**Introduction**

Grafting is an old technique that has been used for the production of individuals with higher resistance to stresses, precocious flowering plants, controlling of plant architecture, and or …. (Kondhare et al., 2021; Yang et al., 2015). Potato/tomato heterografting, watermelon/bottle gourd, or fruit grafting are some examples that heterografting helped plants to survive under stresses or have better growth in different situations (Wang et al., 2020; Zhang et al., 2022). Grafting causes changes in the heterograft traits but the molecular mechanisms behind that had been unknown for decades (Kondhare et al., 2021). There must exist a communication system between different compartments within cells, adjacent cells, and different organs that could transport the environmental or developmental signals that also transport back and forward signals among rootstock and scion in heterografts (Spiegelman et al., 2013; Turnbull & Lopez‐Cobollo, 2013; Xia & Zhang, 2020). Long-distance transportation occurs in the vasculature system transporting different molecules, including sugars, hormones, proteins, amino acids, and RNAs (Turgeon & Wolf, 2009). Unlike other long-distance transport molecules, the biological functions of mobile RNAs have not been completely identified (Xia & Zhang, 2020). The signaling role of RNAs was firstly reported in virus-infected plants, moving virus RNAs through plasmodesmata in plants (Petty et al., 1990; Ryabov et al., 1999). In Arabidopsis, some mobile mRNAs transport from leaf to the floral meristem to regulate the reproductive functions responding to environmental cues (Corbesier et al., 2007; Yoo et al., 2013). Various physiological processes reported having association with mobile mRNAs, such as the regulation of potato tuberization (Ghate et al., 2017; Hannapel & Banerjee, 2017), lateral root formation in parasitic plants (Yoshida et al., 2016), leaf morphology changes in tomato (Haywood et al., 2005), and or floral initiation in Arabidopsis (Huang et al., 2012).

Different methods have been utilized to detect the mobile RNAs (small RNAs or mRNAs), however, transcriptome profiling of scions and rootstocks has efficiently appeared (Li et al., 2022). To choose the species for mobile mRNA detection, the species that are used for heterografting must be compatible for heterografting but with genome sequences distant enough to be unambiguously able to detect mobile mRNAs (Xia & Zhang, 2020). According to the distance between genome sequences, different strategies might be utilized for RNA-seq analysis to detect mobile mRNAs; for those which are not so different such as different genotypes of one species, SNPs could help to identify transcripts from different genotypes. In fact, first SNPs will be identified by mapping the raw reads to the same reference genome, then the transmitted mRNAs are defined if their corresponding reads from one genotype were detected in the other genotype in the heterografts (Wang et al., 2020; Yang et al., 2015). However, for heterografts composed from two distant genome sequences, RNA-seq reads will be mapped against two reference genomes and those reads present in one species that are aligned to the other species but not to the corresponding species’ reference genome will be identified as transmitted mRNAs (Notaguchi et al., 2015; Xia et al., 2018). Indeed in both strategies some criteria must be considered to detect more reliable mobile mRNAs.

Transcriptome assessment of heterografting has become an interesting tool to detect mobile RNAs as long-distance signals in plants (Kondhare et al., 2021). The evolution of C4 plants from their C3 ancestors has been attributed to the process of recruiting and re-organizing pre-existing genes to have new functions in C4 (Burgess et al., 2016; Lyu et al., 2020). Besides that, some morphological changes such as increasing the plasmodesmata intense between mesophyll and bundle sheath cells and the vein density have been well reported in C4 plants compared with their close phylogenetically C3 plants (Khoshravesh et al., 2020). Therefore, heterografting of C3 and C4 plants would be an interesting tool to assess how transmission of RNAs from C3 and C4 parts could pose changes in heterografts compared to their homograft samples. To our knowledge, there is no study on transcriptome profiling of heterografts of C3 and C4 plants, but the previous study investigated the influence of roots and shoots on GSH metabolism and sulfate assimilation in scion and stock tissue of interspecies C3-C4 grafts. The results showed that roots are predominantly controlling the S root-to-shoot translocation. Also, they figured out that C4 plants are adapted to lower sulfate content in the shoot as the C4 scions decreased the *SULT2.1* expression in C3 stock of C4/C3 grafts rather than C3/C3 homograft. In fact, C4 scion induced the expression level of *SULTR2.1* in C3 stock by long-distance signaling (Gerlich et al., 2018). Followed by the previous study, in this paper, we investigated the transcriptome profile of interspecies grafts from *Flaveria bidentis* (C4) and *Flaveria robusta* (C3) by RNA-seq to identification of transmitted RNAs that might be involved in the phenotypic and physiological changes in the heterografts. To do so, we created de novo assemblies from tissues obtained from homografts and determined the differentially expressed genes in homo- and hetero-grafts. Also, by the alignment of short reads from

**Material and method**

**Plant material and sampling**

Interspecies micrografting from *F. robusta* and *F. bidentis* was conducted as explained in previous study (Gerlich et al., 2018). So, we had four different seedlings growing in normal conditions; two homografts (C3/C3, C4/C4) and two reciprocal heterografts, C4 scion/C3 stock (C4/C3), and C3 scion/C4 stock (C3/C4). The RNA extraction was performed for both root and shoot from each seedling type with three biological replication. Sequencing was conducted by …. Company and some information about quality and order

**De novo assembly**

Before running analysis on the raw sequence data, the quality control of samples were checked for adaptor pollination and the law quality bases using FastQC package. Afterwards, trimming of the raw sequences were conducted using Trimmomatic with parameters: SLIDINGWINDOW:4:15, CROP:50, and HEADCROP:10. As there is no reference genome for Flaveria, we had to run Trinity (Grabherr et al., 2011) to create transcriptome de novo assembly using the trimmed fastq files. We needed two assembly files for *F. bidentis* and *F. robusta* separately to be able to detect the transmitted mRNAs, so, we used fastq files from both root and shoot of *F. bidentis* and *F. robusta* homografts to be sure there is no reads related to other species in our assemblies. Trinity was run with paarameters, including –samples\_file, --SS\_lib\_type and default normalization step. The quality of created de novo assemblies was assessed by contig ExN50 using contig\_ExN50\_statistic.pl script and RNA Seq Read Representation using bowtie2. Afterwards, to functionally annotation of obtained contigs, we performed different tools, including Transdecoder (<https://github.com/TransDecoder/TransDecoder>) with default parameters, BLAST against Arabidopsis proteins for sequence homology searching, SignalP (Almagro Armenteros et al., 2019) and tmhmm (Krogh et al., 2001) software tools for predicting signal peptides and transmembrane domains, RNAmmer (Lagesen et al., 2007) to detect rRNAs. Finally, all information obtaining from previous steps for functional annotation of contigs were import into Trinonate SQLite database (https://github.com/Trinotate/Trinotate/wiki).

**Differentially gene expression analysis**

To identify differentially expressed genes in counterpart tissues between heterografts and homografts, we aligned back RNA-seq reads from each tissue to its corresponding assembly (Table 1) to obtain expression values for contigs. RSEM method (Li & Dewey, 2011) was run for evaluating transcript expression. Then DESeq2 R package (Love et al., 2014) was run for differential expression analysis.

Table 1. Samples were aligned back to their corresponding assembly files

|  |  |  |
| --- | --- | --- |
| Tissue | Seedling type | Assembly |
| Root | C3/C3 | C3 |
| Root | C4/C3 | C3 |
| Root | C4/C4 | C4 |
| Root | C4/C3 | C4 |
| Shoot | C3/C3 | C3 |
| Shoot | C3/C4 | C3 |
| Shoot | C4/C4 | C4 |
| Shoot | C3/C4 | C4 |

**Results**

**De novo assembly**

To build a high-quality transcriptome assembly, we first checked the quality of short reads and then trimming of low-quality bases was conducted. For C3 and C4 species, two separate assembly files were built. Only read files from homografts were used to have pure assembly files for each species. Trinity was run with internal normalization and kmer =25.

Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G., & Nielsen, H. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nature biotechnology*, *37*(4), 420-423.

Burgess, S. J., Granero-Moya, I., Grangé-Guermente, M. J., Boursnell, C., Terry, M. J., & Hibberd, J. M. (2016). Ancestral light and chloroplast regulation form the foundations for C4 gene expression. *Nature Plants*, *2*(11), 1-6.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., & Turnbull, C. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *science*, *316*(5827), 1030-1033.

Gerlich, S. C., Walker, B. J., Krueger, S., & Kopriva, S. (2018). Sulfate metabolism in C4 Flaveria species is controlled by the root and connected to serine biosynthesis. *Plant Physiology*, *178*(2), 565-582.

Ghate, T. H., Sharma, P., Kondhare, K. R., Hannapel, D. J., & Banerjee, A. K. (2017). The mobile RNAs, StBEL11 and StBEL29, suppress growth of tubers in potato. *Plant molecular biology*, *93*, 563-578.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., & Zeng, Q. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature biotechnology*, *29*(7), 644-652.

Hannapel, D. J., & Banerjee, A. K. (2017). Multiple mobile mRNA signals regulate tuber development in potato. *Plants*, *6*(1), 8.

Haywood, V., Yu, T. S., Huang, N. C., & Lucas, W. J. (2005). Phloem long‐distance trafficking of GIBBERELLIC ACID‐INSENSITIVE RNA regulates leaf development. *The Plant Journal*, *42*(1), 49-68.

Huang, N. C., Jane, W. N., Chen, J., & Yu, T. S. (2012). Arabidopsis thaliana CENTRORADIALIS homologue (ATC) acts systemically to inhibit floral initiation in Arabidopsis. *The Plant Journal*, *72*(2), 175-184.

Khoshravesh, R., Stata, M., Busch, F. A., Saladié, M., Castelli, J. M., Dakin, N., Hattersley, P. W., Macfarlane, T. D., Sage, R. F., & Ludwig, M. (2020). The evolutionary origin of C4 photosynthesis in the grass subtribe Neurachninae. *Plant Physiology*, *182*(1), 566-583.

Kondhare, K. R., Patil, N. S., & Banerjee, A. K. (2021). A historical overview of long-distance signalling in plants. *Journal of experimental Botany*, *72*(12), 4218-4236.

Krogh, A., Larsson, B., Von Heijne, G., & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of molecular biology*, *305*(3), 567-580.

Lagesen, K., Hallin, P., Rødland, E. A., Stærfeldt, H.-H., Rognes, T., & Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic acids research*, *35*(9), 3100-3108.

Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC bioinformatics*, *12*, 1-16.

Li, W., Chen, S., Liu, Y., Wang, L., Jiang, J., Zhao, S., Fang, W., Chen, F., & Guan, Z. (2022). Long-distance transport RNAs between rootstocks and scions and graft hybridization. *Planta*, *255*(5), 96.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology*, *15*(12), 1-21.

Lyu, M.-J. A., Wang, Y., Jiang, J., Liu, X., Chen, G., & Zhu, X.-G. (2020). What Matters for C4 Transporters: Evolutionary Changes of Phospho enol pyruvate Transporter for C4 Photosynthesis. *Frontiers in plant science*, *11*, 935.

Notaguchi, M., Higashiyama, T., & Suzuki, T. (2015). Identification of mRNAs that move over long distances using an RNA-Seq analysis of Arabidopsis/Nicotiana benthamiana heterografts. *Plant and cell physiology*, *56*(2), 311-321.

Petty, I., French, R., Jones, R., & Jackson, A. (1990). Identification of barley stripe mosaic virus genes involved in viral RNA replication and systemic movement. *The EMBO journal*, *9*(11), 3453-3457.

Ryabov, E. V., Robinson, D. J., & Taliansky, M. E. (1999). A plant virus-encoded protein facilitates long-distance movement of heterologous viral RNA. *Proceedings of the National Academy of Sciences*, *96*(4), 1212-1217.

Spiegelman, Z., Golan, G., & Wolf, S. (2013). Don’t kill the messenger: long-distance trafficking of mRNA molecules. *Plant Science*, *213*, 1-8.

Turgeon, R., & Wolf, S. (2009). Phloem transport: cellular pathways and molecular trafficking. *Annual review of plant biology*, *60*, 207-221.

Turnbull, C. G., & Lopez‐Cobollo, R. M. (2013). Heavy traffic in the fast lane: long‐distance signalling by macromolecules. *New phytologist*, *198*(1), 33-51.

Wang, Y., Wang, L., Xing, N., Wu, X., Wu, X., Wang, B., Lu, Z., Xu, P., Tao, Y., & Li, G. (2020). A universal pipeline for mobile mRNA detection and insights into heterografting advantages under chilling stress. *Horticulture research*, *7*.

Xia, C., & Zhang, C. (2020). Long-distance movement of mRNAs in plants. *Plants*, *9*(6), 731.

Xia, C., Zheng, Y., Huang, J., Zhou, X., Li, R., Zha, M., Wang, S., Huang, Z., Lan, H., & Turgeon, R. (2018). Elucidation of the mechanisms of long-distance mRNA movement in a Nicotiana benthamiana/tomato heterograft system. *Plant Physiology*, *177*(2), 745-758.

Yang, Y., Mao, L., Jittayasothorn, Y., Kang, Y., Jiao, C., Fei, Z., & Zhong, G.-Y. (2015). Messenger RNA exchange between scions and rootstocks in grafted grapevines. *BMC Plant Biology*, *15*(1), 1-14.

Yoo, S. J., Hong, S. M., Jung, H. S., & Ahn, J. H. (2013). The cotyledons produce sufficient FT protein to induce flowering: evidence from cotyledon micrografting in Arabidopsis. *Plant and cell physiology*, *54*(1), 119-128.

Yoshida, S., Cui, S., Ichihashi, Y., & Shirasu, K. (2016). The haustorium, a specialized invasive organ in parasitic plants. *Annual review of plant biology*, *67*, 643-667.

Zhang, G., Zhou, J., Song, J., Guo, X., Nie, X., & Guo, H. (2022). Grafting-induced transcriptome changes and long-distance mRNA movement in the potato/Datura stramonium heterograft system. *Horticulture, Environment, and Biotechnology*, *63*(2), 229-238.