Molecular Cloning of a Novel Sex Pheromone Responsible for the Release of a Different Sex Pheromone in *Closterium peracerosum-strigosum-littorale* Complex

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A sex pheromone, protoplast-release-inducing protein (PR-IP) inducer, of the *Closterium peracerosum-strigosum-littorale* complex is known to induce the release of PR-IP, from mating-type plus (mt+) cells during sexual reproduction. The purified PR-IP inducer was treated with trypsin to obtain internal peptides for determination of partial amino acid sequences. Using these sequences, oligonucleotides were synthesized and used as primers for the combined reverse transcription-PCR. A 296 bp cDNA fragment was amplified, permitting the cloning of corresponding full length cDNA (*CpPI; Closterium peracerosum-strigosum-littorale* complex PR-IP inducer). The deduced amino acid sequence of *CpPI* encodes a protein of 212 amino acid residues of **M**, 23,071 whereas portion of the peptide secreted is predicted to have 142 amino acid residues of **M**, 15,717 and shows no significant similarity with known proteins. The predicted protein has three possible consensus sequences for asparagine-linked glycosylation site. The *CpPI* gene was expressed when mating-type minus (mt−) cells were incubated at a low cell density in the light. Nitrogen deprivation from the medium enhances expression of the *CpPI* gene. An analysis by genomic Southern hybridization revealed that the cDNA probe hybridized to several DNA fragments obtained from both the genome of mt− and mt+ cells. However, in mt− cells, transcripts for the PR-IP inducer could not be detected by Northern hybridization.

Key words: *Closterium* — Conjugation — Gamete —

A considerable amount of work has been reported on sex pheromones in brown algae. These pheromones are volatile and low molecular weight compounds (Maier 1993, 1995). Although their possible mechanisms of action have been proposed so far they have not yet been fully elucidated (Maier and Müller 1986, Maier and Calenberg 1994).

In contrast, only a limited amount of information has been published for green algae. A sexual inducer in *Volvox carteri* is one of the best-characterized pheromonal substances (Al-Hasani and Jaenicke 1992, Kochert and Yates 1974, Mages et al. 1988, Tschochner et al. 1987). It is a glycoprotein, known to be synthesized and released by sexual males at about the same time as sperm packets are released (Starr and Jaenicke 1974). Its possible mechanism of action has been postulated (Gilles et al. 1984, Godl et al. 1995, 1997, Sumper et al. 1993). In addition, a sex pheromone "hurlene" has been reported in the heterogamous green algae, *Chlamydomonas allensworthii* (Starr et al. 1995). It is a plastohydroquinone derivative produced by less-motile female gametes which attracts motile male gametes at a concentration as low as 1 pM.

The involvement of diffusible substances for intercellular communication has been suggested in the sexual reproduction of *Closterium* species, which are unicellular conjugatophycean algae (Coesel and de Jong 1986, Fukumoto et al. 1997, 1998, Hogetsu and Yokoyama 1979, Ichimura 1971, Kato et al. 1981). A "protoplast-release-inducing protein" (PR-IP) was the first sex pheromone isolated from *Closterium* cells (Sekimoto et al. 1990). The PR-IP consists of 19-kDa and 42-kDa subunits, which are released from mt+ cells inducing the release of protoplasts from mt− cells. The induction of protoplast release by PR-IP proceeds only after an appropriate preculture period under...
continuous light, during which time mt" cells differentiate from vegetative cells to gametes (Sekimoto and Fujii 1992). During this period, a receptor for PR-IP appears on mt" cells (Sekimoto et al. 1993b). The presence of a second sex pheromone named "PR-IP inducer" produced by mt" cells has been demonstrated (Sekimoto et al. 1993a). It causes the inhibition of the genes for each PR-IP subunit in mt" cells (Sekimoto et al. 1994). This PR-IP inducer has been purified and preliminary biochemical informations obtained (Nojiri et al. 1995).

In this paper, the isolation of a cDNA encoding the PR-IP inducer from mt" cells is reported and the regulation of its expression described.

Materials and Methods

Strains and vegetative culture—Strains of the heterothallic Closterium peracerosum-strigosum-littorale complex used in this work were NIES-67 (mt") and NIES-68 (mt') obtained from the National Institute for Environmental Studies, the Environmental Agency (Ibaraki, Japan). Clonal cultures were grown in nitrogen-supplemented medium (C medium, Ichimura 1971) as previously described (Sekimoto et al. 1990).

Purification of PR-IP inducer and sequencing of proteolytic peptides—The PR-IP inducer was purified as a single protein with a molecular mass of 18.7 kDa on matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, from a 75-ml aliquot of nitrogen-deficient medium (C medium, Ichimura 1971) in a 300-ml Erlenmeyer flask, in which 0.9 x 10^6 mt" cells had been cultured for 48 h, as previously described (Nojiri et al. 1995). Purified protein was directly subjected to automated Edman degradation using an ABI model 477A protein sequencer connected on-line to a model 120A phenylthiohydantoin (PTH)-analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.). Purified protein was also cleaved with trypsin at 37°C for 12 h after reduction and S-carboxymethylation. The digested peptides were separated on a column Supersphere RP-select B (2 x 120 mm; Merck) with a model 1090M (Hewlett Packard) liquid chromatography system. They were eluted at a flow rate of 0.2 ml min"1 and subjected to MALDI-TOF mass spectrometry on a Reflex MALDI-TOF (Bruker-Franzen Analytik, Bremen, Germany) in linear mode using c-cyano-4-hydroxycinnamic acid as a matrix.

Mating culture for RNA preparation—Vegetatively grown mt" cells were harvested, washed twice with MI medium, and then suspended in MI medium at a cell density of 3 x 10^5 cells ml"1 (high cell density condition) in a tube (27 mm diameter, 11.5 cm long). After 12 h incubation, cells were cultured at a low cell density (0.2 mlmin"1) and subjected to MALDI-TOF mass spectrometry on a Reflex MALDI-TOF (Bruker-Franzen Analytik, Bremen, Germany) in linear mode using c-cyano-4-hydroxycinnamic acid as a matrix.

Northern-hybridization analysis—Total RNA (15 lg lane"1) was fractionated on a 1.17% (w/v) agarose gel that contained 5% (v/v) formaldehyde (Maniatis et al. 1989) and transferred to a nylon membrane (Hibond N; Amersham International, Amersham, Buckinghamshire, U.K.) by capillary action in alkaline transfer solution, PCR amplification was carried out using 0.5 lg of total RNA from mt" cells following reverse transcription with the GeneAmp RNA PCR kit (Perkin-Elmer) according to the manufacturer's instruction. The resulting first strand cDNA was 5-fold diluted and subjected to 2 cycles of amplification (95°C for 0.5 min, 50°C for 0.5 min and 68°C for 1.5 min) followed by 19 cycles of amplification (95°C for 0.5 min and 68°C for 1.5 min) in the presence of both sense (5'-GCCAATCTT-GGCCACACCCAGTGGG-3') and antisense primers (5'-TCCAGGTCGAGCCGAGC-3'). As a control experiment, PCR amplification was carried out using total RNA without reverse transcription.

Analysis of DNA gel blot—Genomic DNA was prepared from vegetative mt" and mt' cells as described previously (Sekimoto et al. 1995). The DNA was digested with EcoRI, fractionated on a 1.17% (w/v) agarose gel, denatured, and transferred to a GeneScreen Plus membrane (NEN Research Products, Boston, MA, U.S.A.) by capillary action in alkaline transfer solution (0.4 M NaOH, 0.6 M NaCl). After blotting, the membrane was incubated in 0.5 M Tris-HCl buffer (pH 7.0) that contained 1 M NaCl for 15 min, followed by baking at 80°C for 2 h. The membrane was then hybridized in a buffer (0.1x SSC, 1% SDS) at 65°C for 2 h. Denatured 32P-labeled full-length cDNA insert, that was labeled with [a-32P]dCTP by the random-priming method (Multiprime DNA labelling system, Amersham) and denatured salmon sperm DNA, were then added to the prehybridization solution, and the filters were incubated at 60°C for 20 h, washed with 2 x SSC, 1% SDS at 60°C, and subjected to autoradiography.
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Table 1  Amino acid sequences of the amino terminus and tryptic peptides of PR-IP inducer

<table>
<thead>
<tr>
<th>Number</th>
<th>Location</th>
<th>Determined amino acid sequences</th>
<th>Molecular mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-term</td>
<td>72-104</td>
<td>LATTxGGSTVTrSVQLPGFDLhsYIFxMTFYN-</td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>72-84</td>
<td>LATTCCGGSTVTr</td>
<td>1,323.5</td>
</tr>
<tr>
<td>#2</td>
<td>161-191</td>
<td>xAAGNVQxFPGETVNilAYwAYDFxPxEEL-</td>
<td></td>
</tr>
<tr>
<td>#3</td>
<td>85-117</td>
<td>SQYLGFDLHSYIFxMTFYNGCAYxVTnPLVcq-</td>
<td></td>
</tr>
<tr>
<td>#4</td>
<td>161-200</td>
<td>xAAGNVQMFPGETVNilAYWADFrPCIEELhFscefyi-</td>
<td></td>
</tr>
</tbody>
</table>

Purified PR-IP inducer was cleaved with trypsin and peptides were separated by reversed-phase HPLC. Separated peptides (#1-4) were subjected to amino acid sequence analysis and to MALDI-TOF mass spectrometry. Only peptide #1 gave a significant signal for the latter. Native protein was also subjected to amino acid sequencing. Lower case letters indicate weakly assigned residues, "x" gave no significant PTH-amino acid signal during sequence analysis.

cubated at 65°C for 20 h, washed with 2 x SSC, 1% SDS at 65°C, and subjected to autoradiography.

Results

Partial amino acid sequences of purified PR-IP inducer and cDNA cloning—PR-IP inducer was purified with near homogeneity through sequential column chromatographic steps (Nojiri et al. 1995). The amino terminal sequence was determined and internal sequence information for the PR-IP inducer was also obtained from tryptic peptides (Table 1). This information allowed the cloning of a cDNA fragment corresponding to the PR-IP inducer mRNA. PCR with a combination of two degenerate primers produced a 296 bp cDNA fragment. Comparison of the deduced amino acid sequences of these cDNA with the amino acid sequences of corresponding peptides indicated that the cDNA fragment might encode part of the PR-IP inducer. The sequences of the remaining 5'- and 3'-stretches were established by cloning of the full-length cDNA using nucleotide sequence information from the fragment (Fig. 1). The complete cDNA sequence was determined from several independent clones.

Analysis of the deduced amino acid sequences of CpPI—One nearly full-length cDNA exhibits a 81 bp 5' untranslated region, followed by a 639 bp open reading frame and a 253 bp 3' untranslated sequence (Fig. 2). This cDNA is designated CpPI (Closterium peracerosum-strigosum-littorale complex PR-IP inducer). The open reading frame predicts a protein of 212 amino acids of Mr 23,071 and a translation product contains the amino terminal sequence and the peptides obtained by tryptic digestion of the purified protein (see also Table 1).

The deduced CpPI protein possesses a hydrophobic signal sequence in the predicted amino-terminal region (Fig. 1, 3), which is consistent with the fact that the PR-IP inducer is an extracellular glycoprotein (Sekimoto et al. 1993a, Nojiri et al. 1995). A putative cleavage site for the signal sequence could be predicted between Ala25 and Arg26 (Von Heijne 1983, Fig. 2). From the amino terminal sequence information of the purified protein, the molecular mass of the peptide portion of the secreted protein is Mr 15,717, consisting of 142 amino acids. The predicted protein has three possible consensus sequences [N-X-(T/S)-X; X is not a proline] for asparagine-linked glycosylation sites (amino acid residues 99-102, 109-112, 203-206; Fig. 2). Two of those asparagine residues are located in regions giving no significant PTH-amino acid signal during sequence analysis.
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**Fig. 2** Nucleotide and deduced amino acid sequences of CpPI. The sequence was determined from both DNA strands. Peptides sequences determined from purified PR-IP inducer are underlined. Double line (= = =), consensus sequence for asparagine-linked glycosylation site; double underline, putative polyadenylation signal; black box with white letter, amino-terminal residue of native PR-IP inducer secreted from *Closterium* mt" cells; arrow, a putative cleavage site of signal peptide.

Analysis (Table 1), indicating a likely addition of glycans. The protein coding region of cDNA has a relatively high GC-content (63.7%).

A computer search using the nucleotide sequence and the deduced amino-acid sequences failed to reveal any homology to known proteins.

**RT-PCR and Northern-hybridization analysis of CpPI—RT-PCR** analysis revealed that transcripts of the CpPI gene could be observed within 2 h of incubation in nitrogen-deficient mating medium at a low cell density in the light (Fig. 4). No expression was observed in cells that had been cultured in nitrogen-deficient medium at a high cell density (0 h incubation of low cell density culture). Only a low level of expression was observed in cells cultured in nitrogen-supplemented medium.

Northern-blot experiments revealed that the CpPI gene was highly expressed when mt" cells were incubated in nitrogen-deficient mating medium in the light (Fig. 5). The transcript size was estimated to be approximately 1.0 kb, which is very close to the size of cloned cDNAs.
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Fig. 3 Hydrophilicity/hydrophobicity plot of the deduced amino acid sequence of CpPI. The profile was generated by the method of Hopp and Woods (1981) with a window of 5 amino acids.

Expression was observed in cells that had been cultured in nitrogen-deficient medium at a low cell density in darkness and vegetative cells (nitrogen-supplemented medium, grown at a relatively high cell density in the light, Fig. 5).

Genomic-Southern hybridization of CpPI—Southern hybridization revealed that the cDNA probe hybridized to EcoRI-digested genomic DNA fragments of 12.5, 10.0, 8.5 and 6.0 kb (Fig. 6). Ten- and 6.0-kb fragments gave more intense signal than others. Moreover, these four fragments identified in the genome of mt− cells were also detected in the genome of mt+ cells, even though no transcript for CpPI was detected by Northern hybridization in the latter case (Fig. 7).

Discussion

Amino acid sequence information of purified PR-IP inducer led us to the successful cloning of full-length cDNA (CpPI). The predicted amino acid sequence contains the sequence of both the amino-terminal end and peptides obtained by trypsin cleavage of the purified protein and shows the presence of three possible asparagine-linked glycosylation sites. Since native PR-IP inducer is a glycosylated protein (Nojiri et al. 1995) and the mt− cell-specific gene expression (Fig. 7) coincides with release of PR-IP inducer from mt−

Fig. 4 Time course of the expression of CpPI gene. Total RNA was isolated from mt− cells that had been incubated in nitrogen-deficient medium for 0, 2, 4, 8, 12 and 24 h or in nitrogen-supplemented medium for 8 h (N+) at a low cell density in the light. (A) PCR amplification of CpPI mRNA after reverse transcription, in the presence of both sense and antisense specific oligonucleotides. Arrow indicates amplified band (435 bp). (B) Same experiment as (A) except that no reverse transcription was carried out before PCR amplification.

Fig. 6 DNA gel blot analysis of Closterium mt+ and mt− cells. Genomic DNA was digested with EcoRI, fractionated on an agarose gel, transferred to a nylon membrane and hybridized with the 32P-labeled full-length CpPI cDNA.
cells (Sekimoto et al. 1993a), we conclude that CpPI encodes the PR-IP inducer.

The CpPI protein contains a relatively long pre-peptide (70 amino acids). It is well known that some proteins have a signal sequence for targeting them into the endoplasmic reticulum followed by an additional peptide for maturation, activation or regulated secretion (pre-pro type) of the protein itself, however, at present there is no biochemical information concerning the intermediate form of the PR-IP inducer before secretion.

In a previous paper, we showed the necessity of light for the release of the PR-IP inducer from mt− cells (Sekimoto et al. 1993a). As illustrated in Figure 5, light is also required for the expression of the CpPI gene in mt− cells. During sexual reproduction, light is also indispensable for the differentiation of vegetative mt− cells to gametes (Sekimoto and Fujii 1992), the synthesis of the putative receptor for the 19-kDa subunit of PR-IP on mt− cells (Sekimoto et al. 1993b). As shown in Figure 4, gene expression of CpPI also depends on the cell density, however, this has yet to be analyzed in detail.

CpPI cDNA hybridizes to genomic DNA fragments from both mt− cells and mt+ cells (Fig. 6), although no expression of the CpPI gene was detected in Northern-blot in the latter case (Fig. 7). This indicates that sex-specific regulation of gene expression must occur, in addition to the environmental regulation. In a previous paper, we had demonstrated a parallel result for cDNAs of PR-IP subunits hybridized to respective genomic DNA fragments from mt+ and mt− cells, while both PR-IP subunit genes were synchronously expressed only in mt+ cells which had been incubated with PR-IP inducer (Sekimoto et al. 1994). Recently, 5′-upstream regions from both PR-IP subunit genes were cloned and some sequences which are possibly involved in the synchronized sex-specific expression of both subunit genes have been postulated (Endo et al. 1997). In the yeast Saccharomyces cerevisiae, mating type is determined by a single locus, which encodes a regulatory protein that controls the expression of pheromone and pheromone-receptor genes (Bölker and Kahmann 1993). It is thought that the gene expression of both PR-IP and the inducer of Cloustonium may be closely regulated by the mating-type locus in the genome. Similar to the case of PR-IP, isolation of the 5′-upstream region of the CpPI gene and its molecular analysis will be important for elucidating mating-type specific gene expression and the sex-determination mechanism in this organism.

In addition, four genomic DNA fragments, which varied in the intensities of signal, were labeled by CpPI cDNA (Fig. 6), whereas no EcoRI-recognition sequence could be found in the cDNA (Fig. 1). As it is unlikely that the pheromone is encoded by a DNA region of more than 30 kb, this result indicates the existence of more than one related gene in the genome. In the case of Volvox carteri, pheromorphins sharing homology with the sex-inducing pheromone were shown to be encoded by a multigene family (Godl et al. 1995).

We have reported a new sex pheromone involved in sexual reproduction of Closterium ehrenbergii (Fukumoto et al. 1997). This pheromone is responsible for induction of sexual cell division of the mt+ cells. Most recently, we have successfully purified the substance and determined the partial amino-acid sequence (Fukumoto et al., unpublished data). Surprisingly, it shows high level of homology to predicted CpPI protein. Since native PR-IP inducer has no
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In plants, many pheromones involved in sexual reproduction have been reported, however, no pheromone gene has been cloned which is responsible for the expression of another sex pheromone gene. The present study provides such data for the Closterium peracerosum-strigosum-littorale complex and should assist our attempts to elucidate the intercellular and intracellular communication of a plant cell at the molecular-biological level.

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