis and defense-related processes, resulting in plants with shorter stem and internode distances, more round leaves and denser hairs; altogether, such phenotypes made the plants tolerant to drought [18, 20]. Overexpression of ATHB-6, a drought-induced HD-Zip I gene from maize, has activated the expression of critical genes in the ABA-dependent and ROS signals pathways and improved tolerance to drought in maize [21]. Overexpression of Zmhdz10, also an HD-Zip I gene from maize, in rice and arabidopsis led to enhanced tolerance to drought and salt stresses [22]. At last, previous research using class I HD-ZIP homeobox genes showed that they get highly regulated under salt stress and that their overexpression in transgenic rice and cotton plants improved salt stress sensitivity [17, 45]. The three HD-Zip genes characterized in this present study belong to the HD-Zip I (gene 10), HD-Zip II (gene 15), and HD-Zip IV (gene 12) subfamilies.

The remaining two of the selected genes belong to the HSF family. Heat shock transcription factors (HSF) are a TF class of great importance that play crucial roles in protecting plants against the effects caused by various abiotic stresses [46]. HSFs operate by inducing the accumulation of heat-shock proteins (Hsps) and by mediating the activation of other heat-responsive genes involved in cell protective mechanisms and the homeostasis of reactive oxygen species (ROS) [47]; and are reported as responsive to heat, drought, cold, and salt stresses [48]. There are three classes of HSFs, denominated A, B, and C. The HSFs from class-A have multiple acidic motifs (AHAs) at the C-terminus and function as transcriptional activators, and those from class-B contain a tetrapeptide -LFGV- in the C-terminus, which is assumed to function as a repressor motif by interaction with an unknown co-repressor, while the function of those from class-C is still unknown [47, 49].

In Arabidopsis, Pérez-Salamó et al. [50] showed that estradiol-dependent induction of HSFA4A confers enhanced tolerance to salt and oxidative agents, whereas inactivation of that gene results in hypersensitivity to salt stress. The overexpression of LlHsfA3A or LlHsfA3B from lily (*Lilium longiflorum*) in Arabidopsis showed hypersensitivity to salt stress, and a lack of sucrose exacerbated this salt sensitivity [49]. In the present study, gene 3 - coding for an HSFA3 protein - is negatively regulated in both scenarios, while gene 5 - coding for an HSFC2b protein - was positively regulated.

Regarding the expression profile of the selected genes measured by RNA-Seq and qPCR techniques, 18 out of 20 showed similar (qualitatively) expression profiles in the two scenarios; only genes 12 and 13 differed. Such similarity in behavior in the initial responses of plants to salt stress and drought got reported previously [48, 51-53], corroborating our results.

Last, and based on the study by McCoy et al. [54], we applied GENIE3 to mine 306 public oil palm transcriptome datasets and to construct a gene regulatory network (GRN) featuring epigenetic regulators and transcription factors from the oil palm genome. Such a study [55] used 1,333 unique regulators and 27,642 target genes from the oil palm reference genome [56]. A closer look at this oil palm GRN, and focusing on the 20 TFs genes characterized in the present study, allowed the identification of the genes regulating the TFs and those under regulation by them. Gene 10 (LOC105048648), a candidate gene concerning its potential capability to promote resistance to both salinity and drought stresses by heterologous overexpression, is a target for 16 epigenetic regulators and transcription factors while targeting 278 genes (Supplementary Table 5). A gene expressing a protein belonging to the Transcription activator GLK1/2-like (IPR044825) family is the one with the highest score among those potentially regulating gene 10, followed by another expressing a protein from the ADP-ribosylation factor GTPase-activating protein AGD11/12/13 (IPR044518) family.

4. Materials & Methods

4.1. RNA-Seq data generation

The RNA-Seq raw sequence data used in this study are available in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information under *Elaeis guineensis* Transcriptome_Drought and Salinity Stresses - BioProject PRJNA573093, BioSample SAMN12799239.

All information regarding the plant material, growth conditions, and abiotic (salt and drought) stress conditions used in the studies that generated RNA-Seq data are in Salgado et al. [23, 24]. In summary, all plants used in this study were clones regenerated from embryogenic callus obtained from the leaves of an adult plant belonging to the AM33 genotype, and all experiments were carried out in a greenhouse at Embrapa Agroenergia (www.embrapa.br/en/agroenergia), in Brasília, DF, Brazil (S-15.732 °, W-47.900 °), using young oil palm plants in the growth phase known as bifid saplings.

In the salinity stress study, on the 12th day after the imposition of the stress, we collected the apical leaf from three control (0.0 g of NaCl per 100 g of the substrate) and three salt-stressed plants (2.0 g of NaCl per 100 g of the substrate) [23]. In the water deprivation study, apical leaves from control and drought-stressed plants were collected 14 days after the imposition of treatments, with three replicates per treatment [24]. In both studies, the plant tissue was immediately immersed in liquid nitrogen and then stored at -80 °C until RNA extraction, library preparation, and sequencing. Total RNA isolation, and RNA quality and quantity analysis, were performed as described by Salgado et al. [23, 24]. The GenOne Company (Rio de Janeiro, RJ, Brazil) performed the RNA-Seq using an Illumina HiSeq platform and the paired-end strategy.

4.2. RNA-Seq data analysis

Details on the RNA-Seq analyses performed using OmicsBox version 1.3 [56] can be found in Salgado et al. [23, 24]. The high-quality RNA-Seq data got aligned to the oil palm reference genome [57] - files downloaded from NCBI (BioProject PRJNA192219; BioSample: SAMN02981535) in March and October 2020 - using default parameters from OmicsBox version 1.3 through software STAR [58].

To quantify expression at the gene or transcript level, we used the default parameters of OmicsBox version 1.3 through HTSeq version 0.9.0 [59]. The pairwise differential expression analysis between the experimental conditions used edgeR version 3.28.0 [60], applying a simple design and an exact statistical test; without using a filter for low-count genes [23, 24].

4.3. Genes selection and annotation

The differentially expressed genes identified in Salgado et al. [23, 24] underwent annotation using the GhostKOALA annotation tool. Based on the results of the KO0300 – Transcription Factor group, we carried out a manual curation using the following conditions: 1°) Selection of the most relevant families according to the literature; 2°) Selection of differentially expressed genes within these previously selected groups; 3°) Genes that are present in both stresses; and 4°) The genes that showed a higher level of differential expression, both Up and Down.

All information regarding intron and exon regions for each gene came from the NCBI (https://www.ncbi.nlm.nih.gov/). To identify the cis-elements in the promoter region, we got a stretch of 1,432 base pairs before the ATG start codon of each gene from the oil palm reference genome [56], and the known elements characterized using the database PlantCARE [61]; the statistical sieve was Score ≤ 5. To analyze the structure of proteins and promoters related to transcription factors, we applied the MEME software (https://meme-suite.org/meme/tools /meme), with a maximum of 10 found motifs. Motifs that did not pass the statistical sieve (E-value ≤ 0.05) got excluded from the analysis.

After obtaining the protein sequences of the selected genes from the oil palm reference genome [56], the proteins domains got characterized through the NCBI Conserved Domain Search (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Those domains that did not pass the statistical sieve (E-value ≤ 1) got excluded from the analysis. For the functional analysis of proteins, we used the EggNOG platform [62]. For the multiple sequence alignment, we applied the ClustalW program [63]; for both the protein and promoter region data. Subsequently, the phylogenetic trees got built using the maximum likelihood method, using a bootstrap of 1000 replicates, using the MEGA11 software [64].

The figures referring to the results of the cis-element analysis; motifs, protein domains, and intron and exon regions got generated through the Python library "DNA Features Viewer" (https://edinburgh-genome-foundry.github.io/DnaFeaturesViewer/).

4.4. RNA extraction, reverse transcriptase-PCR and quantitative real-time PCR analyses

Total RNA isolation, and RNA quality and quantity analysis, were performed as described by Salgado et al. [23, 24]. After quantified, the extracted total RNA served as a template for reverse transcription using the commercial kit SuperScriptR (Invitrogen) following the manufacturer's protocol. The positive control gene was the EgEfMPOB00119 40S mRNA of ribosomal protein S23, a constitutive gene in *E. guineensis*.

For primer design, fasta files from the selected genes got downloaded from NCBI and exported to PerlPrimer software to design primers (Table 2). qPCRs were performed in 96-well optical plates on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with a PowerUpTM SYBRTM Green Master Mix, REF A25741 (Thermo Fisher Scientific), following the manufacturer's instructions. The qPCR reaction conditions were as follows: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. A melting curve got created to analyze the specificity of the reactions. Then, three biological replicates got performed for each sample. The expression level of the untreated sample got set to 1 for normalization.

4.5. Pair-by-pair comparison and correlation analysis

We performed a pair-by-pair comparison between the different stresses and methods used to analyze and validate the genes; the input data were Log_2 (Fold Change). The correlation analysis got carried out using the data set referring to the differential expression of genes (DEGs) common to the two scenarios studied (salinity and drought); obtained by qPCR.

Table 1. Pair of primers used in the qPCR analysis of the 20 genes prospected.

Gene	Forward Primer	Reverse Primer
Gene 01	GATAAGAATGGTCTGAAGAAGGG	CAATTGCTGACCACTTGTTCC
Gene 02	GGAGGACCAGAAACTTGTAGAC	ATTAGAGACCATCTGTTGCCC
Gene 03	TTTTCGCAAGATTGATGCTG	TCATGAGATGCTCCTGTTGC
Gene 04	GGTAGACCTCTCTGCATCG	CCTACCACCAGCTGAAAATACC
Gene 05	GCTCAACACCTATGGGTTTCG	ACCTTCTCTTCGATCTTCCTCTG
Gene 06	CACCACTTAAGATCCGTTGCC	GCTATCAAATCCCACCTGTCAC
Gene 07	ACGAGAAGCTCATTAACTACATCC	CTATCAATGACCACCGATTGCC
Gene 08	GGACCAAGGAGGAAGATGAC	AGAGACCATTTGTTGCCGAG
Gene 09	CTAAGTGCTCAGGCTTGAAGAG	GTCCTCCAGTAGTTCTTTATCTCG
Gene 10	TCGAGCGTGGTGTTTAATG	CGGAGCAGTACCAAGAGAGG
Gene 11	TTTAGGCAATAGGTGGGCAG	TATTGAGCTTCTCCACAGACAG
Gene 12	AGTCACAGTTCACAAGAGCAC	CATTCAGAGCACGAAGGAGAG
Gene 13	AGCTAGCAGGATTGCTTAGG	CTGGTCAACAGTTTCAGCG
Gene 14	CAAGGGTTCATGGACACCAG	TTCGACCATTTGTTTCCAAGC
Gene 15	GACACAAGCCATGATTGAAG	TAGCGAGCAACCCTTTTATCTC
Gene 16	CAAGCAAGCTGGTCTATTGAGG	CGGCTGGTTTGGATTCACTC
Gene 17	CTTCACTCTAGCGATATCTACCG	AACGTTTCAACCCTGCTCTC
Gene 18	CCATGTTGTTCAAAGGAGGG	GCAATTAGAGACCATCTGTTTCC
Gene 19	CCTACTAATACTGGGCTGATGAG	GGTGTTCCAATAGTTCTTGATGTC
Gene 20	GGTGGGAATTCTTCCTCTTC	TAATTCAGAGACCAACCCAACC
Control Gene*	CCAGGGTTCAGCTGATTAAG	TCGTCCAAATCCAGCAATC

* EgEfMPOB00119

Table 2. Profile of transcription factor genes from the leaf of oil palm plants differentially expressed under salt and water stress accordingly to RNA-Seq analysis. Expression Profile (EP), Fold Change (FC), Log$_2$ (FC), and False Discovery Rate (FDR).

Gene	Family	Protein ID	Description	Salt Stress				Drought Stress			
				EP	FC	Log$_2$(FC)	FDR	EP	FC	Log$_2$(FC)	FDR
1	MYBP	XP_010904805.1	transcription factor MYB102	Up	4.2	2.1	0.000	Up	50.2	5.6	0.000
2	MYBP	XP_010906239.1	transcription repressor MYB5-like	Down	-9.6	-3.3	0.000	Down	-21.2	-4.4	0.000
3	HSFF	XP_010912232.1	heat stress transcription factor A-3	Down	-6.3	-2.7	0.000	Down	-6.8	-2.8	0.000
4	NFYC, HAP5	NP_001306836.1	nuclear transcription factor Y subunit C-2-like	Up	4.3	2.1	0.000	Up	18.7	4.2	0.000
5	HSFF	XP_010915439.1	heat stress transcription factor C-2b	Up	2.3	1.2	0.000	Up	11.6	3.5	0.000
6	MYBP	XP_010915677.1	transcription factor TRY	Down	-8.8	-3.1	0.000	Down	-5.6	-2.5	0.000
7	MYBP	XP_010916352.1	myb-related protein 308	Down	-4.7	-2.2	0.000	Down	-3.1	-1.6	0.000
8	MYBP	XP_010920127.1	myb-related protein 308	Down	-5.5	-2.5	0.000	Down	-5.4	-2.4	0.000
9	MYBP	XP_010926024.1	transcription factor MYB62	Up	7.0	2.8	0.000	Up	23.2	4.5	0.000
10	HD-ZIP	XP_010926338.1	homeobox-leucine zipper protein HOX8	Down	-1.8	-0.8	0.016	Down	-8.4	-3.1	0.000
11	MYBP	XP_010926549.1	myb-related protein 306	Up	1.8	0.8	0.029	Up	9.3	3.2	0.000
12	HD-ZIP	XP_019708739.1	homeobox-leucine zipper protein ROC8	Up	2.6	1.4	0.027	Down	-3.5	-1.8	0.007
13	MYBP	XP_010932583.1	transcription factor MYB20	Up	3.5	1.8	0.001	Down	-2.9	-1.6	0.001
14	MYBP	XP_010933430.1	myb-related protein Zm1-like	Up	2.7	1.5	0.022	Up	10.0	3.3	0.000
15	HD-ZIP	XP_010933626.2	homeobox-leucine zipper protein HAT4	Up	2.2	1.1	0.024	Up	4.4	2.1	0.000
16	MYBP	XP_010935898.1	transcription factor MYB30	Up	10.4	3.4	0.000	Up	15.7	4.0	0.000
17	MYBP	XP_010936941.1	transcription factor MYB4	Down	-17.2	-4.1	0.000	Down	-7.7	-2.9	0.000
18	MYBP	XP_010936942.1	anthocyanin regulatory C1 protein-like	Down	-3.8	-1.9	0.002	Down	-5.7	-2.5	0.000
19	MYBP	XP_010939500.1	myb-related protein 306	Up	3.7	1.9	0.000	Up	5.2	2.4	0.000
20	NFYB, HAP3	XP_010939223.1	nuclear transcription factor Y subunit B-3	Down	-1.7	-0.7	0.002	Down	-1.9	-0.9	0.000

5. Conclusion

To the best of our knowledge, no report shows that oil palm (*E. guineensis*) has salinity or drought stress tolerance. All transcriptome data collected and analyzed in the present study and in Salgado et al. [23, 24] derived from the apical leaf of young oil palm plants submitted to very high salinity levels in the soil or water deprivation. When we collected those leaves, the plants showed severe symptoms (browning, scorched, dying) as a consequence of being for almost two weeks under stress. Consequently, we assume that those plants were not showing any signs of tolerance; on the contrary, they were affected by high salinity in the soil or water deprivation and would die if kept longer under those conditions.

Starting from this premise and remembering that the initial goal of this study was to get more insights on the role of transcription factors in the response of young oil palm plants to those two stresses, it is necessary to state that the development of plants tolerant/resistant to those abiotic stresses was also in the horizon during this study. That is why a search for molecular symptoms that might allow us to set up strategies for genetic engineering/editing got performed.

In conclusion, the results presented here shed light on the role of 20 TFs from four different TF families during the response of young oil palm plants to severe levels of salinity in the soil or water deprivation. The behavior of such a group of TFs made it very clear that there is a high level of similarity in the molecular response to both stresses. That similarity allows the search for genes that might grant strategies aiming to develop oil palm plants with a tolerance to both salinity and drought at once. As an example, and based on the results obtained by our group - as well as on the scientific literature available regarding some of the genes here characterized and the success of some of them in achieving tolerance to those abiotic stresses through overexpression or silencing - we postulate that the overexpression of gene 10, an HD-Zip I gene from oil palm, is a valuable strategy to obtain plants with increased tolerance to drought and salinity. However, one must state that once this gene seems to be regulated by 16 and regulating 278 genes, such behavior might trigger several pleiotropic effects once overexpressed in the oil palm genome or heterologously.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Supplementary Table 1a: Annotation of drought stress-responsive transcription factors in oil palm leaves using the Ghostkoala tool. Supplementary Table 1b: Annotation of drought stress-responsive transcription factors in oil palm leaves using the Ghostkoala tool. Supplementary Table 2: Annotation of the domains of the 19 proteins that were positive in the NCBI, description of the type, superfamily and start-end of the domains. Supplementary Table 3: Classification of the gene ontology (GO) of palm oil genes, being F: molecular function, P: biological process, C: cellular component. Supplementary Table 4: Description of the family and differential expression profile of the 20 oil palm genes expressed under salt and water stress using the qPCR technique. Supplementary Table 5: List of regulators and targets of gene 10 (LOC105048648), and respective scores.

Author Contributions: Conceptualization: C.A.F.d.S., P.G. and M.T.S.J. Methodology, investigation, data curation, and formal analysis: R.C.T., A.P.L., F.F.S., and T.L.C.d.S. Funding acquisition and supervision: M.T.S.J. Writing—original draft: F.F.S, C.A.F.d.S., and P.G. Writing—review and editing: M.T.S.J. All authors have read and agreed to the published version of the manuscript.

Funding: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The grant (01.13.0315.00 DendêPalm Project) for this study was awarded by the Brazilian Ministry of Science, Technology, and Innovation (MCTI) via the Brazilian Innovation Agency FINEP. The authors confirm that the funder had no influence over the study design, the content of article, or selection of this journal.

Data Availability Statement: The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Acknowledgments: The authors acknowledge funding to F.F.S. and T.L.C.S. by the Coordination for the Improvement of Higher Education Personnel (CAPES), a Foundation within the Ministry of Education in Brazil, via the Graduate Program in Plant Biotechnology, Federal University of Lavras (UFLA).

Conflicts of Interest: Authors APL, RCT, CAFdS, PB, and MTSJ were employed by company The Brazilian Agricultural Research Corporation. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Statista, 2023. Available online: www.statista.com (accessed on June 30, 2023).
2. Brasil. Ministério da Agricultura, Pecuária e Abastecimento. 2028. Diagnóstico da Produção Sustentável da Palma de Óleo no Brasil / Ministério da Agricultura, Pecuária e Abastecimento - MAPA. http://www.abrapalma.org/pt/wp-content/uploads/2014/12/DIAGNOSTICO_PALMA.pdf
3. Corley, R. H. V., Rao, V., Palat, T., and Praiwan, T. (2018). Breeding for drought tolerance in oil palm. J. Oil Palm Res. 30, 26–35.
4. Zaman, M., Shahid, S. A., & Heng, L. (2018). Guideline for salinity assessment, mitigation and adaptation using nuclear and related techniques. Springer International Publishing. https://doi.org/10.1007/978-3-319-96190-3
5. Machado, R., & Serralheiro, R. (2017). Soil salinity: Effect on vegetable crop growth. Management practices to prevent and mitigate soil salinization. Horticulturae, 3(2), 30. https://doi.org/10.3390/horticulturae3020030
6. Shahid, S. A., Zaman, M., & Heng, L. (2018). Soil salinity: Historical perspectives and a world overview of the problem. Em M. Zaman, S. A. Shahid, & L. Heng, Guideline for Salinity Assessment, Mitigation and Adaptation Using Nuclear and Related Techniques (p. 43–53). Springer International Publishing. https://doi.org/10.1007/978-3-319-96190-3_2
7. Ivushkin, K., Bartholomeus, H., Bregt, A. K., Pulatov, A., Kempen, B., & De Sousa, L. (2019). Global mapping of soil salinity change. Remote Sensing of Environment, 231, 111260. https://doi.org/10.1016/j.rse.2019.111260
8. Angon, P. B., Tahjib-Ul-Arif, Md., Samin, S. I., Habiba, U., Hossain, M. A., & Brestic, M. (2022). How do plants respond to combined drought and salinity stress? —A systematic review. Plants, 11(21), 2884. https://doi.org/10.3390/plants11212884
9. Wang, W., Pang, J., Zhang, F., Sun, L., Yang, L., Fu, T., Guo, L., & Siddique, K. H. M. (2022). Salt-responsive transcriptome analysis of canola roots reveals candidate genes involved in the key metabolic pathway in response to salt stress. Scientific Reports, 12(1), 1666. https://doi.org/10.1038/s41598-022-05700-2
10. Zhang, H., Zhu, J., Gong, Z., & Zhu, J.-K. (2022). Abiotic stress responses in plants. Nature Reviews Genetics, 23(2), 104–119. https://doi.org/10.1038/s41576-021-00413-0
11. Wang, J., Ye, Y., Xu, M., Feng, L., & Xu, L. (2019). Roles of the SPL gene family and miR156 in the salt stress responses of tamarisk (*Tamarix chinensis*). BMC Plant Biology, 19(1), 370. https://doi.org/10.1186/s12870-019-1977-6
12. Javed, T., Shabbir, R., Ali, A., Afzal, I., Zaheer, U., & Gao, S.-J. (2020). Transcription factors in plant stress responses: Challenges and potential for sugarcane improvement. Plants, 9(4), 491. https://doi.org/10.3390/plants9040491
13. He, M., He, C.-Q., & Ding, N.-Z. (2018). Abiotic stresses: General defenses of land plants and chances for engineering multistress tolerance. Frontiers in Plant Science, 9, 1771. https://doi.org/10.3389/fpls.2018.01771
14. Hajyzadeh, M., Turktas, M., Khawar, K. M., & Unver, T. (2015). Mir408 overexpression causes increased drought tolerance in chickpea. Gene, 555(2), 186–193. https://doi.org/10.1016/j.gene.2014.11.002
15. Chaudhari, R. S., Jangale, B. L., Krishna, B., & Sane, P. V. (2023). Improved abiotic stress tolerance in Arabidopsis by constitutive active form of a banana DREB2 type transcription factor, MaDREB20.CA, than its native form, MaDREB20. Protoplasma, 260(3), 671–690. https://doi.org/10.1007/s00709-022-01805-7
16. Belamkar, V., Weeks, N. T., Bharti, A. K., Farmer, A. D., Graham, M. A., & Cannon, S. B. (2014). Comprehensive characterization and RNA-Seq profiling of the HD-Zip transcription factor family in soybean (*Glycine max*) during dehydration and salt stress. BMC Genomics, 15(1), 950. https://doi.org/10.1186/1471-2164-15-950
17. Bhattacharjee, A., Khurana, J. P., & Jain, M. (2016). Characterization of rice homeobox genes, oshox22 and oshox24, and over-expression of oshox24 in transgenic arabidopsis suggest their role in abiotic stress response. Frontiers in Plant Science, 7. https://doi.org/10.3389/fpls.2016.00627
18. Gong, S., Ding, Y., Hu, S., Ding, L., Chen, Z., & Zhu, C. (2019). The role of HD-Zip class I transcription factors in plant response to abiotic stresses. Physiologia Plantarum, 167(4), 516–525. https://doi.org/10.1111/ppl.12965
19. Sharif, R., Raza, A., Chen, P., Li, Y., El-Ballat, E. M., Rauf, A., Hano, C., & El-Esawi, M. A. (2021). Hd-zip gene family: Potential roles in improving plant growth and regulating stress-responsive mechanisms in plants. Genes, 12(8), 1256. https://doi.org/10.3390/genes12081256
20. Dezar, C. A., Gago, G. M., González, D. H., & Chan, R. L. (2005). Hahb-4, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to *Arabidopsis thaliana* plants. Transgenic Research, 14(4), 429–440. https://doi.org/10.1007/s11248-005-5076-0

21. Jiao, P., Jiang, Z., Wei, X., Liu, S., Qu, J., Guan, S., & Ma, Y. (2022). Overexpression of the homeobox-leucine zipper protein ATHB-6 improves the drought tolerance of maize (*Zea mays* L.). Plant Science, 316, 111159. https://doi.org/10.1016/j.plantsci.2021.111159

22. Zhao, Y., Ma, Q., Jin, X., Peng, X., Liu, J., Deng, L., Yan, H., Sheng, L., Jiang, H., & Cheng, B. (2014). A novel maize homeodomain–leucine zipper (Hd-zip) i gene, zmhdz10, positively regulates drought and salt tolerance in both rice and arabidopsis. Plant and Cell Physiology, 55(6), 1142–1156. https://doi.org/10.1093/pcp/pcu054

23. Salgado, F. F., Vieira, L. R., Silva, V. N. B., Leão, A. P., Grynberg, P., Do Carmo Costa, M. M., Togawa, R. C., De Sousa, C. A. F., & Júnior, M. T. S. (2021). Expression analysis of miRNAs and their putative target genes confirm a preponderant role of transcription factors in the early response of oil palm plants to salinity stress. BMC Plant Biology, 21(1), 518. https://doi.org/10.1186/s12870-021-03296-9

24. Salgado, F. F., Da Silva, T. L. C., Vieira, L. R., Silva, V. N. B., Leão, A. P., Costa, M. M. D. C., Togawa, R. C., De Sousa, C. A. F., Grynberg, P., & Souza, M. T. (2022). The early response of oil palm (Elaeis guineensis Jacq.) plants to water deprivation: Expression analysis of miRNAs and their putative target genes, and similarities with the response to salinity stress. Frontiers in Plant Science, 13, 970113. https://doi.org/10.3389/fpls.2022.970113

25. Chen, X., Chen, Z., Zhao, H., Zhao, Y., Cheng, B., & Xiang, Y. (2014). Genome-wide analysis of soybean hd-zip gene family and expression profiling under salinity and drought treatments. PLoS ONE, 9(2), e87156. https://doi.org/10.1371/journal.pone.0087156

26. Stephenson, T. J., McIntyre, C. L., Collet, C., & Xue, G.-P. (2007). Genome-wide identification and expression analysis of the NF-Y family of transcription factors in *Triticum aestivum*. Plant Molecular Biology, 65(1–2), 77–92. https://doi.org/10.1007/s11103-007-9200-9

27. Roelofs, D., Morgan, J., & Stürzenbaum, S. (2010). The significance of genome-wide transcriptional regulation in the evolution of stress tolerance. Evolutionary Ecology, 24(3), 527–539. https://doi.org/10.1007/s10682-009-9345-x

28. Qin, F., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2011). Achievements and challenges in understanding plant abiotic stress responses and tolerance. Plant and Cell Physiology, 52(9), 1569–1582. https://doi.org/10.1093/pcp/pcr106

29. Yamaguchi-Shinozaki, K., & Shinozaki, K. (2007). Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Em J. A. Goode & D. Chadwick (Orgs.), Novartis Foundation Symposia (p. 176–189). John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470515778.ch13

30. Ni, Z., Hu, Z., Jiang, Q., & Zhang, H. (2013). GmNFYA3, a target gene of miR169, is a positive regulator of plant tolerance to drought stress. Plant Molecular Biology, 82(1–2), 113–129. https://doi.org/10.1007/s11103-013-0040-5

31. Xu, Z., Gongbuzhaxi, Wang, C., Xue, F., Zhang, H., & Ji, W. (2015). Wheat NAC transcription factor TaNAC29 is involved in response to salt stress. Plant Physiology and Biochemistry, 96, 356–363. https://doi.org/10.1016/j.plaphy.2015.08.013

32. Song, S., Xu, Y., Huang, D., Miao, H., Liu, J., Jia, C., Hu, W., Valarezo, A. V., Xu, B., & Jin, Z. (2018). Identification of a novel promoter from banana aquaporin family gene (MaTIP1;2) which responses to drought and salt-stress in transgenic Arabidopsis thaliana. Plant Physiology and Biochemistry, 128, 163–169. https://doi.org/10.1016/j.plaphy.2018.05.003

33. Yu, Y., Xu, W., Wang, J., Wang, L., Yao, W., Xu, Y., Ding, J., & Wang, Y. (2013). A core functional region of the RFP1 promoter from Chinese wild grapevine is activated by powdery mildew pathogen and heat stress. Planta, 237(1), 293–303. https://doi.org/10.1007/s00425-012-1769-9

34. Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., & Lepiniec, L. (2010). MYB transcription factors in Arabidopsis. Trends in Plant Science, 15(10), 573–581. https://doi.org/10.1016/j.tplants.2010.06.005

35. Roy, S. (2016). Function of MYB domain transcription factors in abiotic stress and epigenetic control of stress response in plant genome. Plant Signaling & Behavior, 11(1), e1117723. https://doi.org/10.1080/15592324.2015.1117723

36. Wang, X., Niu, Y., & Zheng, Y. (2021). Multiple functions of myb transcription factors in abiotic stress responses. International Journal of Molecular Sciences, 22(11), 6125. https://doi.org/10.3390/ijms22116125

37. Gao, S., Zhang, Y. L., Yang, L., Song, J. B., & Yang, Z. M. (2014). AtMYB20 is negatively involved in plant adaptive response to drought stress. Plant and Soil, 376(1–2), 433–443. https://doi.org/10.1007/s11104-013-1992-6

38. Gong, Q., Li, S., Zheng, Y., Duan, H., Xiao, F., Zhuang, Y., He, J., Wu, G., Zhao, S., Zhou, H., & Lin, H. (2020). SUMOylation of MYB30 enhances salt tolerance by elevating alternative respiration via transcriptionally upregulating AOX1a in Arabidopsis. The Plant Journal, 102(6), 1157–1171. https://doi.org/10.1111/tpj.14689

39. Lee, S. B., & Suh, M. C. (2015). Advances in the understanding of cuticular waxes in *Arabidopsis thaliana* and crop species. Plant Cell Reports, 34(4), 557–572. https://doi.org/10.1007/s00299-015-1772-2

40. Nelson, D. E., Repetti, P. P., Adams, T. R., Creelman, R. A., Wu, J., Warner, D. C., Anstrom, D. C., Bensen, R. J., Castiglioni, P. P., Donnarummo, M. G., Hinchey, B. S., Kumimoto, R. W., Maszle, D. R., Canales, R. D., Krolikowski, K. A., Dotson, S. B., Gutterson, N., Ratcliffe, O. J., & Heard, J. E. (2007). Plant nuclear factor Y (Nf-y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proceedings of the National Academy of Sciences, 104(42), 16450–16455. https://doi.org/10.1073/pnas.0707193104

41. Leyva-González, M. A., Ibarra-Laclette, E., Cruz-Ramírez, A., & Herrera-Estrella, L. (2012). Functional and transcriptome analysis reveals an acclimatization strategy for abiotic stress tolerance mediated by Arabidopsis nf-ya family members. PLoS ONE, 7(10), e48138. https://doi.org/10.1371/journal.pone.0048138

42. Li, L., Yu, Y., Wei, J., Huang, G., Zhang, D., Liu, Y., & Zhang, L. (2013). Homologous HAP5 subunit from Picea wilsonii improved tolerance to salt and decreased sensitivity to ABA in transformed Arabidopsis. Planta, 238(2), 345–356. https://doi.org/10.1007/s00425-013-1894-0

43. Kumar, R. (2014). Role of micrornas in biotic and abiotic stress responses in crop plants. Applied Biochemistry and Biotechnology, 174(1), 93–115. https://doi.org/10.1007/s12010-014-0914-2

44. Zhao, H., Wu, D., Kong, F., Lin, K., Zhang, H., & Li, G. (2017). The *Arabidopsis thaliana* nuclear factor y transcription factors. Frontiers in Plant Science, 07. https://doi.org/10.3389/fpls.2016.02045

45. Li, Y., Yang, Z., Zhang, Y., Guo, J., Liu, L., Wang, C., Wang, B., & Han, G. (2022). The roles of HD-ZIP proteins in plant abiotic stress tolerance. Frontiers in Plant Science, 13, 1027071. https://doi.org/10.3389/fpls.2022.1027071

46. Guo, M., Liu, J.-H., Ma, X., Luo, D.-X., Gong, Z.-H., & Lu, M.-H. (2016). The plant heat stress transcription factors (Hsfs): Structure, regulation, and function in response to abiotic stresses. Frontiers in Plant Science, 7. https://doi.org/10.3389/fpls.2016.00114

47. Ohama, N., Sato, H., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2017). Transcriptional regulatory network of plant heat stress response. Trends in Plant Science, 22(1), 53–65. https://doi.org/10.1016/j.tplants.2016.08.015

48. Li, W., Wan, X.-L., Yu, J.-Y., Wang, K.-L., & Zhang, J. (2019). Genome-wide identification, classification, and expression analysis of the hsf gene family in carnation (*Dianthus caryophyllus*). International Journal of Molecular Sciences, 20(20), 5233. https://doi.org/10.3390/ijms20205233

49. Wu, Z., Liang, J., Wang, C., Zhao, X., Zhong, X., Cao, X., Li, G., He, J., & Yi, M. (2018). Overexpression of lily HsfA3s in Arabidopsis confers increased thermotolerance and salt sensitivity via alterations in proline catabolism. Journal of Experimental Botany, 69(8), 2005–2021. https://doi.org/10.1093/jxb/ery035

50. Pérez-Salamó, I., Papdi, C., Rigó, G., Zsigmond, L., Vilela, B., Lumbreras, V., Nagy, I., Horváth, B., Domoki, M., Darula, Z., Medzihradszky, K., Bögre, L., Koncz, C., & Szabados, L. (2014). The heat shock factor a4a confers salt tolerance and is regulated by oxidative stress and the mitogen-activated protein kinases mpk3 and mpk6. Plant Physiology, 165(1), 319–334. https://doi.org/10.1104/pp.114.237891

51. Zhu, J.-K. (2002). SALT AND DROUGHT STRESS SIGNAL TRANSDUCTION IN PLANTS. Annual Review of Plant Biology, 53(1), 247–273. https://doi.org/10.1146/annurev.arplant.53.091401.143329

52. Rasmussen, S., Barah, P., Suarez-Rodriguez, M. C., Bressendorff, S., Friis, P., Costantino, P., Bones, A. M., Nielsen, H. B., & Mundy, J. (2013). Transcriptome responses to combinations of stresses in arabidopsis. Plant Physiology, 161(4), 1783–1794. https://doi.org/10.1104/pp.112.210773

53. Hazzouri, K. M., Flowers, J. M., Nelson, D., Lemansour, A., Masmoudi, K., & Amiri, K. M. A. (2020). Prospects for the study and improvement of abiotic stress tolerance in date palms in the post-genomics era. Frontiers in Plant Science, 11, 293. https://doi.org/10.3389/fpls.2020.00293

54. McCoy, R.M.; Julian, R.; Kumar, S.R.V.; Ranjan, R.; Varala, K.; Li, Y. A Systems Biology Approach to Identify Essential Epigenetic Regulators for Specific Biological Processes in Plants. Plants 2021, 10, 364. https://doi.org/10.3390/plants10020364

55. Carvalho da Silva, T.L.; Grynberg, P.; Togawa, R.C.; Souza Jr., M.T. The Brazilian Agricultural Research Corporation, Embrapa Agroenergy, Brasília, DF, Brazil. 2023, status (manuscript in preparation; to be submitted).

56. OmicsBox – Bioinformatics Made Easy, BioBam Bioinformatics, March 3, 2019, https://www.biobam.com/omicsbox

57. Singh, R., Ong-Abdullah, M., Low, E.-T. L., Manaf, M. A. A., Rosli, R., Nookiah, R., Ooi, L. C.-L., Ooi, S., Chan, K.-L., Halim, M. A., Azizi, N., Nagappan, J., Bacher, B., Lakey, N., Smith, S. W., He, D., Hogan, M., Budiman, M. A., Lee, E. K., … Sambanthamurthi, R. (2013). Oil palm genome sequence reveals divergence of interfertile species in Old and New worlds. Nature, 500(7462), 335–339. https://doi.org/10.1038/nature12309

58. Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. Bioinformatics, 29(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

59. Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq—A Python framework to work with high-throughput sequencing data. Bioinformatics, 31(2), 166–169. https://doi.org/10.1093/bioinformatics/btu638

60. Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). Edger: A bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics, 26(1), 139–140. https://doi.org/10.1093/bioinformatics/btp616

61. Lescot, M. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Research, 30(1), 325–327. https://doi.org/10.1093/nar/30.1.325

62. Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S. K., Cook, H., Mende, D. R., Letunic, I., Rattei, T., Jensen, L. J., von Mering, C., & Bork, P. (2019). Eggnog 5. 0: A hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Research, 47(D1), D309–D314. https://doi.org/10.1093/nar/gky1085

63. Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22(22), 4673–4680. https://doi.org/10.1093/nar/22.22.4673

64. Tamura, K., Stecher, G., & Kumar, S. (2021). Mega11: Molecular evolutionary genetics analysis version 11. Molecular Biology and Evolution, 38(7), 3022–3027. https://doi.org/10.1093/molbev/msab120