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Brief Communication

Single-cell mass spectrometry reveals heterogeneous triterpenic acid accumulation in apple callus-derived cells

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Plant cell cultures are a promising platform for large-scale production of many natural products used in the pharmaceutical, cosmetic and food industries (Ozyigit *et al*., 2023). However, despite a few isolated success stories, the use of plant cultures in industrial processes is still limited. Although yields of certain metabolites can be increased significantly by biotic elicitation (Laezza *et al*., 2024), overall, the biotechnological application of plant cell cultures is hindered by inherent heterogeneity and genetic instability (Miguel and Marum, 2011). The application of single-cell omics technologies to plant cultures (Yin *et al*., 2024) could provide valuable information for the development and optimization of high-yielding cell cultures. We set out to use our recently established platform for single-cell mass spectrometry (scMS) (Vu *et al*., 2024) to determine whether the levels of commercially important natural products are produced at similar levels across a population of callus-derived cells.

In this study, we developed a callus culture from leaves of the Annurca apple (*Malus pumila* Miller cv Annurca), a species known to accumulate high levels of triterpenic acids, natural products that possess a wide range of pharmacological properties (Mioc *et al*., 2022). The interest in these compounds has resulted in increasing commercial demand. However, isolation from plant materials is expensive and often not environmentally sustainable (Gubser *et al*., 2021), prompting us to develop plant cell cultures for production. After initial metabolomic analysis of the developed callus culture to identify triterpenic acids of interest, we elicited the callus using near-UV radiation (365 nm) for 15 days, which substantially increased the yield of triterpenic acids. We then analysed small populations of individual NUV-elicited and non-elicited cells using our scMS method (Vu *et al*., 2024). scMS revealed that triterpenic acid profiles vary substantially among individual cells of elicited and non-elicited callus, providing a highly resolved understanding of how natural product levels vary among individual cells of plant callus.

Based on the exact mass and fragmentation patterns, our metabolomic analysis revealed the presence of six known triterpenic acids in the callus (Table S1). Ursolic acid (UA) along with the downstream derivatives corosolic acid (CA), euscaphic acid (EA) and annurcoic acid (AA) were detected, in addition to oleanolic (OA) and maslinic acid (MA) (Figure 1a). With the exception of AA, the identity of these molecules was confirmed by comparison with commercial standards, and their accurate quantification was performed by UHPLC-tandem mass spectrometry (Table S2). AA was putatively assigned based on exact mass and comparison of MS^2 fragmentation with previously reported data (Figures S1 and S2) (Sut *et al*., 2018). The amounts of individual compounds measured in the callus were 2.09 ± 0.21 (UA), 4.54 ± 1.02 (CA), 4.73 ± 0.73 (EA), 0.47 ± 0.06 (OA) and 2.32 ± 0.32 (MA) mg g^{-1} fresh weight (FW). AA was not quantified due to lack of a standard. To explore the possibility of boosting triterpenic acid production in the callus, which is usually grown in the dark, we exposed callus cultures to both light and light supplemented with NUV radiation (365 nm), which are known to act as abiotic elicitors (Murthy *et al*., 2024). We observed that near-UV radiation increased the amounts of certain triterpenic acids after 15 days of exposure. Most notably, the levels of CA and EA increased by 2.6- and 2.8-fold, respectively, following elicitation (Figure S3; Table S3).

We then applied our scMS method (Vu *et al*., 2024) to assess the heterogeneity of triterpenic acid accumulation in the cells constituting the callus, as well as to assess the effect of near-UV elicitation on individual cells (Figure 1b). After enzymatic dissociation of the callus to release individual cells (protoplasts) approximately 10 000 protoplasts were distributed and trapped onto a microwell membrane with 50 μm micropore size and imaged by bright-field microscopy to record size and morphology. Single cells were picked using a microfluidics-based robot and transferred to a 96-well plate (Figure 1b). After lysis by osmotic shock and solubilization of the released metabolites by addition of methanol, the resulting single cell extracts were measured in negative ionization mode. A set of 40 cells from callus grown in the dark and 40 cells treated with near-UV was successfully analysed. Extracted ion chromatograms of the targeted analytes from control and NUV-elicited cells showed that our analytical method was suitable for the detection of triterpenic acids in single cells (Figures 1c and S4). Chromatographic separation of UA and OA, and also CA and MA, which are structural isomers, proved challenging with our single cell setup. Thus, quantification of UA and CA was based on combined peak areas. Previous studies have shown that the short process of releasing the protoplasts (2.5 h) does not significantly impact the levels of natural products (Li *et al*., 2023); however, we cannot exclude that small amounts of metabolites leaked from the cells during the process. With these

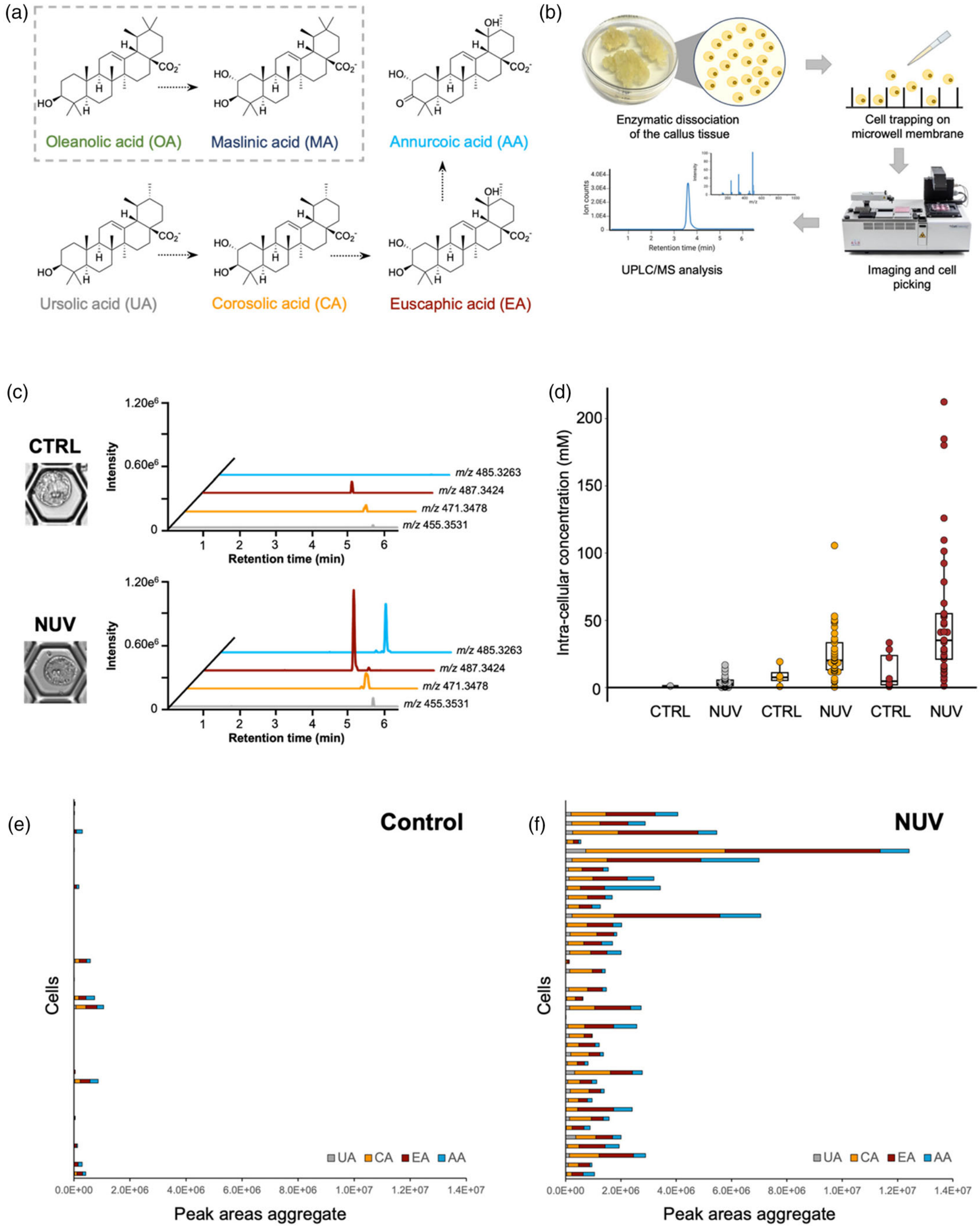

Figure 1 Single cell mass spectrometry (scMS) analysis of callus-derived cells for the detection and quantification of selected triterpenic acids. (a) The biosynthetic relationships of triterpenic acids detected and quantified in the callus tissue. (b) Overview of the scMS platform. Cells constituting the callus tissue are enzymatically dissociated ('protoplasting') and trapped in the 50 μm-wide pores of a microwell membrane. A cell picking robot is then used to image the cells and individually transfer them into 96-well plates. Cells are lysed and further solubilized with organic solvent. The cell extracts are analysed by UHPLC-HRMS for detection and quantification of the metabolites of interest. (c) Extracted ion chromatograms of UA (*m/z* 455.3531), CA (*m/z* 471.3478), EA (*m/z* 487.3424), AA (*m/z* 485.3263) in control (CTRL) and near-UV (NUV) treated cells. (d) Comparison of the intra-cellular concentrations of UA, CA and EA in control and near-UV treated single cells. (e) Aggregate peak areas of UA, CA, EA and AA measured in control cells. (f) Aggregate peak areas of UA, CA, EA and AA measured in near-UV treated. All traces, graphs and compounds are colour-coded: UA in grey, CA in orange, EA in brown and AA in light blue.

caveats, we were able to quantify the intracellular concentrations of UA, CA and EA in single cells (Figure 1c,d, Tables S4 and S5). UA, CA and EA were detected only in a small number of cells derived from the control callus grown in the dark, but in the few cells that did contain these compounds, the intracellular concentrations were substantial (between 0.69 and 33.43 mM). CA and EA were the most abundant compounds, consistent with the bulk analysis of the entire callus. Elicitation with near-UV light resulted in increased accumulation of triterpenic acids in individual cells (Figure 1c–f), reaching levels above 200 mM in the case of EA and 100 mM in the case of CA. The data distribution of the measured concentrations of CA and EA was broad, but the median values were 20.19 and 35.19 mM, respectively (Figure 1d). Hence, production of CA and EA seemed to be favourably affected by NUV treatment, hinting at an increased rate of hydroxylation of the precursor, UA, by biosynthetic enzymes that have not yet been identified (Figure 1a). Remarkably, the near-UV elicitation also impacted the number of cells that accumulated the targeted triterpenic acids. The limit of detection was estimated to be an intracellular concentration of approximately 0.1 mM, assuming an average cell volume of 13 pL.

AA is a distinctive compound of Annurca apple and a major contributor to the diversity of triterpenic acids in the leaf-derived callus. Since we could not include this metabolite in our quantification study because of the lack of a reference compound, we evaluated the aggregate peak areas of the targeted metabolites detected in the cells (Figure 1e,f). AA production was also affected by near-UV radiation, which again points to stimulation of UA biosynthesis and of downstream oxidative enzyme activities (Figure 1a). The effect of near-UV elicitation increased the homogeneity of the cell population in terms of triterpenic acid level; specifically, a larger proportion of cells produced detectable levels of triterpenic acids (Figure 1e,f). To corroborate this finding, we performed additional analysis of a larger number (96 per group) of elicited and non-elicited cells (Figures S5 and S6), which showed a similar pattern. Overall, the control cells produced limited amounts of triterpenic acids, as per their aggregate peak areas, and these were only detectable in approximately 20% of the cells. Following elicitation, the number of cells in which these compounds accumulated increased to 70%; however, a great variability in the quantity of metabolites and their relative ratios still existed.

In conclusion, we used a scMS method to analyse cells of a callus tissue that produces significant amounts of pharmaceutically important triterpenic acids. We measured the intracellular concentrations of three triterpenic acids of interest in a population of these cells. We showed that individual callus cells accumulated variable amounts of the target compounds, revealing the extent of metabolic heterogeneity within the callus cell population. Abiotic elicitation, specifically NUV, dramatically increased the levels of triterpenic compounds. Although the majority of NUV elicited cells sampled contained triterpenic acids, the ratio of the specific triterpenic acids varied substantially across the cell population. Single cell mass spectrometry analyses provide a starting foundation for understanding the molecular mechanisms responsible for metabolic heterogeneity in plant cell cultures, which could in turn facilitate efforts to improve these cell cultures for commercial purposes.

Author contributions

C.L. and L.C. designed and performed the experiments. S.H. assisted with UHPLC–MS method development; J.W. helped with preparing and picking the cells. V.D'A. provided support with the development of callus cultures and with paper revisions; C.L., L.C., M.M.R. and S.E.O'C. conceptualized the study and wrote the paper.

Competing interest statement

No competing interests to declare.

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References

Gubser, G., Vollenweider, S., Eibl, D. and Eibl, R. (2021) Food ingredients and food made with plant cell and tissue cultures: State-of-the art and future trends. *Eng. Life Sci.* **21**, 87–98.

Laezza, C., Imbimbo, P., D'Amelia, V., Marzocchi, A., Monti, D.M., Di Loria, A., Monti, S.M. *et al*. (2024) Use of yeast extract to elicit a pulp-derived callus cultures from Annurca apple and potentiate its biological activity. *J. Funct. Foods* **112**, 105988.

Li, C., Wood, J.C., Vu, A.H., Hamilton, J.P., Rodriguez Lopez, C.E., Payne, R.M.E., Serna Guerrero, D.A. *et al*. (2023) Single-cell multi-omics in the medicinal plant *Catharanthus roseus*. *Nat. Chem. Biol.* **19**, 1031–1041.

Miguel, C. and Marum, L. (2011) An epigenetic view of plant cells cultured in vitro: somaclonal variation and beyond. *J. Exp. Bot.* **62**, 3713–3725.

Mioc, M., Milan, A., Malița, D., Mioc, A., Prodea, A., Racoviceanu, R., Ghiulai, R. *et al*. (2022) Recent advances regarding the molecular mechanisms of triterpenic acids: A review (part I). *Int. J. Mol. Sci.* **23**, 7740.

Murthy, H.N., Joseph, K.S., Paek, K.Y. and Park, S.Y. (2024) Light as an elicitor for enhanced production of secondary metabolites in plant cell, tissue, and organ cultures. *Plant Growth Regul.* **104**, 31–49.

Ozyigit, I.I., Dogan, I., Hocaoglu-Ozyigit, A., Yalcin, B., Erdogan, A., Yalcin, I.E., Cabi, E. *et al*. (2023) Production of secondary metabolites using tissue culture-based biotechnological applications. *Front. Plant Sci.* **14**, 1132555.

Sut, S., Poloniato, G., Malagoli, M. and Dall'Acqua, S. (2018) Fragmentation of the main triterpene acids of apple by LC-APCI-MSn. *J. Mass Spectrom.* **53**, 882–892.

Vu, A.H., Kang, M., Wurlitzer, J., Heinicke, S., Li, C., Wood, J.C., Grabe, V. *et al*. (2024) Quantitative Single-Cell Mass Spectrometry Provides a Highly Resolved Analysis of Natural Product Biosynthesis Partitioning in Plants. *J. Am. Chem. Soc.* **146**, 23891–23900.

Yin, R., Chen, R., Xia, K. and Xu, X. (2024) A single-cell transcriptome atlas reveals the trajectory of early cell fate transition during callus induction in Arabidopsis. *Plant Commun.* **5**, 100941.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figures S1–S6 Supplementary Figures.
Tables S1–S5 Supplemantary Tables.
Data S1 Methods.