Methods

An experimental method to facilitate the identification of hybrid sporophytes in the moss Physcomitrella patens using fluorescent tagged lines

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Summary

• The sequencing of the Physcomitrella patens genome, combined with the high frequency of gene targeting in this species, makes it ideal for reverse genetic studies. For forward genetic studies, experimental crosses and genetic analysis of progeny are essential.
• Since P. patens is monoicous, producing both male and female gametes on the same gametophore, and undergoing self-fertilization at a high frequency, the identification of crossed sporophytes is difficult. Usually spores from many sporophytes from a mixed culture must be tested for the production of recombinant progeny.
• Here, we describe the use of transgenic lines that express a fluorescent transgene constitutively, to provide a direct visual screen for hybrid sporophytes.
• We show that segregations in crosses obtained with this technique are as expected, and demonstrate its utility for the study of the rate of outcrossing between three isolates of P. patens.

Introduction

The sequencing of the Physcomitrella patens genome (Rensing et al., 2008) and the demonstration of efficient gene targeting (Schafer & Zryd, 1997) make reverse genetic study of gene function straightforward and have catalyzed its establishment as an important model system for studies of gene function, gene regulation, and comparative genomics (Cove et al., 2006; Quatrano et al., 2007). Classical genetic studies have a long history in P. patens and its relatives (von Wettstein, 1924). Because mosses, like yeasts, have a dominant haploid phase in their life cycle, a recombinant mapping population can be created between two lines with only a single cross. Dominance variance is absent for traits expressed in the haploid (gametophytic) part of the life cycle, meaning that the phenotype of a plant directly reflects its underlying genotype. Haploidy also facilitates the study of epistasis among loci with modest-sized mapping populations (McDaniel et al., 2007, 2008).

Forward genetics has, however, played a limited role in the development of P. patens as a model system. P. patens, like all members of the Funariaceae, is monoicous – a single haploid gametophyte produces both antherozoids (sperm) and eggs. Fertilization results in a diploid sporophyte that remains attached to its maternal parent throughout its life span. In self-fertile mosses, it is therefore not obvious whether a given sporophyte is the product of self-fertilization or outcrossing. Early workers avoided this problem by making crosses between species with sporophytes of different sizes, so that hybrids were easily identifiable by their intermediate phenotype (von Wettstein, 1932). However, mapping studies using these crosses were not feasible because the survival rate of the interspecific recombinants was very low, often < 0.1% (von Wettstein, 1932; McDaniel et al., 2010). Mutagenesis of P. patens (Engel, 1968; Ashton & Cove, 1977; Courtice et al., 1978; Knight et al., 1991) allowed the isolation of auxotrophic mutants. Some vitamin-requiring strains were found to be unable to
produce sporophytes unless higher amounts of nutrient supplementation were employed (Courtice et al., 1978). However, different combinations of these strains were able to cross with one another, provided the mutations were complementary. These data suggested that the sporophyte must, at least to some extent, be metabolically independent of the gametophyte, but complementation within hybrid sporophytes allowed their development (Courtice et al., 1978). This strategy was of limited use for forward genetics because it only facilitated crossing between strains with complementing auxotrophies. The alternative has been to isolate many individual sporophytes from a mixed culture of the two parental lines, and test spores from each sporophyte for the production of recombinant progeny. These restrictions have tended to limit the use of forward genetics in recent studies of P. patens. The sequenced genome of the Gransden strain (Rensing et al., 2008), and recent efforts to identify polymorphisms between it and the more-recently collected Villersexel strain (von Stackelberg et al., 2006; Kamisugi et al., 2008), increase the need for the facilitation of forward genetics analysis.

Here, we describe the use of transgenic lines that express a fluorescent transgene constitutively, for a rapid visual screen for hybrid sporophytes. We show that this technique can identify polymorphisms between it and the more-recently collected Villersexel strain (von Stackelberg et al., 2006; Kamisugi et al., 2008), increase the need for the facilitation of forward genetics analysis.

Materials and Methods

Moss strains

Details of the origins of P. patens strains used in this study are given in Supporting Information, Table S1.

Moss culture

Vegetative cultures of P. patens were grown according to Cove et al. (2009). P. patens tissue was grown on cellophane disks overlying 0.7% agar (A9799, Plant Cell Culture Agar; Sigma, St Louis, MO, USA) in 90 mm Petri dishes containing BCD medium and 5 mM (di)ammonium tartrate (Cove et al., 2009). These cultures were grown at 25°C in 16 h days (light intensity 60–80 μmol m⁻² s⁻¹).

Sexual reproduction was induced as described in Engel (1968) as modified by Cove et al. (2009). Inocula of protocormal tissue were grown in Magenta jars containing BCD medium, modified with a reduced potassium nitrate content of 400 μM, for 5 wk at 25°C under continuous light, after which the plants were transferred to 15°C with an 8 h day (60–80 μmol m⁻² s⁻¹) to induce gametangia formation. After 2 wk, 20 ml of sterile water was added to the culture and decanted 24 h later. The watering treatment was repeated at 3 wk post-transfer. Sporophytes were observed 3 wk after the first watering (10 wk after the inoculation of the culture). Sporophytes were picked when capsules were becoming brown, usually 2–3 wk later. Sporophyte fluorescence was observed using an Olympus SZX 12 stereomicroscope (Olympus America, Melville, NY, USA).

To ensure uniform spore germination, the spores were stored at 4°C in the dark for at least 7 d before performing the spore germination assay. The germination rate for each sporophyte was established from a sample of at least 500 spores. To assess the significance of the difference in germination rate between selfed and outcrossed sporophytes, we conducted a t-test for unequal sample sizes and assuming unequal variances between the samples.

Plasmid construction

pTHU1-Gateway vector (Fig. S1a) use allows efficient targeting to P. patens genomic site 108 (Schaefer and Zryd, 1997) of a cassette containing a gene coding resistance to hygromycin, and a cDNA expression cassette driven by a maize ubiquitin promoter. In order to facilitate cloning of a cDNA of interest, we cloned the Gateway cassette (Invitrogen) blunt fragment into the HindIII site of p108Ub-Nos (Bezanilla et al., 2005), after filling this in with DNA polymerase (Klenow fragment), and sequenced to confirm the proper orientation of the cassette.

pT2N2x35S-Gateway vector (Fig. S1c) allows efficient targeting to P. patens site 166780 of a cassette containing a gene coding resistance to the G418 antibiotic and a cDNA expression cassette driven by a 2x35S promoter. To build it, we amplified by PCR 1 kb of upstream and downstream flanking sequences from genomic DNA using the primers 5'-Fw and 5'-Rev and 3'-Fw and 3'-Rev, respectively (Table S2). The fragments obtained were subsequently cloned into TOPO 2.1 cloning vector. The 5'-fragment was subcloned using Not 1 and SphI restriction enzymes into the plasmid pLox2NpIIIF (a generous gift from D. S. Schaefer) to produce the pT1N vector. The 5'-fragment was then subcloned using SalI and XhoI restriction enzymes into pT1N to produce the pT2N. A 2x35S sequence was amplified by PCR using the primers 2x35S-Fw1 and 2x35S-Rev1, using pPZP221 (Hajdukiewicz et al., 1994) as template and subcloned into pTOPO 2.1 to create p2x35S. Using Not1 and HindIII restriction enzymes, the 2x35S fragment was cloned unto pBlue-sGFP (S65T)-NOS SK to create p2x35S-NOS SK. A Gateway cassette 2x35S (Invitrogen) was then cloned into Sma1 restriction site of p2x35S-NOS SK to create p2x35S-Fw2 and Nos-Rev and cloned into pTOPO 2.1 to create pXho-2x35S-Gateway-Xho. Finally, the XhoI restriction enzyme fragment from pXho-2x35S-Gateway-Xho was subcloned into the SalI site of pT2N to obtain pTN2x35S-Gateway.
Green fluorescent protein and mCherry cDNA were cloned into pTHUBI-Gateway and pT2N2x35S-Gateway using the Clonase LR approach (Invitrogen) to create, respectively, pTHUBI-mCherry (Fig. S1b) and pT2N2x35S-GFP (Fig. S1d).

**Transformation procedure**

Protoplasts were produced following Cove *et al.* (2009); 1-wk-old protonema was treated with 0.5% Driselase (D8037; Sigma) in 8.5% mannitol for 45 min, passed through a 100 μm sieve, incubated for 15 min and passed through a 50 μm sieve. The sieved protoplasts were washed twice in 8.5% mannitol, and transformed as described in Perroud & Quatrano (2006) using 15 μg of pTHUBI-mCherry or pT2N2x35S-GFP plasmids cut using the *SwaI* restriction site. Hygromycin or G418 (Sigma) was added at 25 μg l⁻¹ to the media to select for antibiotic-resistant cells. Stable transformants were selected for uniform fluorescent protein accumulation,

**Fig. 1** (a–d) Protonemata of *Physcomitrella patens* strains, accumulating fluorescent protein. (a, b) *P. patens* Villersexel line accumulating red-fluorescent protein, (Vx::mCherry), (c, d) *P. patens* Gransden lines accumulating green fluorescent protein (Gd::GFP). (e–p) Gametophore and sporophyte of selfed and crossed strains, accumulating fluorescent protein. (e, h, k, n) Gd::GFP selfed: (f, i, l, o) female Gd::GFP crossed with male Vx::mCherry; (g, j, m, p) Vx::mCherry selfed. (e–g) Bright field view; (h–j) green fluorescent signal observed with Olympus 460–490 excitation/510–550 emission filter set; (k–m) red fluorescent signal observed with Olympus 545–580 excitation/610 wide band pass filter set; (n–p) merged green and red fluorescent signal.
and tested for single locus insertion by Southern hybridization.

Results

To generate the fluorescent-tagged lines of *P. patens*, we inserted a transgene that expresses constitutively a fluorescent protein (either mCherry or green fluorescent protein, GFP), driven by either the maize ubiquitin or 2x35S promoter (Figs 1, S1). The transgenic protein was easily visible under a fluorescence stereomicroscope at ×10 to ×90 magnification. To evaluate the effects of transgene expression on normal development, we cultivated in parallel, under identical conditions, the transgenic and wild-type lines from which they were derived (Fig. 1e–p). The *P. patens* isolates expressing either transgene exhibited similar developmental timing to the wild-type isolate, indicating that in our laboratory conditions (Cove et al., 2009), the transgene did not have a detectable effect on this component of fitness. After 35 d of growth at 25°C in continuous light, gametophores were fully developed in the Villersexel, Kaskaskia and Gransden wild-type cultures as well as in the transgenic cultures. The cultures were then transferred to 15°C, with an 8 h day, to induce gametangia production. Archegonia were evident in all cultures after 15 d culture at 15°C. Sporophytes were detected on both the wild-type strains and the transgenic strains after 35 d at 15°C.

Sporophytes on wild-type plants resulting from fertilization by a transgenic plant inherit the transgene through the paternal line. We counted the total number of sporophytes produced by a wild-type strain, and the number of sporophytes on that strain that were expressing the fluorescent transgene (i.e. outcrossed) in crosses among the Kaskaskia, Villersexel and Gransden strains (Table 1). The mean outcrossing rate between the wild-type Villersexel and transgenic Villersexel plants was 8%, while the outcrossing rate between the Kaskaskia wild-type isolate and transgenic Villersexel was 3%. Based on these few crosses, this difference is not statistically significant. When the Gransden strain was crossed with transgenic Villersexel plants, the outcrossing rate was variable, but reached as high as 71% (Table 1). We also evaluated the germination rate for spores from 17 selfed and six outcrossed sporophytes (Fig. 2). The germination rates were variable in both cases, but we observed a significantly lower germination rate in outcrossed compared with selfed sporophytes (*P* < 0.001).

The procedure was then utilized to identify crossed sporophytes on co-cultures of the Villersexel mCherry transgenic line and a number of *P. patens* mutant strains. Although we have only rarely seen sporophytes on these *P. patens* strains in the years since they were mutagenized, we identified at least one hybrid sporophyte for each of the strains we crossed. Table S1 gives details of the strains that have been crossed to the Villersexel mCherry transgenic line. The segregations observed for each of the allele pairs involved in each cross are given in Table 2.

**Table 1** Outcrossing rates in crosses between transgenic and wild-type strains of the Gransden (Gd), Villersexel (Vx), and Kaskaskia (Ka) isolates

<table>
<thead>
<tr>
<th>Parent A</th>
<th>Parent B</th>
<th>Sporophytes on A</th>
<th>% crossed</th>
<th>n</th>
<th>Sporophytes on B</th>
<th>% crossed</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Gd</td>
<td>Vx::mCherry</td>
<td>71</td>
<td>262</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gd</td>
<td>Vx::GFP</td>
<td>39</td>
<td>432</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gd::mCherry</td>
<td>Gd::GFP</td>
<td>0</td>
<td>315</td>
<td>0</td>
<td>370</td>
<td></td>
<td></td>
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<tr>
<td>Gd::mCherry</td>
<td>Vx::GFP</td>
<td>22</td>
<td>369</td>
<td>0</td>
<td>185</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gd::GFP</td>
<td>Vx::mCherry</td>
<td>12</td>
<td>97</td>
<td>0</td>
<td>155</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vx</td>
<td>Vx::mCherry</td>
<td>4</td>
<td>162</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vx</td>
<td>Vx::GFP</td>
<td>12</td>
<td>173</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vx::mCherry</td>
<td>Ka</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>446</td>
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<td></td>
</tr>
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**Fig. 2** Distribution of spore germination rates in selfed and crossed progeny of *Physcomitrella patens* strains. Each grey spot marks a spore germination rate for a selfed sporophyte (Villersexel and Gransden, *n* = 17) or a hybrid between Villersexel and Gransden (*n* = 6); the white bar indicates the mean in each class, the significance of the difference between the means (*P* = 0.0001) was assessed using a *t*-test.

Discussion

The moss *P. patens* is now widely used in studies of plant genomics and gene function. Here we describe the development of fluorescent transgenic lines in *P. patens* that accelerate the identification of crossed sporophytes, promoting the development of a forward genetics research program using this model system. We also note that forward genetics in the *P. patens* system is facilitated by the weak male fertility of the Gransden laboratory strain. While the Gransden strain produced selfed sporophytes under our laboratory conditions, we detected no crossed sporophytes in crosses between the Villersexel and Gransden strains, where the Gransden strain had been the male parent (Table 1). This indicates that Gransden antherozoids are poor competitors...
Table 2 Segregation of individual allelic pairs in crosses involving transgenic lines

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<tr>
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<tbody>
<tr>
<td>Segregations</td>
<td>Mutant</td>
<td>c2 for</td>
<td>Mutant</td>
<td>c2 for</td>
<td>Mutant</td>
<td>c2 for</td>
</tr>
<tr>
<td></td>
<td>transgene</td>
<td>1:1</td>
<td>transgene</td>
<td>1:1</td>
<td>transgene</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>(1 d.o.f.)</td>
<td>+</td>
<td>(1 d.o.f.)</td>
<td>+</td>
<td>(1 d.o.f.)</td>
</tr>
</tbody>
</table>

- + : mCherry: 222 208 0.46
- + : GFP: – – – 92 108 1.28
- + : pabA3: – – – 206 177 2.02
- + : thiA1: – – – 206 177 2.02
- + : ptrC4: – – – 168 160 0.20
- + : ptrB2: – – – 270 203 9.49**

| GD, Gransden; Vx, Villersexel; c2, chi-square value indicated in italic; d.o.f., degree of freedom; ** significant value. |
| Rows identify the segregation for the allele pair indicated, and the value of the chi-squared for an expected 1 : 1 ratio. |
| Columns identify the segregations within progeny for the cross between the strain indicated and Vx::mCherry. |

- GD, Gransden; Vx, Villersexel; c2, chi-square value indicated in italic; d.o.f., degree of freedom; ** significant value. |
the other segregations, only the segregation of ptrC in the cross between the "thiA1 pabA3 ptrC4" Gransden strain and Villersexel mCherry transgenic deviated significantly from a 1:1 ratio. This may be due to the ptrC allele or a linked allele reducing viability. The techniques described here, combined with the weak male fertility of the Gransden strain, pave the way for an extended forward genetics program in P. patens using the Gransden and trangenic Villersexel strains.

Acknowledgements
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References