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The Sex-Inducing Pheromone and Wounding Trigger the Same Set of Genes in the Multicellular Green Alga *Volvox*

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The sex-inducing pheromone of the multicellular green alga *Volvox carteri* is a glycoprotein that triggers development of males and females at a concentration $<10^{-16}$ M. By differential screening of a cDNA library, two novel genes were identified that are transcribed under the control of this pheromone. Unexpectedly, one gene product was characterized as a lysozyme/chitinase, and the other gene product was shown to encode a polypeptide with a striking modular composition. This polypeptide has a cysteine protease domain separated by an extensin-like module from three repeats of a chitin binding domain. In higher plants, similar protein families are known to play an important role in defense against fungi. Indeed, we found that the same set of genes triggered by the sexual pheromone was also inducible in *V. carteri* by wounding.

INTRODUCTION

The sex-inducing pheromone (sex inducer) of the multicellular green alga *Volvox carteri* is a glycoprotein that is probably among the most potent biological effector molecules known. At a concentration of $<10^{-16}$ M, the pheromone converts asexually growing males and females to the sexual pathway, producing in the next generation sperm- and egg-containing organisms, respectively (Starr and Jaenicke, 1974; Tschochner et al., 1987; Mages et al., 1988).

The asexually growing organism of *V. carteri* is composed of only two cell types exhibiting a complete division of labor: 2000 to 4000 biflagellate *Chlamydomonas*-like somatic cells are arranged in a monolayer at the surface of a hollow sphere (Starr, 1969, 1970), and 16 much larger reproductive cells ("gonidia") lie just below the somatic cell sheet. Eleven or 12 rapid and synchronous cleavage divisions of a gonidium generate all of the cells of an adult organism. *Volvox* cells are surrounded and held together by a glycoprotein-rich extracellular matrix (ECM) (for review, see Kirk et al., 1986; Sumper and Hallmann, 1998).

Many lines of evidence indicate that the ECM plays a key role in the sexual induction process. The earliest biochemical responses to the pheromone detected thus far are structural modifications within the ECM (Wenzl and Sumper, 1982, 1986; Gilles et al., 1983). In particular, the volvocacean ECM contains members of the newly described pherophorin family (Sumper et al., 1993; Godl et al., 1995, 1997). Pherophorins are glycoproteins that contain a C-terminal domain with homology to the sex-inducing pheromone. Some pherophorins are incorporated into the ECM constitutively, but under the influence of the pheromone, synthesis of other members of the pherophorin family is initiated, and from a subset of pherophorins (pherophorin II), this pheromone-like domain becomes proteolytically liberated from the parent glycoproteins. It has been proposed that this modification and processing of the ECM are part of the signal amplification process required to achieve the exquisite sensitivity observed for this sexual induction system.

A molecular–genetic approach was initiated to detect additional genes that might be induced under the influence of the sex-inducing pheromone. By differential screening of a cDNA library from sexually induced females, two novel clones were detected. They encode polypeptides that are not members of the pherophorin family. These clones were characterized in detail and found to encode a chitinase/lysozyme and a cysteine protease that is combined with three repeats of a chitin binding domain. Chitinases and chitin binding proteins are known to play an important role in a plant's defense against fungi (Boller, 1988; Bowles, 1990; Linthorst, 1991). Therefore, our completely unexpected observation prompted us to compare the genes activated by the sex-inducing pheromone with those induced by wounding. Surprisingly, all genes known thus far to be under the control of the sex-inducing pheromone are also activated in response to wounding.

RESULTS

Differential Screening

Total RNA was isolated from *V. carteri* spheroids (female HK10) harvested at various times after the addition of the

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sex-inducing pheromone. RNA samples isolated 3, 6, and 12 hr after the application of the pheromone were pooled, and a cDNA library (λZAP) was constructed from the corresponding poly(A)$^+$ mRNA preparation. By differential screening of the cDNA library with cDNA derived from asexual versus sexually induced organisms, two novel clones were detected in addition to members of the pherophorin II family described previously (Sumper et al., 1993; Godl et al., 1995, 1997). The kinetics of mRNA accumulation in response to the sex-inducing pheromone was analyzed by RNA gel blotting for both of these novel clones (denoted as clone A and clone B). As shown in Figure 1, hybridizing RNA corresponding to clone A started to accumulate ~2 hr after pheromone treatment. Only ~20 min after the application of pheromone was transcription of the clone B gene strongly initiated. No significant signals were observed in asexually growing organisms. The RNAs detected by clone A and clone B cDNAs are 1.4 and 3.7 kb in length, respectively.

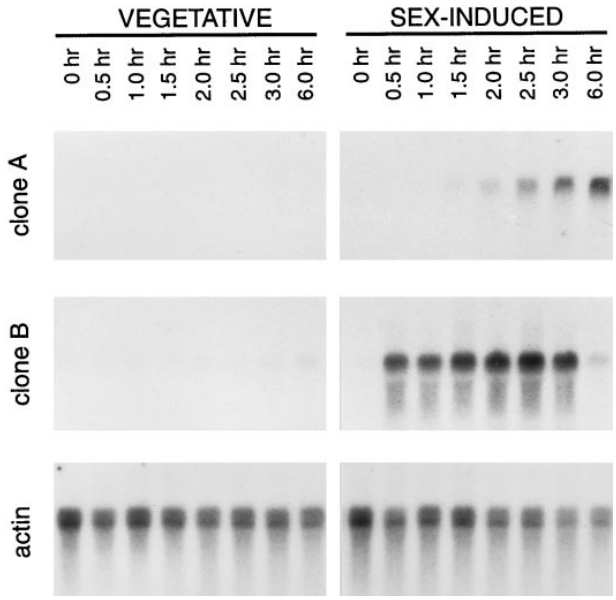

Figure 1. RNA Gel Blot Analysis of Clone A and Clone B mRNAs.

The accumulation of mRNA in vegetative or sexually induced organisms (by treatment with 10^{-12} M sex-inducing pheromone at 0 hr) was determined. Equal amounts of total RNA isolated after different periods of incubation from vegetative or sexually induced *Volvox* organisms were hybridized with clone A, clone B, and actin (Cresnar et al., 1990) to separate cDNA probes. The actin probe was used as a control.

Deduced Amino Acid Sequences of Clones A and B

To extend the cDNA sequence information obtained from the originally isolated cDNAs, rapid amplification of cDNA ends polymerase chain reaction (Frohman et al., 1988) was used to obtain the needed 5′ stretches. This technique resulted in the complete cDNA sequence for clone A (verified by primer extension experiments), whereas a stretch of unusually high GC content within the cDNA sequence of clone B caused premature termination of reverse transcription. Therefore, genomic DNA encoding the clone B gene was cloned and partially sequenced to circumvent this problem in that particular area. 5′ upstream sequences were again collected by rapid amplification of cDNA ends polymerase chain reaction. Finally, a full-length cDNA sequence of clone B was produced by reverse transcription of mRNA with Taq polymerase in the presence of Mn^{2+} (Myers and Gelfand, 1991), followed by amplification of clone B cDNA by polymerase chain reaction.

The deduced amino acid sequences for clones A and B are shown in Figures 2A and 2B. Both amino acid sequences have striking features. The sequence encoded by clone A is 309 amino acid residues in length and includes a typical signal sequence, and its N-terminal half is composed of two nearly perfect repeats of a 48–amino acid sequence motif. These repeats are separated by a typical spacer element (SGGGGSTPTSTAPPAR). Similar repeats located in phage lysozymes (Garvey et al., 1986; Paces et al., 1986), a muramidase (Chu et al., 1992), and an autolysin from *Streptococcus faecalis* (Beliveau et al., 1991) were identified as top-scoring hits in a BLASTP search (Altschul et al., 1990) of the Swiss-Prot protein sequence database. The region of identities covers almost the entire length of the 48–amino acid repeat and is documented in Figure 3. This repeat is characteristic of enzymes of the lysozyme/chitinase family (Joris et al., 1992), which also includes vacuolar and secreted chitinases from plants (Holm and Sander, 1994). These data indicate the function of a chitinase for the clone A–encoded protein, and indeed, this enzymatic activity could be demonstrated for the clone A polypeptide (see below).

A highly modular composition is a striking feature of the deduced amino acid sequence of clone B. Three repeats of a domain that is 48 amino acid residues in length constitute the C-terminal part. Proline-rich sequences with Ser-$(Pro)_n$ motifs characteristic of extensin modules separate these domains from each other. Again, a homology search revealed a close relation of this repeating unit to a well-known protein family, namely, to chitin binding proteins (Raikhel et al., 1993). In Figure 4A, the chitin binding domain of proteins such as wheat germ agglutinin, hevein, and the wound-induced proteins from potato (Stanford et al., 1989) are aligned with the clone B–derived sequences. The degree of homology found in the diagnostic region defined as “inner conserved core region” identifies the protein encoded by clone B as a member of this protein family. As shown below, the recombinantly produced clone B polypeptide indeed exhibits chitin binding activity. The N-terminal sequence of the *Volvox* chitin binding protein shares high homology with cysteine proteases such as a human cathepsin H (30%

identity and 65% similarity). The crucial amino acid residues engaged in catalysis (the catalytic triade) are completely conserved in the *V. carteri* polypeptide (asterisks in Figure 4B). This remarkable combination of a protease domain, extensin-like spacers, and several chitin binding domains suggests that this polypeptide may be specialized for the degradation of chitin-linked proteins. A similar domain combination, however, with different types of spacer elements, has recently been described for a prokaryotic enzyme produced by *Streptomyces griseus* (Sidhu et al., 1994).

Recombinantly Produced Clone A and Clone B Polypeptides

The complete cDNA from clone A and a fragment from clone B encoding all three putative chitin binding modules were cloned into the *Escherichia coli* expression vector pET19b. Different protein fractions of induced *E. coli* cells were analyzed. Figures 5A and 5B show the overproduction of a 31-kD clone A polypeptide and a 26-kD clone B polypeptide in *E. coli* BL21(DE3). Both polypeptides were found in the insoluble fraction. Both recombinant proteins containing a $(His)_{10}$ tag were purified under denaturing conditions by chromatography on chelating Sepharose and were used for the generation of antibodies in rabbits. For activity assays, the denatured polypeptides were first treated under conditions known to favor renaturation of cysteine-rich polypeptides (Rudolph, 1990).

Matrix Localization of Clone A and Clone B Polypeptides

The ECM of *V. carteri* can be fractionated easily into components derived either from the cell-free interior of the spheroid (deep zone according to the nomenclature of Kirk et al. [1986]) or from the material associated with the somatic cell sheets. Mild mechanical stress, as occurs when *Volvox* spheroids are forced through a hypodermic needle, slits the spheroids, producing hemispheres or smaller fragments of cellular sheets. The material of the deep zone is thereby selectively released. After low-speed centrifugation, the cell-free supernatant containing the material from the deep zone and the pellet containing somatic cell sheets and gonidia were subjected to SDS-PAGE and analyzed by protein gel blotting. As documented in Figure 6A, both clone A and clone B proteins can be identified as components of the deep zone fraction of the ECM. No clone A protein and only low levels of clone B protein are detectable in deep zone extracts from asexually growing spheroids compared with extracts from sexually induced organisms (Figure 6B). By reverse transcription–polymerase chain reaction analysis, transcripts of both clones A and B were found to be produced exclusively in somatic cells (data not shown).

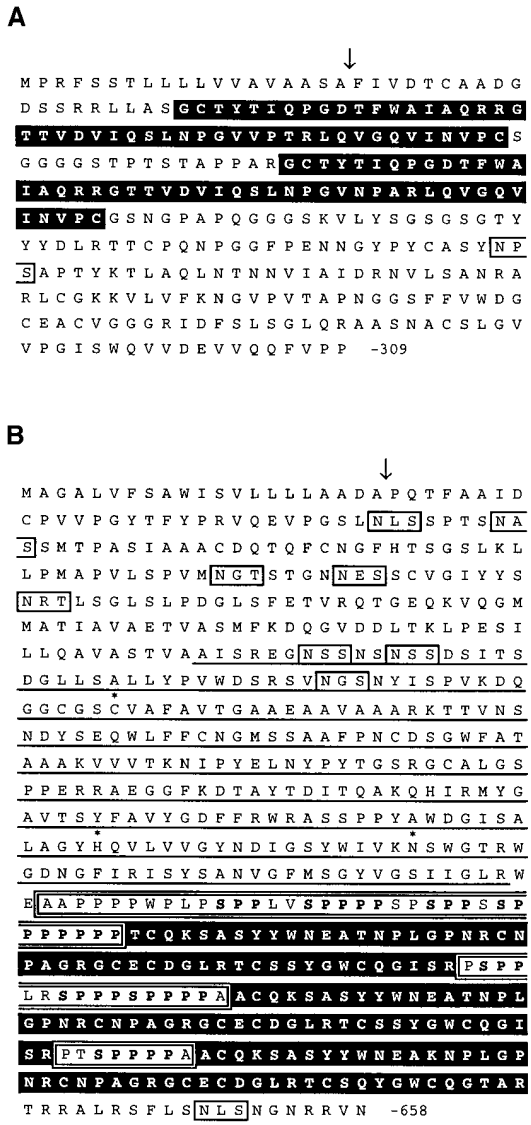

Figure 2. Deduced Amino Acid Sequences.

(A) Deduced amino acid sequence from clone A cDNA.
(B) Deduced amino acid sequence from clone B cDNA and genomic DNA.
Black boxes indicate a repeated sequence motif. Potential N-glycosylation sites are boxed. Arrows mark the potential cleavage sites of the signal peptide. In the sequence derived from clone B, the underlined sequence represents the stretch with homology to cysteine proteases, asterisks above individual amino acid residues mark the conserved catalytic triade, spacer elements are boxed, and typical extensin motifs (Ser-$[Pro]_n$) are shown in boldface letters.

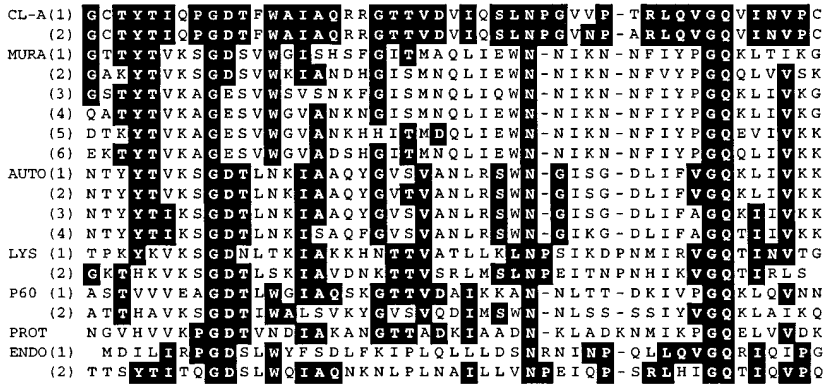

Figure 3. Aligned Amino Acid Sequences of Repeated Domains from Several Lysozme/Chitinase Enzymes and the Repeated Domains of Clone A.

Residues in black boxes show identities to the repeated domains (1) and (2) of clone A. Gaps (–) were introduced to allow maximal alignment. CL-A, clone A; MURA, *Enterococcus hirae* muramidase; AUTO, *Streptococcus faecalis* autolysin; LYS, phage lysozyme; P60, *Listeria monocytogenes* protein p60; PROT, *Staphylococcus aureus* protein A; ENDO, *Bacillus sphaericus* endopeptidase.

Functional Analysis of Clone A and Clone B Polypeptides

A deep zone extract from sexually induced *V. carteri* spheroids was analyzed for the presence of any chitinase enzyme by using an in-gel activity assay (Trudel and Asselin, 1989). For that purpose, deep zone extract (Figure 7A, lane 1), supernatant (lane 2), and immunoprecipitate (lane 3) obtained from deep zone extract with the clone A antibody as well as a control supernatant (lane 4) and precipitate (lane 5) obtained with preimmune serum were applied to an SDS–polyacrylamide gel (12%; containing 0.01% glycol chitin). Electrophoresis was conducted without heat denaturation and in the absence of thiol reagents. In addition, an authentic chitinase was applied in lane 6 (Figure 7A). As seen from Figure 7A, lane 3, the clone A antibody indeed precipitates a chitinase activity, and this enzyme exhibits the deduced molecular mass of the clone A product (30 kD). The preimmune serum was unable to precipitate this chitinase.

The putative chitin binding specificity of the repeating unit motif of the clone B polypeptide was assayed with the clone B polypeptide fragment recombinantly produced in *E. coli.* The polypeptide was mixed with BSA and incubated with insoluble chitin, according to the protocol of Kuranda and Robbins (1991). As documented in Figure 7B, BSA was recovered quantitatively in the supernatant, whereas the clone B polypeptide could be recovered only from the chitin pellet after heating in the presence of SDS, indicating the expected affinity of the polypeptide for this particular polysaccharide.

An in-gel protease assay using casein as substrate failed to detect proteolytic activity in immunoprecipitates obtained from deep zone extract by using the clone B antibody. The reason for this failure is unclear. Perhaps the clone B protein exhibits a high substrate specificity.

Wounding Induces Clone A and Clone B Genes

Chitinases and chitin binding proteins are thought to play an important role in plant defense reactions against pathogen attack. The high similarity of clone B–derived domains with the wound-inducible proteins WIN1 and WIN2 from potato (Stanford et al., 1989) led us to investigate whether the expression of clone A and clone B mRNAs is, in addition to sexual induction, responsive to wounding. First, *V. carteri* spheroids were slit by using the mild mechanical stress of forcing them through a hypodermic needle. Three or 8 hr later, total RNA was extracted from the broken spheroids and submitted to RNA gel blot analysis. As shown in Figure 8, expression of both clone A and clone B mRNAs is not detectable in asexually growing *V. carteri* colonies. In contrast, wounding induced the transcription of these genes at a rate almost as high as that induced by the sex-inducing pheromone.

Wounding Induces Pherophorin II Transcription

Transcription of the pherophorin II gene family was shown previously to be a characteristic response of *V. carteri* somatic cells to the application of the sex-inducing pheromone. Therefore, it was of interest to investigate whether these pheromone-controlled genes are also induced by wounding. The same type of experiment as described above was performed (RNA was extracted 60 min after wounding or pheromone application), and the resulting RNA gel blot was probed with a pherophorin II–specific cDNA. Surprisingly, even the expression of these pherophorin II proteins containing a pheromone-homologous domain in their C-terminal half was strongly induced in response to wounding (Figure 8).

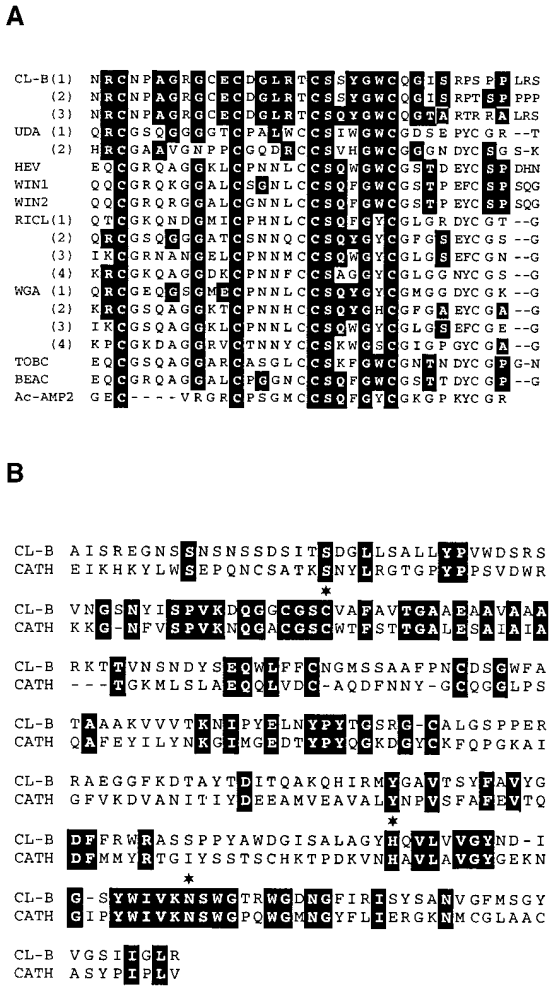

Figure 4. Sequence Comparison of the C- and N-Terminal Partial Sequences of Clone B with Chitin Binding Proteins and Cathepsin H.

(A) Alignment of the repeated C-terminal domains (1 to 3) of clone B with repeated domains (1, 2, 3, and 4, respectively) of several chitin binding proteins.
(B) Alignment of a clone B partial sequence (underlined in Figure 2) and human cathepsin H (CATH). Asterisks above individual amino acid residues mark the conserved catalytic triade.
Residues in black boxes show identities to sequences derived from clones A and B, respectively. Gaps (–) were introduced to allow maximal alignment. CL-B, clone B; UDA, *Urtica dioica* agglutinin; HEV, hevein; WIN, wound-induced proteins from potato; RICL, rice lectin; WGA, wheat germ lectin; TOBC, basic chitinase from tobacco; BEAC, basic chitinase from bean; Ac-AMP2, antimicrobic peptide from *Amaranthus caudatus.*

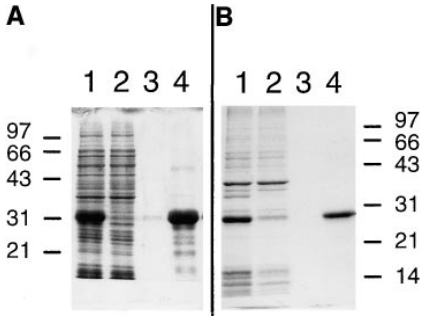

Figure 5. Expression of Clone A– and Clone B–Encoded Polypeptides Tagged with $(His)_{10}$ in *E. coli.*

(A) Clone A–encoded polypeptide.
(B) Clone B–encoded polypeptide.
Sequential steps of the purification analyzed by SDS-PAGE (12% polyacrylamide gel) and Coomassie Brilliant Blue R 250 staining. Lanes 1, dissolved pellet containing inclusion bodies; lanes 2, flowthrough of the chelating chromatography column; lanes 3, final wash fraction; and lanes 4, eluted fraction from the chelating chromatography column. The positions of molecular mass standards (given in kilodaltons) are indicated at left and right.

Wounding Does Not Induce Production of Pheromone

Induction of pherophorin II synthesis in response to wounding could indicate that wounding triggers production of sex-inducing pheromone in much the same way as it was demonstrated in response to heat shock (Kirk and Kirk, 1986). The most sensitive indicator for the presence of sex-inducing pheromone is an asexually growing *V. carteri* spheroid that responds to pheromone concentrations $<10^{-16}$ M with the initiation of sexual development. Therefore, a simple experiment was performed to check for the production of pheromone in response to wounding. A single intact *V. carteri* colony was added to a large excess of fragmented *V. carteri* spheroids (somatic cell sheets and isolated gonidia produced by passing spheroids through a hypodermic needle), and this indicator organism was observed for 24 hr. This simple type of experiment was repeated many times, and the indicator *V. carteri* colonies were never observed to initiate sexual development. Therefore, production of sex-inducing pheromone in response to wounding is extremely unlikely.

DISCUSSION

A particularly fascinating but unsolved problem is the molecular mechanism that enables the sex-inducing pheromone of *V. carteri* to act at a concentration as low as 10^{-16} M. The earliest biochemically detectable responses to the pheromone are structural modifications within the ECM. In

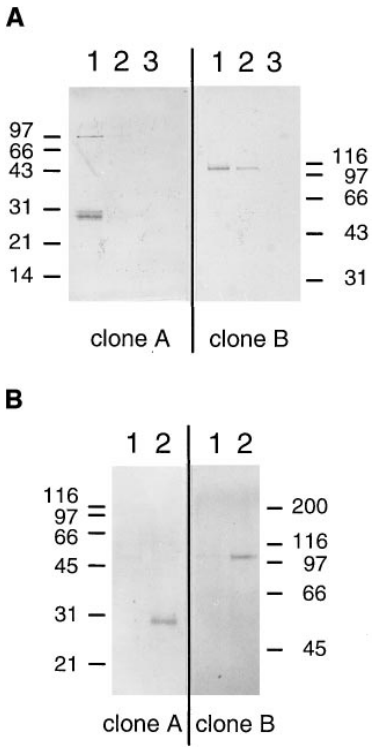

Figure 6. Cellular Localization of the Clone A– and Clone B–Encoded Protein.

(A) Protein gel blot analysis of extracts from sexually induced organisms. Sexually induced *V. carteri* organisms were disrupted by mild mechanical stress and fractioned by filtration, resulting in a cell-free extract containing the ECM material of the deep zone (interior of the sphere) (lanes 1), a preparation of somatic cell sheets (lanes 2), and pure reproductive cells (lanes 3). Samples of 2 μg of protein were separated on 12% (clone A) or 8% (clone B) SDS–polyacrylamide gels and electroblotted to a polyvinylidene fluoride membrane. Immunodetection was performed with the corresponding antiserum (1:10^4 dilution). The positions of molecular mass standards (given in kilodaltons) are indicated at left and right.

(B) Protein gel blot analysis of deep zone ECM extracts from vegetative and sexually induced organisms. Deep zone extracts (2 μg of protein) from vegetative (lanes 1) and sexually induced (lanes 2) organisms were processed and analyzed as described in **(A)**. The positions of molecular mass standards (given in kilodaltons) are indicated at left and right.

particular, induction of the biosynthesis of members of the pherophorin family that contain a domain with homology to the sex-inducing pheromone has been studied in detail (Sumper et al., 1993; Godl et al., 1995, 1997). These biosynthetic activities are restricted exclusively to the somatic cell population of the organism. The ultimate target of the pheromone's message, however, is the reproductive cell, which will divide eventually to produce a sexual offspring. Up to now, the biochemical response reactions of the reproductive cell remained undetected. Therefore, we took a molecular–genetic approach to identify additional genes that are triggered by the pheromone.

Differential screening for genes operating under the control of the sex-inducing pheromone resulted in the identification

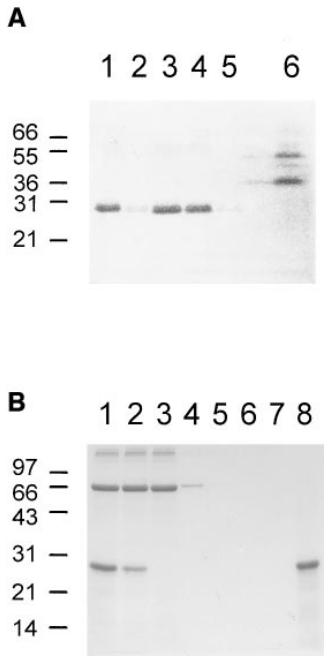

Figure 7. Assay of Chitinase Activity of the Clone A–Encoded Protein and Chitin Binding Properties of the Repeated Domains of Clone B.

(A) Chitinase activity of the clone A–encoded protein. All samples in lanes 1 to 5 were derived from equivalent amounts of an extract from deep zone ECM from sexually induced organisms. Lanes were loaded as follows: lane 1, unfractionated extract; lane 2, the supernatant, and lane 3, the precipitate produced after treatment of the extract with anti–clone A antibody; lane 4, the supernatant, and lane 5, the precipitate produced after treatment of the extract with preimmune serum. An authentic chitinase (18 microunits) from *Serratia marcescens* was applied in lane 6. SDS-PAGE (12% polyacrylamide gel containing 0.01% glycol chitin) was without heat denaturation of samples and in the absence of thiol reagents. The gel was incubated for 48 hr. Lytic zones were resolved by UV illumination after staining with calcofluor white M2R. The positions of molecular mass standards (given in kilodaltons) are indicated at left.

(B) An equimolar mixture of a recombinantly produced repeating domain polypeptide encoded by clone B (amino acid residues 472 to 658) and BSA was treated with insoluble chitin and analyzed by SDS-PAGE on a 12% polyacrylamide gel with Coomassie blue staining. Lane 1 contains the protein mixture without chitin; lane 2, supernatant immediately after the addition of chitin; lane 3, supernatant after incubation for 4 hr; lanes 4 to 7, wash fractions of the insoluble chitin; and lane 8, proteins liberated from insoluble chitin after elution with hot SDS. The positions of molecular mass standards (given in kilodaltons) are indicated at left.

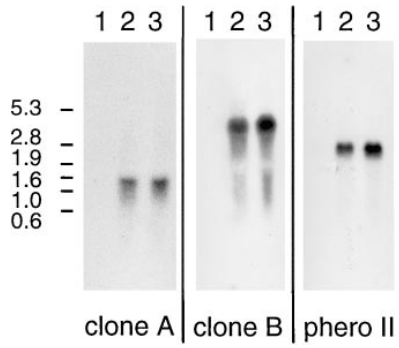

Figure 8. RNA Gel Blot Analysis of Clone A, Clone B, and Pherophorin II mRNA.

Accumulation of mRNA after treatment with the sex-inducing pheromone (10^{-12} M) and after wounding was determined. Equal amounts of total RNA isolated from asexually growing *Volvox* organisms (lanes 1), from organisms treated with the sex-inducing pheromone (lanes 2), and from organisms incubated after wounding (lanes 3) were hybridized with the corresponding cDNA probes. RNA was extracted after 6 hr for clone A analysis, after 3 hr for clone B analysis, and after 1 hr for pherophorin II (phero II) analysis. The positions of molecular mass standards (in kilobases) are indicated at left.

of two novel genes encoding extracellular proteins that are again produced by the somatic cells. One encodes a member of the lysozyme/chitinase family, and the other encodes a putative cysteine protease combined with three chitin binding modules. The chitin binding domains of the latter protein show extensive identity to the corresponding domains of other proteins, such as the class I chitinases (Linthorst et al., 1990), hevein (Broekaert et al., 1990), and the wound-induced WIN proteins from potato (Stanford et al., 1989). Induced expression of these proteins in cells of higher plants by wounding, microbial infection, and activity of the plant stress hormones has been implicated as a defense mechanism against pathogen infection. Very unexpectedly, all genes known thus far to be under the control of the *V. carteri* sex-inducing pheromone (including members of the pheromone-related pherophorins) also were found to be inducible by wounding (for instance, simply by slitting this multicellular algal organism). Therefore, the puzzling conclusion from this work is that the sex-inducing pheromone and wounding either produce the same extracellular signal or use a common signal transduction pathway. What might be the biological reason for this fact?

Volvox normally grows asexually as long as the environment offers optimum conditions. *V. carteri* lives in temporary ponds that dry out in the heat of late summer. Asexual *Volvox* would die in minutes once the pond dries out. However, *V. carteri* is able to escape this catastrophe by switching to the sexual life cycle shortly before the water disappears, producing dormant zygotes that survive the drought. Therefore, switching to the sexual pathway is the essential prerequisite to responding successfully to environmental stresses such as heat and drought. Indeed, Kirk and Kirk (1986) were able to demonstrate that production of the sex-inducing pheromone can be triggered in somatic cells by a short heat shock applied to asexually growing organisms. Therefore, induction of sexuality obviously is an essential element of response reactions to environmental stress. In this context, it appears to make sense that sexual induction and wounding use a common signal transduction pathway.

An alternative possibility can be envisaged for the biological role of clone A and clone B proteins. Rather than acting as disease-preventing agents, they could serve a function in ECM assembly during *Volvox* development. Because wounding as well as pheromone application cause repair and/or remodeling reactions within the ECM, upregulation of these gene products might be expected. However, this scenario would require the existence of chitin-like macromolecules within the *Volvox* ECM. To our knowledge, chitin or chitin-like substances have never been observed as ECM constituents of any member within the order Volvocales. Chitin-like macromolecules, however, have been identified as cell wall components in certain strains of *Chlorella* (Kapaun and Reisser, 1995). Therefore, this alternative possibility for the biological role of clone A and clone B proteins appears unlikely but cannot be ruled out.

In higher plants, several chemical signals have been identified that regulate the wounding response, including oligosaccharides (Bishop et al., 1984), plant hormones (Kernan and Thornburg, 1989; Pena-Cortés et al., 1993), the octadecapeptide systemin (Pearce et al., 1991), and components of the octadecanoid pathway (Vick and Zimmerman, 1983). In *Volvox*, the sex-inducing pheromone and wounding are likely to produce the same primary signal, but its chemical nature is as yet unknown.

METHODS

Volvox carteri Strains and Culture Conditions

Wild-type *Volvox carteri* HK10 (female) was obtained from R.C. Starr (Culture Collection of Algae, University of Texas, Austin). Synchronous cultures were grown in *Volvox* medium (Provasoli and Pintner, 1959) at 28°C in an 8-hr-dark/16-hr-light (10,000 lux) cycle (Starr and Jaenicke, 1974).

Screening of a cDNA Library

A *V. carteri* cDNA library was prepared from polyadenylated RNA, extracted from sexually induced organisms, and purified according to Kirk and Kirk (1985) by using the λZAP cDNA synthesis kit (Stratagene, La Jolla, CA). Replica filters were probed with ^{32}P-labeled cDNA prepared from polyadenylated RNA extracted from sexually induced or asexually growing *V. carteri* spheroids. Hybridization was performed according to standard procedures (Sambrook et al., 1989). Nucleotide sequences were determined by the dideoxy chain

termination method (Sanger et al., 1977). Synthetic oligonucleotides were used to sequence clone A and clone B cDNAs in both directions. The sequence data have been deposited in the GenBank database under accession numbers AF058716 (clone A) and AF058717 (clone B).

Screening of a Genomic Library

Hybridization of an existing *V. carteri* λ-EMBL 3 genomic library (Ertl et al., 1989) was done as described by Sambrook et al. (1989), using a ^{32}P-labeled cDNA of clone B.

RNA Gel Blot Analysis

RNA (10 μg) was size fractionated on a 1.0% denaturing gel, blotted, and cross-linked onto Hybond-N membrane (Amersham) and hybridized with a ^{32}P-labeled cDNA of clone A, clone B, and pherophorin II, respectively. The RNA gel blot was washed in 0.2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS at 65°C.

Expression of cDNA in *Escherichia coli* and Antibody Preparation

cDNA encoding amino acid position 20 to 309 of clone A or position 472 to 658 of clone B was ligated into the NdeI and BamHI sites of *E. coli* expression vector pET19b (Novagen, Lugano, Switzerland), and the recombinant plasmid was transformed into *E. coli* BL21(DE3). After induction with isopropyl β-D-thiogalactoside, a fusion protein containing the $(His)_{10}$ tag was found to be located in inclusion bodies. After solubilization in 6 M urea, the protein was purified to near homogeneity by chromatography on chelating Sepharose, according to a Novagen protocol. The purified material was used to raise polyclonal antibodies in rabbit. Refolding of urea-denatured proteins was performed in 0.6 M argininophosphate in the presence of 5 mM glutathione and 0.5 mM glutathione disulfide, according to Rudolph (1990).

Wounding of *V. carteri* Spheroids

V. carteri spheroids were slit into hemispheres and smaller fragments by forcing a concentrated suspension through a 0.5-mm hypodermic needle.

Immunoprecipitation

Precipitation of the clone A polypeptide was done following the procedure of Harlow and Lane (1988). A deep zone extract of sexually induced *V. carteri* spheroids was mixed with polyclonal immune serum at a ratio of $1{:}10^{-4}$ (v/v) and incubated on a rotary shaker overnight at 4°C. Antigen–antibody complexes were collected by binding to protein A–Sepharose and centrifugation. After several washes with 50 mM Tris-HCl, pH 7.5, the antigen was released by treatment with SDS.

Chitinase and Chitin Binding Assay

The chitinase assay in polyacrylamide gels was performed by the technique described by Trudel and Asselin (1989). The assay for chitin binding activity followed the procedure described by Kuranda and Robbins (1991).

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