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DEVELOPMENT OF AN EFFICIENT PROTOPLAST ISOLATION AND TRANSFECTION PROTOCOL FOR HIGHBUSH BLUEBERRY

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Highbush blueberry (Ericaceae) is an economically important fruit crop due to its unique flavour, rich in nutrients and anthocyanins that positively affect human health with antioxidant and anti-inflammatory capacities. In a climate change scenario, new strategies are needed to increase the plant resilience; among the New Plant Breeding Techniques (NPBTs), the CRISPR/Cas9 system represents a useful tool for target gene editing, improving the health of the plants rapidly.

The most convenient and routinely applied method for gene delivery is *Agrobacterium*-mediated gene transfer with antibiotic selection and stable genomic integration, however, editing plant genomes without introducing foreign DNA into cells may alleviate regulatory concerns related to genetically modified plants. The protoplast technology, used as a versatile cell-based model system to elucidate the biological functions of genes and proteins, could represent an opportunity to develop DNA-free genome editing, through transfection with RNPs, a pre-assembled complex of purified Cas9 protein and guide RNAs.

Here we describe an optimized protocol for protoplast isolation from callus and leaves of highbush blueberry cv Berkley. The enzyme solution optimized for cell wall digestion contained Cellulase Onozuka RS (1%), Macerozyme R10 (0.5%) and Hemicellulase R10 (1.5%). After overnight incubation at room temperature, a yield of 2·10□6 protoplast·ml□-1 was obtained, with 97% of

viability. Isolated protoplasts were then transfected using PEG/Ca□2+ solution and with the plasmid pAVA393:GFP. The transient expression of the green fluorescent protein was evaluated using the fluorescent microscope. The transfection efficiency of the protoplasts resulted higher than 50% after 24 h from the transfection event. After 48 hours from transfection a minimal loss of viability of the protoplasts was observed. The protoplasts, cultured in liquid medium for callus induction (containing thidiazuron 10 µM and 1-naphthaleneacetic acid 10 µM), showed microcolonies just after 10 days in culture. Experiments for callus development and *de novo* shoot organogenesis are under investigation. Our results showed that the highbush blueberry protoplasts can be a valuable cell-based systems to be used for functional genomics and, moreover, a valuable tool to be explored for DNA-free genome editing, using gRNA:Cas9 ribonucleoproteins (RNPs).