Establishing Protoplast-Based Genetic Transformation for Sweet Sorghum (*Sorghum bicolor*) Through Cryopreservation of Microspores

Abstract

Sweet sorghum is a versatile crop known for its ability to thrive in hot, dry, and less arable regions, and its stalks contain easily extractable sugar water that can be directly fermented into ethanol making it valuable for biofuel production through sustainable agriculture. However, the development of double haploids (DH) as an efficient plant breeding approach, has been elusive for *Sorghum bicolor*. To overcome challenges towards DH production, microspore-based techniques have been extensively explored where immature pollen have not yet developed exine wall. The unique microsporogenesis stages offer an ideal opportunity for gametophytes' genetic transformation and androgenesis using methods typically reserved for plant protoplasts. Unlike mature plant cells, these microspores do not produce phenolic compounds which otherwise hinder in vitro culture advancement. However, sweet sorghum's short flowering window limits the access to fresh microspores, necessitating the development of a cryopreservation protocol to ensure year-round availability of viable gametophytes. This study presents the preservation protocols for microspores and their subsequent transformation as natural protoplasts. Panicles containing microspores at requisite developmental stages were harvested from sweet sorghum plants based on flag leaf emergence time. Released free floating microspores were suspended in MS5501 (PhytoTech Labs, Lenexa, KS) media supplemented with 5% DMSO and 5% glycerol, then cryopreserved using Mr. Frosty® containers to ensure controlled freezing (-80°C) for long term storage. Following post-thaw recovery, a protoplast-based transformation method was employed to successfully introduce naked DNA (pY31-GFP-GUS) carrying a green fluorescent protein reporter gene utilizing polyethylene glycol protocol which facilitated direct uptake and expression for genetically modified gametophytes. This standardized approach would streamline the production of stable, genetically enhanced DH sweet sorghum plants by innovating a robust pathway.