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A droplet-vitrification protocol enabled cryopreservation of doubled haploid explants of *Malus x domestica* Borkh. 'Golden Delicious'

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ABSTRACT

High throughput cryopreservation is a key step in the development of an apple t-DNA mutant library. The aim of this study was to develop an easy, efficient and rapid droplet vitrification protocol to cryopreserve doubled haploids of *Malus* x *domestica* Borkh. 'Golden Delicious'. The doubled haploid genotype proved to be less tolerant to cryopreservation than 'Golden Delicious'. Apices improved the quality of regeneration and the facility of dissection compared to meristems. About 50% of the explants treated with PVS2 for 40 min survived after immersion in liquid nitrogen, and 10% showed sustainable development. Phytotoxicity was observed mostly after immersion in the vitrification solution. Hyperhydricity was regularly observed in these experiments and decreased final efficiency. The use of meta-topolin instead of 6-benzylaminopurine (BAP) reduced hyperhydricity by almost 50%. We demonstrated that doubled haploid apple apices can be cryopreserved, but further improvements are needed before this technique can be used at high throughput.

1. Introduction

The recent publication of the genome sequence of the apple 'Golden Delicious' (Velasco et al., 2010) opens a new era in the breeding of this crop and should accelerate the identification of gene-trait relationships. In this context, the creation of an apple 'Golden Delicious' t-DNA mutant library would be an invaluable tool for functional genetic studies of many traits. However, *Agrobacterium*-mediated insertion of t-DNA occurs only on one DNA strand and apple is a heterozygous species. Therefore, the strategy we propose is the transformation of a haploid genotype derived from 'Golden Delicious', which will make it possible to identify all insertional mutations. Several haploid genotypes derived from 'Golden Delicious' have been produced in our group (Zhang and Lespinasse, 1991). The starting material for such a project is thus available. The first step in this strategy will be the efficient transformation of an apple haploid clone. The second step will be to subject the transgenic haploid lines to chromosome doubling to build a collection of homozygous doubled haploid (DH) mutants. Finally, maintaining a collection of several thousand apple DH mutants in greenhouse or in the field can be challenging. Therefore DH mutant lines will be cryopreserved to reduce both the required storage space and maintenance costs. Given the large number of plants required to establish this collection, the cryopreservation protocol needs to be highly efficient, easy and rapid.

Currently, many plants can be cryopreserved using different techniques (Sakai et al., 2008). In apple, a dozen papers reported successful cryopreservation of in vitro cultured explants using encapsulation-dehydration, vitrification or droplet-vitrification (Benelli et al., 2013). Because of the positive results obtained with this last technique, we hypothesised that a well-adapted droplet-vitrification protocol would allow efficient cryopreservation of apple DH lines. DH plants are homozygous lines created by chromosome doubling of haploid plants. As apple is self-incompatible, these artificial homozygous lines suffer from varying degrees of inbreeding depression (Höfer et al., 2008) and, to our knowledge, cryopreservation of haploids or doubled haploids has never been reported. Therefore our study focused on cryopreservation of a 'Golden Delicious'-derived DH line, a possible bottleneck in the establishment of an apple DH t-DNA mutant collection.

Abbreviations: BAP, 6-benzylaminopurine; DMSO, dimethyl sulfoxide; EG, ethylene glycol; GD, Golden Delicious; DH, doubled haploid; IBA, indole-3-butyric acid; LN, liquid nitrogen; LS, loading solution; MS, Murashige and Skoog; mT, meta-topolin; PVS, plant vitrification solution; RS, recovery solution.

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2. Material and methods

2.1. Plant material

All experiments were performed on a DH 'Golden Delicious' derivative, X9273, created at INRA ANGERS (France). The DH was obtained after spontaneous duplication of a haploid individual selected among the progeny of a selfed derivative from 'Golden Delicious'. The apple *Malus* x *domestica* Borkh. 'Golden Delicious' (GD) was used as control. The DH and GD shoot cultures were micropropagated as described previously (Righetti et al., 2014).

2.2. Cryopreservation

Two types of explants were extracted from DH and GD in vitro apical shoots acclimatized for four months at 2 °C, in the dark: 2–3 mm apices and 0.3–0.4 mm meristems (Fig. 1). Explants were dipped in the loading solution (glycerol 18.4% w/v, sucrose 13.7% w/v) for 20 min at 23 °C, then transferred to PVS2 (glycerol 30.0% w/v, dimethyl sulfoxide 15% w/v, ethylene glycol 15.0% w/v, sucrose 13.7% w/v) at 0 °C. Explants were transferred into drops of PVS2 placed on aluminium foil strips, which were then dipped in cryovials filled with liquid nitrogen (LN) for minimum one hour. To thaw cryopreserved samples from LN, the aluminium foil strips were directly plunged in a recovery solution (sucrose 41% w/v) for 15 min at 23 °C. Then the explants were placed on a filter paper on solid Murashige and Skoog medium (Murashige and Skoog 1962). with 102.6 g/l sucrose, 7 g/l Difco Bacto agar and incubated in the dark for 24 h. The explants were then transferred on micropropagation medium and incubated for six days, under the standard micropropagation conditions, before being transferred on new medium for three weeks. Later on, explants were subcultured once a month.

2.3. Hyperhydricity control

In order to control the hyperhydricity observed on DH shoots, two factors were tested during micropropagation: the type of vessel used and the effect of cytokinin. To test the type of vessel, explants were placed in Petri dishes (75 ml dish containing 30 ml of medium, 10 explants per dish) which were tightly sealed with paraffin film, or in small baby food jars (130 ml jar containing 25 ml medium, 5 explants per jar) or in test tubes (50 ml tube containing 10 ml medium, one explant per tube) with ventilated caps. To evaluate the effects of cytokinin, BAP was replaced by meta-Topolin (mT) at concentrations of 0.5 mg/l or 1 mg/l, in the Petri dishes. For this trial, only meristem explants of DH were used because of their particular sensitivity to hyperhydricity. The explants were plated on the micropropagation media under light, immediately after their excision.

2.4. Parameters evaluated and statistics

During each trial, three parameters were evaluated: (i) survival (the number of green shoots more than 3 (for meristems) or 6 (for apices) mm in length divided by the total number of explants), (ii) hyperhydricity (the number of hyperhydric surviving shoots divided by the number of surviving shoots), and (iii) regeneration (the number of green shoots more than 6 mm in length with several developed leaves divided by the number of surviving shoots). Final regrowth efficiency was calculated as the number of non hyperhydric shoots more than 6 mm in length with several developed leaves divided by the total number of explants. For each treatment, 30 explants were dissected. A generalized linear model in binomial family was performed with R software, using chi-square test. The non-parametric statistic test of Kruskal Wallis was used for pairwise comparison ($p < 0.05$).

3. Results

3.1. Effect of genotype and explant type on shoot regrowth after immersion in liquid nitrogen

The first trial revealed that shoot regrowth after immersion in liquid nitrogen was affected by both genotype and explant type. Comparison of apex explants from DH and GD indicated a significant genotype effect for all parameters except regeneration (Table 1, p value of chi-square test <0.001). The survival of the GD apices was not affected by the PVS2 treatment, and only moderately reduced after treatment in LN. Among the surviving GD explants, the rate of regeneration was very high, with a very low frequency of hyperhydricity in all treatments. The final efficiency was 93% after PVS2 and 63% after LN.

The survival of DH apices was significantly reduced after the PVS2 step, and only 47% of DH apices survived after immersion in LN. The regeneration rate of the surviving DH apices was high in all treatments. Despite the low hyperhydricity in the control treatment, the rate of hyperhydricity of DH apices was very high after immersion in PVS2 or in LN. Hyperhydricity was observed as soon as the first leaves became visible and did not disappear until the end of the experiment. The final efficiency was low in all the treatments which included a PVS2 or LN step, due to the frequent hyperhydricity of this genotype.

This experiment also revealed that the type of DH explant (meristems or apices) had a significant influence on hyperhydricity and final efficiency (Table 1, p value of chi-square test <0.001). In the control treatment, meristems were much more affected by hyperhydricity than apices, reducing their final efficiency. However, the behaviour of apex and meristem DH explants was similar after PVS2 and LN treatments.

3.2. Effect of the type of vessel and of cytokinin on hyperhydricity of explants

The second trial was designed to reduce the frequent hyperhydricity observed in DH meristem explants during the first trial. During this experiment, DH meristems were dissected, and their regrowth was measured after two months without dehydration and freezing. The type of vessel significantly affected the regeneration rate (Fig. 2A) with the highest regeneration rate being obtained with Petri dishes, but it had no influence on hyperhydricity. The final efficiency of the three types of vessel was similar and lower than the final efficiency of the control treatment during the first trial on DH meristems (Table 1).

The most important effects were observed in the cytokinin assay (Fig. 2B). Shoots regenerated in the two media with mT were significantly less hyperhydric than those regenerated in the medium containing BAP. The final efficiency was significantly higher on the medium with 1 mg/l mT, compared to the medium with 0.5 mg/l BAP. Overall, in this experiment, the highest rate (50%) of well-developed and non hyperhydric explants was obtained in Petri dishes containing medium 1 mg/l mT. This result was much higher than the final efficiency observed during the first trial on DH meristems (27%, Table 1).

4. Discussion

The aim of this work was to develop an easy and rapid efficient droplet-vitrification protocol for doubled haploid (DH) in vitro apple shoot cultures. This is a key point in our long-term goal of