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Characterization of an actin gene family in *Palmaria palmata* and *Porphyra purpurea* (Rhodophyta)

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Abstract: The actin gene family was investigated in *Palmaria palmata* and *Porphyra purpurea,* two members of the Rhodoplantae. Respectively four and two partial actin gene sequences were isolated from *P. purpurea* and *P. palmata* by PCR using actin primers deduced from the conserved regions of many classes of organisms. In *P. purpurea*, the partial actin gene *puract1, puract2* and *puract3* were shown to code for proteins whereas *puract4* is a pseudogene. This result confirms the presence of several actin genes already suspected in the genus *Porphyra*. In *P. palmata*, *palmact1* presents a greater identity with actin sequences from other red algae than *palmact2,* the second gene characterized in this species. Sequencing of the gene *palmact1* revealed the existence of an intron in the coding region. To our knowledge this is the first time that two actin encoding genes have been observed in a member of the Florideophyceae. The two genes were both expressed in tetrasporophytic fronds, which raises the question as to their function. We discuss the alternative possibilities that these two genes may possess distinct roles in the cell, or fulfill similar functions in cellular mechanisms. The presence of an actin multigenic family was further demonstrated in four additional species of Rhodophyta suggesting that actin multigenic families are a relatively common feature in red algae. Phylogenetic analyses strongly support the occurrence of several independent duplication events of actin genes in the Rhodophyta.

Résumé : *Mise en évidence d'un famille de gènes d'actine chez* Palmaria palmata *et* Porphyra purpurea *(Rhodophyta)*. La présence d'une famille multigénique d'actine a été étudiée chez *Palmaria palmata* et *Porphyra purpurea*, deux membres des Rhodoplantae. Respectivement quatre et deux séquences partielles de gènes codant l'actine ont été isolées chez *P. purpurea* et *P. palmata* par PCR en utilisant des amorces oligonucléotidiques déduites à partir de régions conservées chez de nombreux organismes. Chez *P. purpurea*, les gènes partiels d'actine *puract1*, *puract2* et *puract3* codent des protéines tandis que *puract4* est un pseudogène. Ce résultat confirme la présence de plusieurs gènes codant l'actine pour le genre *Porphyra*, ce qui avait déjà été suspecté au cours de précédentes études. Chez *Palmaria palmata*, *palmact1* présente une identité supérieure avec les séquences d'actines des autres taxa d'algues rouges inclus dans cette analyse qu'avec *palmact2*, le second gène caractérisé chez cette espèce. Le séquençage du gène *palmact1* a révélé l'existence d'un intron dans la région codante. A notre connaissance, c'est la première fois que deux gènes codant l'actine ont été observés chez un membre des Florideophyceae. De plus, ces deux gènes sont exprimés dans la génération sporophytique du cycle de *P. palmata*

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ce qui soulève la question de leurs fonctions. Nous discutons les différentes alternatives d'une coexpression ou d'une tissus-spécificité de ces deux gènes. La présence d'une famille multigénique d'actine a été démontrée chez quatre espèces additionnelles de Rhodophytes suggérant ainsi que cette famille multigénique est relativement commune chez les algues rouges. Des analyses phylogénétiques supportent fortement l'occurrence de plusieurs duplications indépendantes des gènes d'actine chez les Rhodophytes.

Keywords: Actin genes, Cytoskeleton, Expression, *Palmaria palmata, Porphyra purpurea*

Abbreviation: dNTP: desoxynucleoside triphosphate

Introduction

Palmaria palmata (Linnaeus) Kuntze (Palmariales: Florideophyceae) and *Porphyra purpurea* (Roth) C. Agardh (Bangiales: Bangiophyceae) are multicellular marine red algae common along the rocky shores of the English Channel. Different from most Florideophyceae, *Palmaria palmata* exhibits a biphasic life cycle with only one diploid phase (Van Der Meer & Todd, 1980). Mature sporophytic fronds release some haploid spores that give rise directly to male and female gametophytes. Spermatia released from the mature male gametophyte fertilize the female gametophyte and, subsequently, a sporophytic frond develops. Male gametophytes and sporophytes are macroscopic and morphologically similar, whereas female gametophytes remain microscopic. *Palmaria palmata* was included in the genus *Rhodymenia*, Rhodymeniales, until a detailed anatomical investigation by Guiry (1974) revealed novel features of tetrasporangial development, which prompted him to resurrect the genus *Palmaria* for this species and assign it to the new family Palmariaceae. Guiry (1978) subsequently designated ordinal status to this lineage, Palmariales, and recent advances in the molecular systematics of the Florideophyceae (Harper & Saunders, 2001; Saunders & Hommersand, 2004) have demonstrated that the order Palmariales belongs to the Nemaliophycidae along with the Acrochaetiales, Balbianales, Balliales, Batrachospermales, Colaconematales, Corallinales, Nemaliales and Rhodogorgonales. Only a few nuclear encoded proteins have been investigated so far in the Nemaliophycidae. The taxonomy of the Bangiales has been less convoluted as this order has been well defined based on morphological characters and a heteromorphic life cycle in which a macroscopic gametophyte alternates with a microscopic conchocelis sporophyte. Nevertheless, defining species boundaries as well as the inference of intergeneric relationships is difficult within this order owing to a paucity of reliable morphological characters. The relationship between the two genera recognized so far, *Porphyra* and *Bangia*, is challenged by recent phylogenies (Broom et al., 2004) and new genera have been proposed (Müller et al., 2005; Nelson et al., 2005). Nuclear encoded proteins have been extensively studied in the commercial species *Porphyra yezoensis* Ueda, and 20,000 ESTs are available (Nikaido et al., 2000) but few data are available for the other species.

Actin constitutes a family of highly conserved proteins that are ubiquitous in eukaryotic cells and serves many cellular functions, notably including maintenance of the general architectural organization of the cell. The last decade has seen significant advances in the study of the actin cytoskeleton in red algae. Specific labelling of microfilaments by antibodies or phalloidin coupled with fluorescent stains (McDonald et al., 1993) as well as ultrastructural studies have demonstrated the presence and structure of the actin cytoskeleton in *Porphyra leucosticta* Thuret in Le Jolis (McDonald et al., 1992), *Audouinella botryocarpa* (Harvey) Woelkerling, *Tiffaniella snyderae* (Farlow) I.A. Abbot, *Griffithsia pacifica* Kylin (Garbary & McDonald, 1996a), *Ceramium strictum* Harvey (Garbary & McDonald, 1996b), and *Antithamnion kylinii* N.L. Gardner (Babuka & Pueschel, 1998). These investigations have established that actin microfilaments are a component of the contractile ring involved in the cytokinetic mechanism of red algae, and also that they are involved in the movement of material within the cell (Garbary & McDonald, 1996a). Chemical inhibitors have been employed to produce discernible changes in the structure and function of microfilaments. For example during fertilization, gamete fusion has been inhibited in *Bostrychia moritziana* (Sonder *ex* Kuetzing) J. Agardh (Wilson et al., 2002a) as well as processes such as mitosis, cellulose deposition on the cell surface, and migration of the nuclei in the trichogyne in *Aglaothamnion oosumiense* Itono (Kim & Kim, 1999). In addition, actin inhibitors result in the cessation of movement of chloroplasts (Russel et al., 1996; Wilson et al., 2002b) and nuclei (Garbary et al., 1992). At present, a single actin gene has been characterized in the florideophyte *Chondrus crispus* Stackhouse (Bouget et al., 1995) and *Porphyra yezoensis* (Kitade et al., 2002), whereas two gene

copies have been reported in *Cyanidium caldarium* Tilden Geitler (Takahashi et al., 1998). However, Kitade et al. (2002) noted the existence of several other actin genes for *Porphyra yezoensis*. These genes can be identified in the sequence database of individual ESTs (http://www.kazusa.or.jp/en/plant/porphyra/EST/ Nikaido et al., 2000). Takahashi et al. (1995) described an actin gene in the bangiophyte *Cyanidioschyzon merolae* P. De Luca, R. Taddei & L. Varano but recently an actin-like protein has also been characterized (Matsuzaki et al., 2004). As suggested by Garbary and McDonald (1998), it would be interesting to develop more molecular approaches to study gene sequences and gene regulation leading to actin formation in the red algae.

Actin genes generally occur in complex gene families in higher eukaryotes including animals and land plants (Bhattacharya et al., 2000) and in single copies in alveolata (Leander & Keeling, 2004), many green algae (Bhattacharya et al., 1998) and other algal divisions such as the Heterokontophyta (Bhattacharya et al., 1991; Goodner et al., 1995). In land plants, the multiplicity of actin genes seems to play a key role in evolution and there may be a close relationship between the number of actin genes and the complexity of reproductive structures (Bhattacharya et al., 2000). In other reports, phylogenetic analyses and the expression pattern of actin gene families separate vegetative and reproductive classes of genes. Meagher et al. (1999a) therefore hypothesized that these genes are probably required for the development of the corresponding tissue types.

Red algae are a sister group to the green lineage (Moreira et al., 2000); it is of great interest to investigate actin genes in a member of the Florideophyceae, which have more complex morphological organization than the Bangiophyceae. In this context, we have characterized two actin encoding genes in *P. palmata*, and the expression of these genes has been demonstrated. Finally, we have also investigated the diversity of the actin gene family previously characterized in the Bangiophyceae.

Material and methods

Seaweeds

Palmaria palmata and *Porphyra purpurea* were field collected throughout the intertidal zone at four different locations along the French shore of the Atlantic Ocean and the English Channel (Table 1). *Porphyra purpurea* fronds were identified according to the definition given by Brodie & Irvine (1997). Thalli were cleaned with absorbent paper and rinsed with distilled water. Apical tips from *P. palmata* and the centre of a blade of *P. purpurea* were removed, and examined under a dissecting microscope to confirm the absence of epiphytes or endophytes. Samples for DNA extraction were lyophilized and stored at -20°C whereas *P. palmata* samples for RNA extraction were conserved at -70°C. Prior to conservation, samples for RNA extraction were forthwith analysed for ploidy to distinguish male gametophytes from sporophytes by flow cytometry performed with a FACSort (Becton Dickinson, San José, California) as described by Ar Gall et al. (1996).

Primers

Two specific oligonucleotide primers for the conserved coding sequences TFQQMWI and TNWDDM of most members of the actin family were kindly provided by J.P. Cadoret and J.M. Escoubas (DRIM, Montpellier):
AVI1, 5'-TAATCCACATCTGCTGGAAGG-3' and AVI2, 5'-TCACCAACTGGGATGACATGG-3'

For expression studies, two pairs of specific oligonucleotide primers were designed to differentiate each actin gene in *P. palmata*:
ACT1 sense, 5'-CGCCTTTTACTCGGAGCTCC-3' and ACT1 antisense primer, 5'-TGGAGCCTCCAATCCACACG-3'
ACT2 sense, 5'-ACATCCAGTTCTCCTCACCG-3' and ACT2 antisense primers, 5'-AAACGCTGTACTGGCGTTCG-3'

Table 1. Species studied and collection details.
Tableau 1. Liste des taxons inclus et détails des récoltes.

Taxon	Locality	Date	Collector	Experiment
P. palmata	Concarneau, Brittany 47° 86 66N, 3° 91 66W	16.05.2000	L. Le Gall	PCR
P. palmata	Roscoff, Brittany 48° 47 23N, 4° 20 11W	04.10.2001	L. Le Gall	RT-PCR
P. palmata	Cap Levy, Normandy 49° 41 86N, 1° 28 45W	22.11.2000	L. Le Gall	PCR & Sequencing
P. purpurea	Asnelle, Normandy 49° 33 33N, 0° 58 33W	13.03.2002	L. Le Gall	PCR & Sequencing

Amplification of actin genes from genomic DNA

Genomic DNA was extracted as described by Winnepenninckx et al. (1993). Briefly, tissue was ground in liquid nitrogen, resuspended in high salt buffer containing 0.1 $g.mL^{-1}$ cetyltrimethylammonium and 0.1 $mg.mL^{-1}$ proteinase K, incubated for 30 min at 60°C and subsequently extracted with an equal volume of chloroform / iso amyl alcohol (24:1) by centrifugation. Propanol-2 was added to the supernatant to precipitate genomic DNA. Finally, DNA was spooled out with a glass rod, washed with 75% ethanol/10 mM ammonium acetate, air-dried and resuspended in sterile MilliQ water. Amplification reactions were performed with 50 ng of genomic DNA in a total volume of 50 µL with 0.2mM of each dNTP, 0.4mM of each primer and 1.5U of Pfu DNA Polymerase (Promega). Amplification was performed for 40 cycles at 95°C for 1 min and a final extension step at 72°C for 10 min. PCR products were resolved on a 1.5 % agarose gel. Subsequently, fragments were gel-purified, and subcloned using a pGEM-T Easy System II kit (Promega). Two to thirteen clones were sequenced with an ABI Prism BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems).

Amplification of RNA transcript of actin genes

Total RNA was extracted from sporophytic fronds of *P. palmata* using the Tri Reagent™ kit (Sigma) and reverse-transcribed into oligo$(dT)_{17}$ primed cDNA with 200 U Moloney murine leukaemia virus reverse transcriptase (Promega). Amplification was then performed with the specific primer pairs ACT1 and ACT2 using the reaction mix described above for 30 cycles at 95°C for 1 min, 53°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min. PCR products were resolved on a 1.5% agarose gel. PCR fragments were gel-purified and sequenced as previously described.

Database consultation

A search for partial actin cDNA from *Porphyra yezoensis* was performed in the database of individual expressed sequence tags posted on line (Nikaido et al., 2000). Twenty seven ESTs showed sequence similarity to actin genes from red algae and plants available in public DNA databases. These sequences were assembled automatically using Sequencher™4.2.2 and three different contig were obtained. Contig 1 resulted from an assemblage of the individual ESTs AV432091 and AV430571, and the reverse complementary EST AV432885, AV433811 and AV437974. Constig 2 resulted from an assemblage of the reverse complementary EST AV436298, AV432544, and AV430104. The third contig, resulting from the assemblage of the ESTs AV434968, AV 431540, AV430782, AV432289, AU194907, AU187444, AV435141, AU186696, matched the *P. yezoensis* actin available in the public database EMBL (accession number AB039831). Phylogenetic signals of contig 1 (3'half of the gene) and contig 2 (5' half of the gene) were analyzed individually resulting in similar branching pattern. The two contigs were therefore concatenated in a final alignment.

Gene phylogenetic analysis

The final alignment contained 15 genes (840 nucleotide sites) belonging to 9 taxa. Nucleic acid sequences were manually aligned with the assistance of the program MacClade 4.06. Parsimony and distance analyses were completed in PAUP 4.0b10 for the Macintosh (Swofford, 2001). Maximum parsimony was implemented using 10 random additions under heuristic tree search (tree bisection and reconnection). Distance analyses used the general time reversible model and the tree was constructed with neighbor joining. Bootstrap resampling (2000 replicates) was performed for both distance and parsimony to estimate robustness (Felsenstein, 1985). Trees were outgroup-rooted with actin genes belonging to the three Cryptophyta species *Cryptomonas ovata* Ehrenberg (AF284836), *Guillardia theta* D.R.A. Hill & R. Wetherbee (AF284835) and *Pyrenomonas helgolandii* (AF284834), a sister group to red and green lineage actins (Stibitz et al., 2000)

Results

Characterization of actin genes from Palmaria palmata

To identify actin genes in *P. palmata*, amplification was performed from genomic DNA using highly conserved primers AVI1 and AVI2 (Fig.1) for samples from Concarneau (Atlantic ocean). Two distinct bands were observed, one with the expected size of 835 bp and a second of approximately 950 bp. The same result was obtained with *P. palmata* collected at Cap Levy (English Channel) (data not shown). The PCR products from the Cap Levy *P. palmata* were subcloned, two clones of *palmact1* (950 bp) and ten clones of *palmact2* (835 bp) were sequenced. Nucleotide and deduced amino acid sequences of the corresponding proteins (Fig. 2) indicated that the two PCR fragments encoded two actin proteins (accession numbers AJ496179 and AJ496180, respectively). BLAST analyses (Altschul et al., 1997) with both nucleotide and protein databases demonstrated that the two actin genes in *P. palmata* are related to ß actin forms (data not shown). The *palmact1* gene contains an additional sequence, which explains the size difference observed between the two PCR fragments. Furthermore, this sequence displays putative donor, branch and acceptor sites of splicing (Liaud et al., 1995). To confirm splicing of this putative intron (96 bp),

corresponding cDNA was sequenced. Sequences for *palmact1* and *palmact2* had 77% identity throughout the nucleotide coding region and the deduced amino acid sequences exhibited 83.8% identity. An alignment with other species shows that the *P. palmata* differ from other red algal actin genes by 6% to 29% at the amino acid level (Fig. 3 and Table 2). Comparison of *Palmact1* and *Palmact2* with the actin protein from *Chondrus crispus* and *Porphyra yezoensis* indicates that *Palmact2* is more divergent from these proteins than *Palmact1* (Table 2).

Expression of the two actin encoding genes in sporophytic fronds

Total RNA extracted from sporophytic fronds of *P. palmata* was used to study the expression of actin encoding genes by RT-PCR with the specific primer pairs ACT1 and ACT2 designed to differentiate between the two actin genes and to yield PCR products of different size (Fig. 2). The results of these analyses are shown in Figure 4. Both *palmact1* and *palmact2* were amplified, demonstrating that the two genes are expressed in sporophytic fronds. As a control, amplification was performed under the same conditions on total RNA without reverse transcription to check for contamination with genomic DNA. The *palmact2* band displayed the expected size of approximately 700 bp and the size of *palmact1* band is about 770 bp, which corresponds to the length of the expected fragments without the intron. The expression level of the *palmact1* gene appears to be higher than *palmact2*, but this would require further investigation. Similar amplifications were performed with male gametophytic fronds, but very low signals were observed (data not shown).

Presence of numerous actin genes in Porphyra

Amplification from genomic DNA of the red macroalga *Porphyra purpurea* was performed under the same conditions as described for *P. palmata*. Only one PCR product (approximately 830 bp) was observed, and subcloned (Fig. 1). Thirteen clones were sequenced and at least 4 different actin related genes were identified. One of these genes, *puract4* (accession number DQ111779), is clearly a pseudogene because of the presence of several stop codons. Three of these sequences, *puract1* (accession number DQ111776), *puract2* (accession number DQ111777) and *puract3* (accession number DQ111778) putatively encode a protein product as DNA sequences do not present unexpected stop codons. These three genes are closely related, but cannot be considered as multiple copies of the same gene because they have one to three differences at the amino acid level. They do not reflect intraspecific nor allelic variation because only a single haploid frond was selected for the DNA extraction.

Phylogenetic analyses

Distance and maximum parsimony analyses were completed to resolve relationships among red algal actin genes and determine the origin of these actin gene families. The neighbour joining tree (Fig. 5) shows that a hypothesis of several independent gene duplications in the course of the red algal evolution is strongly supported. Indeed, our data supports independent duplication events in the bangiophytes *Porphyra* and *Cyanidium caldarium*, as well as in the florideophyte *P. palmata*. This gene phylogeny shows recent duplications in the Bangiophyceae whereas actin genes characterized in *P. palmata* seem to have duplicated more anciently. In the Florideophyceae, strong support was acquired for *Chondrus crispus* actin gene orthology with *palmact1* and paralogy with *palmact2*. *Palmact2* produced the longest branch for the florideophyte gene included in this analysis (162 changes) perhaps indicating that this gene

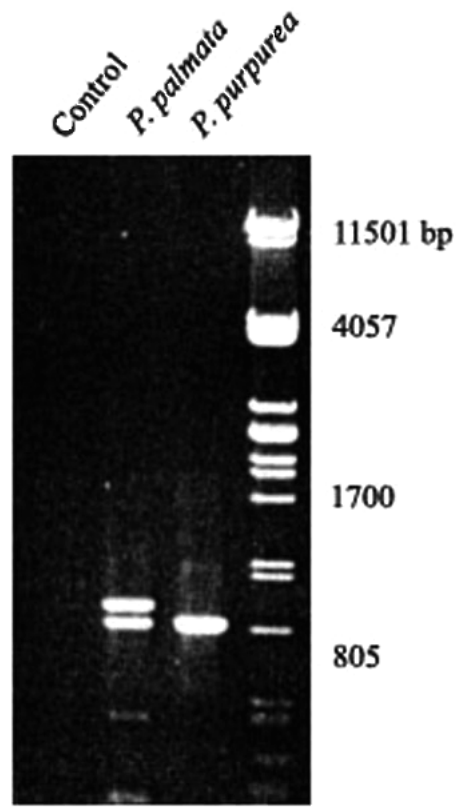

Figure 1. Amplification of genomic DNA from *Palmaria palmata* with the specific primers AVI1 and AVI2. PCR products were visualised on a 1.5% agarose gel. Left lanes = control and PCR experiment; right lane = size standard.

Figure 1. Amplification de l'ADN génomique de *Palmaria palmata* avec les amorces oligonucléotidiques spécifiques AVI1 et AVI2. Les produits de PCR sont visualisés sur un gel d'agarose. Puit gauche = témoin et expérience; puits droit = marqueurs de taille.

Figure 2. Nucleotide sequences of two genomic PCR fragments encoding actin and the corresponding amino acid sequences in *Palmaria palmata*. The intron in *palmact1* is indicated in small letters and putative splicing sites are indicated in bold. Specific oligonucleotides used for the PCR expression study are delineated by horizontal arrows.

Figure 2. Séquences nucléotidiques des deux fragments génomiques codant l'actine de *Palmaria palmata* amplifiés par PCR et séquences protéiques correspondantes. L'intron dans la séquence codante de *palmact1* est indiqué en minuscule et les sites d'épissages putatifs sont indiqués en gras. Les amorces oligonucléotidiques utilisées lors de l'étude de l'expression par PCR sont délimitées par des flèches horizontales.

palmact1

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ACC AAC TGG GAT GAC ATG GAG AAG ATC TGG CAC CAC GCC TTT TAC TCG GAG CTC      54
 T   N   W   D   D   M   E   K   I   W   H   H   A   F   Y   S   E   L      18
▶
CGC GTT GAC CCG CAG GAG CAC CCC GTT CTT CTC ACC GAG GCT CCG CTG AAT CCC     108
 R   V   D   P   Q   E   H   P   V   L   L   T   E   A   P   L   N   P      36

AAG GCC AAT CGC GAG CGC ATG ACA CAG ATC ATG TTC GAG AGC TTT AAC ATC CCC     162
 K   A   N   R   E   R   M   T   Q   I   M   F   E   S   F   N   I   P      54

GCC TTT TAC GTC GCC ATC CAG GCG GTT CTT TCG CTG TAT GCC TCC GGG CGG ACG     216
 A   F   Y   V   A   I   Q   A   V   L   S   L   Y   A   S   G   R   T      72

TCC GGT ATC GTT CTT GAT TCA GGC GAT GGT GTC ACG CAT ACC GTC CCC ATC TAT     270
 S   G   I   V   L   D   S   G   D   G   V   T   H   T   V   P   I   Y      90

GAG GGT TAC TCG CTT CCC CAC GCT GTT CTC AGG ATC GAT CTT GCC GGT CGT GAT     324
 E   G   Y   S   L   P   H   A   V   L   R   I   D   L   A   G   R   D     108

TTG ACC GGA TGG ATG GCC AAG CTC CTC ATC CAG CGC GGC TAC TCT TTC ACC ACC     378
 L   T   G   W   M   A   K   L   L   I   Q   R   G   Y   S   F   T   T     126

TCT GCC GAG CTT GAA ATT GTC CGC GAC ATT AAG CAA CAG CTT TGC TTC GTT GCC     432
 S   A   E   L   E   I   V   R   D   I   K   Q   Q   L   C   F   V   A     144

GAG GAC TTC GAC AAG GAA CTT GCC TCC GCC TCG TCG AAC TCT TCG CTC GAG AAG     486
 E   D   F   D   K   E   L   A   S   A   S   S   N   S   S   L   E   K     162

GAG TAT GAG CTT CCC GAC GGT CAG GTC ATC TCC GTC GCT GAG GAG CGA TTC CAG     540
 E   Y   E   L   P   D   G   Q   V   I   S   V   A   E   E   R   F   Q     180

TGT CCT GAG GCC CTT TTC AAG CCC GAG ATG CTT GGG AAC GAA ATG AGC GGC ATG     594
 C   P   E   A   L   F   K   P   E   M   L   G   N   E   M   S   G   M     198

CAC CTT ACT TCC TTC AAC TCA ATC ATG AAG ACT GAC ATT GAT ATC CGT AAG GAT     648
 H   L   T   S   F   N   S   I   M   K   T   D   I   D   I   R   K   D     216
                                                5' Donor
CTG TAC AAT AAC ATT GTC ATG TCT GGC GGG TCT ACT gtaagttgctaagcggttctgatt     707
 L   Y   N   N   I   V   M   S   G   G   S   T                           228
                    Branch site                          3'Acceptor
ttttggttctctttcctgggaacttccgaagctgactcgcttgtcttcatttcttgtcttggcccgtctcag     779

ATG TTC GCC GGC ATT AGC TCT AGG GTG CAG AAA GAG ATT GAG ACT CTT GCC CCT     834
 M   F   A   G   I   S   S   R   V   Q   K   E   I   E   T   L   A   P     246
                                                                 ◀
GCG AGC ATG AAG ATT AAG GTT ATC GCG CCT CCC GAG CGT CAG TAC TCC GTG TGG     888
 A   S   M   K   I   K   V   I   A   P   P   E   R   Q   Y   S   V   W     264

ATT GGA GGC TCC ATC CTC TCT TCC CTC ACC ACC TTC CAG CAG ATG TGG ATT A       942
 I   G   G   S   I   L   S   S   L   T   T   F   Q   Q   M   W   I
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palmact2

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ACC AAC TGG GAT GAC ATG GAA AAG ATT TGG AGC CAC GCT TTT TCG TCG GAG CTC      54
 T   N   W   D   D   M   E   K   I   W   S   H   A   F   S   S   E   L      18
                     ▶
CGC GTC GAG CCC GAG GAA CAT CCA GTT CTC CTC ACC GAA GCG CCC CTC AAC CCG     108
 R   V   E   P   E   E   H   P   V   L   L   T   E   A   P   L   N   P      36

AAG GCC AAC CGC GAG CGC ATG ACC CAG ATC ATG TTC GAG ACC TTC AAC ATC CCC     162
 K   A   N   R   E   R   M   T   Q   I   M   F   E   T   F   N   I   P      54

GCC TTC TAC GTC GCC ATC CAG GCC GTC CTC AGC CTC TAT TCG TCC GGC CGG ACG     216
 A   F   Y   V   A   I   Q   A   V   L   S   L   Y   S   S   G   R   T      72

TCC GGG ATC GTC ATC GAC TCT GGC GAC GGC GTG ACA CAC ACT GTC CCC ATC TAC     270
 S   G   I   V   I   D   S   G   D   G   V   T   H   T   V   P   I   Y      90

GAG GGT TAC TCA CTT CCA CAC GCC GTC CTC CGC ATC GAC CTC GCC GGC CGT GAC     324
 E   G   Y   S   L   P   H   A   V   L   R   I   D   L   A   G   R   D     108

CTG ACC GGG TTC GTG TCC AAG CTT CTC ATG CAG CGC GGC CAC TCG TTC ACC ACC     378
 L   T   G   F   V   S   K   L   L   M   Q   R   G   H   S   F   T   T     126

TCC GCC GAG CTC GAG ATT GTG CGC GAC ATC AGG CAG CAG CTC TGC TTC GTC GCC     432
 S   A   E   L   E   I   V   R   D   I   R   Q   Q   L   C   F   V   A     144

GAA GAC TAC AAG CAG GAG CTC GCG GCC GCA GAC AGC AAC TCG GCG CTC ATG AAG     486
 E   D   Y   K   Q   E   L   A   A   A   D   S   N   S   A   L   M   K     162

GAG TAC GAG CTC CCC GAC GGT AAC GTC ATC AGC ATC GGC CAG GAA CGC TTC CAG     540
 E   Y   E   L   P   D   G   N   V   I   S   I   G   Q   E   R   F   Q     180

TGC CCC GAG GCC CTC TTC AAC CCG AGC CTC CTC GGC CTC GAA GCC ACC GGC ATG     594
 C   P   E   A   L   F   N   P   S   L   L   G   L   E   A   T   G   M     198

CAC GAG ACC TGC TTC AAG TCC GTC ATG AAG ACC GAC ATT GAC ATC CGA AAG GAC     648
 H   E   T   C   F   K   S   V   M   K   T   D   I   D   I   R   K   D     216

CTC TAC GCC AAC GCC GTC ATG TCC GGA GGC AGC ACC ATG TTC AAA GGC ATC GCA     702
 L   Y   A   N   A   V   M   S   G   G   S   T   M   F   K   G   I   A     234

GCA CGA GTA TCG AAA GAG CTC GAA ACG CTC GCC CCG AAA ACT GTC AAA ATC AAG     756
 A   R   V   S   K   E   L   E   T   L   A   P   K   T   V   K   I   K     252
                                 ◀
GTC ATC GCG CCC CCC GAA CGC CAG TAC AGC GTT TGG ATC GGC GGC TCC ATC CTC     810
 V   I   A   P   P   E   R   Q   Y   S   V   W   I   G   G   S   I   L     270

TCC TCC CTC TCC ACC TTC CAG CAG ATG TGG TTA                              843
 S   S   L   S   T   F   Q   Q   M   W   L                              281
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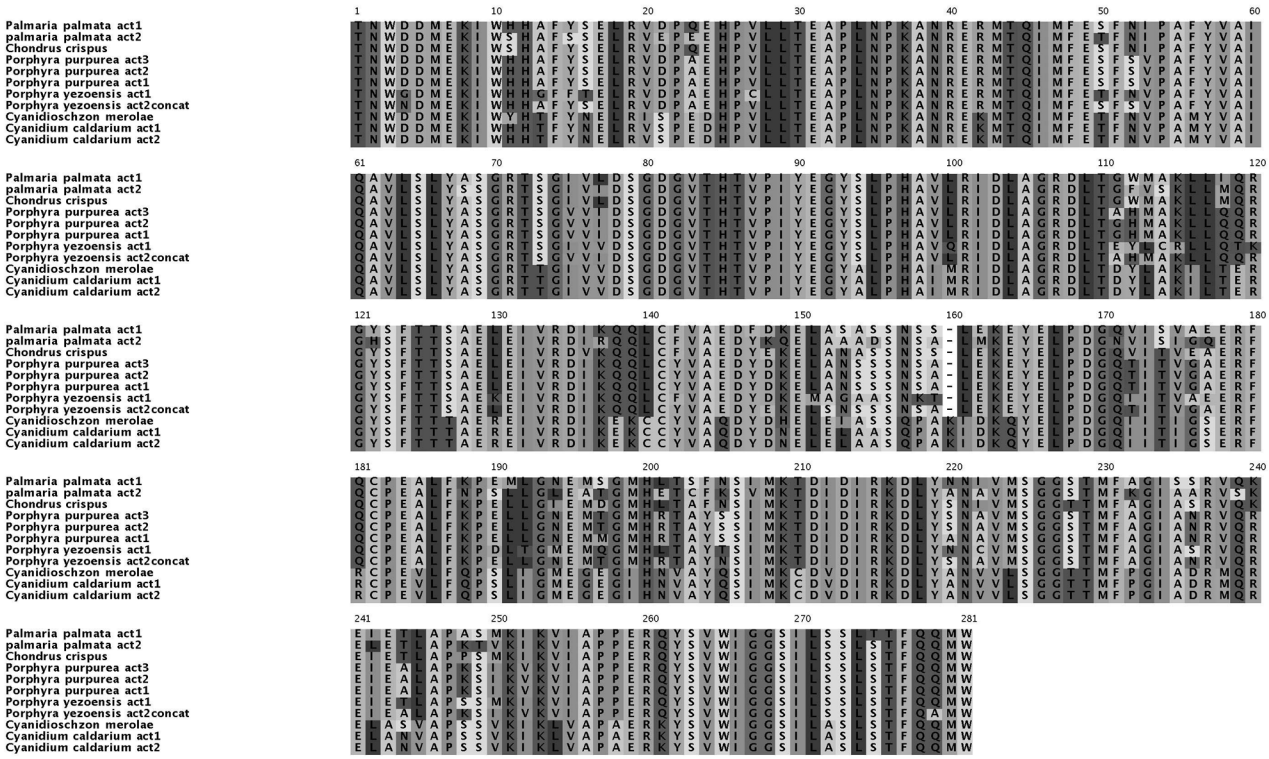

Figure 3. Comparison of actin proteins from various Rhodophyta. Amino acid sequences were manually aligned to maximise similarity with the assistance of the program MacClade 4.06.

Figure 3. Comparaison de séquences protéiques d'actine de différentes rhodophytes. Les séquences ont été manuellement alignées pour optimiser leur similarité avec l'assistance du programme MacClade 4.06.

Table 2. Summary of the percentage of amino acid divergence in actin genes from six taxa of the Rhodoplantae.
Tableau 2. Résumé de la divergence des séquences protéiques d'actines provenant de six taxons de Rhodoplantae.

Taxon		Gene names / ref	AA Divergence (%) 1	2	3	4	5	6	7	8	9	10	11
Palmaria palmata	1	*Palmact1* / AJ496179	-	**16**	6	12	11	11	14	12	29	29	29
	2	*Palmact2* / AJ496180		-	15	17	16	16	21	18	29	28	28
Chondrus crispus	3	P53499			-	10	10	10	15	11	27	27	27
Porphyra purpurea	4	*Puract1* / DQ111776				-	**0.4**	**1**	15	2	26	26	26
	5	*Puract2* / DQ111777					-	**1**	15	2	26	26	26
	6	*Puract3* / DQ111778						-	15	3	26	26	26
Porphyra yezoensis	7	*Act1* / AB039831							-	**16**	26	25	25
	8	*Act2* / Nikaido et al. (2000)								-	27	27	27
Cyanidioschison merolae	9	P53500									-	2	2
Cyanidium caldarium	10	Takahashi et al. (1998)										-	**0**
	11												-

has evolved under different selective pressure, which is a common feature displayed by paralogous genes after duplication (Jordan et al., 2004). Actin genes in the two species of *Porphyra* were associated solidly as a sister group to *C. crispus* and *palmact1* and not resolved as a unique actin gene family.

Discussion

Our results demonstrate that actin is encoded by two different genes in *P. palmata.* The possibility of one gene coming from a red algal contaminant was virtually eliminated as attention was paid to thallus cleaning. Moreover

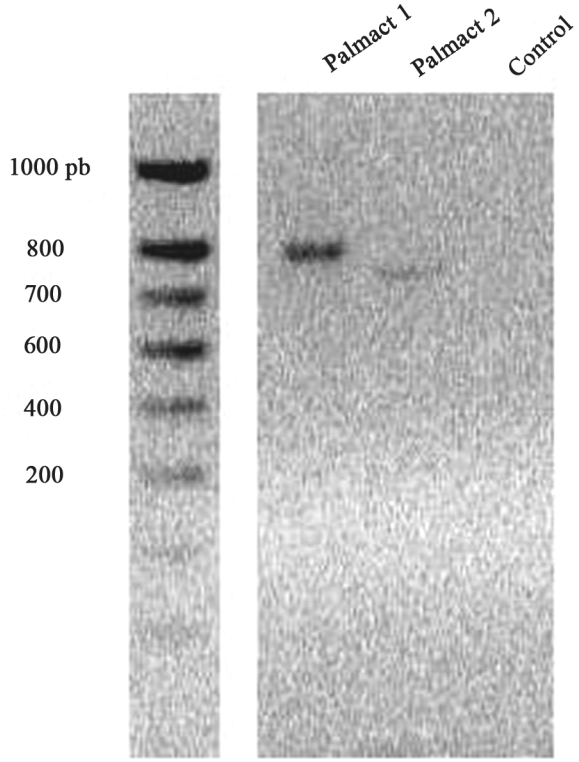

Figure 4. RT-PCR from RNA extracts of sporophytic fronds of *Palmaria palmata* with specific primer for each gene pairs, ACT1 and ACT2. PCR products were visualised on a 1.5% agarose gel. (Only brightness and contrast have been adjusted).

Figure 4. RT-PCR réalisée à partir d'ARN extrait de la génération sporophytique de *Palmaria palmata* avec des amorces oligonucléotidiques spécifiques de chacun des deux gènes caractérisés, ACT1 et ACT2. Les produits de PCR sont visualisés sur un gel d'agarose a 1,5%. (Seuls la luminosité et le contraste ont été ajustés).

congruent results were observed with *P. palmata* collected from three different locations and seasons. To our knowledge, this is the first time that the expression of two actin genes has been reported in a member of the Florideophyceae. These two genes present some significant differences in their organization as *palmact1* is characterized by the presence of an intron in the coding region. The presence of an intron in an almost identical position to that in *palmact1* has been shown in the chlorophytes *Chlamydomonas reinhardtii* P.A. Dangeard (Sugase et al., 1996) and *Volvox carteri* F. Stein (Cresnar et al., 1990), and in the heterokont *Costaria costata* (C. Agardh) Saunders (Bhattacharya et al., 1991). These observations are congruent with the model of Bagavathi & Malathi (1996) of the evolution of intron distribution in which it is argued that the ancestral actin gene contained several introns, some or all of which may have been lost in certain species. It must also be pointed out that actin genes have most often been characterized from cDNA libraries, in which case the possible presence of introns will have been overlooked.

Our results demonstrate the presence of two actin genes in *P. palmata* and the characterization of this multigenic family in other members of the Palmariales is now in progress. These results raise important questions about the function and origin of these genes. *Palmact1* and *palmact2* are simultaneously expressed in tetrasporophytic fronds of *P. palmata*. In relative terms, *palmact1*, which shows more identities throughout its coding region with actin genes from the rhodophytes *Chondrus crispus* (Bouget et al., 1995) and *Porphyra yezoensis* (Kitade et al., 2002), appears to be more strongly expressed than *palmact2*. However, both of these genes exhibit a low level of expression by RT-PCR, possibly due to the presence of RT inhibitors as previously reported in various Rhodophyta (Loya et al., 1995; Ohta et al., 1998; Mizushina et al., 2001). The expression of the two actin encoding genes implies that neither represents a pseudogene, a phenomenon which is common in multigenic families (Moniz de Sa & Drouin, 1996).

Different hypotheses can be advanced to explain the function of the two actin proteins. Kondrashov et al. (2002) hypothesized that gene duplications were beneficial as protein should be over expressed and/or better regulated in response to environmental conditions. Gene duplications are also traditionally considered to be a major evolutionary source as they lead to the emergence of new functions or differentially regulated subfunctions. In unicellular organisms, or within the cell of complex organisms, genes may be expressed in different temporal patterns or genes can be co-expressed. Moreover, in the case of multicellular organisms actin genes can be expressed with a different spatial pattern.

When genes are co-expressed, different forms of actin proteins may have distinct sub-cellular functions or play a role in the dynamic behaviour of actin microfilaments (Meagher et al., 1999b). In *P. palmata*, *palmact1* and *palmact2* were both expressed in the sporophytic thalli, but expression studies at the cellular level are required to confirm the hypothesis that co-expression actually occurs. It should be noted that *P. palmata* thalli are composed of differentiated cortical and medullar cells, which may have different spatial patterns of gene expression. In *Arabidopsis thaliana*, actin genes are differentially expressed in vegetative and reproductive tissues (An et al., 1999) indicating that actin genes are separated into two functional groups. In our expression study fronds were not fertile, suggesting that *palmact1*and *palmact2* are not clearly separated into such functional groups; it would nevertheless be interesting to analyze the level of expression of each gene in vegetative and reproductive tissues. Moreover, within each tissue, cells are not synchronous in their cell cycle (Goff &

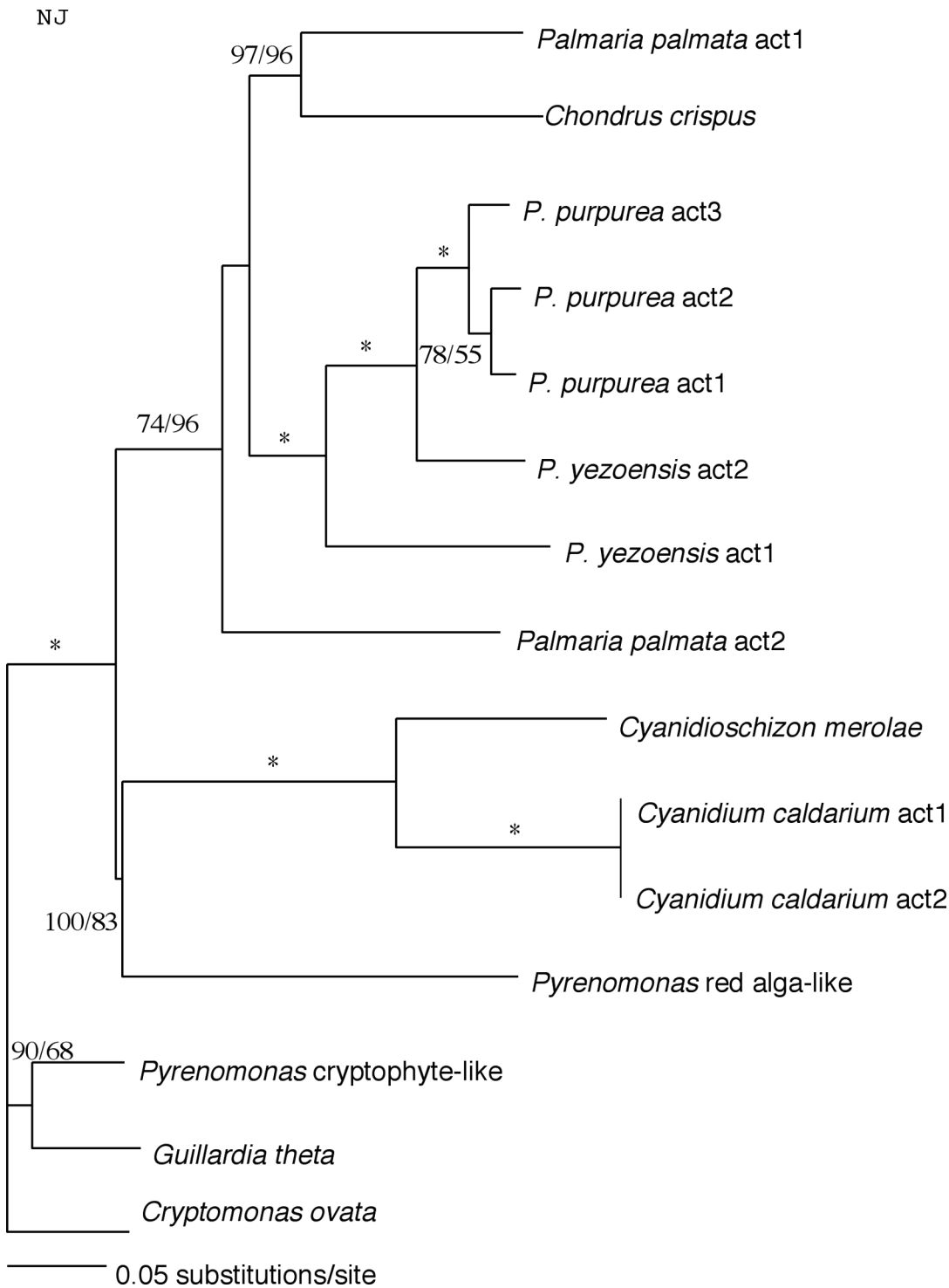

Figure 5. Phylogenetic analyses of red algal actin nucleotide sequences. The tree was inferred with distance methods and has been outgroup-rooted with three cryptophyte actin sequences. The values at internal nodes result from distance and parsimony bootstrap analyses respectively (2,000 replications). * indicates 100% support in both analyses.

Figure 5. Analyses phylogénétiques des séquences oligonucléotidiques d'actines d'algues rouges. L'arbre a été obtenu par la méthode de distance et a été enraciné avec trois séquences d'actine de Cryptophytes. Les valeurs indiquées aux nœuds internes résultent des analyses de bootstrap réalisées respectivement par les méthodes de distance et de parcimonie (2 000 réplications). * indique 100% de support pour les deux analyses.

Coleman, 1990), and considering the key role of actin in the mechanism of cytokinesis, it could be argued that the temporal expression pattern of actin genes may be correlated with the cell cycle.

The presence of several actin genes has been correlated with the complexity of cellular mechanisms or morphological structure. In the genus *Nannochloris* (Chlorophyta) the number of actin genes seems to be well correlated with the mode of cell division (Yamamoto et al., 2001). In land plants, genes encoding actin occur in increasingly more complex gene families through the course of evolution and a relationship has been postulated between actin gene duplication and concomitant morphological complexity of tissues during plant evolution (Bhattacharya et al., 2000). In the division Rhodophyta, however, both unicellular and macroalgae exhibit either a single or several actin genes. Hence, there does not appear to be an obvious relationship between the number of actin genes present in genomes and the organisational complexity of the organism.

Our phylogenetic studies of actin genes raises strong evidence for several recent duplication events in the Bangiophyceae. The two actin copies found in *C. caldarium* are nearly identical and Takahara et al. (2000) observed that they result from a recent duplication of a quite long part of the genome containing at least the actin ORF and its flanking regions. In *P. yezoensis* the two genes observed so far exhibit some significant differences at the protein level suggesting that they may fulfil distinct functions as we hypothesize for *P. palmata*, whereas the three genes characterized in *Porphyra purpurea* are closely related resulting presumably from a fairly recent duplication. It must be stressed, however, that actin genes have been investigated in only a few red algal species, and that exhaustive investigations of the whole potential range of actin genes are rare. Recently, *P. yezoensis* has been chosen as the best candidate for macroalgal genomic initiative (Waaland et al., 2004); leading, hopefully, to a complete nuclear genome sequence that would provide substantial advances in the study of the red algal actin gene family. Our results clearly demonstrated that the actin gene family observed in *P. palmata, Cyanidium caldarium* and the genus *Porphyra* proceed from distinct duplication events. While such a series of events may be complex, it should not be so surprising, because gene duplication is a common feature in nuclear genome evolution (Lynch & Conery, 2000). Furthermore, duplicated actin genes appear to be often retained by selection; which is likely due to their high biological import in red algal cells. Indeed, Jordan et al. (2004) have shown that the retension of duplicated genes by selection depends on the importance of gene function.

Our results have clearly demonstrated that actin genes occur as multigene families in taxonomically distant species of red algae leaving unresolved the question of the functions of the different actin proteins. We also showed that several independent duplication events have happened but that the data set of actin genes in the Rhodophyta is at present too limited to permit hypotheses about the history of the actin multigene families in this division. Nevertheless, this study highlights that the presence of paralogous actin genes in some Rhodophyta complicates the use of actin genes as markers of species evolution in this lineage, as indeed does the fact that high selective pressure on actin genes acts to restrict the number of random mutations (An et al., 1999). Furthermore, we hope to have removed the long standing belief that red algae have a single actin gene and we expect that future studies addressing the function and the evolution of actin genes will provide a more complete understanding of the occurrence and origin of multiple actin encoding genes in the Rhodophyta.

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