Characterization and Molecular Cloning of Conjugation-Regulating Sex Pheromones in Homothallic Closterium

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Conjugation-regulating pheromones were analyzed in homothallic Closterium for the first time. Members of the Closterium peracerosum–strigosum–littorale complex are unicellular charophycean algae in which there are two modes of zygospore formation: heterothallism and homothallism. A homothallic strain of Closterium (designation, kodama20) forms selfing zygospores via the conjugation of two sister gametangial cells derived from one vegetative cell. Conjugation-promoting and -suppressing activities, against cells at very low (1 \times 10^2 \text{ cells ml}^{-1}) and normal (1 \times 10^4 \text{ cells ml}^{-1}) cell density, respectively, were detected in the medium in which cells of a normal density had been cultured. Pheromone activities decreased to 20% after incubation at 60°C for 10 min. The release and action of the pheromones was dependent on light. The culture medium was subjected to gel filtration, and both active substances had an apparent molecular mass of 17 kDa; this was similar to that previously reported for the heterothallic sex-specific pheromone protoplast-release-inducing protein (PR-IP) Inducer. cDNAs encoding the orthologs of PR-IP Inducer were isolated from the homothallic strain. Recombinant PR-IP Inducers produced by yeast cells showed conjugation-promoting activity. These results indicate that conjugation of the homothallic strain is regulated by an ortholog of a heterothallic sex-specific pheromone.

Keywords: Closterium • Conjugation • Homothallism • Homothallism • Sex pheromone • Sexual reproduction • Zygospore.

Abbreviations: C. psl, Closterium peracerosum–strigosum–littorale; mt+, mating-type plus; mt−, mating-type minus; PNGase F, peptide-N-glycosidase F; PR-IP, protoplast-release-inducing protein; RACE, rapid amplification of cDNA ends; SCD, sexual cell division; SCD-IP, sexual cell division-inducing pheromone.

Introduction

Sexual reproduction results in increased genetic diversity, because it involves the union of genetic material contributed by two different gametes, usually from two different organisms. The Closterium peracerosum–strigosum–littorale (C. psl) complex is a unicellular, isogamous, charophycean alga that is closely related to land plants (McCourt et al. 2004, Turmel et al. 2006). It exhibits two types of dormant zygospore formation. Some populations form zygospores within single clonal cells (homothallic), whereas others form zygospores between different clones (heterothallic; Graham and Wilcox 2000).

The heterothallic strains have two sexes, or mating types (mt): plus (mt+) and minus (mt−). Once these strains are mixed in nitrogen-depleted medium, the sexual reproductive process proceeds as follows: (i) cell division resulting in the formation of two gametangial cells from a vegetative cell of each mt sex (sexual cell division; SCD), (ii) formation of a sexual pair between mt+ and mt− gametangial cells, (iii) formation of conjugation papillae, (iv) release of gametic protoplasts from each member of a pair; (v) formation of zygospores by protoplast fusion. At least two sex pheromones mediate these steps (Akatsuka et al. 2003, Akatsuka et al. 2006, Sekimoto 2000, Sekimoto 2005, Tsuchikane et al. 2003, Tsuchikane et al. 2005).

Protoplast-release-inducing protein (PR-IP) Inducer is a glycoprotein with a molecular mass of 18.7 kDa (Nojiri et al. 1995). It is released from mt− cells and induces mucilage secretion (Akatsuka et al. 2003), SCD (Tsuchikane et al. 2005) and the production of PR-IP (Sekimoto et al. 1994a, Sekimoto 2002) in mt+ cells. PR-IP is a glycoprotein, consisting of 19- and 42-kDa subunits, that induces the release of gametic protoplasts from mt− cells (Sekimoto et al. 1990), mucilage secretion and SCD of mt− cells (Akatsuka et al. 2006). The cDNAs that encode these sex pheromones have been cloned from heterothallic strains (NIES-67 and NIES-68; mating group I-E, Sekimoto et al. 1994b,
1994c, 1998a). We previously identified conjugation-related and pheromone-responsive genes (Seikimoto et al. 2006).

The heterothallic C. psl. complex is composed of several reproductively isolated mating groups (Watanabe 1977, Watanabe and Ichimura 1978), whose phylogenetic relationships are known (Tsuchikane et al. 2008). The cDNAs that encode orthologs of PR-IP Inducer have been cloned from two mating groups (groups II-A and II-B; Tsuchikane et al. 2008).

We recently observed the detailed conjugation process in a homothallic strain in culture (Tsuchikane et al. 2010). All the zygospores were formed within a single clonal cell, but most were produced by the pairing and fusion of two sister gametangial cells that originated from one vegetative cell. For conjugation to occur, cell density in the culture was critical; conjugation proceeded well at 10^3–10^5 cells per 2 ml. When cell density was high (1×10^5 cells per 2 ml) conjugation rarely occurred, and at extremely low cell densities (1×10^2 cells per 2 ml) the process was delayed, but progressed well after some proliferation. In addition, we detected a conjugation-promoting factor in a high cell density culture, which promoted conjugation in 1×10^5-cell cocultures. The cocultures were separated by a filter membrane that allowed the passage of dissolved chemicals, but not Closterium cells (Tsuchikane et al. 2010). However, no biochemical characteristics of the substance were reported.

In this study, we characterized the conjugation-promoting and -suppressing activities of homothallic cells in cell-free conditioned media, and established a suitable bioassay system. To elucidate the identity of the active substances, we cloned cDNAs that encode the orthologous PR-IP Inducer. We demonstrated the biological activity of the recombinant pheromones and discuss the role of sex pheromones in homothallic strains.

### Results

#### Detection and characterization of conjugation-promoting and -suppressing activities

Various numbers of cells were cultured in conditioned media for 72 h. When 1×10^2 cells were incubated, the number of gametangial cells, protoplasts and zygospores increased compared with those incubated in mating (MI) medium (Fig. 1A, B). In contrast, when 1×10^4 cells were cultured in the same conditioned medium, zygospore formation was suppressed (Fig. 1C, D). When 1×10^5 cells were cultured in the same conditioned medium, zygospore formation was equal to that of cells cultured in MI media (Fig. 1E, F). These conjugation-promoting and -suppressing activities were further characterized.

Conjugation-promoting and -suppressing activities were rare in conditioned medium from dark-grown cultures, and when conditioned medium was used in assays carried out in darkness (data not shown). Incubation of the conditioned medium at 60°C for 10 min decreased the promotion and suppression activities to 20% of their initial level (Fig. 2). To estimate the molecular mass of these active substances, conditioned medium was concentrated using an anion-exchange column, and subjected to gel filtration. The active substances were eluted at a position corresponding to an apparent molecular mass of 17 kDa (Fig. 3A, B). These data indicate that the conjugation-promoting and -suppressing molecules were proteins or glycoproteins. Furthermore, the biochemical characteristics of the active substances (light dependency, heat stability, and apparent molecular mass) were similar to those of the previously isolated heterothallic sex pheromone, PR-IP Inducer (Seikimoto et al. 1993, Nojiri et al. 1995).

#### Isolation of cDNA that encodes an ortholog of the PR-IP Inducer

Using a combination of degenerate and rapid amplification of cDNA ends (RACE)–PCR, cDNA that encodes the orthologous PR-IP Inducer was successfully isolated from kodama20 (AB558136; CpPI-kd01; Fig. 4A). The sequence of the full-length cDNA contained an open reading frame with coding capacity for a protein of 227 amino acid residues. The alignment of putative sex pheromones indicated that two of three putative N-glycosylation sites in the heterothallic strains of the C. psl. complex were conserved at almost the same position in CpPI-kd01 (Fig. 4A).

The cDNA fragment that encodes the entire secreted portion of the PR-IP Inducer was amplified from the first-strand cDNA population. The cDNA fragments corresponding to the coding region were collected, ligated into the multiple cloning site of the YEpFLAG-1 expression vector and introduced into Escherichia coli. Plasmids were isolated from 16 independent clones and the sequences of those clones were analyzed. Four additional sequences that also encode the orthologous PR-IP Inducer were obtained and named CpPI-kd02, 03, 04 and 05 (AB558137, AB558138, AB558139 and AB558140, respectively; Fig. 4B). Of the 16 clones, two, eight, four, one and one of them were found to match CpPI-kd01, 02, 03, 04 and 05, respectively. Using the alignment of putative mature peptide sequences, Bayesian trees were generated (Fig. 5). The groups containing the heterothallic sex pheromones CpPI-A and CpPI-B and the homothallic CpPI-kd01, 02, 03, 04 and 05 constituted robust clades [1.00% posterior probability; 99 and 100% bootstrap probability for maximum likelihood (ML) and neighbor-joining (NJ), respectively]. In further experiments, CpPI-kd01, 02 and 03 were selected and used for the preparation of recombinant PR-IP Inducer.

#### Assay of conjugation-promoting and -suppressing activities of the recombinant PR-IP Inducer

Yeast cells that had been transformed with the expression vector YEpFLAG1 containing DNA fragments of the secreted portion of the PR-IP Inducer (CpPI-kd01, 02, 03) were cultured in YP4 High Stability Expression Media for 72 h. The cultured media were analyzed by SDS–PAGE followed by Western blotting with the anti-FLAG M2 antibody. The recombinant pheromones exhibited a pattern of indistinct bands with apparent...
molecular masses around 20–100 kDa (Fig. 6A). To check the degree of glycosylation of pheromones, affinity-purified pheromones were exposed to peptide-N-glycosidase F (PNGase F), which removes oligosaccharides from N-linked glycoproteins. The bands shifted to give a polypeptide band with an apparent molecular mass of 18.3 kDa corresponding to the deduced molecular mass of the non-glycosylated FLAG-tagged polypeptide (17,271.86 Da; Fig. 6).

When 1×10^2 vegetative kodama20 cells were incubated in MI medium containing various concentrations of the purified recombinant PR-IP Inducer, the number of zygospores increased in a dose-dependent manner, reaching a maximum
at $3 \times 10^{-8}$ M recombinant PR-IP Inducer (Fig. 7). Promoting activities were not observed at a higher concentration ($10^{-7}$ M; Fig. 7). Conjugation-suppressing activity in $1 \times 10^4$-cell cultures was not be detected at any concentration of recombinant PR-IP Inducer, even at $3 \times 10^{-8}$ M, the concentration suitable for conjugation promotion (data not shown).

**Discussion**

Our previous report suggested the presence of a conjugation-promoting factor in the homothallic strain, kodama20 (Tsuchikane et al. 2010). In the present study, we detected both conjugation-promoting and -suppressing activities in conditioned media; they were critically dependent on cell density (Fig. 1). The formation of zygospores in $1 \times 10^4$-cell cultures was promoted, and that in $1 \times 10^2$-cell cultures was suppressed by incubation in the same conditioned medium. Both conjugation-promoting and -suppressing active pheromones were heat labile (Fig. 2), produced under light conditions and had apparent molecular masses of 17 kDa (Fig. 3A, B). These characteristics are similar to those of the sex pheromone PR-IP Inducer, previously isolated from the heterothallic *C. psl.* complex (Sekimoto et al. 1993, Nojiri et al. 1995). We isolated cDNAs that encode orthologous PR-IP Inducers from the sexually isolated mating groups IIA and IIB (Tsuchikane et al. 2008). Because the homothallic and heterothallic pheromones are probably closely related, cDNAs from kodama20 that encode the orthologous PR-IP Inducer were analyzed.

In kodama20, at least five genes (CpPI-kd01, 02, 03, 04 and 05) possibly encode the orthologous PR-IP Inducer. According to Bayesian analysis, these deduced proteins are a monophyletic group, and gene duplication occurred after the divergence of the heterothallic group and the homothallic kodama20 (Fig. 5). Based on the alignment of 1,506 group I introns that interrupt nuclear SSU rDNAs, kodama20 appears to be closely related to the heterothallic mating group II-B, which was partially isolated from group II-A (Tsuchikane et al. 2010). However, our present
analysis indicates that the sister relationship between heterothallic CpPIs and homothallic CpPIs is not coincident with the phylogenetic relationships based on the group I introns. It seems likely that these CpPI-kds have mutated positively in the homothallic strain. Further isolation of genes that encode the PR-IP Inducer in other homothallic strains is important to clarify the phylogenetic relationship of sex pheromones between heterothallic and homothallic strains.

The recombinant PR-IP Inducers (CpPI-kd01, 02 and 03) were expressed in yeast and exhibited patterns of indistinct

**Fig. 4** Comparison of the deduced amino acid sequences of PR-IP Inducers from homothallic and heterothallic strains. (A) Alignment of deduced amino acid sequences of sex pheromones. CpPI-A, CpPI-B, CpPI-E and CpPI-kd20: PR-IP Inducer from groups II-A, II-B, I-E and kodama20, respectively; SCD-IP, sexual cell division-inducing pheromone isolated from *C. ehrenbergii*. Putative extracellular secreted portions (mature peptide portion of the sex pheromones) are underlined. Frame with broken line, consensus sequences for the asparagine-linked glycosylation sites. (B) Alignment of deduced amino acid sequences of CpPI-kds in kodama20.

**Fig. 5** Bayesian tree of the deduced amino acid sequences of the PR-IP Inducer using 140 amino acids of the putative mature sex pheromones. Numbers indicate posterior probabilities from Bayesian analysis (left), and bootstrap values from ML (middle) and NJ (right) analyses. Branch lengths represent amino acid substitutions per site.
bands (Fig. 6). This is due to the asparagine-linked glycosylation system of yeast, and corresponds to previous reports (Sekimoto 2002). All recombinant PR-IP Inducers showed conjugation-promoting activities in $1 \times 10^5$-cell cultures (Fig. 7), indicating that these orthologous pheromones play significant roles in sexual reproduction in homothallic strains. In contrast, these recombinant pheromones showed no conjugation-suppressing activity in $1 \times 10^4$-cell cultures, even at a concentration suitable for conjugation promotion in $1 \times 10^2$-cell cultures. Although the characteristics of the conjugation-suppressing pheromone are quite similar to the previously characterized heterothallic PR-IP Inducer, the active substance may be different than the orthologs. Alternatively, the recombinant proteins produced in yeast may not possess the activity required to suppress zygospore formation in the homothallic strain, i.e. it may only be present in the native orthologous PR-IP Inducer produced in Closterium. This possibility awaits confirmation; a recently developed gene-introduction system for Closterium cells would be useful for this, because it allows the production of native recombinant pheromones (Abe et al. 2008).

We previously reported that optimum cell density (between $1 \times 10^3$ and $1 \times 10^4$ cells per 2 ml) is required for the progress of conjugation in homothallic strains (Tsuchikane et al. 2010). At a low cell density ($1 \times 10^2$ cells per 2 ml), neither gametangial cell formation nor conjugation was observed in the first 3 d of cultivation in MI medium. However, these cells proliferated in the nitrogen-depleted condition to about $1 \times 10^3$ cells within 3 d. Then, remarkably, zygospore formation was observed 4 d after the beginning of the incubation. In contrast, incubation at a higher cell density ($10^5$ cells per 2 ml MI medium) showed a low conjugation ratio. It seems that cells discern and regulate their density to achieve conjugation through a mechanism similar to the quorum sensing observed in some types of bacteria (Camilli and Bassler 2006). Therefore, the PR-IP Inducer may not be released into the medium unless cell density is optimal. To clarify this possibility, it necessary to isolate a putative ‘autoinducer’-like substance.

In the heterothallic strain, PR-IP Inducer is released from one mt cell in nitrogen-depleted medium in the light; it induces
SCD and the production of PR-IP in complementary mt cells (Tsuchikane et al. 2005). In homothallic kodama20, the pheromone is also produced in nitrogen-depleted medium in the light, and it stimulates the formation of gametangial cells by SCD. Presently, we have no information about orthologous PR-IP in homothallic strains; however, it is possible to speculate that the production of orthologous PR-IP is stimulated by homothallic PR-IP Inducer and is required for conjugation.

During homothallic conjugation, most of the observed zygospores originate from fusion of two sister gametangial cells produced from one vegetative mother cell. The gametangial cells seem to be able to discern their own sister, although all the gametangial cells derived from different vegetative cells are theoretically clonal (Tsuchikane et al. 2010). Our data indicate that SCD is a segregative process that produces complementary mt in homothallic strains that are equivalent to those in heterothallic strains; however, SCD is not thought to involve meiosis. If this is true, homothallic PR-IP Inducer may be functional during sex differentiation in the homothallic strain, and the putative orthologous PR-IP would be produced in only one of the sister gametangial cells. To clarify this hypothesis, we are attempting to isolate cDNAs that encode orthologous subunits of PR-IP.

Materials and Methods

Strains and vegetative culture
The origin of the axenic homothallic clone of C. psalpiniformis (kodama20) used in the present study was previously published (Tsuchikane et al. 2010). Clonal culture was grown in 300-ml Erlenmeyer flasks containing 150 ml of nitrogen-supplemented medium (Tsuchikane et al. 2005). In homothallic kodama20, the pheromone is also produced in nitrogen-depleted medium in the light, and it stimulates the formation of gametangial cells by SCD. Presently, we have no information about orthologous PR-IP in homothallic strains; however, it is possible to speculate that the production of orthologous PR-IP is stimulated by homothallic PR-IP Inducer and is required for conjugation.

Preparation of conditioned medium
Vegetative cells in late logarithmic phase (at 14 d) were centrifuged and washed three times with nitrogen-depleted MI medium in a 300-ml Erlenmeyer flask and incubated under continuous light, unless otherwise stated. After 72 h of incubation, the numbers of cells in conjugation processes (gametangial cells, protoplast-releasing cells and zygospores) were counted. To test the thermal stability of the conjugation-promoting or -suppressing activities, the same conditioned media were incubated at different temperatures for 10 min, immediately cooled on ice just after treatment and used for assays.

Gel filtration of conjugation-regulating activity
The conditioned media (1,083 ml) were filtered through filter paper (Filter Paper Qualitative; Advantest MFS Inc., Dubline, CA, USA) and mixed with 57 ml of 1M Tris–HCl buffer (pH 8.0) to a final concentration of 50 mM Tris–HCl. These media were applied to a DEAE–Sepharose CL-6B (Amersham Pharmacia Biotech, Little Chalfont, UK) column (25 mm diameter, 70 mm long), equilibrated with 50 mM Tris–HCl buffer (pH 8.0). Proteins were eluted with the same buffer supplemented with 250 mM NaCl at a flow rate of 10 ml h⁻¹. The elute was applied to a Sephacryl S-100 HR (Amersham Pharmacia) column (2.5 mm diameter, 750 mm long), equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl at a flow rate of 5 ml h⁻¹. Each 1.8-ml fraction was collected, and small aliquots of each were added to MI medium for assay of conjugation-promoting or -suppressing activity.

Isolation of total RNA
Total RNA was isolated from cells that had been incubated in MI medium under continuous light for 48 h, as described previously (Tsuchikane et al. 2008).

Cloning and sequencing of cDNA that encodes the orthologous PR-IP Inducer
The synthesis of cDNA from total RNA was performed with the TAKARA RNA LA PCR Kit (AMV) ver. 1.1 (Takara Bio, Ohtsu, Shiga, Japan) according to the manufacturer’s instructions. 3’ RACE was performed using a degenerate primer [DP-1: 5’-T(A/T)CAAC(A/C)T(C/G)AC(C/G)TTCTACAAC-3’; Tsuchikane et al. 2008] and cDNA adapter-specific primer (5’-GTTTCCAGTGCAGC-3’). The PCP conditions were 95°C for 1 min; 35 cycles at 95°C for 1 min, 58°C for 0.5 min, and 72°C for 3 min; and 72°C for 3 min. After direct determination of the nucleotide sequences of the amplified products, the 5’ regions of the genes were amplified with a gene-specific primer (5’-CATAGTCTGACGACACCTC-3’) and adapter primer (5’-GACTGGAGACGAGGACCAT-3’) using the GenRacer Kit (Invitrogen, Carlsbad, CA). The PCR products were separated on a 1.5% agarose gel, collected using the SUPREC 01 column (Takara), ligated into pGEM T-easy vector (Promega, Heidelberg, Germany) and transformed into an E. coli strain (DH5α). Plasmid DNA including target insert DNA was extracted from the E. coli suspension using the GFX Micro Plasmid Prep Kit (Amersham Biosciences Corp., Piscataway, New Jersey) according to the manufacturer’s instructions. The origin of the axenic homothallic clone of C. psalpiniformis (kodama20) used in the present study was previously published (Tsuchikane et al. 2010). Clonal culture was grown in 300-ml Erlenmeyer flasks containing 150 ml of nitrogen-supplemented medium (Tsuchikane et al. 2005). In homothallic kodama20, the pheromone is also produced in nitrogen-depleted medium in the light, and it stimulates the formation of gametangial cells by SCD. Presently, we have no information about orthologous PR-IP in homothallic strains; however, it is possible to speculate that the production of orthologous PR-IP is stimulated by homothallic PR-IP Inducer and is required for conjugation.

Preparation of conditioned medium
Vegetative cells in late logarithmic phase (at 14 d) were centrifuged and washed three times with nitrogen-depleted MI medium (Ichimura 1971). Cells in this suspension were counted under a microscope using a custom-made counting chamber (0.25 × 0.25 mm) to determine cell density. The counts were repeated at least three times to ensure accuracy. The total cell number was adjusted to approximately 3.6 × 10⁷ in 72 ml of MI medium in a 300-ml Erlenmeyer flask and incubated under continuous light, unless otherwise stated. Medium from the culture was passed through a membrane filter (MILLEX HV; Millipore, Bedford, MA, USA) to remove cells after incubation.

Biological activity of conditioned medium and the recombinant PR-IP Inducer
Vegetative cells were washed three times with fresh MI medium and then cultured in MI medium (control), conditioned medium or MI medium containing various concentrations of purified recombinant PR-IP Inducer at a density of 1 × 10⁶, 1 × 10⁷ or 1 × 10⁸ cells per 2 ml in a test tube (17.5 mm diameter, 130 mm length) under continuous light, unless otherwise stated. After 72 h of incubation, the numbers of cells in conjugation processes (gametangial cells, protoplast-releasing cells and zygospores) were counted. To test the thermal stability of the conjugation-promoting or -suppressing activities, the same conditioned media were incubated at different temperatures for 10 min, immediately cooled on ice just after treatment and used for assays.

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PCR products were treated with both and confirmed. Amplified products and the FLAG fusion junction were verified. Subcloned plasmids were collected and the sequences of the translation initiation of a yeast FLAG fusion protein was determined. The DNA fragment that encodes the entire secreted portion of the PR-IP Inducer was amplified from the first-strand cDNA population. The primers used were indkd20Ecoupper (5′-AAGGAATTCCTCGCCACCACCTCGCG-3′) and indkdBamlower (5′-CTAGGATCCCTAAATGACGGCCCGGGCT-3′). The sequence of the indkd20Ecoupupr-sense primer was designed to allow introduction of the EcoRI restriction site immediately upstream of the coding regions. The sequence of the indkdBamlower primer was designed to allow introduction of the translational stop codon (TAG) and BamHI restriction sites immediately after the 3′ coding regions. The respective PCR products were treated with both EcoRI and BamHI and ligated into the multiple cloning sites of the YEpFLAG-1 expression vector (Sigma, St Louis, MO, USA). The multiple cloning sites were directly preceded by the 24-bp DNA coding sequence of the α-factor signal peptide. Subcloned plasmids were collected and the sequences of the amplified products and the FLAG fusion junction were verified and confirmed.

**Construction of the expression plasmid for recombinant proteins**

The DNA fragment that encodes the entire secreted portion of the PR-IP Inducer was amplified from the first-strand cDNA population. The primers used were indkd20Ecoupper-sense (5′-AAGGAATTCCTCGCCACCACCTCGCG-3′) and indkdBamlower (5′-CTAGGATCCCTAAATGACGGCCCGGGCT-3′). The sequence of the indkd20Ecoupupr-sense primer was designed to allow introduction of the EcoRI restriction site immediately upstream of the coding regions. The sequence of the indkdBamlower primer was designed to allow introduction of the translational stop codon (TAG) and BamHI restriction sites immediately after the 3′ coding regions. The respective PCR products were treated with both EcoRI and BamHI and ligated into the multiple cloning sites of the YEpFLAG-1 expression vector (Sigma, St Louis, MO, USA). The multiple cloning sites were directly preceded by the 24-bp DNA coding sequence of the α-factor signal peptide. Subcloned plasmids were collected and the sequences of the amplified products and the FLAG fusion junction were verified and confirmed.

**Expression and purification of recombinant proteins**

Correct constructs were introduced into host cells of Saccharomyces cerevisiae (strain BJ3505; Sigma) using the lithium acetate transformation method, in accordance with the manufacturer’s instructions. As a negative control, transformants with control plasmids (YEpFLAG-1) were also produced. To induce the production and secretion of recombinant PR-IP Inducer, transformed yeast cells were cultured with YP4 high-stability expression medium (Sigma). Recombinant PR-IP Inducer was purified from cultured medium using an anti-FLAG M2 affinity gel column (Sigma). The eluate was subjected to ultrafiltration (Ultrafree-CL, 5000 MWCO; Millipore) and the buffer was changed to MI medium. The protein content was determined using bovine serum albumin as the standard, according to the method of Bradford (1976).

**Deglycosylation of recombinant proteins**

Deglycosylation of recombinant PR-IP Inducer was performed as previously described (Sekimoto 2002).

**SDS–PAGE and immunoblot analysis**

SDS–PAGE and immunoblotting using anti-FLAG M2 antibody were performed as previously described (Tsuchikane et al. 2005).

**Flamingo staining**

Proteins on the gel were stained with Flamingo Fluorescent Gel Stain (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer’s instructions, and observed using a fluorescent imaging analyzer (Molecular Imager FX Pro; Bio-Rad).

**Sequence analysis**

Sequences of PR-IP Inducers (CpPi-A, CpPi-B and CpPi-E; DDBJ accession numbers BAG74746, BAG74747 and BAA88710, respectively) and SCD-IP (BAC80145) in Closterium ehrenbergii (NIES-229; Fukumoto et al. 2003) were obtained from DNA databases. Alignment of deduced amino acid sequences was performed with Clustal X (version 1.81; Thompson et al. 1997). Gaps were removed from the aligned sequences for phylogenetic analysis. For Bayesian analysis, we applied a WAG model (Whelan and Goldman 2001). Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Two runs with four chains of Markov Chain Monte Carlo (MCMC) iterations were performed for 10^6 generations, keeping one tree every 100 generations. The first 25% of generations were discarded as burn-in, and the remaining trees were used to calculate a 50% majority-rule tree and to determine the posterior probabilities of the individual branches. The local bootstrap probability of each branch was estimated by the resampling-of-estimated-log-likelihood (RELL) method (Kishino et al. 1990, Hasegawa and Kishino 1994). Phylogenetic trees were rooted with sequences of SCD-IP from C. ehrenbergii.

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