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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*.

Keywords

forward genetics; hybridization; mutants; reverse genetics; self-fertilization

Introduction

The sequencing of the *Physcomitrella patens* genome (Rensing *et al.*, 2008) and the demonstration of efficient gene targeting (Schaefer & 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

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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 does not interfere with expected segregation ratios in crosses among three different isolates of *P. patens*, and demonstrate the utility of this system for mapping induced mutations. We also provide the first experimental estimates of the outcrossing rate in a monoicous bryophyte.

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 overlaying 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Sexual reproduction was induced as described in Engel (1968) as modified by Cove *et al.* (2009). Inocula of protonemal 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$) 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

pTHUBI-Gateway vector (Fig. S1a) use allows efficient targeting to *P. patens* genomic site108 (Schaefer and Zrýd, 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 *Hind*III site of p108Ubi-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* site146870 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 in TOPO 2.1 cloning vector. The 3'-fragment was subcloned using Not I and *Spe*I restriction enzymes into the plasmid pLox2NptIIIF (a generous gift from D. S. Schaefer) to produce the pT1N vector. The 5'-fragment was then subcloned using *Sal*I and *Xho*I 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 *Not*I and *Hind*III 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 *Sma*I 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 *Xho*I restriction enzyme fragment from pXho-2x35S-Gateway-Xho was subcloned into the *Sal*I 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 *Swa*I 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, 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.

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 relative to those of the Villersexel strain. The inability of Gransden antherozoids to fertilize eggs on the gametophores of other plants may have been a feature of the original Gransden population. However, for a male-sterility mutation to invade a hermaphroditic population, the mutation must confer elevated female fertility or the magnitude of inbreeding depression must be high (Charlesworth & Charlesworth, 1978). In our experiments, the Gransden strain did not produce more sporophytes than the Villersexel strain, indicating the loss of male fertility has not been compensated by a gain in female fertility. Similarly, Taylor *et al.* (2007) found no evidence of inbreeding depression in the related hermaphrodite *Funaria hygrometrica*, suggesting this explanation lacks support. Alternatively, the reduced ability of the Gransden strain to act as a male parent in outcrosses may result from deleterious mutations that arose during the long period of laboratory cultivation since the strain was isolated (by H. Whitehouse in 1962).

The relatively high outcrossing rate in the Gransden × Villersexel cross suggests that our experimental conditions are favorable to cross-fertilization. For this reason, we believe that the maximum outcrossing rate in natural populations may be similar to the *c.* 3% for the Villersexel × Kaskaskia cross or the *c.* 8% for the Villersexel × Villersexel cross, although we acknowledge that mating in nature is likely to be influenced by other factors. Silencing of the transgene in hybrid sporophytes could cause us to underestimate the outcrossing rate, but the transgene segregated at 1 : 1 in the progeny of nearly all crosses, suggesting there is no silencing at the protonemal stage, and we have never seen silencing in any transgenic selfed sporophytes. This outcrossing estimate is also consistent with multilocus genealogical evidence in the *Physcomitrella* — *Physcomitrium* species complex (McDaniel *et al.*, 2010) and allozyme heterozygosity in natural populations of several self-fertile mosses (Eppley *et al.*, 2006), both suggesting that outcrossing is quite rare. Nevertheless, the fact that we and others have reported natural or experimental hybrids between distantly related lineages of in the moss family *Funariaceae* (Britton, 1895; Andrews, 1918; von Wettstein, 1932; Bauer & Brosig, 1959; Pettet, 1964; McDaniel *et al.*, 2010) suggests that there are few F₁ barriers to hybridization in this group.

In spite of the lack of F₁ barriers, the mean spore germination rate that we observed was 25% in crosses between genetically distinct parents, compared with > 80% in self-fertilization (Fig. 2). This is consistent with the segregation distortion (presumably from spore death) in the Gransden × Villersexel mapping population reported by Kamisugi *et al.* (2008). However, this still leaves > 1000 viable spores per sporophyte, and therefore this choice of parents is unlikely to interfere with most mapping projects. Moreover, the transgene had no detectable effect on the viability of the recombinants since the segregation ratio of the transgene did not deviate from the expected 1 : 1 ratio (Table 2). For the other segregations, only the segregation of *pttC* in the cross between the *thiA1 pabA3 pttC4* Gransden strain and Villersexel mCherry transgenic deviated significantly from a 1 : 1 ratio. This may be due the *pttC* 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 transgenic Villersexel strains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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.

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.

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

Table 1

Parent A	Parent B	Sporophytes on A		Sporophytes on B	
		% crossed	n	% crossed	n
Gd	Vx::mCherry	71	262	–	–
Gd	Vx::GFP	39	432	–	–
Gd::mCherry	Gd::GFP	0	315	0	370
Gd::mCherry	Vx::GFP	22	369	0	185
Gd::GFP	Vx::mCherry	12	97	0	155
Vx	Vx::mCherry	4	162	–	–
Vx	Vx::GFP	12	173	–	–
Vx::mCherry	Ka	–	–	3	446

Table 2
Segregation of individual allelic pairs in crosses involving transgenic lines

Vx::mCherry crossed to:		Kaskasia		Gd::GFP		Gd::gtr-C5 <i>pabA3</i>		Gd::ptrB2 <i>pabA3</i>		Gd::thiA1 <i>pabA3 ptrC4</i>		Cd::ptrB2 <i>thiA1</i>								
Segregations	+	Mutant or transgene	c2 for 1 : 1 (d.o.f.)	+	Mutant or transgene	c2 for 1 : 1 (d.o.f.)	+	Mutant or transgene	c2 for 1 : 1 (d.o.f.)	+	Mutant or transgene	c2 for 1 : 1 (d.o.f.)	+	Mutant or transgene	c2 for 1 : 1 (d.o.f.)					
+	+	mCherry	222	208	0.46	93	107	0.98	196	187	0.21	161	167	0.11	236	237	0.00	133	144	0.44
+	+	GFP	-	-	-	92	108	1.28	-	-	-	-	-	-	-	-	-	-	-	-
+	+	<i>pabA3</i>	-	-	-	-	-	-	206	177	2.20	184	144	4.88	237	236	0.00	-	-	-
+	+	<i>thiA1</i>	-	-	-	-	-	-	-	-	-	-	-	-	230	243	0.36	130	147	1.04
+	+	<i>gtrC4</i>	-	-	-	-	-	-	206	177	2.20	-	-	-	-	-	-	-	-	-
+	+	<i>ptrB2</i>	-	-	-	-	-	-	-	-	-	168	160	0.20	-	-	-	132	145	0.61
+	+	<i>ptrC4</i>	-	-	-	-	-	-	-	-	-	-	-	-	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.