

Life-cycle-generation-specific developmental processes are modified in the *immediate upright* mutant of the brown alga *Ectocarpus siliculosus*

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Development of the sporophyte and gametophyte generations of the brown alga *E. siliculosus* involves two different patterns of early development, which begin with either a symmetric or an asymmetric division of the initial cell, respectively. A mutant, *immediate upright* (*imm*), was isolated that exhibited several characteristics typical of the gametophyte during the early development of the sporophyte generation. Genetic analyses showed that *imm* is a recessive, single-locus Mendelian factor and analysis of gene expression in this mutant indicated that the regulation of a number of life-cycle-regulated genes is specifically modified in *imm* mutant sporophytes. Thus, *IMM* appears to be a regulatory locus that controls part of the sporophyte-specific developmental programme, the mutant exhibiting partial homeotic conversion of the sporophyte into the gametophyte, a phenomenon that has not been described previously.

KEY WORDS: Brown algae, *Ectocarpus siliculosus*, Immediate upright, Initial-cell division, Life cycle, Phaeophyceae

INTRODUCTION

The brown algae evolved multicellularity independently of animals and higher plants, and they include several lineages that rival higher plants in their complexity (de Reviers, 2003). Brown algae develop from cells that are released into the surrounding seawater (de Reviers, 2002), and the early stages of development are therefore easily observable. The growth of the initial cell can be markedly different in different species, involving either unipolar or bipolar germination (emergence of either one or two germ tubes, respectively). Moreover, bipolar germination can result in different growth patterns. When germination produces a symmetric filament, this leads to the development of a prostrate, basal structure before the erect thallus is formed (termed mediate differentiation or heterotrichy, see Table 1). By contrast, if an asymmetric structure is formed following germination, this leads to the immediate development of an erect thallus without the formation of a prostrate, basal structure (immediate differentiation) (Pedersen, 1984; Fletcher, 1987).

Asymmetric cell divisions are defined in terms of developmental fate, so that a cell division may be asymmetric even if there is no morphological difference between the daughter cells at the time of division, provided that each cell adopts a different cell fate (Horvitz and Herskowitz, 1992; Scheres and Benfey, 1999). Similarly, a symmetric division is defined as one that generates daughter cells that are destined to acquire the same developmental fate (Morrison and Kimble, 2006). Zygotic/initial

cell divisions frequently elaborate polarity that underlies a major body axis [Berleth and Chatfield, 2002 (<http://www.aspb.org/publications/arabidopsis/>); Schneider and Bowerman, 2003; Huynh and St Johnston, 2004]. Within the brown algae, efforts to dissect the processes of polarisation and the first cell division have concentrated on the Fucales, where it has been shown that polarity can be established in response to a range of external vectors with unidirectional light being the strongest stimulus (Quatrano, 1997).

The filamentous brown alga *Ectocarpus siliculosus* (Dillwyn) Lyngbye has been proposed as a general model for the brown algae (Peters et al., 2004a) and its genome has been sequenced at Genoscope (<http://www.genoscope.cns.fr/spip/Ectocarpus-siliculosus,740.html>). The life cycle of *E. siliculosus* involves an alternation between two macroscopic generations that differ morphologically (sporophytes produce few laterals and develop from a branched prostrate base, whereas gametophytes are more richly branched and devoid of a prostrate base) (Kornmann, 1956; Müller, 1964). In this study, we show that, unlike the sporophyte, which is formed by mediate differentiation following bipolar germination and symmetric division of the initial cell (Peters et al., 2004b), the *E. siliculosus* gametophyte exhibits an asymmetric initial cell division and immediate differentiation of an erect thallus. Therefore, the alternation of generations in *E. siliculosus* involves an alternation between two fundamentally different patterns of initial cell division: symmetric and asymmetric.

A spontaneous mutant, *immediate upright* (*imm*), exhibited several phenotypic traits characteristic of the gametophyte generation during the sporophyte generation of its life cycle, including asymmetric initial cell division. This mutant produced functional meiospores, demonstrating that symmetric initial cell division is not essential for an individual to become a functional sporophyte. However, specific alterations in the expression of generation-specific genes were detected in this mutant using a microarray approach, indicating that *imm* is a bona fide life cycle mutant and hence that the phenotypic traits that are modified in *imm* are normally under life cycle control in wild-type algae.

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Table 1. Definitions of terms used in the text

Term used	Definition and comments
Gametophyte	The gamete-producing generation of a plant life cycle.
Sporophyte	The spore-producing generation of a plant life cycle.
Partho-sporophyte	Sporophyte generation produced by direct germination of gametes that have not undergone fusion.
Plurilocular sporangium	Multi-chambered reproductive structure containing mitotically produced spores that reproduce the sporophyte. Found on the sporophyte.
Unilocular sporangium	Single-chambered reproductive structure containing meiotically produced spores that develop as gametophytes. Found on the sporophyte.
Plurilocular gametangium	Multi-chambered reproductive structure containing gametes. Found on the gametophyte.
Meio-spore	Spore generated via a meiotic division in a unilocular sporangium.
Mito-spore	Spore generated without meiosis in a plurilocular sporangium.
Rhizoid	Root-like filament consisting of narrow (3-5 μm) cells.
Mediate differentiation	Establishment of a prostrate, basal structure before development of an erect thallus. Associated with symmetric initial cell division and exhibited by the <i>Ectocarpus</i> sporophyte. Also termed heterotrichy.
Immediate differentiation	Direct development of an erect thallus. Associated with asymmetric initial cell division and exhibited by the <i>Ectocarpus</i> gametophyte.

MATERIALS AND METHODS

Algal strains and culture conditions

The *Ectocarpus siliculosus* strains used were meiotic offspring of a field sporophyte collected in 1988 in San Juan de Marcona, Peru. The gametophyte clone used for genome sequencing, Ec 32, is from the same lineage (Table 2). The strains were cultivated in 7-8 ml polystyrene Petri dishes, occasionally on glass cover-slips, normally at 15°C in white fluorescent light of 10-30 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photon fluence rate. Additional light regimes used were: natural diffuse light at 5°C, dim (1 $\mu\text{mol m}^{-2}\text{s}^{-1}$) white light at 15°C and bright (10-30 $\mu\text{mol m}^{-2}\text{s}^{-1}$) white light at 10 and 20°C. Day length was 10 hours light: 14 hours dark, except for the 5°C condition in which day length varied according to the season. The culture medium was half-strength, Provasoli-enriched (Starr and Zeikus, 1993), autoclaved seawater. For the study of germination, spores or gametes were allowed to settle on cover-slips. Zygotes were produced and isolated as described by Peters et al. (Peters et al., 2004b). The sex of gametophytes was determined by microscopic observation of zygote formation in hanging-drop preparations (Kawai et al., 2005) in which the strains to be tested had been combined with fertile thalli of male and female reference strains. Genetic analysis of meiotic offspring followed Müller (Müller, 1991).

Photopolarisation tests

To determine the response of the gametophyte and the sporophyte to a light stimulus, algae were grown at low density in 5 cm (7-8 ml) Petri dishes under unidirectional white light. The orientation of germination was scored according to four quadrants (i.e. towards the light, away from the light or in

one of the two quadrants perpendicular to the light). Algae that germinated into the quadrant away from the light were scored as exhibiting negative phototropy.

Microarray analysis of gene expression

A microarray was constructed by spotting PCR amplified inserts from two suppression subtraction hybridisation (SSH) libraries, enriched for genes expressed preferentially during the sporophyte and gametophyte generations, respectively, onto glass slides. The SSH libraries were produced in a differential screen between the partho-sporophyte and gametophyte generations of the life cycle using the PCR Select kit (Clontech, Mountain View, CA, USA). Oligonucleotides corresponding to flanking vector sequences were used to amplify inserts from 600 clones randomly chosen from each library and these 1200 PCR products were spotted in triplicate on each microarray. RNA was extracted as described by Apt et al. (Apt et al., 1995) and its quality was monitored on a Bioanalyser using an RNA 6000 Nano Assay kit (Agilent, Santa Clara, CA, USA). Labelled cDNA targets were synthesised from 15 μg of total RNA with an oligo(dT) primer and by indirect labelling using the CyScribe cDNA Post Labelling kit (Amersham, Piscataway, NJ, USA). Hybridised microarrays were scanned with a GenePix 4000 (Molecular Devices Corporation, Downingtown, PA, USA) and analysed using GenePix Pro 5.1. All hybridisations were carried out in triplicate. Data corresponding to a set of 132 probes that did not show differential expression during the sporophyte and gametophyte generations were used to normalise the data obtained from the different hybridisation experiments. The average value for the coefficient of variation was 0.29 for

Table 2. *Ectocarpus* strains used in this study

Name	Generation, sex	Germination phenotype*	Genotype	Description	Origin	Year	Other names
Ec 17	SP	Wild type	<i>IMM IMM</i>	Field isolate	San Juan de Marcona, Peru	1988	CCAP [†] 1310/193, SAm120Sp [‡]
Ec 25	GA f	Wild type	<i>IMM</i>	Meiotic offspring from Ec 17	Laboratory	2002	CCAP 1310/3
Ec 32	GA m	Wild type	<i>IMM</i>	Meiotic offspring from Ec 17	Laboratory	2002	CCAP 1310/4
Ec 137	GA m	<i>imm</i>	<i>imm</i>	Meiotic offspring from Ec 17, germination mutant	Laboratory	2002	CCAP 1310/319
Ec 372	SP	Wild type	<i>imm IMM</i>	Cross 25f wt × 137m <i>imm</i>	Laboratory	2003	CCAP 1310/320
Ec 419	GA f	<i>imm</i>	<i>imm</i>	Meiotic offspring from 372	Laboratory	2003	CCAP 1310/321
Ec 420	GA m	<i>imm</i>	<i>imm</i>	Meiotic offspring from 372	Laboratory	2003	CCAP 1310/322
Ec 421	GA f	Wild type	<i>IMM</i>	Meiotic offspring from 372	Laboratory	2003	CCAP 1310/323
Ec 423	GA m	Wild type	<i>IMM</i>	Meiotic offspring from 372	Laboratory	2003	CCAP 1310/324
Ec 428	SP	<i>imm</i>	<i>imm imm</i>	Product of cross 419f <i>imm</i> × 420m <i>imm</i>	Laboratory	2003	CCAP 1310/325
Ec 429	SP	Wild type	<i>imm IMM</i>	Cross 419f <i>imm</i> × 423m wt	This work	2004	CCAP 1310/326
Ec 430	SP	Wild type	<i>imm IMM</i>	Cross 421f wt × 420m <i>imm</i>	This work	2004	CCAP 1310/327
Ec 432	SP	Wild type	<i>IMM IMM</i>	Cross 421f wt × 423m wt	Laboratory	2003	CCAP 1310/328

*Germination of settled gametes for gametophytes or of zygotes or mito-spores for sporophytes.

[†]CCAP, Culture Collections of Algae and Protozoa (marine) reference number, Dunstaffnage Marine Laboratory, Oban, Scotland.

[‡]Designation in Stache-Crain et al. (Stache-Crain et al., 1997).

SP, sporophyte; GA, gametophyte; *imm*, immediate upright mutation.

the wild-type partheno-sporophyte samples and 0.35 for the *imm* mutant partheno-sporophyte samples. Full descriptions of the array design, probe sequences, RNA extraction, cDNA labelling, hybridisation and normalisation protocols are available via the ArrayExpress database (accession number E-MEXP-848). Statistical analysis was carried out using the Statistical Analysis of Microarrays (SAM) method (Tusher et al., 2001) in the TIGR MeV package, version 3.1. The cDNA fragments corresponding to probes that had been shown to be significantly, differentially expressed by this analysis were sequenced and compared with the EST and genomic sequence data available for *Ectocarpus siliculosus*.

Quantitative PCR

Total RNA was extracted from diploid sporophytes, partheno-sporophytes and gametophytes of both wild-type and *imm* strains using the Plant RNeasy extraction kit (Qiagen, Courtabouef, France), and treated with RNase-free DNase-I according to the manufacturer's instructions (Qiagen). The concentration and quality of the RNA was determined by spectrophotometry and agarose gel electrophoresis. Two milligrams of total RNA were reverse-transcribed using the Superscript II RT kit (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Oligonucleotide sequences were designed for each gene using both Primer Express TM1.0 (PE Applied Biosystems, Foster City, CA, USA) and Oligo 4.0 (Rychlik and Rhoads, 1989). cDNAs were amplified using the IQ Sybrgreen supermix (Biorad Laboratories, Hercules, CA, USA) on a Chromo4 System thermocycler (BioRad Laboratories). The amplification efficiency was tested using a genomic dilution series and was always at least 80%. The specificity of amplification was checked with a dissociation curve. Details of the oligonucleotides used can be provided on request. *E. siliculosus* genomic DNA was used as a quantification reference. A dilution series ranging from 37 to 48671 copies of the *E. siliculosus* genome was prepared and tested for each gene amplification. The *EF1 α* gene was chosen as a constitutively expressed control based on tests carried out on three *Ectocarpus* genes (encoding actin, *EF1 α* and α -tubulin). Normalisation was carried out using the *EF1 α* data and the geNorm method (Vandesompele et al., 2003). The normalised data were expressed as the mean \pm s.d. calculated from three independent biological experiments.

RESULTS

Gametophytes and sporophytes of *E. siliculosus* exhibit markedly different patterns of early development

The sexual life cycle of *E. siliculosus* involves an alternation between two independent generations: the sporophyte and the gametophyte (Fig. 1). To compare the early development of these two generations, gametophytes were raised from meiospores of a heterozygous, field-isolated, sporophyte (strain Ec 17) and their development in culture compared with that of sporophytes that were also derived from the Ec 17 strain (see Table 1 for a glossary of the terms used in this section). A total of 120 gametophytes were isolated and all exhibited the same pattern of early development. Germination was bilateral and an asymmetric division of the initial meiospore cell resulted in the occurrence of different cell types in the two emerging germ tubes. The first germ tube developed into a rhizoidal filament 3-5 μ m in diameter (Fig. 2A), whereas the second germ tube formed an upright filament (Fig. 2B). The rhizoid had a wavy appearance and, as it originated 2-7 days before the emergence of the second germ tube, it was often quite long by the time that the upright filament elongated (Fig. 2C). The cells of the upright filament were characterised by broader (initially 10 μ m in diameter but enlarging to 20-40 μ m in higher thallus parts) and regularly cylindrical cells (Fig. 2B,D). The upright filaments produced laterals from the distal end of filament cells (Fig. 2E) to form a richly branched thallus (Fig. 2F). After 3-5 weeks in white light, gametophytes became fertile with plurilocular gametangia forming at terminal or lateral positions on the upright filaments (Fig. 2G). On

older gametophytes, additional rhizoids were formed as laterals from the basal ends of upright filaments (Fig. 2H,I). Gametophytes adhered only weakly to glass or polystyrene, easily becoming detached and floating off into the medium. The overall development of the gametophyte was of the immediate differentiation type because the erect thallus developed directly without prior establishment of a prostrate basal structure.

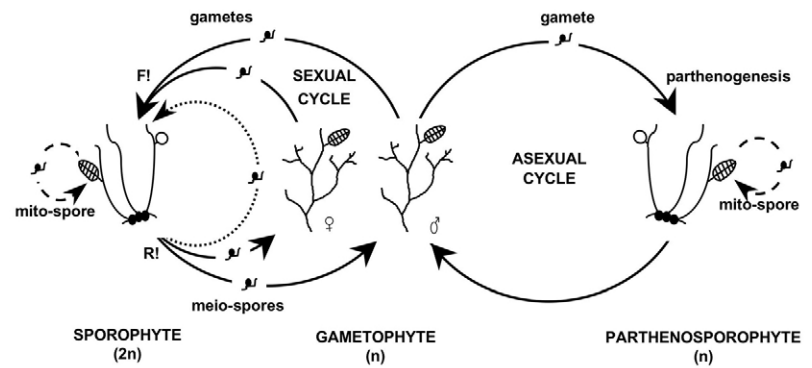
E. siliculosus sporophytes can be produced in a number of different ways (Fig. 1): (1) via gamete fusion and zygote production; (2) by mito-spores produced by the plurilocular sporangia of sporophytes; (3) from a minority of the meio-spores produced by unilocular sporangia (the majority producing gametophytes); or (4) by parthenogenesis from settled unfertilised gametes (referred to as partheno-sporophytes). The early development of sporophytes produced by each of these alternative pathways was compared with the early development of the gametophyte generation of the same strain, described above. All sporophytes showed the same pattern of development, except that the growth of partheno-sporophytes was slower during the first few days. Germination was bipolar, as in the gametophyte. However, in contrast to the situation observed in the gametophyte, the two daughter cells of the sporophyte initial cell exhibited symmetric cell fates, producing the two ends of a symmetric prostrate filament (Fig. 3A-E). Because of these identical cell fates, the initial cell division in the sporophyte is defined as symmetric despite the morphological asymmetry of the initial cell at the time of cell division (Fig. 3B). This follows the definition given by Morrison and Kimble (Morrison and Kimble, 2006).

The cells of the prostrate filament became rounder and their cell walls thickened as they became older (i.e. in the central region of the filament; Fig. 3E,F). Laterals with the same morphology as the initial filament were produced from the rounded cells, and grew along the surface of the substratum (Fig. 3F) or up into the medium. Upright filaments with cylindrical cells (20-30 μ m diameter) developed on the prostrate base after 4-5 weeks to produce an erect thallus (Fig. 3G,H). The upright filaments usually emerged from older regions of the prostrate base. The mature sporophyte consisted of a well-developed, prostrate basal system from which emerged the upright filaments (Fig. 3I). Compared with gametophyte germlings, the prostrate sporophyte thalli were not easily detached from either polystyrene or glass surfaces. Plurilocular sporangia developed on upright filaments after 7 weeks, in terminal or lateral positions (Fig. 3J). Occasionally, plurilocular sporangia developed on the prostrate structure, in some cases even before the emergence of upright filaments (not shown). Unilocular sporangia formed only on upright filaments, in terminal or lateral positions, a week after the plurilocular sporangia (Fig. 3K). The upright filaments were branched (not shown), although to a lesser extent than those of a gametophyte, and, in older thalli, also produced rhizoids (not shown). Rhizoids originated only from the upright filaments and were not produced by the filaments of the prostrate structure (Fig. 3H). Overall, the sporophyte exhibited mediate differentiation because a prostrate, basal structure was formed before the development of the erect thallus (i.e. the upright filaments).

In summary, observation of the early development of the two generations of the *E. siliculosus* life cycle showed that they exhibited markedly different patterns of development. These two patterns of development involved either asymmetric or symmetric divisions of the initial cells of gametophytes or sporophytes, respectively. The gametophyte formed two types of vegetative structures (rhizoids and upright filaments) and one kind of reproductive structure (plurilocular gametangia), the sporophyte three types of vegetative

Fig. 1. Life history of *Ectocarpus* in culture.

The sexual cycle (left) involves an alternation between the diploid sporophyte and haploid dioecious (male and female) gametophytes. The sporophyte produces meio-spores, via a meiotic division (R!), in the unilocular (single-chambered) sporangia. The meio-spores are released and develop as gametophytes, which produce gametes in plurilocular gametangia. Fusion of male and female gametes produces a zygote (F!). The zygote develops as a diploid sporophyte, completing the sexual cycle. Unfertilised gametes can enter a parthenogenetic asexual cycle by germinating without fusion to produce a partheno-sporophyte (right). The partheno-sporophyte produces spores in unilocular sporangia and these develop as gametophytes, completing the parthenogenetic, asexual cycle. The parthenogenetic, asexual pathway is shown only for a male, but female gametes can also develop parthenogenetically. Two additional pathways of asexual reproduction are possible. The first involves the production, by sporophytes, of mito-spores in plurilocular sporangia. These spores reproduce the sporophyte stage (dashed lines). In addition, a proportion of the meio-spores may develop as sporophytes rather than gametophytes (dotted line). This latter phenomenon is also observed with the partheno-sporophyte but this has been omitted to simplify the diagram. R!, meiotic reduction; F!, gamete fusion.



structures (prostrate filaments, rhizoids and upright filaments) and two kinds of reproductive structure (plurilocular and unilocular sporangia).

Responses of the germinating sporophyte and the gametophyte to unidirectional light

Initial cell (zygote) photopolarisation has been extensively studied in members of the Fucales group (Corellou et al., 2005; Robinson and Miller, 1997; Fowler et al., 2004; Brownlee and Bouget, 1998). In this group, photopolarisation causes the zygote to germinate in a negatively phototropic manner, emitting a rhizoid on the side opposite the light source. The markedly different germination patterns of the *E. siliculosus* sporophyte and gametophyte led us to test their responses to unidirectional light. When grown in unidirectional light, the majority of individuals of both the sporophyte and gametophyte generations germinated in a negatively phototropic manner (Fig. 4). However, this response was significantly less marked in the sporophyte generation, where only 73% of individuals germinated away from the light, compared with 93% for the gametophytes ($\chi^2=25.68$, $P<0.001$).

The sporophyte of the *imm* mutant exhibits a gametophyte-like pattern of early development

The ability of *E. siliculosus* to produce partheno-sporophytes from unfertilised, haploid gametes allows the phenotypic effects of mutations to be detected directly in both the gametophyte and the (partheno-)sporophyte generations. Phenotypic analysis of partheno-sporophytes derived from the 120 gametophytes produced by the Ec 17 sporophyte identified one gametophyte strain (Ec 137) that produced partheno-sporophytes with an aberrant germination pattern. The partheno-sporophytes of this strain exhibited asymmetric division of the initial cell rather than the usual pattern of symmetric division of the initial cell (exhibited by the partheno-sporophytes derived from its sibling gametophytes). The same aberrant developmental phenotype was seen in diploid sporophytes homozygous for the mutant locus (see below for the production of such thalli). The aberrant germination pattern involved the production of a first germ tube that developed into a thin (3–5 μm diameter) filament that resembled the rhizoid produced by the germinating gametophyte (compare Fig. 5A with Fig. 2A). The second germ tube developed into an upright filament (initially 10 μm in diameter, but widening to 20–30 μm in the upper part of the filament), again similar to that produced by germinating gametophytes

(compare Fig. 5B with Fig. 2D). Based on this phenotype, the mutant was named *immediate upright (imm)*. The aberrant germination pattern was observed for all developing gametes of Ec 137 and was not modified by cultivation at 5, 10 or 20°C.

The aberrant early development of the *imm* mutant had a significant effect on the morphology of the alga later in development. After 6 weeks growth, the *imm* mutant had produced a well-developed erect thallus attached to the substratum with rhizoids (Fig. 5C), whereas wild-type sporophytes had, after the same period of growth, developed a dense, prostrate, basal system from which only the first upright filaments emerged (Fig. 3I). This morphological difference between wild-type and *imm* sporophytes was already visible under the light microscope after 1–2 weeks growth and the two types of growth habit could be distinguished with the naked eye after 4–5 weeks. This is illustrated by the macroscopic views shown in Fig. 5D,E.

Despite the resemblance of the *imm* partheno-sporophyte to the wild-type gametophyte, further analysis showed that it retained its sporophyte identity. Mature *imm* partheno-sporophytes produced not only plurilocular but also unilocular sporangia, reproductive structures that are only produced by the sporophyte generation (Fig. 5F,G). Both of these structures can either emerge directly from a filament or be borne on a short branch, in both the wild type and in the *imm* mutant. Mating combinations with gametophyte reference strains showed that the spores released from the plurilocular sporangia were indeed spores and not gametes (they were incapable of fusing with fertile gametes of either sex). Even during early development, and despite their resemblance to the gametophyte, *imm* partheno-sporophytes exhibited several features that were typical of the wild-type sporophyte. For example, *imm* partheno-sporophytes adhered firmly to the substratum and were difficult to detach. Moreover, when grown in unidirectional light, the negative phototropic response was less marked than that detected normally for the gametophyte and more similar to that of the wild-type sporophyte, with only 68% of the individuals tested germinating away from the light (Fig. 4). It was also noted that, although the first germ tube developed as a filament in the same manner as in the gametophyte, it was less wavy in appearance (compare Fig. 5B with Fig. 2C,D).

The meio-spores produced in the unilocular sporangia of the *imm* partheno-sporophytes developed into phenotypically normal gametophytes that produced functional gametes (data not shown). All asexually produced sporophytes, derived either from mito-

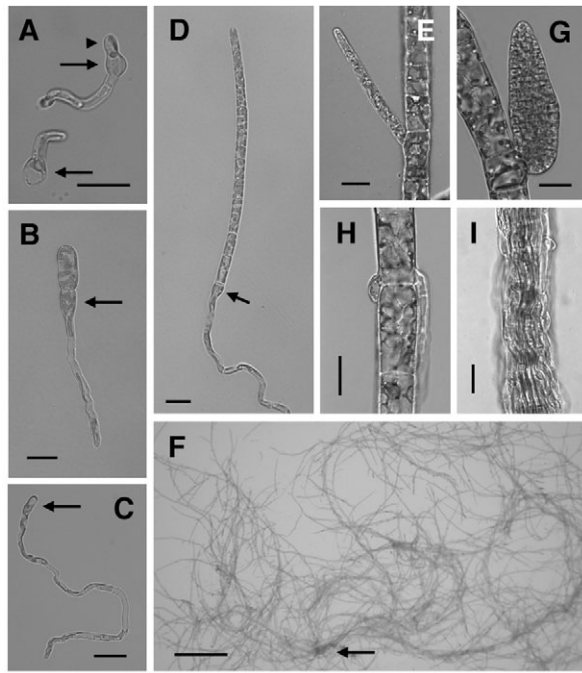


Fig. 2. Development of the *Ectocarpus* gametophyte. (A) Two germinating meiospores, the uppermost showing initiation of the second germ tube (arrowhead). (B) Germinating meiospore after 4 days growth, with a rhizoid developed from the first (lower) germ tube and an upright filament from the second (upper) germ tube. (C) Five-day-old germinating meiospore in which only the rhizoid has developed so far. (D) Gametophyte 1 week after germination; upright filament still unbranched. The cell corresponding originally to the meiospore is indicated by an arrow in A-D. (E) Branching of the upright filament. (F) Mature, richly branched gametophyte; arrow indicates the base of the thallus. (G) Gametangium. (H) Beginning of the formation of additional rhizoids from basal ends of cells of the upright filament. (I) Rhizoids covering an older upright filament. Scale bars: in F, 200 μm ; in all other micrographs, 20 μm .

spores produced in the plurilocular sporangia of the *imm* partheno-sporophytes or via the gametophyte generation by parthenogenetic germination of gametes, exhibited the *imm* phenotype.

Taken together, these experiments showed that the *imm* mutant carried a heritable character that induces a modification of the early development of the sporophyte, causing it to resemble the gametophyte morphologically. The *imm* mutation had no observable effect on the development of the gametophyte generation. The appearance of the *imm* mutation in one of 120 gametophytes derived from the Ec 17 sporophyte indicates that this mutation arose spontaneously in this generation of gametophytes.

***imm* behaves as a stable recessive single-locus Mendelian allele**

The *imm* mutant (strain Ec 137) was crossed with a sister gametophyte (Ec 25) whose gametes showed a wild-type pattern of parthenogenetic development (see Fig. 6 for a summary of the crosses carried out for the genetic analysis). Three zygotes were raised from this cross and the resulting sporophytes all showed a wild-type pattern of early development. The development of one of these sporophytes (strain Ec 372) was followed to maturity and no

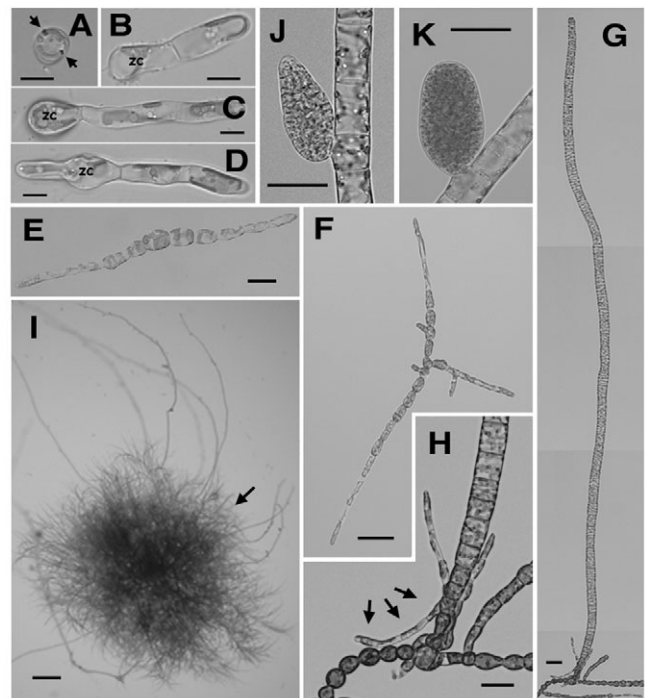


Fig. 3. Development of the *Ectocarpus* sporophyte. (A) Zygote, the two eyespots are visible (arrows). (B,C) Development of the first germ tube. (D) Initiation of the second germ tube. (E) Prostrate filament after 2 weeks, older cells (including the original zygote cell) round up. (F) Branching of the prostrate filament. (G) Upright filament developed from a prostrate base. (H) Transition between prostrate base and upright filament of the thallus shown in G. Note the 'string of pearls' shape of the cells of the prostrate filament, contrasting with the regular cylindrical shape of the cells of the upright filament; arrows indicate a rhizoid formed near the base of the upright filament. (I) Mature, six-week-old sporophyte with a well-developed prostrate basal system (arrow) and upright filaments emerging from it. (J) Plurilocular sporangium (mitosporangium). (K) Unilocular sporangium (meiosporangium). zc, original zygote cell. Scale bars: 5 μm in A-D; 20 μm in E,H; 40 μm in F,G,J,K; 100 μm in I. A-D are reproduced, with permission, from Peters et al. (Peters et al., 2004b).

differences were observed compared with the developmental pattern of a wild-type sporophyte (data not shown). This indicated that the *imm* mutation was recessive and was complemented by the wild-type allele in the diploid sporophyte.

To analyse the segregation of the *imm* locus in subsequent generations, 15 unilocular sporangia were isolated individually from the diploid sporophyte Ec 372. These sporangia, each of which contained more than 100 meio-spores (derived from a single meiosis followed by at least 5 mitotic divisions), produced 15 'families' of gametophytes, which again exhibited a germination pattern typical of wild-type gametophytes. For each of these families, between 15 and 31 gametophytes were sub-isolated and their sex and the germination pattern of their gametes (i.e. the partheno-sporophyte generation) were recorded (see Table S1 in the supplementary material). Overall, 155 gametophytes produced partheno-sporophytes with a wild-type germination pattern and 191 produced partheno-sporophytes with the *imm* germination pattern. These figures are consistent with a 1:1 segregation ratio and Mendelian inheritance of a single-locus recessive mutation ($\chi^2=3.746$, the deviation from 1:1 has a probability greater than 0.05).

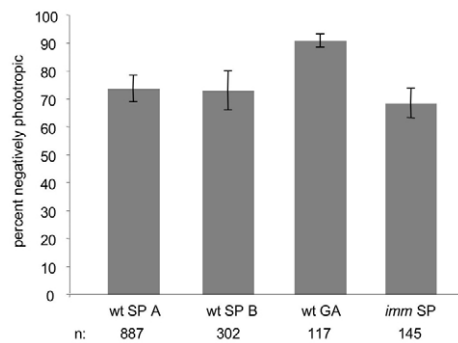


Fig. 4. Photopolarisation of mutant and wild-type *Ectocarpus* germlings in response to unidirectional light. Error bars show standard deviations. The negative phototropic response of the wild-type gametophyte was significantly more marked than those of the wild-type and *imm* sporophytes ($\chi^2=25.68$, $P<0.001$). wt SP A, wild-type partheno-sporophytes; wt SP B, wild-type sporophytes from mitospores; wt GA, wild-type gametophytes; *imm* SP, *imm* partheno-sporophytes, n, number of individuals scored in each population.

Several additional crosses were performed to analyse further the inheritance of the *imm* mutation. Using the gametophytes derived from the diploid sporophyte Ec 372, crosses were performed with all possible combinations of wild-type and *imm* mutant gametes (Fig. 6). Eleven diploid sporophytes, homozygous for *imm*, were raised from the cross Ec 419 (*imm*) \times Ec 420 (*imm*). Without exception, they showed the *imm* germination pattern, indicating full penetrance of the *imm* mutation in this diploid context (provided the dominant, wild-type *IMM* allele is absent). From one of these sporophytes (Ec 428), we isolated five unilocular sporangia. From each of the families of gametophytes that developed from them, we selected six gametophytes and recorded their sex and the germination pattern of their gametes. Regardless of their sex, all of these gametophytes produced partheno-sporophytes with the *imm* germination pattern.

Five diploid sporophytes were raised from the cross Ec 421 (*IMM*) \times Ec 423 (*IMM*) and all showed the wild-type germination pattern. From one of these sporophytes (Ec 432) we isolated five unilocular sporangia and again analysed the sex and gamete germination patterns of six gametophytes from each family. Regardless of sex, all showed the wild-type germination pattern.

Finally, from the reciprocal crosses Ec 419 (*imm*) \times Ec 423 (*IMM*) and Ec 421 (*IMM*) \times Ec 420 (*imm*) we raised nine and three zygotes, respectively, and these all showed a wild-type germination pattern. A sporophyte from each cross (Ec 429 and Ec 430, respectively) was raised and eight (for Ec 429) and 11 (for Ec 430) families of gametophytes corresponding to single unilocular sporangia were isolated. The mutant germination pattern was observed in the germlings from ~50% of the 18-20 gametophytes analysed per family.

In none of the above analyses did a single gametophyte produce populations of partheno-sporophytes with both types of germination pattern.

The analyses of progeny from the three *imm/IMM* heterozygous sporophytes (Ec 372, Ec 429 and Ec 430) were used to test for linkage between *IMM* and the sex locus. For this, the phenotypes of the progeny from each unilocular sporangium were used to determine the segregation pattern of the two loci during the single meiotic event that had occurred in each sporangium (see Table S2 in the supplementary material). No statistical deviation from a 1:1:1:1

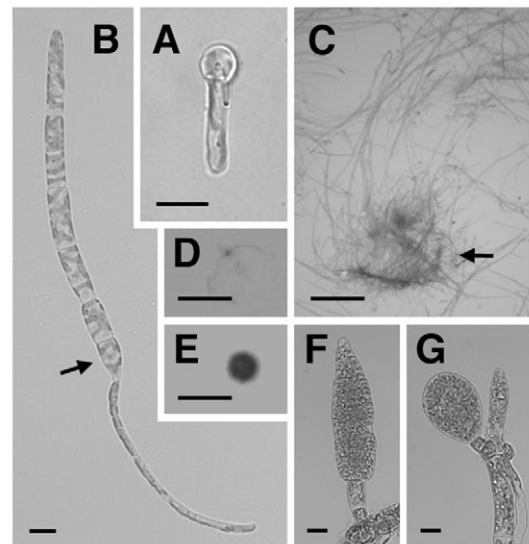


Fig. 5. Development of the *imm* mutant sporophyte. (A) First germ tube of germinating zygote cell homozygous for *imm*. (B) Germling at a later stage, with a rhizoid developed from the first germ tube (below) and an upright filament from the second germ tube (above). The cell corresponding to the original zygote is indicated by an arrow. (C) Mature, six-week-old *imm* mutant sporophyte with well developed, richly branched upright filaments. The arrow indicates the point of attachment to the substratum. (D,E) Macroscopic views of five-week-old thalli of *imm* and wild-type sporophytes, respectively, illustrating the marked difference in morphology. The wild type has formed a dense prostrate basal system. The *imm* mutant lacks this structure; it possesses a well-developed, but diffuse, erect thallus which is not as easily visualised with the naked eye. (F) Plurilocular sporangium on an *imm* mutant sporophyte. (G) Unilocular sporangium on an *imm* mutant sporophyte. Scale bars: 10 μm in A,B; 250 μm in C; 1 cm in D,E; 20 μm in F,G.

ratio was detected in any of the three crosses (e.g. for strain Ec 372, $\chi^2=0.266$, $P>0.9$), indicating that *IMM* segregated independently of the sex locus.

Taken together, these data demonstrate that the *imm* mutation was stably inherited as a recessive Mendelian factor through several generations of the life cycle and that the *IMM* locus was not linked to the sex locus.

Gene expression in the sporophyte of the *imm* mutant indicates that it is partially converted into a gametophyte

The sporophyte generation of the *imm* mutant exhibited several morphological features typical of the gametophyte generation during its early development, such as asymmetric initial cell division, and yet remained functionally a sporophyte (producing spores but not gametes). To determine whether these similarities with the gametophyte generation were due to a partial conversion of the sporophyte into a gametophyte, we assayed the expression of two libraries of sporophyte- and gametophyte-upregulated genes (isolated by suppression subtraction hybridisation) in the partheno-sporophyte of the *imm* mutant using a microarray approach. cDNA fragment inserts from 600 clones from each of the two SSH libraries were arrayed on glass slides and hybridised with fluorescently labelled cDNA. Fig. 7A shows that the relative abundances of the transcripts corresponding to the sequences spotted on the microarray were similar in two independent total RNA samples from wild-type

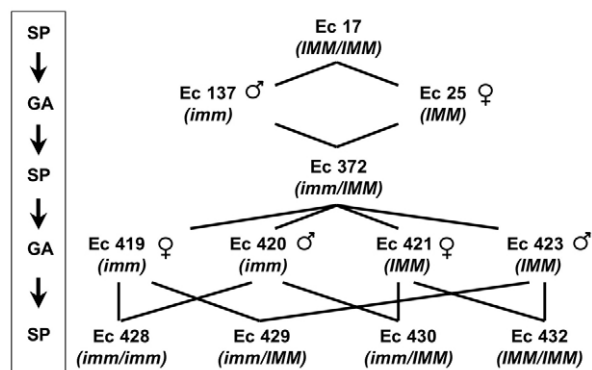


Fig. 6. Summary of the crosses carried out for the genetic analysis of the *imm* mutation. Refer to Table 2 for the strain codes (e.g. Ec 17). The genotype is shown in brackets. SP, sporophyte, GA, gametophyte.

partheno-sporophytes of the strain Ec 432. By contrast, when cDNA from the partheno-sporophyte stage of the *imm* mutant was compared with cDNA from the same wild-type partheno-sporophyte, there was a marked tendency for a subset of the transcripts corresponding to the gametophyte-expressed SSH library to be more abundant in the mutant cDNA sample and for a subset of the transcripts corresponding to the sporophyte-expressed SSH library to be less abundant (Fig. 7B). A statistical test, carried out using the Statistical Analysis of Microarrays (SAM) method (Tusher et al., 2001), identified 80 clones whose corresponding transcripts were significantly more abundant in the *imm* partheno-sporophyte RNA sample and 62 clones whose transcripts were significantly less abundant. Seventy-nine of the 80 clones corresponding to genes upregulated in the *imm* mutant (99%) were from the gametophyte-expressed SSH library, whereas 60 of the 62 clones corresponding to downregulated genes (97%) were from the sporophyte-expressed SSH library (Fig. 7C). Taken together with the similarity between the early morphogenesis of the *imm* mutant sporophyte and that of the wild-type gametophyte (Fig. 3), these data provide convincing evidence that there is a partial switch from the sporophyte to the gametophyte developmental programme in the *imm* mutant sporophyte.

To further investigate the observed modifications in gene expression in the *imm* mutant, the SSH clones corresponding to genes that had been identified as significantly up- or downregulated in the *imm* mutant partheno-sporophyte compared with the wild-type partheno-sporophyte were sequenced. The sequences of the SSH clones were compared with each other, with a collection of 26,000 *E. siliculosus* EST sequences generated from an immature partheno-sporophyte cDNA library and with a 7× assembly of the *E. siliculosus* genome sequence (A.F.P., D.S., S.M.C., B.C., N. Aiach, B. Segurens, B. Setterblad, J. Weissenbach, P. Wincker and J.M.C., unpublished). This analysis showed that the 142 differentially regulated SSH clones corresponded to 40 different genes (27 downregulated genes and 13 upregulated genes). These genes are listed in Table 3 (and the corresponding SSH clones are listed in Table S3 in the supplementary material). All but four of the genes that were downregulated in the *imm* mutant compared with the wild type were represented in the collection of 26,000 partheno-sporophyte ESTs, whereas only one of the 13 upregulated genes was represented in this collection of sequences (Table 3). This supports the data from the microarray analysis indicating that a group of genes that are normally expressed during the gametophyte stage are

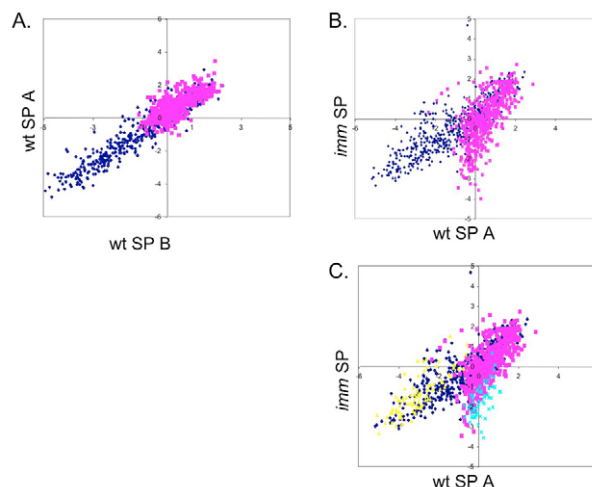


Fig. 7. Microarray analysis of the expression of genes corresponding to two subtraction libraries enriched for sporophyte- and gametophyte-specific cDNAs in wild-type and *imm* partheno-sporophytes. Relative abundance of transcripts corresponding to genes identified by suppression subtraction hybridisation in (A) two independent wild-type partheno-sporophyte samples and (B) a wild-type partheno-sporophyte and an *imm* mutant partheno-sporophyte sample. Sequences corresponding to the sporophyte SSH library are shown in pink, sequences corresponding to the gametophyte SSH library are shown in blue. (C) The same graph as shown in B except that genes that are significantly upregulated in the *imm* mutant partheno-sporophyte compared with the wild type are highlighted in yellow and genes that are significantly downregulated in light blue. wt SP A and wt SP B, two independent wild-type partheno-sporophyte cDNA targets; *imm* SP, *imm* mutant partheno-sporophyte cDNA target.

upregulated in the *imm* partheno-sporophyte, whereas a group of sporophyte-generation genes are correspondingly downregulated. When the selected upregulated and downregulated genes were compared with public databases, over half (23 genes) either did not match sequences in the database or matched only hypothetical proteins (Table 3).

Quantitative PCR analysis

Table 3 lists the genes that were identified, by the microarray analysis, as being significantly downregulated (IDW genes) or upregulated (IUP genes) in the *imm* partheno-sporophyte compared with a wild-type partheno-sporophyte. The abundances of mRNAs corresponding to ten of the IDW genes and to nine of the IUP genes were measured in the sporophyte and gametophyte generations of both the *imm* mutant and wild-type strains using quantitative PCR.

The results for the wild-type partheno-sporophyte and gametophyte samples showed that most of the IDW genes exhibited a sporophyte-specific or sporophyte-preferential pattern of expression, whereas most of the IUP genes exhibited a gametophyte-specific or gametophyte-preferential pattern of expression (Fig. 8). The difference between the expression levels in the two generations was very marked in most cases, with a maximum fold difference of 6975 for *IUP9*. Altogether, the quantitative PCR analysis allowed the validation of 16 genes (nine for the sporophyte and seven for the gametophyte) that showed a differential pattern of expression between the two generations (Fig. 8).

We compared the expression levels of these genes in the partheno-sporophyte and gametophyte of the *imm* mutant with the values obtained for the wild type (Fig. 8). With only a few exceptions (*IDW7*, *IDW9*, *IDW11*, *IUP8* and *IUP9*), the genes were expressed at a similar level in *imm* and wild-type gametophytes. This is consistent with the fact that we did not detect any visible phenotype of the *imm* mutation in the gametophyte generation. In addition, in the *imm* partheno-sporophyte, most of the genes assayed showed an expression level that was either comparable with that of the wild-type gametophyte (*IDW1*, *IDW2*, *IDW3*, *IUP1*) or was intermediate between the abundances assayed in the two generations of the wild type (*IDW4*, *IDW5*, *IDW6*, *IDW7*, *IDW8*, *IUP2*, *IUP3*, *IUP4*, *IUP5*, *IUP6*, *IUP7*, *IUP8*, *IUP9*). For the last five genes, the transcripts

were markedly less abundant in the *imm* partheno-sporophyte compared with the wild-type gametophyte but they were, nonetheless at least 28 times more abundant than in the wild-type partheno-sporophyte (see Table S4 in the supplementary material). The quantitative PCR, therefore, confirmed that there was an increase in the abundance of the transcripts of gametophyte-expressed genes and a decrease in the abundance of sporophyte-expressed genes in the *imm* sporophyte.

Quantitative PCR analyses were carried out on partheno-sporophytes because, as mentioned above, we observed no morphological or developmental differences between partheno-sporophytes and diploid sporophytes derived from gamete fusions. This was the case for both the wild-type Ec 32 strain and for the *imm* mutant. To further verify that the two stages of the life cycle behaved

Table 3. Genes identified as being upregulated or downregulated in the *imm* mutant partheno-sporophyte compared to a wild-type partheno-sporophyte

Gene	Up- or down-regulated	SSH library	Number of times detected by microarray	Number of ESTs*	Best database match (with accession number and E value)
IDW1	Down	SS [†]	2	2	Hypothetical protein (Q4C2K2, 2e-07)
IDW2	Down	SS	4	13	Hypothetical protein (Q4C2K2, 3e-07)
IDW3	Down	SS	2	4	Hypothetical protein (Q4C2K2, 6e-07)
IDW4	Down	SS	4	8	FCP [‡] (O81933, 2e-12)
IDW5	Down	SS	1	0	Helicase (Q5NAA4, 1e-45)
IDW6	Down	SS	1	3	No match
IDW7	Down	SS	1	7	Peroxidase / catalase (Q6D5X9, 9e-18)
IDW8	Down	SS	7	4	EsV-1-163 [¶] (Q8QKV3, 9e-16)
IDW9	Down	SS	16	4	FCP (Q40296, 5e-92)
IDW10	Down	SS	1	6	FirrV-1-B30** (Q6XM06, 3e-14)
IDW11	Down	SS	1	7	Peptidoglycan-binding (Q4ZVL4, 2e-06)
IDW12	Down	SS	1	52	FCP (Q40296, 1e-93)
IDW13	Down	SS	1	10	EF1A (P56331, 1e-13)
IDW14	Down	SS	2	13	Phosphoglycerate mutase (Q3CHP4, 3e-05)
IDW15	Down	SS	2	2	No match
IDW16	Down	SS	1	4	FCP (Q40296, 4e-91)
IDW17	Down	SS	1	0	No match
IDW18	Down	SS	3	9	FCP (Q40296, 5e-93)
IDW19	Down	SS	1	6	No match
IDW20	Down	SS	1	6	FCP (Q40298, 6e-33)
IDW21	Down	SS	1	6	FCP (Q5K278, 6e-11)
IDW22	Down	SS	2	4	FCP (Q40296, 7e-38)
IDW23	Down	SS	2	1	No match
IDW24	Down	SS	1	26	FCP (Q40296, 1e-92)
IDW25	Down	SS	1	0	No match
IDW26	Down	GS [‡]	1	3	Nucleotide sugar epimerase (Q7NLQ3, 3e-18)
IDW27	Down	GS	1	0	No match
IUP1	Up	GS	1	0	No match
IUP2	Up	GS	1	0	No match
IUP3	Up	GS	1	0	No match
IUP4	Up	GS	5	0	No match
IUP5	Up	GS	2	0	Hypothetical protein (Q4DJM6, 2e-16)
IUP6	Up	GS	23	0	No match
IUP7	Up	GS	4	0	No match
IUP8	Up	GS	1	0	No match
IUP9	Up	GS	37	1	No match
IUP10	Up	GS	1	0	Nucleotide sugar epimerase (Q7NLQ3, 3e-18)
IUP11	Up	GS	1	0	No match
IUP12	Up	GS	1	0	No match
IUP13	Up	SS	1	0	No match

*Number of times each gene was represented in a collection of 26,000 ESTs produced from an immature partheno-sporophyte cDNA library.

[†]Sporophyte-gene-enriched library.

[‡]Gametophyte-gene-enriched library.

[§]FCP, fucoxanthin chlorophyll a/c protein.

[¶]EsV-1-163, *Ectocarpus* virus 1 gene 163.

**FirrV-1-B30 *Feldmannia* virus 1 gene B30.

The partial cDNA sequences corresponding to these genes have been deposited in the dbEST database and have the following GenBank Accession Numbers: EV406070 to EV406109.

in the same manner, we also measured transcript abundances for five of the genes tested in Fig. 8 at both stages for both the wild type and the *imm* mutant. The results of this experiment (see Fig. S1 in the supplementary material) indicate, as expected, that partheno-sporophytes and zygote-derived sporophytes exhibit very similar patterns of gene expression and that the deregulation of generation-specific genes observed in the *imm* partheno-sporophyte also occurred in a *imm/imm* diploid sporophyte.

DISCUSSION

Early development of the wild type sporophyte and gametophyte

Here, we show that the sporophyte and gametophyte generations of *E. siliculosus* exhibit markedly different patterns of early development and correlate this difference with the mode of division of the initial cells during each generation. The early stages of gametophyte development involve asymmetric division

of the initial cell and immediate differentiation, whereas the sporophyte arises via a symmetric initial cell division and exhibits mediate differentiation.

Explaining the stability of haploid-diploid life cycles has been particularly problematic because, depending on the niche of the organism, either the haploid or the diploid generation is expected to present certain advantages and theoretical models predict that this should lead to a dominance of one generation over the other under a wide range of conditions (Mable and Otto, 1998). It has been suggested, however, that if the two generations are adapted to different ecological niches, this could stabilise a haploid-diploid life cycle (Stebbins and Hill, 1980; Willson, 1981) and this suggestion is supported by more recent theoretical work (Hughes and Otto, 1999). It is possible that the difference in morphology between the wild-type sporophyte and gametophyte of *Ectocarpus* reflects an adaptation to different ecological niches, with the dense, more robust thallus of the sporophyte, particularly its prostrate base, being better adapted for persisting in less favourable conditions during most of the year and the more fragile gametophyte being short-lived and adapted for producing gametes over a period of a few weeks. Future work on the ecology of the two generations of the *Ectocarpus* life cycle might therefore provide a means to test theoretical hypotheses concerning the stability of haploid-diploid life cycles.

Phenotypic analysis of the *imm* mutant

The gametophyte generation of the *imm* mutant was phenotypically wild type, but the sporophyte generation exhibited a gametophyte-like pattern of germination and early development. Microarray analysis of gene expression in the mutant strain showed that the morphological resemblance of the *imm* sporophyte to a gametophyte was correlated with a specific upregulation of a subset of the genes that are normally expressed during the gametophyte generation and a corresponding downregulation of a subset of sporophyte-generation genes. This analysis, therefore, provided evidence that the mutant was affected in processes that are regulated during the life cycle. In addition, phenotypic analysis of the *imm* mutant showed that the development of mature sporophytes with the appropriate reproductive structures (unilocular and plurilocular sporangia) was not contingent on the wild-type sporophyte pattern of early development (involving symmetric division of the initial cell and mediate differentiation). These two, temporally separate parts of the sporophyte developmental programme were genetically uncoupled in the *imm* mutant. Hence, not only does *E. siliculosus* exhibit alternation between developmental programmes involving symmetric and asymmetric initial cell divisions during the sporophyte and gametophyte phases of the life cycle, respectively, but both symmetric (in the wild type) and asymmetric (in *imm* individuals) initial cell divisions can give rise to the sporophyte generation. Moreover, because the *imm* mutation represents a single locus, germination and subsequent cell division patterns might have been modified quite easily during evolution in the brown algal lineage. This may help explain the variety of germination patterns observed in different brown algal species.

Microarray analysis identified 40 genes whose level of expression was modified in the *imm* mutant. This number is an underestimate because only a small subset of the *Ectocarpus* gene set was represented on the array. These data suggest that *IMM* may be regulating a cascade of downstream genes to mediate its influence on early sporophyte development. Analysis of the sequences of the genes whose expression patterns were altered in the *imm* mutant provided only limited information about the molecular events underlying the observed phenotypes.

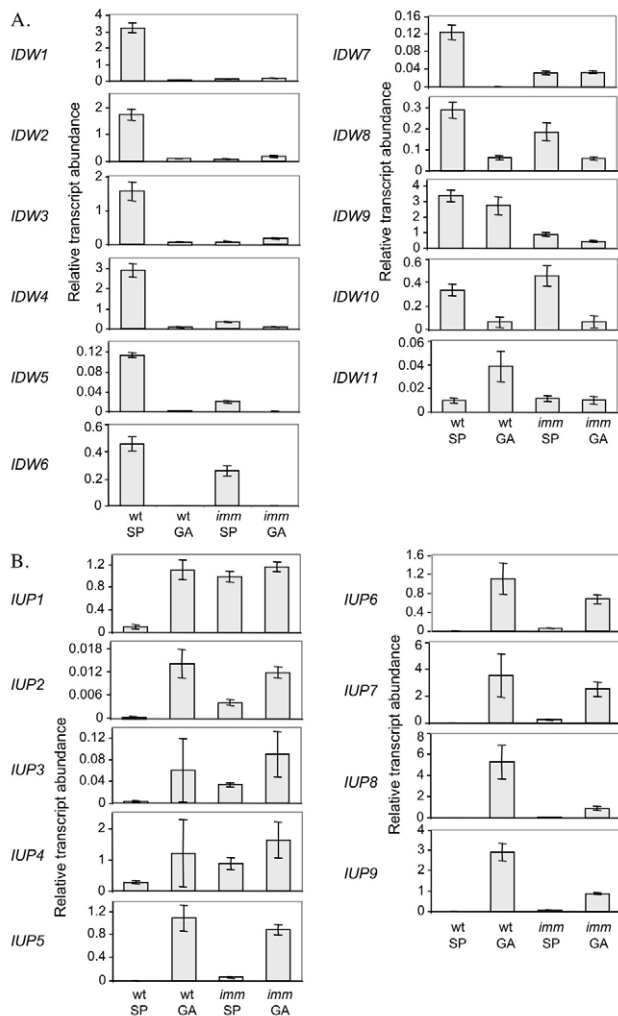


Fig. 8. Quantitative PCR analysis of the abundances of gene transcripts in young partheno-sporophytes and gametophytes of both the wild type and the *imm* mutant strain. The genes assayed are described in more detail in Table 3. Error bars show standard deviations. wt SP, wild-type partheno-sporophyte; wt GA, wild-type gametophyte; *imm* SP, *imm* partheno-sporophyte; *imm* GA, *imm* gametophyte.

Current efforts aimed at developing *E. siliculosus* as a model species (Peters et al., 2004a; Coelho et al., 2007; Charrier et al., 2008) are expected to provide a means to investigate, at the molecular level, the mechanisms behind the phenomena described in this study.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/8/1503/DC1>

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