biology and life sciences forum

MDPI

Proceeding Paper

Quantification and Immunolocalization of Auxin in *Prunus dulcis* (Mill.) D. A. Webb Micrografts †

Sandra Caeiro [1,*], Tércia Lopes [1], Ana Pedrosa [1], André Caeiro [1], Rita Pires [2], Ana Faustino [2,3], Armindo Rosa [4], António Marreiros [4], Jorge Canhoto [1], Liliana Marum [2,3] and Sandra Correia [1,*]

1 University of Coimbra, Centre for Functional Ecology, Department of Life Sciences, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal; tercia.lopes.95@gmail.com (T.L.); anasimoespedrosa@gmail.com (A.P.); andrecaeiro91@gmail.com (A.C.); jorgecan@uc.pt (J.C.)
2 Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL)/Instituto Politécnico de Beja (IPBeja), 7801-908 Beja, Portugal; rita.pires@cebal.pt (R.P.); anafaustino@uevora.pt (A.F.); liliana.marum@cebal.pt (L.M.)
3 MED—Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento, CEBAL, 7801-908, Beja, Portugal
4 Direcção Regional de Agricultura e Pescas do Algarve, Apartado 282, Patacão, 8001-904 Faro, Portugal; armirosa@drapalg.min-agricultura.pt (A.R.); marreiro@drapalg.min-agricultura.pt (A.M.)
* Correspondence: caeirosandra@hotmail.com (S.C.); sandraimc@ci.uc.pt (S.C.)
† Presented at the 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants, 1–15 December 2021; Available online: https://iecps2021.sciforum.net/.

Citation: Caeiro, S.; Lopes, T.; Pedrosa, A.; Caeiro, A.; Pires, R.; Faustino, A.; Rosa, A.; Marreiros, A.; Canhoto, J.; Marum, L.; Correia, S. Quantification and immunolocalization of auxin in *Prunus dulcis* (Mill.) D. A. Webb micrografts. *Biol. Life Sci. Forum* **2021**, *1*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Feibo Wu

Published: 29 November 2021

Abstract: Almond (*Prunus dulcis* (Mill.) D. A. Webb) is a traditional culture in Portugal, which gained a renewed interest due to the installation of new orchards. Grafting remains the main method used for almond propagation. The successful establishment of a graft union between two parts (scion and rootstock), requires auxins, which are involved in wound response and vascular regeneration. This work aimed at the quantification and immunolocalization of indole-3-acetic acid (IAA) in almond micrografts before micrografting (T0) and 21 days after micrografting (T2). The results are a step forward to understand of how auxin is involved in graft compatibility in almond.

Keywords: almond; auxin; immunohistochemistry; indole-3-acetic acid; in vitro culture; micrografts

1. Introduction

In recent years almond production has increased due to the strong tendency of consumers towards plant-based products. One of the main almond propagation methods is grafting [1], a technique that joins two parts, scion and rootstock to improve fruit quality, increase production, increase tolerance to biotic and abiotic stresses and/or improve edaphoclimatic adaptation [2]. Nevertheless, grafting is a challenging technique, and after wounding the plants, the scion is brought in contact with the rootstock, and through cell dedifferentiation and division the *callus* is formed. Then, the full regeneration of functional vascular tissues establishes a connection between organs of different plants [3]. During grafting, hormonal signaling is involved in graft union formation, scion-rootstock communication and plant growth and development [4]. Among other plant growth regulators (PGRs), auxins are reported to be involved in the development of successful graft unions [5].

Since auxins are transported from stem apical regions to the roots, the plant vasculature transport dynamics are affected after cutting, during grafting, causing an accumulation of auxins in the scion and a depletion in the stock [3]. In *Arabidopsis thaliana* it was shown that auxin accumulation in the grafted zone is followed by cell differentiation and vascular reconnection between scion and rootstock [6].

In spite of its practical importance and biological relevance the mechanisms involved in scion-rootstock interactions are still poorly understood, but the role of auxins during grafting has been discussed [5,7,8].

In order to evaluate auxin role in grafting of *P. dulcis,* the quantification and immunolocalization of endogenous indole-3-acetic acid (IAA) in almond micrografts (homografts and heterografts) were performed. This study will contribute to increase knowledge of auxins influence on micrografting in *Prunus* spp.

2. Materials and Methods

2.1. Plant Materials

Canhota, a Portuguese traditional variety, and bitter almond plants were in vitro established from seedlings and micropropagated in MS (Murashige and Skoog, 1962) medium supplementedd with 6-Benzylaminopurine (BAP, 0.1 mg/L), 3% (*w*/*v*) sucrose and 0.7% (*w*/*v*) agar. The pH was adjusted to 5.7 and autoclaved for 20 min at 121 °C. Cultures were kept in a a growth chamber at 25 ± 1 °C and 16 h light/8 h dark photoperiod.

2.2. Micrografts

Micrografts were established using bitter almond homografts and bitter almond (rootstock) x Canhota (scion) heterografts. Under sterille conditions, apical shoot segments with approximately 1 cm were used as scion and basal shoot segments with the same length were used as rootstocks. A 'v' cut was made and the scion was inserted in the stock. For each combination 10 micrografts were placed on plastic containers (125 × 65 × 80 mm Combiness box, with white filters) containing MS medium supplemented with 3% (*w*/*v*) sucrose and 0.7% (*w*/*v*) agar, and kept in the same conditions.

2.3. IAA Quantification

The IAA content was accessed using the colorimetric method described by Anthony and Street (1969). The plant material (3–60.5 mg tissue fresh weight (FW)) was gorunded in a mortar with liquid nitrogen. A volume of Na-phosphate buffer 0.01 M (pH 7.0) 3-fold greater than the FW was added and after centrifugation (17,000 rpm; 12 min), the supernatant was recovered, and Na-phosphate buffer 0.01 M (pH 7.0) was added until the final volume of 1 mL. To the diluted sample, 2 mL of 100% (*w*/*v*) trichloroacetic acid (TCA) and 2 mL of Ehrlich's reagent (Sigma-Aldrich, MO, USA) were added in order. A blank solution of Na-phosphate buffer 0.01 M was prepared simultaneously. Reaction occurred for 20 min in the dark, and the absorbance was measured at 530 nm in a Jenway 7305 spectrometer. For each segment 3 replicates were used, and the results obtained in μg of IAA per mg of fresh tissue.

2.4. IAA Immunolocalization

Samples were fixated by complete immersion in a solution of ice-cold 4% (*w*/*v*) paraformaldehyde, overnight on an orbital shaker under gentle shaking. For sample dehydration, the fixative was replaced by ice-cold filtered 10% (*w*/*v*) sucrose solution in 1× phosphate-buffered saline (PBS) pH 7.4, applied o/n at 4 °C, which was then replaced by a new ice-cold filtered 20% (*w*/*v*) sucrose solution in 1xPBS pH 7.4 for a second overnight period at 4 °C. In every solution replacement vacuum was applied for 20 min, and samples kept in an orbital shaker under gentle shaking. Samples were embedded in Optimum Cutting Temperature compound (O.C.T.) (Tissue-Tek TM, Sakura) and kept at −80 °C before cryosectioning. Sections of 10 μm were cut in a cryostat (CM3050 S Leica Microsystems, Nussloch, Germany) were collected on coated slides and permeabilized with 2% (*w*/*v*) driselase in 8.5% (*w*/*v*) D-mannitol solution at 37 °C for 30 min, before treatment. Samples were pre-treated with blocking solution (10% bovine serum albumin -BSA in 1xPBS for 30 min). Washed with 1xPBS for 5 min and incubated with anti-IAA primary antibody (Ref.: AS09 421 AGRISERA, Vännäs, Sweden), in a 1:200 dilution in 1%

BSA in 1xPBS, overnight in a dark moistened chamber. The secondary antibody, Alexa Fluor® 633 goat anti-rabbit (Molecular Probes, Göttingen, Germany), was diluted 1:200 in 1% BSA in 1xPBS and sections incubated for 1h in a dark moistened chamber. Samples were washed twice with 1xPBS for 2 min and slides assembled with Dako Fluorescence Mounting Medium (Agilent). Negative controls were obtained by omitting the incubation step with the primary antibody. Images were acquired in a Zeiss Axio Observer.Z1 inverted microscope (equipped with a AxioCam HRm and Zen Blue 2012 software (all from Carl Zeiss, Germany)) using a A-Plan 2.5×/0.06 differential interface contrast (DIC) and EC Plan-Neofluar 10×/0.3 Ph1 objectives and R 631/633 nm laser (Colibri 7 LED light source). Images were processed with Fiji Software (version 1.53c).

IAA quantification and immunolocalization assays were carried out before micrografting (T0) and 21 days after micrografting (T2). T0 samples correspond to cut but ungrafted scion and rootstock. Micrografts collected at T2 were segmented in scion, union and rootstock.

2.5. Statistical Analysis

Statistical analysis was performed using Graph Pad Prism (Version 8.4.3 (686)). Tukey's multiple comparison test at $p \leq 0.05$ was used to analyze IAA content in ungrafted scions and rootstock at T0, and in the micrograft segments at T2.

3. Results and Discussion

3.1. IAA Quantification

The established micrograft combinations (Figure 1) resulted in different micrografting success rates after 21 days, with 60% and 90% in bitter almond x bitter almond and bitter almond x Canhota, respectively.

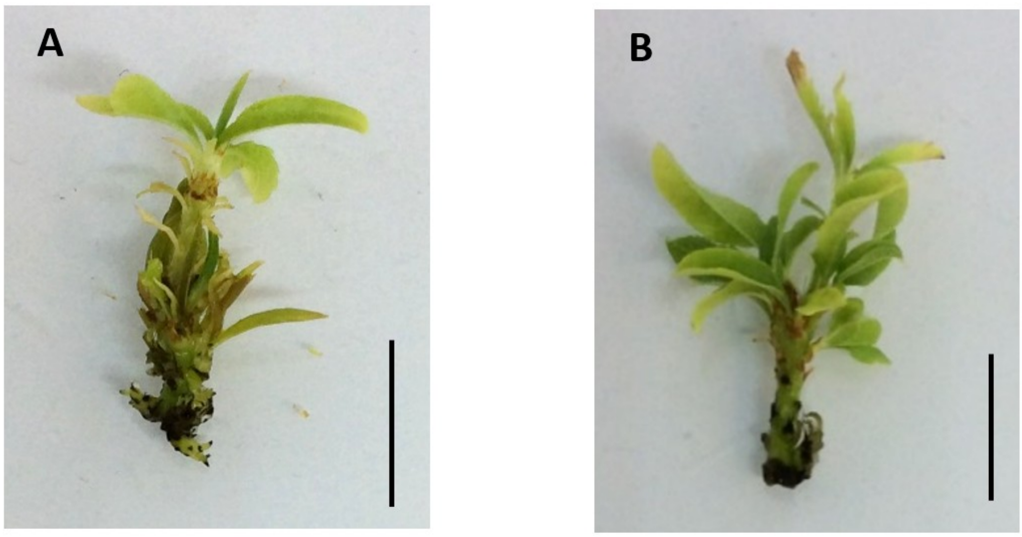

Figure 1. Micrografts 21 days after micrografting. (**A**) Bitter almond x bitter almond and (**B**) bitter almond x Canhota. Scale bar—1 cm.

IAA quantification at T0 showed significant differences ($p \leq 0.05$) between scions (Canhota and bitter almond), and between Canhota scion and bitter almond rootstock (Figure 2). Canhota scion presented an IAA content of 1.292 ± 0.448 μg IAA/ mg FW, lower than the 5.505 ± 1.179 μg IAA/ mg FW observed in bitter almond scion and the 4.107 ± 1.253 μg IAA/ mg FW in bitter almond rootstock.

Chen et al. (2017) analyzed the IAA concentrations of compatible and incompatible combinations of grafted *Litchi chinensis* and revealed an initial decrease of IAA concentrations in compatible grafts. In addition to these observations, here the micrograft combinations established with Canhota, which revealed significantly lower IAA content, presented higher micrografts success. Since both combinations were established with the same rootstock, the difference in micrografting success rates could be linked to the IAA initial levels in scions and rootstock, and initial lower levels of IAA in the scion could lead to a higher micrografting success rate.

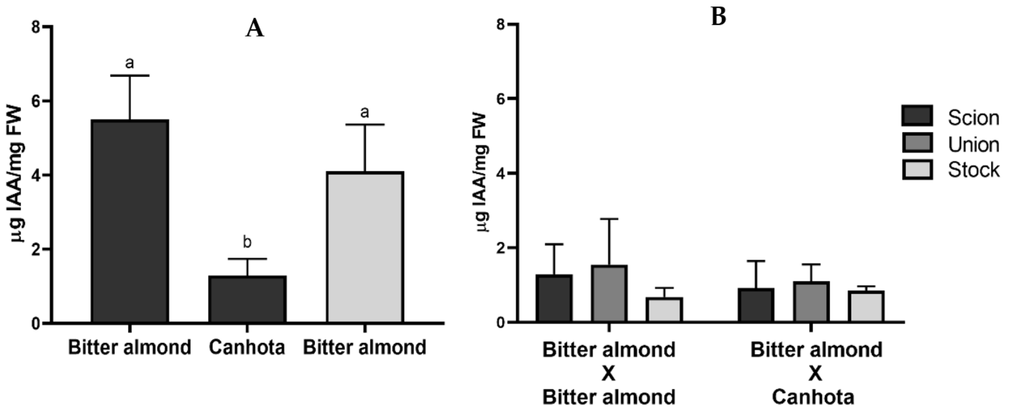

Figure 2. IAA quantification (**A**) in bitter almond and Canhota varieties ungrafted scions and rootstocks (T0) and (**B**) in bitter almond homografts and Canhota grafted onto bitter almond segments at 21 days after grafting (T2) using Ehrlich reaction. IAA is presented in µg of IAA per mg of tissue FW. Error bars correspond to SD (n = 3). Values indicated with different letters were statistically different at $p \leq 0.05$ using Tukey's multiple comparison test.

At T2, the IAA content of micrograft segments (scion, union and rootstock) (Figure 2) was not statistically different ($p > 0.05$) in both bitter almond x bitter almond and bitter almond x Canhota. However, a trend for IAA accumulation at the graft union was observed in both combinations, with 1.547 ± 1.225 µg IAA/mg FW in homografts and 1.103 ± 0.451 µg IAA/ mg FW in heterografts. Auxin has been described in wound response and vascular formation, thus it is unsurprising that auxins are involved in micrografting [7]. This tendency of accumulation at the graft union could be associated with the described role of IAA in the graft compatibility [9], that was proved by some authors by the exogenous application of IAA, which improved grafting success [8]. In *A. thaliana* the accumulation of auxin at the graft union was followed by cell differentiation and vascular reconnection, crucial steps for the success of grafting [6], and even though a tendency for auxin accumulation at the graft union was observed here the following events need further investigation.

3.2. IAA Immunolocalization

At T0, IAA signal was detected in Canhota scion and in bitter almond rootstock (Figure 3). The observation of more intense labelled IAA in Canhota shoot apex is in accordance to the reported auxin synthesis in plants young growing regions, in the shoot apex and young leaves [10]. This analysis was performed immediately after cutting the segments, and the presence of fluorescence in the bitter almond rootstock might indicate that auxin transport, from the mainly synthesis organs to the roots, is not yet affected.

At T2, IAA signal was detected in the scion segment and in the graft union, apparently in the scion part (Figure 4). In the rootstock, auxin is apparently absent close to the graft union, and more intensively labelled closer to the base. The changes in auxin transport comparatively to T0, may have resulted from the vasculature cut. This asymmetric accumulation of auxin, also described in *A. thaliana*, is probably required for an efficient regeneration and reconnection of vascular tissues during grafting [3].

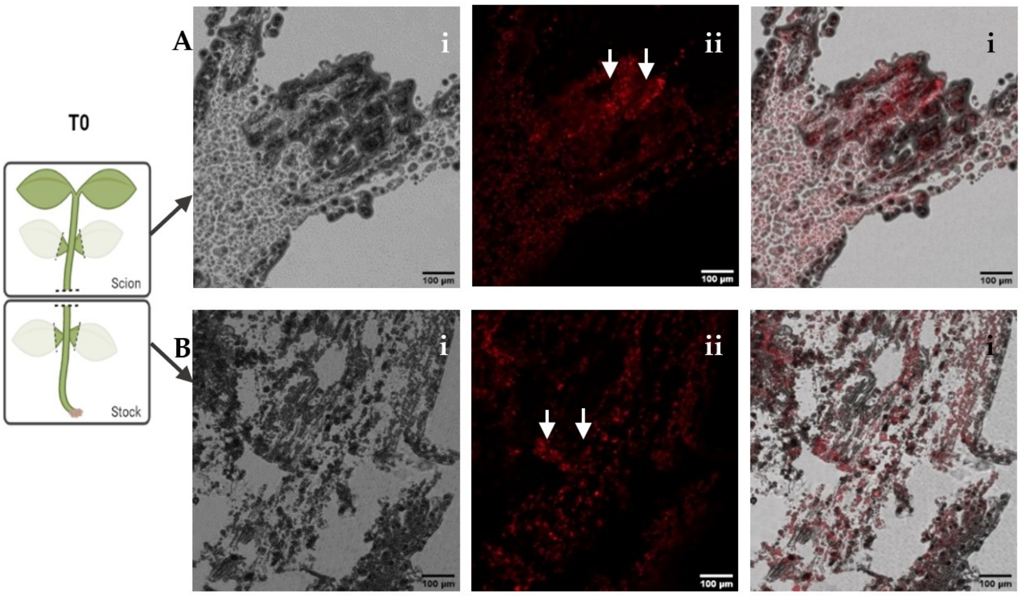

Figure 3. Immunolocalization of IAA in 4% (*v*/*v*) paraformaldehyde fixed histological sections (10 µm) of Canhota scion (**A**) and bitter almond stock (**B**) at T0. (i) Sections observed under transmission light (right column) were used for histological control. (ii) Sections stained with the anti-IAA antibody and Alexa Fluor® 633 (center column). (iii) Merged images. Arrows indicate the places where IAA has been more intensively labelled. Scale bar—100 µm.

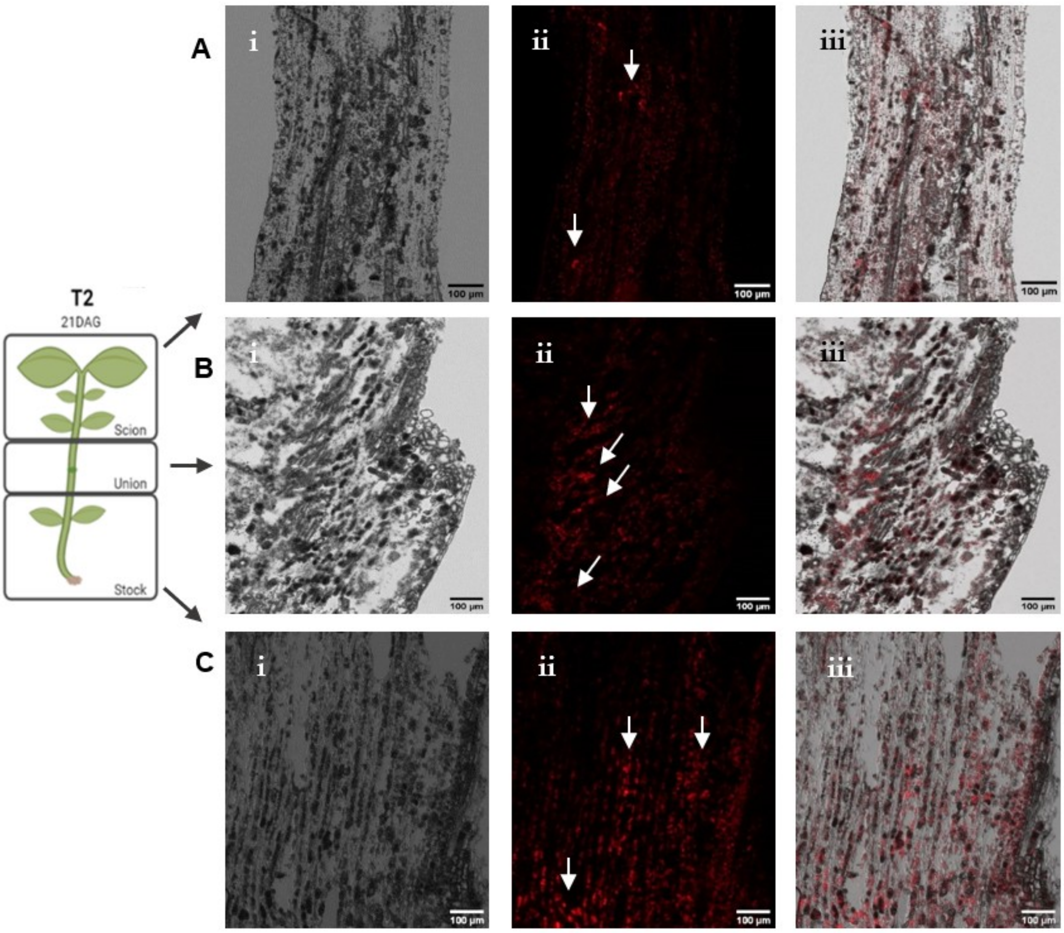

Figure 4. Immunolocalization of IAA in 4% (*v*/*v*) paraformaldehyde fixed histological sections (10 µm) of bitter almond x bitter almond micrografts scion (**A**), graft union (**B**) and stock (**C**) at T2. (i) Sections observed under transmission light (right column) were used for histological control. (ii) Sections stained with the anti-IAA antibody and Alexa Fluor® 633

(center column). (iii) Merged images. Arrows indicate the places where IAA has been more intensively labelled Scale bar—100 μm.

4. Conclusions

Auxin quantification in almond micrografts revealed a possible influence of scion IAA initial levels on micrografting success, and a tendency for IAA accumulation at the graft union 21 days after micrografting. In ungrafted scions and rootstocks, IAA transport dynamics does not appear to be affected by the cut. However, 21 days after micrografting, IAA presents a tendency for accumulation at the graft union, which could be the first signs for the successful establishment of compatible micrografts. These results are a step forward for the investigation of auxin role in grafting of *P. dulcis*, and a base for future studies on the molecular communication between scion and rootstock in almond grafts.

Author Contributions: Conceptualization, L.M. and S.C. (Sandra Correia); methodology, S.C. (Sandra Caeiro), T.L., A.P., A.C., R.P. and A.F.; validation and formal analysis, J.C., L.M. and S.C. (Sandra Correia); investigation, S.C. (Sandra Caeiro), T.L., A.P., A.C., R.P., A.F., A.R. and A.M.; writing—original draft preparation, S.C. (Sandra Caeiro); writing—review and editing, J.C., L.M. and S.C. (Sandra Correia); supervision, project administration and funding acquisition, J.C., L.M. and S.C. (Sandra Correia). All authors have read and agreed to the published version of the manuscript.

Funding: This work was made in the scope of CULTIVAR project (CENTRO-01-0145-FEDER-000020), co-financed by the Regional Operational Programme Centro 2020, Portugal 2020 and European Union, through European Fund for Regional Development (ERDF), and Inov-Amendo-AL: Microenxertia in vitro de amendoeiras selecionadas para a promoção do amendoal no Alentejo (ALT20-03-0246-FEDER-000068) supported by Program Alentejo 2020, through the European Fund for Regional Development (ERDF), within the scope of the Collective Action Support System—Transfer of scientific and technological knowledge. Domain of Competitiveness and Internationalization. This work was also funded by FCT through Contrato—Programa to L. Marum (CEEC-INST/00131/2018), and for UIDB/05183/2020 and UIDB/04004/2020 projects.

Data Availability Statement: The data presented in this study are available within the article.

Acknowledgments: We thank Associação de Viveiristas do Distrito de Coimbra for the plant material provided and Mónica Zuzarte and Teresa Rodrigues for their help with microscopy methodologies.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Aguiar, C.; Pereira, J.A.; Arrobas, M.; Almeida, A.; Bento, A.; Cortés, I.L.; Rodrigues, N.; Rodrigues, M.Â.; Ribeiro, A.C.; Santos, S.A.P.; et al. *Amendoeira: Estado de Produção*; Centro Nacional de Competências dos Frutos Secos: 2017.
2. Gautier, A.T.; Chambaud, C.; Brocard, L.; Ollat, N.; Gambetta, G.A.; Delrot, S.; and Cookson, S.J. Merging genotypes: Graft union formation and scion/rootstock interactions. *J. Exp. Bot.* **2018**, *70*, 747–755. https://doi.org/10.1093/jxb/ery422.
3. Melnyk, C.W. Plant grafting: Insights into tissue regeneration. *Regeneration* **2016**, *4*, 3–14. https://doi.org/10.1002/reg2.71.
4. Aloni, B., Cohen, R., Karni, L., Aktas, H., and Edelstein, M. Hormonal signaling in rootstock–scion interactions. *Sci. Hortic.* **2010**, *127*, 119–126. https://doi.org/10.1016/j.scienta.2010.09.003.
5. Aloni, R. Differentiation of vascular tissues. *Annu. Rev. Plant Physiol.* **1987**, *38*, 179–204. https://doi.org/10.1146/annurev.pp.38.060187.
6. Yin, H.; Yan, B.; Sun, J.; Jia, P.; Zhang, Z.; Yan, X.; Chai, J.; Ren, Z.; Zheng, G. and Liu, H. Graft-union development: A delicate process that involves cell–cell communication between scion and stock for local auxin accumulation. *J. Exp. Bot.* **2012**, *63*, 4219–4232. https://doi.org/10.1093/jxb/ers109.
7. Nanda, A.K., and Melnyk, C.W. The role of plant hormones during grafting. *J. Plant. Res.* **2017**, *131*, 49–58.
8. Saravana Kumar, R.M., Gao, L.X., Yuan, H.W., Xu, D.B., Liang, Z., Tao, S.C., Edqvist, J. Auxin enhances grafting success in *Carya cathayensis* (Chinese hickory). *Planta* **2017**, *247*, 761–772. https://doi.org/10.1007/s00425-017-2824-3.
9. Chen, Z.; Zhao, J.; Hu, F.; Qin, Y.; Wang, X.; and Hu, G. Transcriptome changes between compatible and incompatible graft combination of *Litchi chinensis* by digital gene expression profile. *Sci. Rep.* **2017**, *7*, 3954. https://doi.org/10.1038/s41598-017-04328-x.
10. Friml, J.; Palme, K. Polar auxin transport–old questions and new concepts? *Plant Mol. Biol.* **2002**, *49*, 273–284. https://doi.org/10.1023/a:1015248926412.