High Throughput Cryopreservation of 140 000 Physcomitrella patens Mutants

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Abstract: A high throughput protocol was established to preserve 140 000 mutants of a moss, Physcomitrella patens, a model plant for functional genomics studies, over liquid nitrogen. Regarding the reliable long-term storage of diverse mutant phenotypes, as well as time and cost effectiveness, each working step was optimized: 1) plant preparation, 2) freezing regime, cryogenic conditions, 3) regrowth after thawing. A prerequisite for maximum regrowth was a 1-week preculture of chopped plant material on a supplemented medium prior to freezing. Cryovials as preculture vessels resulted in identical regrowth rates, compared to petri dishes. The cryovial type had a significant influence on regrowth rates. A cooling rate of -1°C/min down to -35°C with a 10 min holding time before transferring plants to -152°C was the most suitable freezing regime. This protocol allows a cryopreservation of 1100 plants during a 5-day working week, practicable by one person. For more than 650 cryopreserved mutants a maximum regrowth rate of 100% was obtained, independently of mutant phenotypes.

Key words: Cryopreservation, functional genomics, germplasm storage, moss, mutant collection, Physcomitrella patens (Hedw.) B.S.G.

Introduction

The moss Physcomitrella patens (Hedw.) B.S.G. is an attractive model to unravel plant gene functions. In contrast to seed plants, a very efficient homologous recombination system allows routine production of targeted gene knockouts (Schafer and Zrój, 1997; Girke et al., 1998; Reski, 1998; Strepp et al., 1998; Girod et al., 1999; Schafer, 2001). Mutant collections, generated via transformation with a gene-disruption library, provide a broad spectrum of diverse mutant phenotypes as basic material for forward genetics approaches to isolate and molecularly characterize genes (Nishiyama et al., 2000; Egener et al., 2002).

To maintain a mutant collection under in vitro conditions, periodic transfers to fresh culture media are required. This involves not only manpower and high costs, but also the hazards of contamination, and sometimes the loss of the entire plant (Bajaj, 1995). Moreover, periodic subculture can result in a loss of genetic and physiological stability (Karthä and Engelmann, 1994). Frequent in vitro subcultures can be avoided by mutant conservation. The production and subsequent storage of seeds can be used as a conservation method, as practiced for higher plants. However, conserving moss spores as equivalents to seeds would exclude all developmental mutant genotypes, e.g., mutants which only form the juvenile, filamentous protonema without the production of reproductive organs. A high frequency of such developmental mutants in a tagged mutant collection was described previously (Egener et al., 2002). An alternative conservation technique is cryopreservation, a method based on the reduction and subsequent arrest of metabolic functions by imposition of ultra-low temperature (Karthä, 1987). Plant cryopreservation offers long-term storage capability, maximal stability of phenotypic and genotypic characteristics, along with minimal storage space and maintenance requirements (Withers, 1985). Slow cooling at a precisely defined rate to an intermediate temperature provides a controlled amount of cryodehydration, important for removal of water from cells to a point where no deleterious ice crystals will be formed at the final storage temperature. Cryodehydration can be continued by an extended incubation at intermediate temperature (holding time) prior to long-term storage in liquid or gaseous nitrogen (Grout, 1995). The process of cryopreservation comprises: (1) plant preconditioning, incubation in a cryoprotectant in which the plant will be frozen and stored; (2) cooling rate, transfer temperature, holding time at this temperature, and storage at ultra-low temperature; (3) and finally the recovery process (Grout, 1995). For the establishment of a cryopreservation protocol, optimization of each of these details is important to reduce regrowth variations between different laboratories and to avoid differences in survival rates due to variations during single protocol steps (Reed et al., 2001).

The present study was performed to establish a protocol suitable for the high throughput cryopreservation of 140 000 Physcomitrella mutants, which were produced by a transposon-based shuttle mutagenesis system (Egener et al., 2002). The requirements for this protocol were: (a) to find a procedure suitable for various (unknown) phenotypes of Physcomitrella...
mutants, differing, e.g., in developmental or metabolic traits; (b) its applicability for high daily throughput cryopreservation; and (c) its adaptation to a 5-day working week. Beside time efficiency, it also resulted in cost saving. The present study was based on two existing protocols, reporting the cryopreservation of Physcomitrella (Grimmley and Withers, 1983; Christianson, 1998). However, these studies dealt only with wild-type plants, did not evaluate in detail plant regrowth after thawing, and were not applicable for high throughput procedures.

Here, we report the effects of plant preparation, preculture performance, preculture vessels, cryovial type, freezing conditions, and the culture medium composition on regrowth rate and regrowth intensity. Each protocol step was optimized for a 5-day working week and for high throughput cryopreservation of 1100 plants/week, practicable by one person.

Materials and Methods

Plant material, culture conditions and culture media

Wild-type Physcomitrella patens (Hedw.) B.S.G., as well as various tagged mutants (Egener et al., 2002), were used. Plants were grown in vitro at a temperature of 25 °C and kept under a 16 h photoperiod with a light intensity of 70 μmol m⁻² s⁻¹ (Philips TDL 38 W/25). They were cultured on solid medium in 6-well petri dishes, using a minimal medium (Knop medium, as described in Reski and Abel, 1985), as well as a complete medium (supplemented Knop medium, as described in Egener et al., 2002). Plants were transferred to fresh medium each month. In preparation for cryopreservation, plants were cultured for one week on a preculture medium. This medium consisted of the complete medium with 920 mg/l ammonium tartrate, supplemented with the following cryo additives: 87 g/l mannitol (according to Grimsley and Withers, 1983), 10 μM abscisic acid (ABA, dissolved in dimethyl sulfoxide [DMSO]), and 100 mM proline (according to Christianson, 1998). Liquid medium was filter sterilised (0.22 μm, Steritop, Millipore). Solid medium was supplemented with 1.2% (w/v) agar. Macro- and microelements, FeSO₄·x·H₂O, glucose and mannitol, were autoclaved, other supplements were filter-sterilized (0.22 μm, Steritop, Millipore) and added to the medium after autoclaving. For all media, the pH was adjusted to 5.8 before autoclaving and before filter-sterilizing, respectively.

Preparation for freezing: Preculture and cryoprotection

During preculture, which started one week before freezing, plants were cultured under the above-described culture conditions on the preculture medium. Six-well petri dishes (solid medium) and cryovials (polypropylene, 2 ml, Greiner, Frickenhausen, Germany) containing 800 μl liquid medium were used as preculture vessels. Prior to freezing, plants were treated for 1 h with a cryoprotectant. Plants precultured on solid medium were transferred into cryovials containing DMSO (5% v/v) + glucose (10% w/v) in distilled water as cryoprotectant (according to Grimsley and Withers, 1983). Plants precultured in cryovials were treated with 300 μl of cryoprotectant consisting of DMSO (20% v/v) + glucose (25% w/v) in distilled water; the cryoprotectant was added directly to the plant material in the cryovials, without removing the preculture medium. The cryoprotectants were filter-sterilized.

Freezing procedure, storage of plants

Two different freezing procedures were performed: (1) A simple procedure which is also practicable without a programmable freezing unit and without special cryo containers. Following the cryoprotection treatment, the cryovials were placed in a 70% ethanol bath which was maintained for 2 h at −20 °C. Subsequently, plants were transferred to −80 °C in a freezer, until thawing. Using this freezing method, plant material is cooled down at approx. −1 °C/min. This method was used to test the influence of preculture, plant preparation, and cryovial type. (2) For a highly standardized routine procedure and the long-term storage of mutants, controlled plant freezing was performed using a programmable freezing apparatus (KRYO 10–16 Ill, Messer Cryotherm, Kirchen/Sieg, Germany) and cryo containers (Chromos 400, Messer Cryotherm). Cryovials were placed in the gaseous phase over liquid nitrogen (−152 °C), with a storage duration between two and three weeks for all experiments. Exact cooling rates were measured in the cooling chamber, the starting temperature was +20 °C. This method was used to test the suitability of cryo vials as preculture vessels, to test the influence of culture medium composition and of regrowth medium composition on plant regrowth, to determine the optimal incubation time in the cryoprotectant, and to assess the optimal freezing regime.

Thawing and assessment of regrowth rate and regrowth intensity

By agitating the cryovials in a water bath at +30 °C, plant material was rapidly thawed and subsequently dropped, without washing, on the regrowth medium consisting of the above-mentioned minimal medium and complete medium, respectively. Differences between treatments were assessed over a period of 4–5 weeks after thawing, by a weekly determination of regrowth rates (%) and regrowth intensities. The regrowth rate provides information about the percentage of plants able to survive the cryopreservation procedure. Regrowth of all thawed plants of one treatment represents a maximum regrowth rate of 100%. Regrowth rates were determined for each treatment, once per week, by referring the actual number of regrown plants to the total number of thawed plants. To obtain information about the growth intensity of thawed plant material, numbers between 0–3 were assigned to each plant once a week, where 0 = no growth, 1 = starting growth/some growth, 2 = good growth, 3 = very good growth.

Experimental design

The effect of different parameters which could influence the success of a cryopreservation protocol was investigated as described below.

Preconditioning: Effect of preculture and plant chopping. Plants were frozen after a 1-week preculture in 6-well petri dishes (treatment A), or without a preculture (treatment B). Additionally, plants were not chopped at all (treatment A), chopped after thawing (treatment B), or chopped before the start of preculture, and prior to freezing (treatment C). All six treatment combinations (Aa, Ab, Ac, Ba, Bb, Bc) were applied to clones of each plant. For plant chopping, scalpels were used. After thawing, plants were regrown on complete medium.
Cryopreservation of Physcomitrella Mutants

Cryogenic conditions: Choice of cryo vial type and storage system. Vials of two different brands were compared (A, B). For each brand, vials with external (a) and internal thread (b) were tested. Chopped plants were used, precultured on solid medium (c) or without preculture (d). All eight treatment combinations (Aac, Aad, Abc, Abd, Bae, Bad, Bbc, Bbd) were applied to wild-type plants. After thawing, plants were regrown on complete medium. The choice of the storage system was dependent on the results of the cryo vial testing and is therefore described in the “results” part of this report.

Freezing regime: Choice of cooling rate, transfer temperature, and holding time. In a first experiment, different cooling rates (−0.5, −1, −2°C/min), down to a terminal freezing temperature of −20°C were tested. In a second experiment, different terminal temperatures (-10, −15, −20, −25, −30, −35, −40°C) and different holding times (10, 30, 60 min) at each of the terminal temperatures were tested, using a cooling rate of −1°C/min. All 21 treatment combinations were applied to clones of each plant. In both experiments, chopped plant material was used, which was precultured for one week on solid medium. Thawed plants were regrown on the complete medium.

Preparation and recovery: Preculture vessel, incubation time, and regrowth medium. Cryo vials were compared to 6-well petri dishes as preculture vessels. Six different incubation times (0, 1, 2, 3, 4, 5 h) in the cryoprotectant prior to freezing were tested for plant material precultured in cryo vials and 6-well petri dishes, respectively. All 12 treatment combinations were applied to clones of each plant. The cryoprotectant was added directly to plants precultured in cryo vials, without removing the preculture medium. Plant material precultured in 6-well petri dishes was transferred to cryo vials containing liquid preculture medium, afterwards the cryoprotectant was added. Following the different incubation times, all vials were frozen together. After thawing, the plant suspensions from each cryo vial were divided into two parts which were transferred to minimal medium or to complete medium, for regrowth. Five weeks after thawing, fresh weight determinations were performed for plants precultured in cryo vials.

Material used prior to preculture: Plant tissue and culture medium. Different moss tissue (stems, leaves, protonema) of wild-type plants was used. Prior to preculture, plants were cultured on minimal or on complete medium. They were chopped and subsequently precultured for 1 week in cryo vials. After thawing, plant material of each cryo vial was divided into two parts and dropped on minimal and complete medium for regrowth.

Statistical analyses

Preconditioning: Effect of preculture and plant chopping. The experiment was repeated three times, independently one from the other. Twenty plants were used per experiment, each with three wild-type plants and 17 different mutants, including two slow-growing types. Mean regrowth rates, mean regrowth intensities, and standard errors were determined for each treatment at each documentation day. Significant differences were evaluated by repeated measures analysis of variance on ranks. All pairwise multiple comparisons were made with the Student–Newman–Keuls method. A p value < 0.05 was considered to be statistically significant. The statistic analysis was performed using the software Sigma Stat (Version 2.03, SPSS Inc., Erkrath, Germany).

Cryogenic conditions: Choice of cryo vial type and storage system. The experiment was repeated six times, independently one from the other. Five wild-type plants were used per experiment. Mean regrowth rates and standard errors were calculated for each treatment at each documentation day. Significant differences were evaluated as described above.

Freezing regime: Choice of cooling rate, transfer temperature, and holding time. Both experiments were repeated three times, independently one from the other. Twenty plants were used per experiment, each with five wild-type plants and 15 different mutants, including two slow-growing plants. Mean regrowth rates, mean regrowth intensities, and standard errors were determined for each treatment at each documentation day. Significant differences were evaluated as described above.

Preparation and recovery: Preculture vessel, incubation time, and regrowth medium. Sixty plants (45 different mutants and 15 wild-type plants) were used. Mean regrowth rates, mean regrowth intensities, and standard errors were determined for each treatment. For each incubation time and for each culture medium, a statistical analysis was performed using the t-test of Sigma Stat (Version 2.03, SPSS Inc., Erkrath, Germany). Mean fresh weights and standard deviations were calculated for plants precultured in cryo vials. Due to the determination of differing fresh weights in dependence of the incubation time (see the “results” part of this report), fresh weights were divided into two groups. Group 1 represents the values obtained for plants incubated for 1, 2, 3 h and group 2 summarizes the fresh weights of plants with incubation times of 4, 5 h. The differences between the mean values of the two groups were tested for significance using the t-test of Sigma Stat (Version 2.03, SPSS Inc., Erkrath, Germany).

Material used prior to preculture: Plant tissue and culture medium. The experiment was repeated three times, independently one from the other. The number of plants per single experiment was 15. The plant regrowth after thawing was evaluated descriptively.

Results

Preconditioning: Effect of preculture and plant chopping. To study the effects of preculture and of plant chopping on regrowth rates and regrowth intensities, plants were frozen after a 1-week preculture (treatment A) or without preculture (treatment B), each with (a) no plant chopping during the whole cryopreservation process (treatments Aa, Ba), (b) plant chopping after thawing (treatments Ab, Bb), or (c) plant chopping before freezing (treatment Bc), or before the start of preculture (treatment Ac). One week after thawing, highly different regrowth rates were observed, dependent on the treatment (Fig. 1). The comparison of treatments Aa vs. Ba, Ab vs. Bb, and Ac vs. Bc showed that a preculture before freezing speeded up plant recovery after thawing. A preculture performance resulted in significantly higher regrowth rates compared to direct freezing (Student–Newman–Keuls method; p < 0.05). Comparing treatments Aa, Ab, Ac resp. Ba, Bb, Bc among themselves showed that an enhancement of regrowth rates was notably enforced by plant chopping. The importance
of plant chopping is especially visible by comparing regrowth rates obtained 4 weeks after thawing (Fig. 1). Nearly equal regrowth rates were obtained for plants treated in the same way (chopped or not chopped, treatment a, b, c), independently of preculture. The regrowth rate of chopped and additionally precultured plants (treatment Ac) was 100%, but at least 91.7 ± 4.4% for chopped plants which were frozen without preculture (treatment Bc). Chopping plants after thawing resulted in regrowth rates of 76.7 ± 6.0% (treatment Ab) and 78.3 ± 4.4% (treatment Bb). The lowest regrowth rates were obtained for plants which were not chopped at all, with regrowth rates of 66.7 ± 1.7% (treatment Aa) and 63.3 ± 4.4% (treatment Ba). The regrowth rates of treatments Ac/Bc (chopping before freezing) were significantly higher compared to the other treatments, as assessed with Student–Newman–Keuls method (p < 0.05: Fig. 1). However, the results demonstrated that regrowth of all thawed plants was indeed achieved, but only when plant material was chopped and subsequently precultured (treatment Ac). In addition, this regrowth rate of 100% was already achieved on the first documentation day, 1 week after thawing. In comparison, maximum regrowth rates for all other treatments were reached only 3 or 4 weeks after thawing (Fig. 1). No changes were observed 5 weeks after thawing (data not shown).

Fig. 1 Influence of a 1-week preculture and of plant chopping on the regrowth rate of thawed plants. Mean values and standard errors are presented. Within a documentation day, different numbers indicate significant differences between treatments (pairwise multiple comparison, Student–Newman–Keuls method; p < 0.05).

Fig. 2 Influence of a 1-week preculture and of plant chopping on the regrowth intensity (0 = no growth, 1 = starting growth/some growth, 2 = good growth, 3 = very good growth) of thawed plants. Mean values and standard errors are presented. Within a documentation day, different numbers indicate significant differences between treatments (pairwise multiple comparison, Student–Newman–Keuls method; p < 0.05).
Table 1  Influence of the cryovial type on the mean regrowth rate (%). Different brands (A, B) and different threads (external, internal; a, b) were compared. Values in parenthesis are standard errors. * Significant different from the other treatments (pairwise multiple comparison, Student–Newman–Keuls method; p < 0.05).

<table>
<thead>
<tr>
<th>Vial type</th>
<th>7 days after thawing</th>
<th>14 days after thawing</th>
<th>21 days after thawing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brand A, external thread</td>
<td>53.3 (4.2)</td>
<td>93.3 (4.2)</td>
<td>100</td>
</tr>
<tr>
<td>(Aa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brand A, internal thread</td>
<td>73.3 (8.4)</td>
<td>93.3 (4.2)</td>
<td>100</td>
</tr>
<tr>
<td>(Ab)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brand B, external thread</td>
<td>66.6 (4.2)*</td>
<td>66.7 (8.4)</td>
<td>66.7 (8.4)</td>
</tr>
<tr>
<td>(Ba)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brand B, internal thread</td>
<td>46.6 (6.6)</td>
<td>93.3 (4.2)</td>
<td>100</td>
</tr>
<tr>
<td>(Bb)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The use of treatment Ac (preculture + chopping) further resulted in significantly higher regrowth intensities compared to all other treatments, as assessed with the Student–Newman–Keuls method (p < 0.05), independent of the documentation day (Fig. 2). At the final documentation, 35 days after thawing, a high mean regrowth intensity of 2.95 ± 0.03 was observed for treatment Ac, in contrast to values of 1.35–2.28 for all other treatments. Chopped plants regrew “lawn-like” during culture on regrowth medium (Fig. 2B). Due to the above-described results, a 1-week preculture of chopped plants was chosen for the final protocol.

Cryogenic conditions: Choice of cryo vial type and storage system. The cryo vial suitability of two different brands (A, B), each with (a) external or (b) internal thread was compared. Plants which were pre cultured before freezing started regrowth during the first week after thawing, independently of brand and mode of thread (data not shown). For plants which were not pre cultured, noticeable differences in regrowth rates were observed 1 week after thawing (Table 1). A very low regrowth rate of 6.6 ± 4.2% was observed for plants frozen in cryo vials of brand B, with external thread (treatment Ba). This regrowth rate differed significantly from the regrowth rates of variants Aa, Ba, and Bb (46.6–73.3%), as assessed by the Student–Newman–Keuls method (p < 0.05). Furthermore, the use of variant Ba resulted in a maximum regrowth rate of 66.7 ± 8.4%, in contrast to the other tested variants with maximum regrowth rates of 100%, 3 weeks after thawing (Table 1). Different suitability of internal and external threads were assessed concerning the handling of cryo vials. Especially for a daily high throughput cryopreservation, there is a high risk of touching an internal thread with the hand when removing the cap. This, of course, connected with a higher contamination risk. Due to this observation and the above-described results, vials of brand A with an external thread (Aa) were chosen for the final protocol.

The long-term storage of Physcomitrella mutants could be performed in liquid nitrogen or in its gaseous phase. Based on the above-described results, combined with storage systems properties known from the literature, we decided to perform the storage in the gaseous phase: Vials with external thread are very suitable for storage in the gaseous phase but not for storage in liquid nitrogen. Furthermore, storage in the gaseous phase avoids sample or bacterial cross-contamination which could occur when utilizing liquid cryogenic storage systems (Berry et al., 1998; Byers, 1998). In addition, storing vials in the gaseous phase is connected with lower costs for nitrogen and reduced risks for workers.

Freezing regime: Choice of cooling rate, transfer temperature, and holding time. All plants started regrowth during the first week after thawing, independently of cooling rate (−0.5, −1, −2 °C/min), terminal freezing temperature (−10, −15, −20, −25, −30, −35, −40 °C) and holding time (10, 30, 60 min) at the terminal freezing temperature prior to plant transfer to the final temperature of −152 °C (data not shown). However, the intensities of plant regrowth after thawing differed depending on the treatment. One week after thawing, a reduced regrowth intensity of 1.15 ± 0.06 was observed for the use of −2°C/min as cooling rate, compared to 1.80 ± 0.07 or 1.83 ± 0.04 for plants cooled down at rates of −0.5 °C or −1 °C/min, respectively (Fig. 3). During the whole regrowth period, no significant differences were obtained for regrowth intensities of plants which were cooled down with one of the slower cooling rates; however, they were always significantly better compared to plants which was cooled down at a rate of −2°C/min (Student–Newman–Keuls method, p < 0.05; Fig. 3). With respect to time efficiency, a cooling rate of −1°C/min was chosen for the final protocol.

The regrowth intensity after thawing was also dependent on the transfer temperature (Fig. 4). A transfer temperature of −35°C speeded up plant recovery after thawing. One week after thawing, regrowth intensity of 1.82 ± 0.11 for plants transferred at −35°C was significantly higher, compared to all other variants, as assessed with the Student–Newman–Keuls method (p < 0.05). For other transfer temperatures, lower than −15°C, nearly equal regrowth intensities (1.52–1.53) were obtained, compared to 1.0 and 1.1 ± 0.07 for the two transfer temperatures higher than −20°C (Fig. 4). Also, during the follow-
ing 2 weeks, best regrowth intensities were observed for plants transferred at −35°C, resulting in maximum values after 4 weeks on regrowth medium. Due to these results, a transfer temperature of −35°C was chosen for the final protocol. Different holding times (10, 30, 60 min) resulted in equal regrowth intensities. With respect to time efficiency, the shortest holding time (10 min) was chosen for the final protocol.

**Preparation and recovery: Preculture vessel, incubation time, and regrowth medium**

Although all plants started regrowth during the first week after thawing - independent of preculture vessel, incubation time, and regrowth medium - differing regrowth intensities were observed for different treatments (Table 2). The comparison of cryo vials and 6-well petri dishes as preculture vessels showed that the differences of regrowth intensities were not significant (t-test, p = 0.37–0.86). Regrowth on complete medium resulted in higher fresh weights (95.6–182.0 mg/plant, Fig. 5) compared to plants regrown on minimal medium (47.5–56.9 mg/plant, data not shown). In contrast to regrowth on minimal medium, a connection of fresh weight and incubation time was obtained for plants cultured on complete medium. Due to the results (Fig. 5), the fresh weights were divided into two groups: group 1 summarized the fresh weights of plants incubated for 1–3 h in the cryoprotectant, group 2 summarized the fresh weights of plants incubated for 0, 4, and 5 h. Comparing the mean values of the groups with the t-test resulted in significantly higher fresh weights of plants incubated for 1–3 h (p < 0.05). Due to these results, an incubation time of 1–3 h and the use of complete medium for regrowth were chosen for the final protocol.

**Table 2** Influence of the preculture vessel (cryo vial, 6-well petri dish) on the mean regrowth intensity, 1 week after thawing. Values in parenthesis are standard errors. A statistical analysis was performed using the t-test for each incubation time and for each culture medium. For all results no significant differences between the preculture vessels were determined (p = 0.37–0.86).

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Regrowth medium: minimal medium</th>
<th>Regrowth medium: complete medium</th>
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<tbody>
<tr>
<td></td>
<td>Cryo vial</td>
<td>6-well petri dish</td>
</tr>
<tr>
<td>0</td>
<td>2.08 (0.10)</td>
<td>2.18 (0.08)</td>
</tr>
<tr>
<td>1</td>
<td>2.03 (0.10)</td>
<td>2.12 (0.08)</td>
</tr>
<tr>
<td>2</td>
<td>1.98 (0.08)</td>
<td>2.08 (0.07)</td>
</tr>
<tr>
<td>3</td>
<td>2.05 (0.08)</td>
<td>2.12 (0.06)</td>
</tr>
<tr>
<td>4</td>
<td>2.00 (0.08)</td>
<td>2.02 (0.07)</td>
</tr>
<tr>
<td>5</td>
<td>2.10 (0.09)</td>
<td>2.20 (0.07)</td>
</tr>
</tbody>
</table>

**Fig. 4** Influence of different transfer temperatures on regrowth intensities. The plant material was cooled down at −1°C/min. Mean values and standard errors are presented. Within a documentation day, different numbers indicate significant differences between treatments (pairwise multiple comparison, Student–Newman–Keuls method; p < 0.05).

**Fig. 5** Influence of incubation time in the cryoprotectant on mean fresh weights of regrown plants on complete medium. Fresh weight determination was performed 5 weeks after thawing. Cryo vials were used as preculture vessels. Error bars are standard deviations.
Material used prior to preculture: Plant tissue and culture medium. The regrowth of chopped adult moss tissue (stems, leaves) after cryopreservation was rare, compared to chopped juvenile filamentous protonema (Figs. 6A, 6B). This was observed for regrowth on minimal and complete medium. Furthermore, chopping protonema was much easier and faster compared to chopping leaves or stems. We conclude that even the choice of the culture medium used prior to the 1-week preculture has an influence on regrowth after thawing: A medium composition preventing the development of adult tissue should be used. We observed that culture on complete medium resulted in plants consisting mainly of protonema (Fig. 6B). In contrast, plants cultured on minimal medium consisted of stems and gametophores (Fig. 6C). For the final protocol, the complete medium was chosen for plant culture prior to preculture due to its influence on plant development, combined with the maintenance of important auxotrophic plants inside a mutant collection.

Final protocol

In short, the successful protocol requires plants cultured on a medium preventing stem/leaf differentiation. A 1-week preculture of chopped plants should be performed directly into cryovials (Greiner) with an external thread, each filled with 800 μl preculture medium, consisting of a complete medium supplemented with mannitol (87 g/l), ABA (10 μM), and proline (100 mM). Before freezing, 300 μl cryoprotectant (DMSO 20%, glucose 25%) is added directly to the plant material in the preculture medium. Freezing is performed 1–3 h after adding the cryoprotectant. Controlled freezing starts at +20 °C with a cooling rate of −1 °C/min down to −35 °C. The holding time is 10 min. Long-term storage is performed in gaseous nitrogen at −152 °C. Rapid thawing is performed in a +30 °C water bath. Plant material and surrounding liquid are transferred to the regrowth medium (complete medium) without any washing steps. This protocol allows cryopreservation of 1100 plants during a 5-day working week, practicable by one person.
Validation

About 140,000 plants were frozen using the final protocol. The mutants differed in various morphological and developmental characteristics. More than 650 mutants were thawed, of which 225 plants were stored between 6 months and 2 years. All thawed mutants started regrowth during the first week after thawing, and for each mutant new colonies were established.

Discussion

An efficient protocol for high throughput cryopreservation of Physcomitrella mutants was established. It is based on two reports on the cryopreservation of this moss (Grimsley and Withers, 1983; Christianson, 1998). However, these reports did not deal with mutants, nor did they specify their analyses of plant regrowth after thawing, and were not optimized for high throughput procedures, crucial for functional genomic approaches. The protocol described here leads to time savings, combined with maximum regrowth rates of mutants differing in various characteristics.

The use of cryo vials as preculture vessels contributed most to time effectiveness, as no further steps, apart from adding the cryoprotectant to the plants, are necessary prior to freezing. Especially, overlay of cellophane on agar (Grimsley and Withers, 1983), centrifugation (Christianson, 1998), and transfer of plants from preculture vessels into cryo vials containing the cryoprotectant (Grimsley and Withers, 1983; Christianson, 1998) could be omitted. These simplifications reduced contamination risks and contributed to cost-saving. Although growth rates of in vitro plant cultures are influenced by the volume and composition of the gaseous atmosphere inside the culture vessel (DeProft et al., 1985; Gould and Murashige, 1985; Belcher et al., 1987), 2 ml cryo vials may not be used as preculture vessels for most plant species. Mosses like Physcomitrella, in contrast, are especially well suited for such a treatment as their habitats are mainly moist, wet soil, and mud on riversides or drained ponds (Ahrens, 2000; Frahm and Frey, 1992).

A saturated plant mutant collection includes strains with unpredictable phenotypes and metabolic changes. Therefore, protocols have to be optimized to minimize selection for the most desired phenotypes (Bajaj, 1995; Reinhold et al., 1998). In general, rapidly dividing tissues have the highest regeneration capacity in vitro (Pierik, 1997). Therefore, we chopped moss plants, and thus induced vigorous growth. A 100% regrowth was achieved if chopped plants were additionally precultured. For several plant species, differences in cryopreservation survival were connected to the cell status, including cell size, degree of vacuolation, cell cycle stage, and some metabolic factors (Withers, 1985). We conclude that chopping leads to sufficient moss material able to survive the freeze-thawing cycle, independent of mutant phenotype and growth properties. The astonishingly high regrowth rate of chopped plants without preculture (91.7±4.4%) may be explained by a natural ability of Physcomitrella to cold harden, implying a certain tolerance to the cryopreservation stress, as has previously been postulated for several seed plants (Karthä and Engelmann, 1994; Grout, 1995). From all developmental stages of Physcomitrella, protonema filaments display the highest level of osmotic stress tolerance (Frank and Reski, 2003). This is in accor-
dance with our recent findings of superior regrowth from chopped protonema when compared to chopped leafy stems.

In contrast to previous reports on Physcomitrella cryopreservation (Grimsley and Withers, 1983; Christianson, 1998), we assessed the regeneration quality of thawed plants: a prerequisite for the detailed optimization of different treatments. Surprisingly, the cryo vial brand significantly influenced regrowth rates, highlighting the importance to test different brands when applying our protocol to other plant species. Although regrowth medium composition influenced recovery after cryopreservation of different plant species (Dussert et al., 1992; Benson et al., 1995; Ryynänen and Häggman, 2001), we did not observe major effects of the different media tested here, indicating broad metabolic tolerance of Physcomitrella in cryopreservation.

In conclusion, the protocol described here allows high throughput cryopreservation of 1100 Physcomitrella plants per day, practicable by one person. This safe and effective long-term storage provides an opportunity to create large mutant collections of this model plant in a variety of different laboratories.

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References


