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A large plant beta-tubulin family with minimal C-terminal variation but differences in expression

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Abstract

Tubulins, as the major structural component of microtubules (MT), are highly conserved throughout the entire eukaryotic kingdom. They consist of alpha/beta heterodimers. Both monomers, at least in multicellular organisms, are encoded by gene families. In higher plants up to eight beta-tubulin isotypes, mostly differing in their very C-termini, have been described. These variable beta-tubulin C-termini have been discussed in the context of functional microtubule diversity. However, in plants, in contrast to vertebrates, functional isotype specificity remains yet to be demonstrated. Unlike higher plants, unicellular green algae in general do not exhibit isotypic variations. The moss *Physcomitrella patens* is a phylogenetic intermediate between higher plants and green algae. We isolated six beta-tubulin genes from *Physcomitrella*, named *PpTub1* to *6*. We show that the exon/intron structure, with the exception of one additional intron in *PpTub6*, is identical with that of higher plants, and that some members of the family are differentially expressed. Moreover, we find that all *Physcomitrella* isotypes are highly conserved and, most strikingly, are almost identical within their C-terminal amino acids (aa). This evolutionary ancient and large beta-tubulin gene family without significant isotypic sequence variation points to a role of differential regulation in the evolution of plant tubulin isotypes.

Keywords: Beta-tubulin isotypes; Evolution of land plants; Moss; *Physcomitrella patens*

1. Introduction

Microtubules (MT), as steady-state dynamic polymers, are ubiquitous eukaryotic cell structures that are involved in a variety of intracellular processes including morphogenesis, cell growth and division. Tubulin, the major structural component of MT, is organized in alpha/beta heterodimers. Both monomers are highly conserved among species. It has long been known that in vascular plants, like in all other multicellular organisms studied so far, the alpha-, and even more pronounced, the beta-tubulins are encoded by multigene families (for review on plant beta-tubulins, see Breviario and Nick, 2000). The members of these families (termed isotypes) differ from each other almost exclusively in their extreme C-termini (for review of C-termini see Sackett, 1995). The C-terminus of alpha- and beta-tubulin extends from the surface of heterodimers and assembled MT (Nogales et al., 1998, 1999), is the major site of interaction with MT-associated proteins (MAP; Littauer et al., 1986) and a target for posttranslational modifications (MacRae, 1997). In consequence, adaptive responses of MT to environmental signals and drugs are related to the very C-termini of plant alpha- and beta-tubulins (Breviario and Nick, 2000).

Taken together, isotype-specific C-terminal variation appears to be a prerequisite for the regulation of MT

Abbreviations: A, adenosine; aa, amino acid(s); bp, basepair(s); C, cytidine; D, A/G/T; cDNA, DNA complementary to RNA; DNase, deoxyribonuclease; EST, expressed sequence tag; G, guanosine; GTP, guanidintriphosphate; GTPase, guanidintriphosphate hydrolase; kb, kilobase(s); MAP, MT-associated protein(s); MT, microtubule(s); ng, nanogram; ORF, open reading frame(s); PCR, polymerase chain reaction; RNase, ribonuclease; RT, reverse transcriptase; T, thymidine; tsp, transcription start point(s); UTR, untranslated region(s).

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dynamics as well as for functional MT diversity. First experimental evidence for such a differential function came from Drosophila, where two beta-tubulin isotypes (beta-2 and beta-3) were found to be functionally non-redundant (Hoyle and Raff, 1990), and where removal of the C-terminal 15 amino acids (aa) of the testis specific beta-2 isotype still allowed for correct MT-doublets assembly but prevented the formation of functional superstructures such as axonemata (Fackenthal et al., 1993). However, so far, conclusive evidence for a functional specificity of plant-tubulin isotypes is lacking. This may be due to the fact that individual MT can be composed of combinations between different isotypes (Hussey et al., 1987).

In contrast to higher plants, green algae—like most other protists—are endowed with only one or two identical or nearly identical isotypes (one or two conservative aa exchanges only), that are encoded by two to three distinct loci (for review, see Ludueña, 1998). This stimulated a long debate, why the growing number of different cell types and specialized cellular functions during the course of evolution is accompanied by a growing number of different beta-tubulin isotypes. Were these isotypes used to fulfill different functions or were the multigene families stabilized because they met different regulatory demands? An increasing number of regulatory studies, also for plants, have revealed, that in each family analysed so far some members are expressed rather constitutively, whereas others show specific patterns of expression and regulation (for reviews, see Breviario, 2000; Ludueña, 1998).

To address the issue of differential function versus differential regulation as evolutionary driving force, we investigated a beta-tubulin gene family from a nonvascular, multicellular plant as a phylogenetic intermediate of higher plants and green algae. In our approach, we used the moss *Physcomitrella patens*. Some of the mosses appeared 450 million years ago (Theißen et al., 2001). It is known from fossil records that there have been only small morphological changes since then, making bryophytes, in more general, the most conservative group of land plants (Miller, 1984), and thus ideal candidates for evolutionary studies. For a general review on *Physcomitrella*, see Reski (1998a).

We isolated all six highly conserved members of the beta-tubulin gene family of *Physcomitrella*, named *PpTub1* to *6*. Remarkably, the conservation includes the very C-termini of the deduced proteins. The organisation of these genes, with one exception (an additional intron in *PpTub6*), is identical when compared to their counterparts in higher plants. Phylogenetic analysis of 58 full-length beta-tubulins from plants, different algae and fungi locates the six *Physcomitrella* tubulins into a monophyletic cluster basal of vascular plants and, on the other side, clearly separated from green algae. Reverse transcriptase (RT)-polymerase chain reaction (PCR) experiments for different culture conditions demonstrated that the transcripts of some beta-tubulins are expressed more or less constitutively, whereas some are clearly differentially regulated. This differential regulation on the background of high C-terminal conservation in this seed-less land plant suggests that it was differential regulation rather than differential function that stabilized multiple beta-tubulin genes and thus drove the evolution of different beta-tubulin isotypes.

2. Materials and methods

2.1. Plant cultivation

P. patens (Hedw.) B.S.G. was grown as described by Richter et al. (2002). Chloronema-enriched cultures were cultivated in liquid Knop medium supplemented with 5 % (w/v) ammonium-tartrate for at least 7 days.

2.2. Preparation of genomic DNA and total RNA

Genomic DNA was isolated from *P. patens* that had been cultivated for 13 days following the CTAB protocol as described in Richter et al. (2002). Total RNA was prepared by grinding tissue in liquid nitrogen using the E.Z.N.A. Plant RNA Kit (PeqLab) following the protocol of the producer.

2.3. Molecular cloning of PpTub1 to 6

Conventional molecular biology protocols were essentially as described by Sambrook et al. (1989). Sequences of all subsequent primers can be given upon request. For a schematic localisation of primers, see Fig. 1. *PpTub1* clones (primers 1 and 4; three independent genomic clones) and *PpTub3* clones (primer 3 and 4; genomic and cDNA, one clone each) were generated with Taq recombinant polymerase (MBI Fermentas). Both cDNA and genomic PCR products were amplified with primers 2 and 4 (*PpTub2*), 297 and 299 (*PpTub4*), 298 and 300 (*PpTub5*), 296 and 336 (*PpTub6*), using the Advantage cDNA Polymerase Mix (Clontech). A *PpTub1* cDNA clone (primers 89 and 91) was generated with Pfu native polymerase (MBI Fermentas), as well as two additional genomic clones of *PpTub4* (primer 297 and 299) and *PpTub5* (primer 298 and 300). In addition, PCR amplificates covering entire genomic regions, e.g. *PpTub6* (primers 236 and 238), were sequenced to confirm intron sequences.

2.4. Two-step RT-PCR

Deoxyribonuclease (DNase, GIBCO/Invitrogen) treated total RNA (400 ng) was used for reverse transcription with oligo-d(T) primers and SUPERSCRIPT II RNase H-RT (GIBCO/Invitrogen), following the manual of the manufacturer. For PCR on generated cDNA, one upstream/forward primer (496), located within exon 3, was used for the amplification of all *PpTub* cDNA. Downstream/reverse primers (*PpTub1* to *6*: primers 426, 427, 428, 299, 512 or

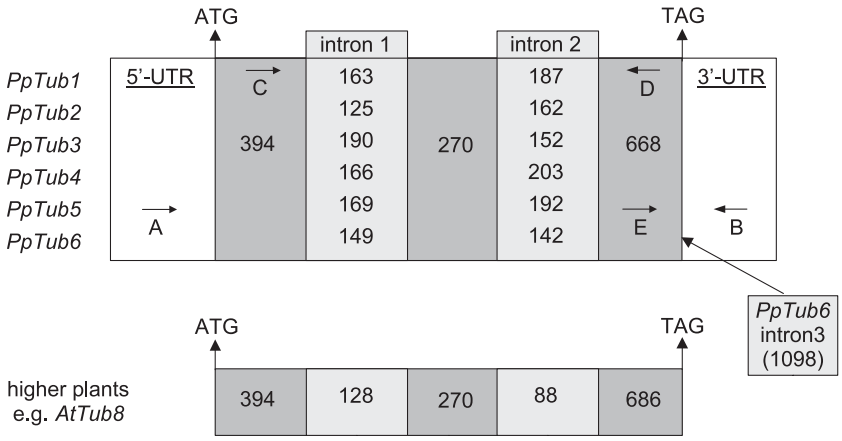

Fig. 1. Schematic, not to scale, overview of the *PpTub1* to *6* transcription units (all numbers in bp). Indicated are 5′-UTR, ORF (dark grey), introns (light grey), 3′-UTR and approximate position of primers (A: primer 2, 89, 236, 296, 297 or 298; B: primer 91, 238, 299, 300, 336, 426, 427, 428, 512 or 546; C: primer 1 or 3; D: primer 4; E: primer 496).

546, respectively) were gene-specific and located within the corresponding 3′-UTR. Gene-specificity for each primer pair was tested in PCR using *PpTub1* to *6* as template (data not shown). The absence of contaminations by genomic DNA was verified by control PCR on each cDNA with primers located on either side of an intron (not shown). The internal standard was based on a PCR with primers against the *Physcomitrella* orthologue of L21 (*PpL21*), coding for a ribosomal protein. This gene has been used as constitutively expressed standard in *Physcomitrella* before (Koprivova et al., 2002). All PCR were repeated at least once on each cDNA using 1 unit Taq recombinant polymerase (MBI Fermentas), 2 mM $MgCl_2$, 0.25 mM of each primer and 1.5–4 nanogram (ng) of initial total RNA in a total volume of 20 µl for one PCR. Mastermixes were upscaled appropriately. Cycling conditions were: an initial step of 1 min at 96 °C, 10 s 96 °C, 10 s 60 °C and 30 s 72 °C as a second step, with as many repetitions as needed, followed by a terminal step of 3 min at 72 °C. Samples were loaded quantitatively on 1.8% (w/v) ethidium bromide-stained agarose gels. After electrophoresis, images were recorded on an UV-screen using the BioDocAnalyze documentation system (Biometra).

2.5. Expressed sequence tag (EST) search

In order to identify all *Physcomitrella* EST with significant homology to beta-tubulins, the GenBank EST database http://www.ncbi.nlm.nih.gov) was searched by NCBI/blastn with the *AtTub1* open reading frame (ORF) as query. Settings were as given by the browser with database "EST others", limit by entrez query "Physcomitrella" and an expected *E*-value range of 0–0.01 (for a public BLAST service against clustered EST data from the moss *P. patens*, see: http://www.cosmoss.org). The resulting EST were grouped for each particular gene by multiple sequence alignments resulting in a 5′- and 3′-contig for each locus. From each contig, 5′- and 3′-UTR sequences were used for additional analogous BLAST searches in order to identify EST outside of the coding regions.

2.6. Software

For primer design, general sequence analysis, and pairwise sequence alignments the Sci Ed Central, Clone Manager Suite (Sci-Ed Software) was used. Lasergene, DNASTAR (Version 5) SeqMan II (DNASTAR) was used for analysing and editing sequencing raw data. Homology searches were carried out by BLAST 2 (Altschul et al., 1997). Multiple sequence alignments for use in tree constructions were performed with CLUSTAL W 1.81 (Thompson et al., 1994), using default parameters. Phylogenetic trees were created with TREECON 1.3 (Van de Peer and De Wachter, 1994) and TREE-PUZZLE 5.1 (Schmidt et al., 2002). Neighbour-joining trees were done using the Tajima and Nei model, InDels consideration and bootstrap resampling (1000 ×). Maximum likelihood analysis was done using the Whelan and Goldman substitution model with data set frequencies, exact parameter estimation, and eight gamma distributed heterogeneity rates.

3. Results

3.1. Molecular cloning of the Physcomitrella beta-tubulin gene family

Based on an alignment of all nine published beta-tubulin genomic sequences from *Arabidopsis thaliana* (*AtTub1* to *9*; Marks et al., 1987; Oppenheimer et al., 1988; Snustad et al., 1992), primers were designed for highly conserved coding regions (primers 1, 3 and 4). In addition, one publicly available EST from *Physcomitrella* (GenBank accession number AJ225393) was used to design a 5′-UTR primer (primer 2). With these primers, PCR on cDNA and genomic DNA was performed. From sequence comparisons between

the cloned cDNA and genomic PCR products, three groups of clones emerged that were all highly similar to known beta-tubulin genes. The sequences within a group were identical, but differed between groups. These three *Physcomitrella* beta-tubulin orthologues were named *PpTub1*, *PpTub2* and *PpTub3* (GenBank accession nos. AY382287, AY382288 and AY382289, respectively).

Furthermore, a detailed analysis of all public *Physcomitrella* EST with high similarity to beta-tubulin genes (177 EST; GenBank accession nos. can be given upon request), led to three new additional upstream and three new additional downstream groups of EST, that were identical within a group, but neither identical to any of the other groups, nor to *PpTub1*, *PpTub2* or *PpTub3*. From the predicted non-coding upstream and downstream regions, specific primers were designed for each new group and used for PCR testing all possible primer combinations. This helped to correlate corresponding upstream and downstream groups to particular loci (not shown), named *PpTub4*, *PpTub5* and *PpTub6* (GenBank accession nos. AY382290, AY382291 and AY382286, respectively). Both genomic and cDNA amplificates of all three new loci were cloned and sequenced. EST and cDNA data of all six genes were used to confirm the genomic sequence data and the intron/exon borders. An overview of all six genomic regions and the approximate position of all primers are given in Fig. 1. It is very likely that the six cloned genes represent the complete beta-tubulin gene family of *Physcomitrella*, since our PCR approach on screening genomic DNA and cDNA did not reveal additional family members, and a high number of EST were analysed (over 100 000 EST in November 2003; see http://www.ncbi.nlm.nih.gov/dbEST). Moreover, no further hits that were different from *PpTub1* to *6* could be detected in EST derived from additional gametophore and even sporophyte-specific cDNA libraries (Rensing, personal communication). The presumed family size of six members is consistent with the fact that gene families in *Physcomitrella* in general are smaller as compared to *Arabidopsis* (Rensing et al., 2002).

3.2. Gene structure and conservation

Number and location of *PpTub* introns are identical compared to introns in *AtTub1* to *9* and beta-tubulins from other higher plants where intron positions are known (*Gossypium hirsutum* AF487511), *Lupinus albus* U47660 and X70184), *Glycine max* M21296 and M21297) and *Pisum sativum* X54844)). *PpTub6*, as the only exception, harbours an additional, unusual large third intron of more than 1 kilobase (kb) in length precisely downstream of the stop codon (Fig. 1). To our knowledge, this intron position has not been described for any other beta-tubulin gene so far. All *PpTub* introns follow the [GT. . .AG] splicing rule. Intron/exon borders of all 13 introns are embedded in consensus sequences for plant splicing sites (Table 1). The introns of *PpTub2* and *3*, like most plant introns (Lorković et al., 2000), are T-rich (over 40%; *PpTub3* intron1 33% only), but the introns of the other *PpTub* genes are G-rich (34% to 51%), in addition to a moderate T-content (27% to 36%).

Table 1
PpTub1 to *6* introns: Consensus sequences of splice sites[a]

	5′-splice site	3′-splice site
intron1 *PpTub1* to *6*	**A G** [G T A_5 T_5 G G_3	T_4 G_5 C A G] **G T**
intron2 *PpTub1* to *6*	**G_3 T** [G T A_5 A G T_5	T G C A G] **T C_4**
intron3 *PpTub6*	**A G** [G T A G G A	A A C A G] **G T**
PpTub consensus	**A_8 G_7/T_6**	T_{10} G_{11} C A G]
	[G T A_{11} A_6/T_5 G T_6	**G_7/T_6 T_9/C_4**
Plant consensus[b]	**A G** [G T A A G T	T G C A G] **G T**

[a] exon sequences bold; numbering if identity not 100%.
[b] Lorković et al. (2000).

When the different *PpTub* genes are compared among themselves, the coding regions are found to be highly conserved, whereas outside of the coding regions the overall similarity drops immediately and significantly (data not shown). This is very similar to the situation in the beta-tubulin family of *Arabidopsis*. However, in both gene families there is one exception: *AtTub2* and *3* are linked in the genome and code for identical proteins (AtTUB2_3, Snustad et al., 1992), and *PpTub1* and *5* do have striking similarities within their 5′-UTR, 3′-UTR and introns (data not shown) and their deduced proteins exhibit only two exchanges within 443 aa (corresponding to 99,5% identity; aa positions 84 (T to I) and 438 (E to D); compare Fig. 2). This indicates that in both species one gene has been duplicated relatively recently.

3.3. Phylogenetic analyses of deduced proteins

From all six genes proper proteins with high similarities to known beta-tubulins could be predicted; compared to e.g. *Arabidopsis*, the aa identities range from 90.7% to 94.8%. Between the different beta-tubulins from *Physcomitrella* identities are even higher, ranging from 96.4% to 99.5% (for comparison, within *Arabidopsis* they are between 90.3% and 96.4%), with PpTUB4 being the most diverged isotype (for an overview of polymorphic sites, see Fig. 2).

The predicted guanidintriphosphate hydrolase (GTPase) and guanidintriphosphate (GTP)-exchange domains are preserved unchanged, with the exception of one single conservative exchange in PpTUB6 (aa position 63). The N-terminal MREI peptide, shown at least in vertebrates to be involved in a posttranscriptional autoregulatory mechanism (Cleveland, 1988) is present in *Physcomitrella*, like in most hitherto studied plant beta-tubulins and even subfamily-II alpha-tubulins. Two N-terminal aa positions (aa 37 and 39) show "hotspots" of nonconservative variation within the *Physcomitrella* beta-tubulin family. The same "hotspots" can be found also in all other beta-tubulin families of higher plants but here are embedded in large regions of variability (approximately aa 20 to 90). Thus, even in this relatively variable N-terminal region, the *Physcomitrella* beta-tubulin

position	18	35	37	39	40	51	52	63	78	84	124	135	268	296	316	321	438	440
aa (majority)	A	T	Q	L	S	Y	N	A	S	I	A	V	V	A	M	M	E	L
PpTUB1			K							T								
PpTUB2																		
PpTUB3				T													D	P
PpTUB4	S	S	K	V	T	F	D		T		V	F	I	S	V	V		
PpTUB5			K														D	
PpTUB6				V				G										

Fig. 2. Polymorphic sites within the *Physcomitrella* beta-tubulin family. A schematic overview is given above, with vertical bars indicacting those aa positions found to be variable. The predicted GTP-exchange domains (grey; positions 62 to 77, 141 to 146 and 178 to 182) and the GTPase domain (black; position 103 to 109) are included. Corresponding aa positions and, where required, differences to the majority for each family member are given in the table below.

isotypes are more conserved as compared to beta-tubulins from higher plants.

Isotype differences would be expected mainly for the very C-termini, the regions of interaction and modification. But the overall conservation among *Physcomitrella* beta-tubulins is extended to the very C-termini, with respect to both, conservation in length and aa sequence. This is in clear contrast to all other beta-tubulin families studied so far in higher plants, where the C-terminal aa show a high variability, sharing only a general acidity. In addition, in beta-tubulins from higher plants only one or at the most two members of each family exhibit the short C-terminus characteristic for green algae as well as for *Physcomitrella* (Fig. 3). In *Arabidopsis*, these isotypes with a shorter C-terminus are believed to be correlated to cold resistance. For instance, *AtTub9* is upregulated in response to low temperature (Chu et al., 1993), and cold-sensitive maize MT can be rendered cold stable by artificial cleavage of the C-terminus of beta-tubulin (for review, see Nick, 2000). Interestingly, the fern *Anemia phyllitidis* shares the C-terminal variability with higher plants (of three known members, one is clearly distinct), but the C-termini of all three isotypes are as short as in green algae and in *Physcomitrella*.

Using 58 full-length isotypes, a neighbour-joining tree was constructed. In this tree, the beta-tubulin family from *P. patens* locates as monophyletic cluster basal of vascular plants and, on the other side, clearly separate from green algae (tree not shown). Moreover, the same tree topology was obtained by using the maximum likelihood method (full-length alignment; tree not shown). To test, whether clustering and location of the *Physcomitrella* family within these trees is related to the fact, that the C-termini of the *Physcomitrella* beta-tubulins differ from those in angiosperms, we constructed an analogous neighbour-joining tree by omitting the C-termini (for removed aa, see Fig. 3). The resulting tree is shown in Fig. 4 and revealed an essentially identical topology as for the trees based on the full-length alignment: the overall grouping of all isotypes remains the same. Red algae, brown algae and Oomycota can always be separated clearly from green plants. With exception of *Physcomitrella*, whose proteins in all trees form a cluster of close paralogues, in all families where more than two isotypes are known, the beta-tubulins of a given species are spread throughout the clade, even though some members colocate close-by each other (e.g. AtTUB4 and 9, or ZmTUB3 and 4). Moreover, in all trees beta-tubulins from monocotyledonous plants colocate with those from dicotyledonous plants and vice versa. Actually, this fact has been used to argue that plant beta-tubulin gene families originated prior to the divergence of monocots and dicots (Ludueña, 1998), yet, as our data indicate, after the separation from the mosses. This conclusion is supported by the conservation of intron/exon structures through all beta-tubulin families of terrestrial plants, irrespective of taxa and including *P. patens*.

3.4. PpTub4 and PpTub6 are differentially regulated

Since the beta-tubulin genes from *Physcomitrella* are not only highly conserved over their entire sequence, but also in their C-terminal regions, we asked whether particular *Physcomitrella* beta-tubulin genes are differentially expressed in different tissues. We approached this question by two-step RT-PCR on total RNA isolated from three different tissue conditions: (1) two independent replica of untreated flask cultures, harvested at different days (both contained about 70–80% protonemata with some buds and leafy stems), (2) two independent replica of chloronema-enriched flask cultures harvested at different days and both of which almost exclusively consists of chloronema cells (90–95%) and (3) adult gametophyte, with the leafy stem only, harvested and pooled from plate-grown moss. From each of these five total-RNA samples, two independent cDNA-preparations were done and each cDNA was used for at least two PCR replicas per primer pair. For each round of PCR, all primer pairs were used in parallel aliquots using the same master-

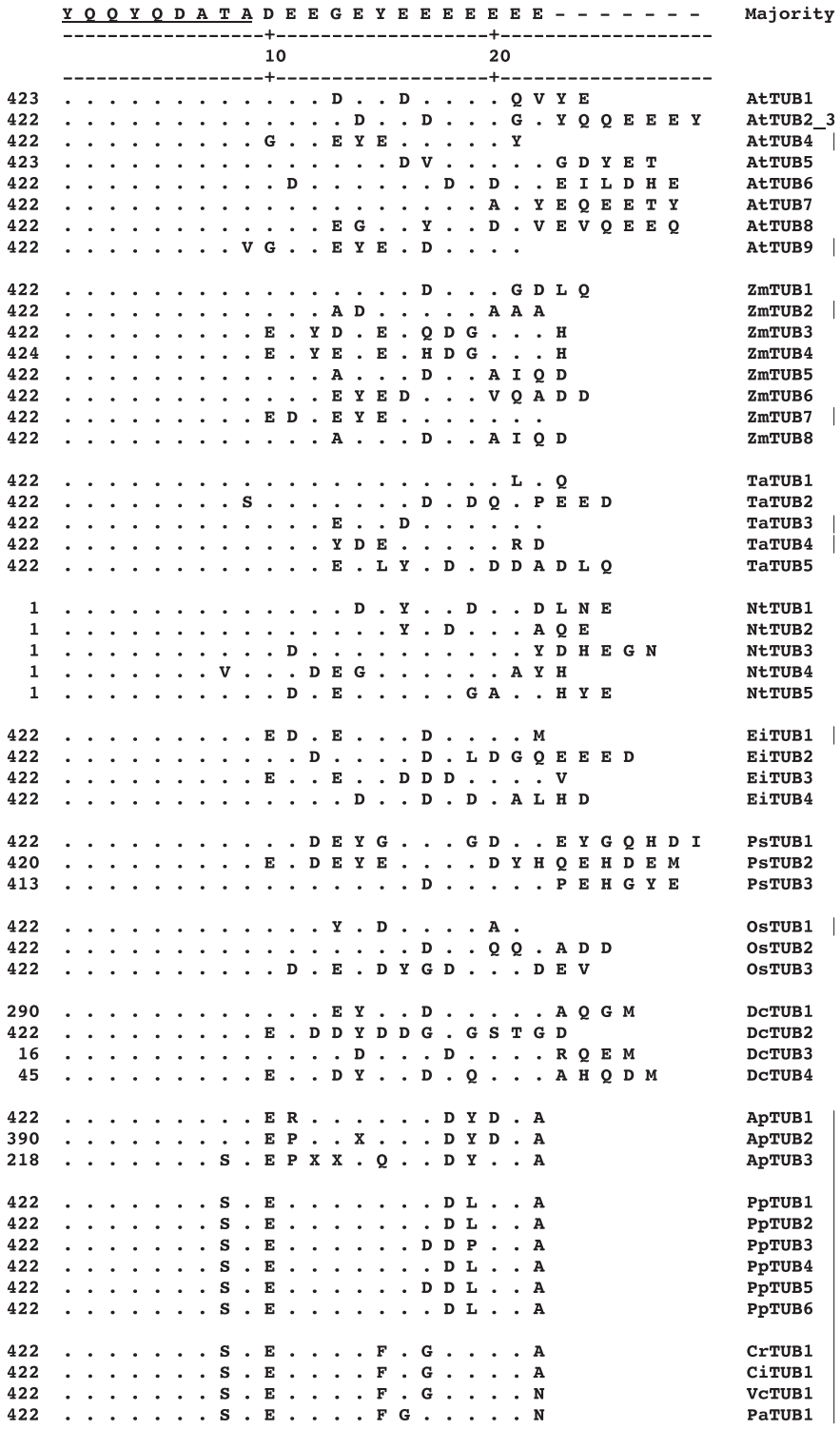

Fig. 3. Alignment (gaps removed) of all beta-tubulin C-termini of green plants with more than two published members. The sequence of the majority is given completely on top (underlined sequences are not removed in the tree construction shown in Fig. 4). Dots represent identical aa. Numbering on the left records the position of the first aa in the alignment; normally position 422, higher numbers indicate N-terminal insertions, lower numbers indicate truncated proteins. Indicated by vertical bars are those members of a family having short C-termini. Shown are beta-tubulin C-termini of *Arabidopsis thaliana* (AtTUB1 to 9), *Zea mays* (ZmTUB1 to 8), *Triticum aestivum* (TaTUB1 to 5), *Nicotiana tabacum* (NtTUB1 to 5), *Eleusine indica* (EiTUB1 to 4), *Pisum sativum* (PsTUB1 to 3), *Oryza sativa* (OsTUB1 to 3), *Daucus carota* (DcTUB1 to 4), the fern *Anemia phyllitidis* (ApTUB1 to 3), *Physcomitrella patens* (PpTUB1 to 6) and the four green algae *Chlamydomonas reinhardtii* (CrTUB1), *Chlamydomonas incerta* (CiTUB1), *Volvox carteri* (VcTUB1) and *Polytomella agilis* (PaTUB1). GenBank accession nos. can be given upon request.

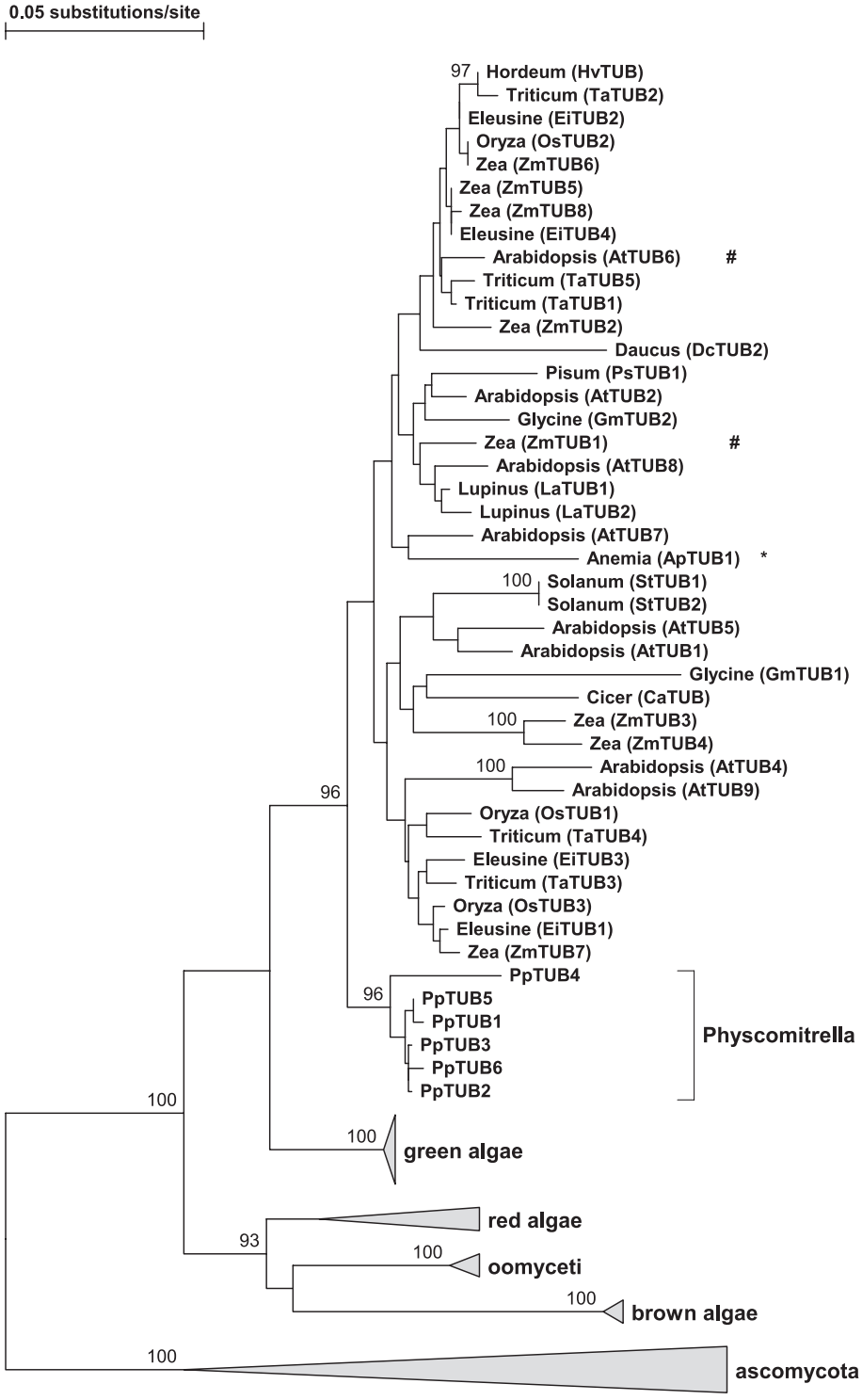

Fig. 4. Neighbour-joining tree of 58 full-length beta-tubulin proteins where the C-termini are not considered (compare Fig. 3). Midpoint rooting at the longest internal branch resulted in an outgroop of Ascomycota. Bootstrap values are given above 90% (value for separation of green algae from higher plants: 82%). Two beta-tubulins from the red algae (*Porphyra purpurea*), two from the brown algae (*Ectocarpus variabilis*), two from Oomycota (*Achlya klebsiana* and *Phytophthora cinnamomi*) and three from Ascomycota (*Saccharomyces cervisiae*, S*chizosaccharomyces pombe* and *Neurospora crassa*) were used. In addition to the six *Physcomitrella* beta-tubulins, four beta-tubulins from green algae (compare Fig. 3) and one from the fern *Anemia phyllitidis* (ApTUB1; marked by *) were included. The remaining beta-tubulins are exclusively collected from flowering plants. Abbreviations not given in Fig. 3 are: *Hordeum vulgare* (HvTUB), *Glycine max* (GmTUB1-2), *Solanum Tuberosum* (StTUB1-2), *Lupinus albus* (LaTUB1-2) and *Cicer arietinum* (CaTUB). Monocotyledonous beta-tubulins located among dicotyledonous beta-tubulins or vice versa are exemplarily indicated (#). GenBank accession nos. can be given upon request.

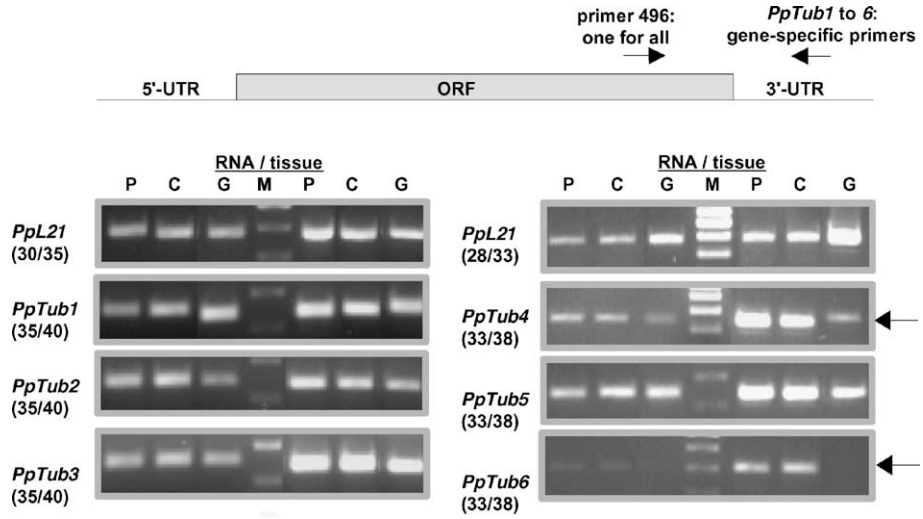

Fig. 5. Abundance of *PpTub1* to *6* transcripts. Representative results of several repetitions of two-step RT-PCR experiments are presented. The experimental design is schematically shown on top, below results of two experiments testing the expression of *PpTub1* to *3* (left) and *PbTub4* to *6* (right) in (P) untreated flask cultures (chloro- and caulonema cells), (C) chloronemata enriched cultures (90 to 95%) and (G) plate grown gametophores (M = marker). *PpL21* codes for a ribosomal protein and served as internal standard. For each gene, amplificates of two cycling numbers (indicated in brackets below the name of the corresponding gene) are shown for each experiment to verify that amplification had not yet reached saturation. Note that expression of *PpTub4* and *PpTub6* are downregulated in adult gametophytes (indicated by arrows).

mixes for all primer pairs, and a minimum of two cycle numbers were checked. The results were highly reproducible and are exemplarily shown in Fig. 5. The transcripts of all six genes were similar in abundance when normal flask cultures and chloronema enriched flask cultures were compared. In plate grown adult gametophytes, four of the six genes (*PpTub1*, *2*, *3*, and *5*) behaved similarly as in the two flask cultures. Interestingly, *PpTub4* and *PpTub6*—the two genes that show structural particularities—are expressed significantly weaker in gametophytes as compared to the two flask cultures. This indicates that the expression of *PpTub1*, *2*, *3*, and *5* is more or less constitutive in the tissues tested, whereas *PpTub4* and *PpTub6* are downregulated in adult gametophytes.

4. Discussion

We used the moss *P. patens* as a phylogenetic intermediate of green algae and higher plants and identified the first large plant beta-tubulin gene family that lacks significant C-terminal isotypic variation. The extreme similarity between the beta-tubulin isotypes from *Physcomitrella* makes it very likely that they are functionally redundant. On the base of functional redundancy, the emergence and stabilization of multigene beta-tubulin families during early evolution of terrestrial plants must be explained by more complex regulatory demands calling for altered temporal and/or spatial expression patterns. It has been proposed earlier that the driving force for the evolution of higher plant beta-tubulin families were such regulatory requirements, rather than functional specificities (Breviario and Nick, 2000). However, so far there has been only circumstantial evidence supporting this idea. The virtual identity of the six *Pptub* gene products allowed us to ask directly, whether it was the potential for differential regulation that stabilized multiple but quasi identical gene products during the early evolution of terrestrial plants. We therefore followed transcript abundance for all six *Pptub* genes through different stages of the *Physcomitrella* lifecycle. All six genes were equally expressed between cultures, containing mainly caulo- and chloronema cells, and chloronema-enriched cultures (containing almost exclusively chloronema cells). During the later lifecycle stages of *Physcomitrella* (adult gametophyte), two of the beta-tubulin genes (*PpTub4* and *PpTub6*) are clearly downregulated, whereas the other four beta-tubulins are expressed to more or less equal levels as during the early stages. It should be noted that *PpTub4* and *PpTub6* are not only unique by a strongly deviating regulation (Fig. 5). PpTUB4, in addition, is the most diverged member within the family (Fig. 2), leading to an outstanding location within the cluster formed by the *Physcomitrella* beta-tubulins in phylogenetic trees (Fig. 4). *PpTub6*, on the other hand, harbours an additional intron just downstream of the stop codon (Fig. 1). The correlation between divergent regulation and an additional intron has to be seen in the light of recent findings by Morello et al. (2002) and Giani et al. (2003). They have shown for tubulin from rice that introns are essential and specific elements for the expression and regulation of tubulin genes.

We expect that additional regulation patterns will emerge, when the expression of the six beta-tubulin genes can be followed through the entire life cycle of *Physcomitrella* and in response to different signals at the cellular level, for instance by the use of promoter-reporter fusions. Nevertheless, already at this level of resolution, we can

observe that the situation in this seed-less organism resembles what is known from seed plants, where constitutively expressed beta-tubulins coexist with genes that are highly regulated. It seems that the *Physcomitrella* beta-tubulins represent a kind of "missing link" between the situation in algae and most fungi, where in general no isotypic beta-tubulin sequence variation can be found, and the situation in higher plants, where several beta-tubulins exist that differ in their C-termini and often in their regulation patterns. In the case of *PpTub4* and, even more pronounced, of *PpTub6*, high-sequence conservation is accompanied by regulatory differences. This means that during evolution differential regulation apparently preceded isotypic sequence variation. In the course of further evolution, the growing number of different cell types and specialized cellular functions seemed to be the driving force for isotypic variations in at least some beta-tubulins, most likely in a concerted co-evolution with MAP. The situation in the fern *Anemia phyllitidis*, where one of the three beta-tubulins already displays a clearly diverged C-terminus might represent an early stage in the evolution of functional beta-tubulin diversity. It might be speculated that the most diverged member within the *Physcomitrella* family (PpTUB4), with an altered pattern of expression, might correspond to an even earlier evolutionary stage. This development would then culminate in the situation found in higher plants, where the combination of differential regulation and isotypic variation has generated a highly versatile system with high potential for environmental adaptivity (for review, see Breviario and Nick, 2000).

It remains to be elucidated what was responsible for the initial multiplication of beta-tubulin genes as precondition for divergent regulatory patterns. One might speculate that mosses as pioneering plants that initially were not sheltered by a protective canopy had to face severe mutational pressure due to UV-B irradiation. And further that multiplication of individual genes might have been a strategy to cope with this situation—tubulins as central elements of cell division would be primary targets for such a mechanism. However, functional specificity of beta-tubulin isotypes in plants remains to be shown. From the experience with alpha-tubulins (Anthony and Hussey, 1998), overexpression of individual beta-tubulin members is expected to be accompanied by severe side effects, for instance the co-suppression of several tubulin isotypes. This would hamper any approach that is targeted on specific isotype functions. Alternatively, one might search for knockouts of individual family members. However, despite intensive research, null mutants for beta-tubulins are generally lacking. On the other hand, loss-of-function mutants for the *Arabidopsis* alpha-tubulins *AtTua6* and *AtTua4* where circumstantially isolated due to a peculiar root-nutation phenotype ('lefty1' and 'lefty2'; Thitamadee et al., 2002). This suggests that functional specificity exists. Compared to other plants, *Physcomitrella* is unique in its efficiency for homologous recombination into its nuclear DNA. This allows for targeted single or multiple gene disruption or replacements (for review, see Reski, 1998b). Based on the data presented here, in *Physcomitrella* functional studies for particular beta-tubulin genes (with *PpTub4* or *6* being the prime candidates) have now become feasible. With the known gene-specific (e.g. intron) sequences, a genomic walk into surrounding genomic regions via an inverse-PCR approach should allow to isolate the regulatory sequences conferring specificity to a particular beta-tubulin gene. By specifically replacing a given beta-tubulin gene by a designed variant, where regulatory sequences have been swapped, removed or modified, it will not only be possible to define the role of a given regulatory sequence (by using appropriate reporters), but also to test the developmental relevance of a given regulation pattern (by analyzing phenotypes of mutants that express beta-tubulin in a changed pattern).

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