

Molecular Characterization of a Zygote Wall Protein: An Extensin-Like Molecule in *Chlamydomonas reinhardtii*

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The green alga *Chlamydomonas reinhardtii* elaborates two biochemically and morphologically distinct cell walls during its life cycle: one surrounds the vegetative and gametic cell and the other encompasses the zygote. Hydroxyproline-rich glycoproteins (HRGPs) constitute a major component of both walls. We describe the isolation and characterization of a zygote-specific gene encoding a wall HRGP. The derived amino acid sequence of this algal HRGP is similar to those of higher plant extensins, rich in proline and serine residues and possessing repeating amino acid motifs, notably X(Pro)₃ and (Ser-Pro)_n. Antiserum against this zygote wall protein detected common epitopes in several other zygote polypeptides, at least one of which is also encoded by a zygote-specific gene. We conclude that there is one set of HRGP wall genes expressed only in zygotes and another set that is specific to vegetative and gametic cells.

INTRODUCTION

In higher plants, the major proteins of the cell wall are hydroxyproline-rich glycoproteins (HRGPs) called extensins (Dougall and Shimbayashi, 1960; Lamport and Northcote, 1960). Sequence analyses of several extensins and extensin-encoding genes (reviewed by Cooper et al., 1987; Showalter and Varner, 1989; Varner and Lin, 1989) have documented a number of common features: the proteins are basic, rich in hydroxyproline (Hyp), lysine (Lys), serine (Ser), tyrosine (Tyr), and valine (Val); they contain repetitive motifs, a common example being Ser(Hyp)₄; and they are highly glycosylated on their Ser and Hyp residues with short arabinose-containing oligosaccharides. By electron microscopy they appear as fibrous rods, consistent with a polyproline-II extended helical conformation.

Lamport originally proposed (1965) that the extensins might be involved in plant cell morphogenesis, analogous to the many roles demonstrated for collagens in animal development (Trelstad, 1984). Since then it has been shown that the HRGPs are encoded by multi-gene families, and that particular transcripts accumulate in the presence of such agents as ethylene, wounding, and pathogen attack (reviewed by Varner and Lin, 1989). Different plant cell types produce varying amounts of extensin in highly specific arrays (Cassab and Varner, 1988), strongly suggesting a role in morphogenetic patterning. To date, however, there are no reported examples of specific extensins associated with specific developmental events in higher plants.

In the green alga *Chlamydomonas reinhardtii*, by contrast, there is considerable evidence that two distinct sets of HRGPs are present in the cell wall at two different stages of the life cycle. The walls of the vegetative and gametic cells are highly ordered structures that carry a chaotrope-soluble crystalline layer composed of well-characterized HRGPs (Catt, Hills, and Roberts, 1978; Goodenough and Heuser, 1985, 1988; Roberts et al., 1985; Goodenough et al., 1986; Adair et al., 1987). When gametes mate, their walls are shed, and the zygotes proceed to elaborate new walls that are denser, thicker, lack the crystalline layer, and are insoluble in chaotropes and SDS (Minami and Goodenough, 1978; Grief, O'Neill, and Shaw, 1987). The glycopolypeptides in both types of cell walls are rich in hydroxyproline (Grief, O'Neill, and Shaw, 1987) but differ markedly in their electrophoretic mobility (Minami and Goodenough, 1978). Therefore, *Chlamydomonas* appears to switch between two HRGP "programs," one expressed by vegetative/gametic cells and the other by zygotic cells. While this could reflect the presence of two separate sets of HRGP genes, the above-cited studies do not rule out other possibilities, such as alternate post-translational modifications of common gene products.

We have recently constructed two cDNA libraries from *C. reinhardtii* zygote mRNA and, by differential screening, have identified eight unique classes of cDNAs expressed only in zygotes (Ferris and Goodenough, 1987; G. Matters, unpublished data). In this paper, we present evidence that one of these cDNA classes, class IV, encodes a zygote wall HRGP, and we examine the expression of this protein in vitro and in vivo. Since this gene is not expressed in

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vegetative or gametic cells, we conclude that a switch in HRGP gene expression accompanies zygote differentiation in *C. reinhardtii*.

RESULTS

Sequence Analysis

The 1060-bp class IV cDNA clone analyzed in this study (Figure 1) was isolated from a λ gt10 cDNA library of 3-hr zygote mRNA using a shorter class IV cDNA (No. 25 described in Ferris and Goodenough, 1987) as a probe. The class IV genomic clone (15.6 kb) presented in Figure 1 was isolated from an EMBL3 λ library of total DNA from *C. reinhardtii* strain CC-620 by screening with nick-translated cDNA 25. Restriction maps of both the 1060-bp class IV cDNA and its corresponding genomic clone (Figure 1) were used to localize the transcribed region within the genomic sequence. This localization was confirmed by probing DNA gel blots (Southern, 1975) of the genomic clone with the nick-translated cDNA (data not shown).

The DNA sequence of the entire class IV cDNA and 2234 bp of the genomic clone was determined and an open reading frame of 606 bp was identified (Figure 2). The open reading frame could encode a protein of 202 amino acids with a derived molecular mass of 19,806 kD; however, there is an apparent signal sequence of 28 amino acids that would yield a molecular mass of 17,014 kD for the mature protein. The signal sequence was delineated using the guidelines set forth by von Heijne (1985).

The candidate initiating methionine (the first in the open reading frame) is found in the sequence ACGAAGAUGC. The scanning model for translation (Kozak, 1989) proposes a consensus sequence for initiation in higher eukaryotes as GCC⁺CCAUGC. An informal survey of various *Chlamydomonas* nuclear genes indicates that only the adenosine positioned three nucleotides upstream of the AUG is highly conserved. This situation is similar to that in yeast mRNA sequences (Kozak, 1989).

Restriction mapping data from both the genomic and cDNA clones indicated that the class IV gene contained an intron that falls upstream of the initiating methionine, starting 141 bp from the 5' transcription start site and ending 10 bp upstream of the methionine (Figure 2). Although this is an odd position for an intron, introns upstream of the coding region have been noted in other genes; *hsp83* in *Drosophila melanogaster* (Hackett and Lis, 1983), and the polyubiquitin gene in both humans and chickens (Schlesinger and Bond, 1987). Incidentally, there is an intron in the 3'-untranslated region of the carrot extensin gene (DC5A1; Chen and Varner, 1985), but no intron was found in the only other full-length genomic clone of an extensin gene (soybean SbPRP1; Hong, Nagao, and Key, 1987). The 5' and 3' splice junctions of the class IV gene's intron match closely the consensus splice sequences for *Chlamydomonas* introns, XG|GTG⁺G_C...CAG|G, as noted in Zimmer et al. (1988).

The genomic sequence following the 3' end of the cDNA revealed a putative polyadenylation signal (TGTA) lying 4 bp beyond the end of the cDNA. Typical of nuclear genes from *Chlamydomonas*, the class IV gene has a long 3'-nontranslated sequence (approximately 400 nucleotides).

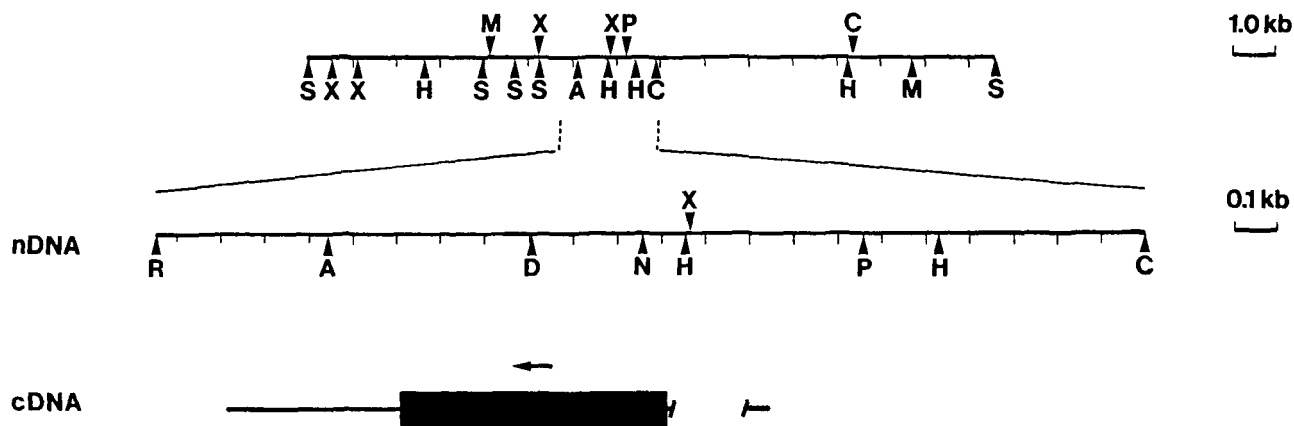


Figure 1. Restriction Map of the 15.6-kb Genomic Clone Showing the Location and Organization of the Class IV Gene.

The top line gives a map of the entire 15.6-kb EMBL3 genomic clone, whereas the second line (nDNA = nuclear DNA) shows an expanded view of the 2.2-kb region subjected to DNA sequence analysis. The position of the class IV cDNA on the genomic restriction map is illustrated by the bottom line (cDNA). The slanted hash marks indicate intron boundaries, and the boxed region corresponds to the open reading frame. The arrow above the box shows direction of transcription. Restriction enzymes are as follows: A = AclI; C = SacI; D = DdeI; H = HindIII; M = SmaI; N = HincII; P = PstI; R = RsaI; S = Sall; and X = XhoI.

GAGCTCTTCAG

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-766 GTGGCTAGGCTGGCAGAGCGGTCGGCGCGCGGGAGCGGCTGTAAGCGCGGAGGAGAC -767
-706 CAGGACGGGCTGGTGGCGGCGAGGCTGATGGCGAGGCGAGGAGCTGGCGCGGAGGAG -707
-646 GGGCGCGGGAGGCGGCTGTCATTGTGCGTTCAAATGTTCCATTCAACAGACACTCGGCTGGC -647
-586 GACTGCGCGAGCGTGGCAGGCTGGCGGCTTGGCGCGCGCGGCGAGGAGCACCTGGAAAGCC -587
-526 TTGGGGGCTTAGGAGGTCGGCGGGGAAGTCGGGACGCTCGGCTCGCGTGGGGCTTGTGGC -527
-466 TGATACCGAGGAGCTGTCGAGGGGTCGGCTGGAGGAGGCGCGGCTGGGAAGAGCTGC -467
-406 TGTGCTCGACGCGGTCGACGAGGAGCGCGCGGGGCTGGGCGGCTGCGGTGGCGGAGT -407
-346 GGGCCGACAGCGGCTGCTGCTGTTATTTCAAAGCAAGCTTAAGCTTGTGATAACAGGGCT -347
-286 CGCATGAAGCGCGGAGAGCAGCGAGCGCCAGAGGAAGGCGAGCGGATGTTGACACATT -287
-226 GCGGGGGTTTGGCGGGGCTCTTAGGCTCTGCGGCTATTCGCTGGCGGTGGCACTGTGCAT -227
-166 ACATATCGGTTAGGAGCACTATGACAAAGCTGCACTCAGACAAAATGCAAAATCGGCAT -167
-106 TTTGATTGTCGAGGACACGACAGTTGAGAGAAATCGCGGCTATTCAGCGGAAGTGTG -107
41
-46 CCGATCCCGCGCACTATATATAGCTGCTGTCGCGATAGCATCTTACTATTAAACGCGACCTC 14
15 CACAGTCAAAGCGGTAGCGCGAGGTCACCGGCGCAGCGCATCCAAGCGATCCTCT 74
75 CCAAAGCGGATTCACGGTGGCGGACCGGCCCATACGTCGCGCTAGCCACATTCCGGATCGC 134
135 TTCCAG /GTGCGTATGGCGGAGTGGTCACCATCAGGCACTTGTGCGTGGCATGCCA 192
193 GTATACCAAGTGGCGGCGCTGGGGCGTATTGATCCATCTTTTGTGATAGCCATGCTCG 252
253 AGCGCGACGCGAAGCTTACCCCACTCCCTTGTATCCGCACTCGCAG\ AGCCAGCAAG 310
311 ATG CGC AAG GTC TCT TGT TTT GGC GGC GTA GTC GCG GCG GTG GCG 355
M R K V S A F G G A L V A A V A
1
356 GTC ATG TCA ATG TTG ACG ATG CCG ACG GCT GCG CTC GCG GCG CGC 400
V M S M L T M P G A A L A A R
16
401 ACC AGC CTG CTC GCA ACC GCC AAC GCA ACC GGC TAC GAC GCG CGC 445
T S L L A T A N A T A Y D A P
31
446 CCT CCT GAC TTC AGG TFG GAG CCG CPT CCC CAC GAG GCT GTG GAG 490
P P D F S F G P P P H E A V E
46
491 CCG CCG CCA GCG AAG GGC GCG GCG GCG TTC ACG CCG CCG CGC CAG 535
P P P A K G A A A F T P P P Q
61
536 GAC TTG TCC ACC GAG CCG CCG CCG GCG GCG TTT GAG CAG ACT TCG 580
D L S T E P P P A E F E Q T S
76
581 CCT CCG CCG TCG GCG TCG CCG TCG GCT TCG CCG TCA TCC TCA GCG 625
P P P S A S R S P S P S S S A
91
626 TCT CCG TCG CCA TCG CCG GCG CTT GTG CCG ATT CCG AAT GTG GCG 670
S P S P S P A L V P I P N V G
106
671 AAG ACT CCG TCT CCG AAG CCG AGC TCG AGC CCG AGC CCG AGC CCG 715
N T A S P N P S S S P S P S P
121
716 AGT CCG AGC CCG AGC CCG GCT GCG ACC TCA AGC CCG AGC CCG ATC 760
S P S P S P A A T S S P S P I
136
761 GCC AGC CCG CCG CCG CCG GCT ACC CTC ACG AAT CAG CCG CCG 805
A S P P P L P A T L T N Q P P
151
806 CCG AGC CCG GTG GAG CAG CTA GGT GCG AAC ACC GAG CCG GCG TCC 850
P S A V D N L G G N T Q A G S
166
851 GCG GCG GCG GCA CGT GGT GGT CTG CTG TTG CTT TCG TGT GTG GCG 895
G A A A R A G L L L L S C V A
181
896 GCG TCG GCG TTG GCT GTG CTG TGA CCAGTGGACCCGTCGAGGTGACGC 945
A W A F A V L
196
946 CGTGTGCTGGGTGTCAAGTGGAGGCTTCAGATAATGACGGGAGAGCTGCTCGGTCT 1005
1006 GTGAGACCTGATGCGCTCGCGGACCATTCACAGATGATCGACCTGAAGTAACG 1065
1066 TCTCTGTGTAGACATTTTACCTGGCTGCTTGAAGCGGCTAGCGGCGGAGCGCGGA 1125
1126 CGCCTTCAACTATCATCTGGCGTTTGAAGCAGTGATGAGCGGCTGTCACAGACGAA 1185
1186 GCGCTGCAAGCGTCCGCGCTAGCTGTGCTCCCGCTTGTATCCGAGCTGCGAGCGA 1245
1246 TTCTGTGCTGTCATCAGACAGTTGCACCATGTTCCGACAGTAGTCCAGATCTAATC 1305
1306 CAGATCATATTTCCAGCGCTCGCGGCTCAGCGCTCAAGCTGCATGCTGTCACATTCCACA 1365
1366 CGCTACTCTTGTCTCTCTGCTGAAGAGTTCTACCGTAATCTGTCAGCCCAATAAGCCG 1425
1426 ACTCACTACCGCTACTTCTGCTGCAACACCGCAGCTAC 1465

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Figure 2. Nucleotide Sequence of the Genomic Region Encoding the Class IV Gene.

The sequence of the noncoding DNA strand is presented. The open reading frame is arranged in triplets with the corresponding amino acids below. Numbering above the sequence is relative to the transcription start site, while numbering below the sequence indicates amino acid position within the open reading frame. Asterisks mark the 5' and 3' ends of the class IV cDNA. A putative promoter sequence is underlined. The intron sequence is demarcated by spaces and slashes. Within the open reading frame, the cleavage site for the putative signal peptide is marked by an arrowhead. A putative polyadenylation signal sequence is double-underlined.

Primer extension was used to localize the transcription start site (Figure 3). A 17-nucleotide primer matching the sequence from base pair 140 to 124 (see Figure 2) was labeled and extended with reverse transcriptase, yielding four strong bands of 121, 122, 124, and 125 nucleotides. Based on some complementary studies using this primer to sequence zygote RNA (data not shown), the adenosine located 123 nucleotides upstream of the primer (a thymidine in the sequence ladder of Figure 3) was selected as the start site, but the adenosine at 126 nucleotides is a potential alternative. Thirty-one bases upstream of the start site there is a TATATA sequence resembling a promoter.

The most striking feature of this gene is its derived amino acid sequence. The gene product is predicted to be 24% proline and 17% serine; it contains eight $X(\text{Pro})_3$ units; and it contains two long stretches of $(\text{Ser-Pro})_x$. These characteristics are hallmarks of the extensin gene family and, indeed, when the National Biomedical Research Foundation protein database (release 19) was searched using the FASTA program (Pearson and Lipman, 1988), the protein with the most significant homology to the class IV product was the extensin precursor of carrot (Chen and Varner, 1985). Therefore, we propose that the class IV

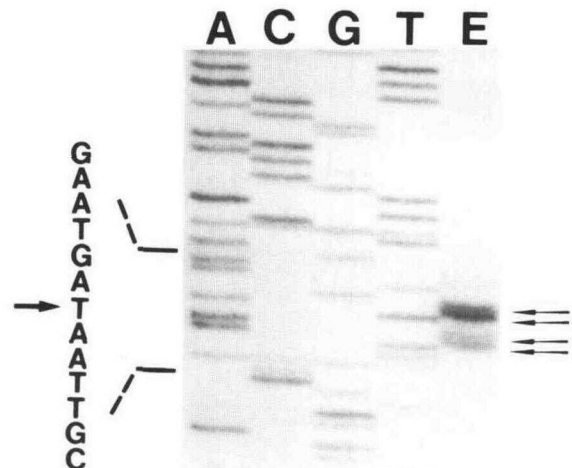


Figure 3. Primer Extension Analysis of the Class IV Gene.

A 17-nucleotide primer was annealed to total RNA and extended with reverse transcriptase to yield the four bands in lane E. The sizes of the four bands are 121, 122, 124, and 125 nucleotides. The same primer was used to generate the adjacent sequence ladder from a single-strand DNA template. The coding strand DNA sequence corresponding to the region around the extension products (nucleotides -7 to +6 in Figure 2) is presented on the left side of the ladder. The putative transcription start site is marked by an arrow.

gene encodes a zygote wall HRGP. This proposal is consistent with the demonstration that the early stages of zygote development are devoted to wall synthesis (Minami and Goodenough, 1978).

In Vitro Protein Studies

To study the expression of the class IV gene, antibody was raised against a fusion protein with the class IV product attached to the carboxyl terminus of β -galactosidase. The antibody was first used for immunoprecipitation from in vitro translations of poly A⁺ RNA. Poly A⁺ RNA from gametes, from 30-min and from 180-min zygotes were translated in a rabbit reticulocyte lysate system using ³⁵S-methionine. The reaction products were denatured, immunoprecipitated first with preimmune serum, then with fusion protein antibody, and analyzed by SDS-PAGE and autoradiography (Figure 4). Three bands are seen in the immunoprecipitates of both the 30-min and 180-min zygote mRNA translations; their sizes are approximately 66 kD, 36 kD, and 34 kD. A fourth band, at approximately 46 kD, is seen only in the 180-min zygote mRNA immunoprecipitate. None of these four bands is immunoprecipitated from the products of the gamete RNA translation (lane 1). The

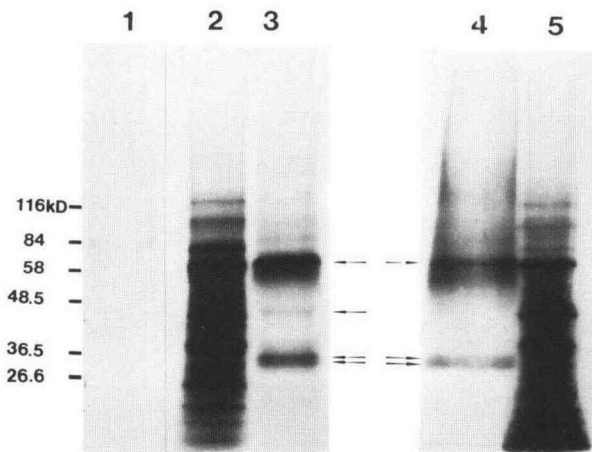


Figure 4. Immunoprecipitation from in Vitro Translations of Poly A⁺ RNA from Gametes and Zygotes.

Poly A⁺ RNA from gametes, 30-min zygotes, and 180-min zygotes was translated in a rabbit reticulocyte lysate system, and the products were immunoprecipitated with the fusion protein antibody. Lane 1 contains the immunoprecipitate from the gamete RNA translation. The total translation products from 180-min and 30-min zygote RNA are shown in lanes 2 and 5, respectively. The corresponding immunoprecipitates from these two samples are found in lanes 3 and 4. The three proteins found in both zygote immunoprecipitates have apparent molecular masses of 66 kD, 36 kD, and 34 kD. A fourth band of 46 kD is seen only in the 180-min zygote RNA immunoprecipitate (lane 3).

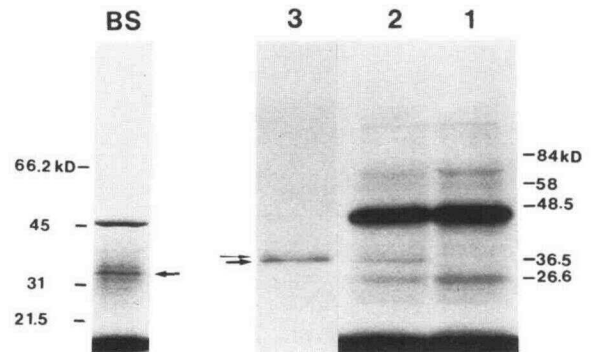


Figure 5. Determination of the Apparent Molecular Weight of the Protein Encoded by the Class IV Gene.

Left panel. In vitro transcription/translation analysis of the class IV cDNA in the Bluescript (BS) plasmid. The 45-kD band is an endogenous product of the rabbit reticulocyte lysate system. An arrow points to the 34-kD class IV product.

Right panel. Hybrid select-translation using the class IV cDNA. In lane 1 are the products of a translation with no exogenous RNA added to the translation system. Lane 2 shows the products of a translation with the hybrid-selected RNA. There is a band (around 34 kD) in lane 2 that appears to be specific to the hybrid-selected RNA. Immunoprecipitation of the hybrid-selected RNA translation products with the fusion protein antibody confirms that two of the bands are related to the class IV protein (lane 3). There is a strong band at 34 kD and a fainter band (thin arrow) at 36 kD.

66-kD band is quite obvious in the lane of total translation products from 180-min (lane 2) and 30-min (lane 5) zygote RNA, but the 34-kD, 36-kD, and 46-kD bands are less apparent. With four zygote-specific products being immunoprecipitated by the fusion protein antibody, it was not clear which corresponded to the class IV protein itself. Moreover, the class IV derived amino acid sequence suggests a molecular mass of ~20 kD, but no bands in this size range were observed. Therefore, several approaches were taken to clarify these unexpected results.

By cloning the entire class IV cDNA into the Bluescript pKS vector, it was possible to transcribe class IV mRNA in vitro using T7 RNA polymerase and then translate this mRNA in a rabbit reticulocyte lysate system. When the products of translation were examined by SDS-PAGE and autoradiography, a major band was visible at approximately 34 kD (Figure 5, left panel). As demonstrated in later experiments, the band at 45 kD is an endogenous rabbit reticulocyte product seen whether or not exogenous mRNA is added to the lysate.

Hybrid select-translation was used to verify the results of the Bluescript transcription/translation. The class IV cDNA was blotted to nitrocellulose and annealed to poly A⁺ RNA from 30-min zygotes. The annealed RNA was washed, released, and translated in a rabbit reticulocyte lysate system. The translation mix was denatured by boiling in SDS and then immunoprecipitated with the fusion

protein antibody. When the immunoprecipitate was examined by SDS-PAGE and autoradiography, a strong signal was seen at 34 kD, with a fainter signal around 36 kD (Figure 5, lane 3 of right panel). The lane with the translation products from a reaction with no added mRNA (lane 1) shows that, although there are several endogenous reticulocyte lysate products, including the major band at 45 kD, none corresponds to the 34-kD product.

These data indicate that the 34-kD band corresponds to the product of the class IV gene with its anomalous electrophoretic mobility presumably due to the high proline content of the protein (see Discussion). The origins of the 36-kD, 46-kD, and 66-kD bands that also immunoprecipitate with the fusion protein antibody remained unresolved. An obvious possibility was that these species represent additional zygote proteins possessing one or more epitopes in common with those found in the class IV product. Therefore, the derived amino acid sequences of the seven other classes of zygote-specific cDNAs analyzed to date (J.P. Woessner, G.L. Matters and P.J. Ferris, unpublished data) were reexamined. As shown in Figure 6, the derived amino acid sequence from the class VI cDNA contains two regions of Ser-Pro repeats that match the two Ser-Pro repeats in the class IV sequence, most notably the (Ser-Pro)₆ from amino acid 130 to amino acid 141.

Hybrid select-translation with class VI cDNA-selected poly A⁺ RNA from 30-min zygotes was performed to identify the protein product corresponding to the class VI cDNA (Figure 7), and a faint band was detected at 66 kD in lane HS (in addition to the endogenous 45-kD species). To demonstrate that this is indeed the 66-kD protein with homology to the class IV product, the class VI hybrid select-translation products were immunoprecipitated with the fusion protein antibody. One band at 66 kD is visible in the immunoprecipitate (Figure 7, lane IM). In summary, then, the data indicate that the 34-kD polypeptide is the class IV gene product, the 66-kD polypeptide is the product of the class VI gene, and the 36-kD and 46-kD species are likely to represent other homologous HRGPs whose corresponding genes have not yet been identified.

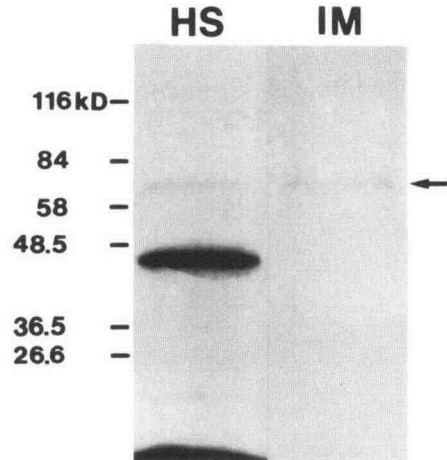


Figure 7. Hybrid Select-Translation with the Class VI cDNA.

The translation of the Class VI hybrid-selected RNA (HS) reveals several bands (left lane). Only one band, at 66 kD, is detected when the translation products are immunoprecipitated (IM) with the fusion protein antibody (right lane). The 45-kD band in the left lane is an endogenous product of the rabbit reticulocyte lysate.

In Vivo Protein Studies

¹⁴C-acetate was added to a culture of 60-min zygotes, and, after a 30-min labeling period, samples of the cell lysate were prepared, immunoprecipitated with the fusion protein antibody, and analyzed by SDS-PAGE and autoradiography (Figure 8, lane CL). In parallel with the in vitro protein results (Figure 4), four bands are detected, corresponding to 66 kD, 46 kD, 36 kD, and 34 kD. By scanning densitometry of both the in vitro and in vivo autoradiographs, the 66-kD protein is about 10 times as abundant as the 34-kD and 36-kD proteins, which, in turn, are both approximately 1.5 times as abundant as the 46-kD protein.

In a parallel experiment, a preparation of the proteins secreted by the 60-min zygotes after the 30-min ¹⁴C-

Class IV	123	Ala Ser Pro Asn Pro Ser Ser Ser Pro Ser Pro Ser Pro Ser Pro Ser Pro Ser Pro Ala	142
		GCG TCT CCC AAC CCC AGC TCC AGC CCC AGC CCC AGC CCC AGT CCG AGC CCC AGC CCT GCT	
Class VI		Ala Thr Pro Ser Pro Ser Pro Ser Pro Ser Pro Ser Pro Ser Pro Ser Pro Ser Pro Ser Pro Ala	
		GCC ACA CCC AGC CCC TCT CCT TCT CCT TCT CCT TCT CCC TCT CCG TCG CCC TCT CCT GCC	
Class IV	98	Ser Pro Ser Pro Ser Ser Ser Ala Ser Pro Ser Pro Ser Pro Ser Pro Ala	112
		TCG CCT TCG CCC TCA TCC TCA GCC TCT CCG TCG CCA TCC CCC GCC	
Class VI		Pro Ser Pro Ser Pro Ser Pro Thr Pro Ser Pro Ala	
		CCA TCG CCC TCG CCC TCG CCC ACG CCC TCG CCC GCC	

Figure 6. Comparison of the Derived Amino Acid Sequences from the Class IV and Class VI cDNAs.

Two regions from the sequences of each cDNA are aligned by homology at the amino acid level. The numbers flanking the class IV sequences correspond to amino acid position. The numbers for the class VI sequence are not provided since we do not have a full-length class VI cDNA yet.

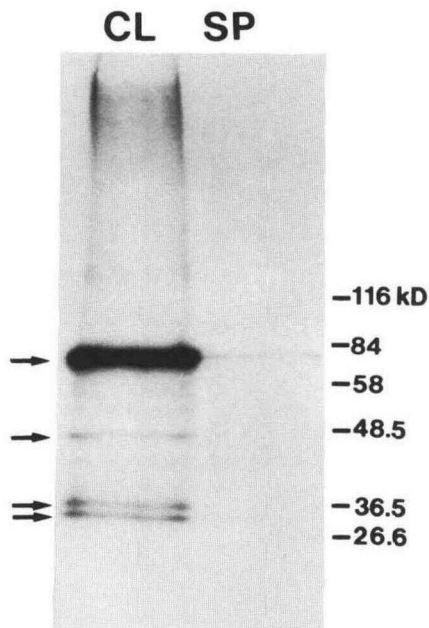


Figure 8. In Vivo Labeling and Immunoprecipitation.

Sixty-minute zygotes were labeled with ^{14}C -acetate for 30 min, and then the cells and supernatant were separated and prepared for immunoprecipitation with the fusion protein antibody. The left lane (CL = cell lysate) shows the immunoprecipitated products from the lysed zygotes. Four major proteins are detected, and their apparent molecular weights are 66 kD, 46 kD, 36 kD, and 34 kD. The right lane is the immunoprecipitate from the secreted polypeptides (SP). Only the 66-kD protein is visible. This band is not a gel artifact originating from the CL lane, as it has been detected on gels of SP where no CL lane was present.

acetate labeling was incubated with the fusion protein antibody. Although such preparations contain abundant glycopolypeptides as analyzed by SDS-PAGE and periodic acid-Schiff staining (data not shown), none of these proteins is immunoprecipitated by the antibody: a 1-month exposure is necessary to detect a faint band at 66 kD (Figure 8, lane SP). Apparently, the epitopes recognized by the fusion protein antibody are masked by glycosylation of the proteins, so that only the immediate translation products in the rough endoplasmic reticulum are susceptible to immunoprecipitation. By this reasoning, the small amount of 66-kD product in the SP lane is most likely nonglycosylated protein derived from the occasional lysed cells contaminating the preparation.

DISCUSSION

The structural role of HRGPs in higher plant cell walls is not well defined due to the sheer number of various

polymer components that interact in a three-dimensional array to create the wall (see review by Varner and Lin, 1989). Hence, much of what we know about structural roles for extensins comes from studies of the much simpler vegetative/gamete wall of *C. reinhardtii* in which HRGPs are the major structural elements (Roberts et al., 1985; Goodenough et al., 1986). This is not the only wall structure found in *Chlamydomonas* cells: as with many fungi and algae, *Chlamydomonas* cells elaborate one type of cell wall when they are haploid or homozygous for mating type and acquire the ability to produce a different cell coat when they are heterozygous for mating type (Goodenough, 1985; Goodenough and Ferris, 1987). Gametes shed their cell walls during the mating reaction, and, for approximately the next 20 hr, the major cellular activity in the zygote is assembly of a new wall. The zygote wall bears little resemblance to the well-studied vegetative/gamete wall, lacking any kind of crystalline structure and resisting solubilization with chaotropes and SDS (Minami and Goodenough, 1978; Grief, O'Neill, and Shaw, 1987).

Early studies with pulse-labeled *Chlamydomonas* cells revealed that gametic fusion results in the synthesis of novel zygote-specific glycoproteins that are secreted into the medium as early as 15 min after fusion (Minami and Goodenough, 1978). Electron micrographs of the secreted proteins prepared by the quick-freeze, deep-etch method (U.W. Goodenough, J.E. Heuser, and J.P. Woessner, unpublished data) show several different-sized molecules, each possessing the fibrous structure characteristic of extensins but lacking the bends typical of the fibrous vegetative/gamete HRGPs (Goodenough et al., 1986). Amino acid analysis of the zygote wall, moreover, indicates that hydroxyproline is present at 22.5 mol % (Grief, O'Neill, and Shaw, 1987), again suggesting that extensin-like proteins are present.

Encouraged by these data, we set out to learn about the HRGPs that assemble to form the zygote wall. On the hypothesis that the unique patterns of zygote protein synthesis represent distinctive changes in gene expression, two cDNA libraries were generated from zygote mRNA and differentially screened with labeled cDNA from gametic and zygotic mRNA (Ferris and Goodenough, 1987; G.L. Matters, unpublished data). Eight classes of zygote-specific cDNA clones have been isolated, seven of which are transcribed within 10 min after mating. Because the predominant cellular activity of early zygotes is cell wall construction, and because zygote glycoproteins begin to appear in the medium 15 min after gametic fusion, it seemed likely that some of the seven cDNA classes would encode wall proteins.

This paper presents evidence that one of these cDNAs, termed class IV, encodes a zygote wall protein. The derived amino acid sequence reveals similarities and differences with typical higher-plant extensins. First, one is immediately struck by the high percentage of proline and serine in the class IV protein (24% and 17%, respectively).

These 2 amino acids are predominant in most extensins, with values as high as 46% proline and 15% serine (Showalter and Rumeau, 1989). Repetitive proline units are also characteristic of extensins. While almost all higher plant extensins have Ser(Pro)₄ as a common repeat, the zygote wall protein has X(Pro)₃. In addition, the zygote wall protein possesses a (Ser-Pro)_x repeat that our work has demonstrated to be a common epitope between at least two different zygote proteins. While this is not a common motif in higher plant extensins, some examples of both (Ser-Pro)_x and Pro-X-Pro-X-Pro have been found (Showalter and Rumeau, 1989). For both the higher plant extensins and the class IV protein, the repetitive proline sequences lead one to predict a fibrous structure for the molecule.

Although both the class IV protein and higher plant extensins have a net positive charge, arginine, not lysine, is the more abundant basic amino acid in the zygote protein. Moreover, the class IV protein possesses only 1 tyrosine, whereas this amino acid is usually much more abundant in higher plant extensins. In general, however, it is becoming clear that, other than the high percentages of serine and proline, the amino acid composition of extensins is quite variable.

Immunoprecipitation experiments with an antibody directed against a class IV fusion protein yielded two unexpected results: first, more than one zygote-specific protein was recognized by the class IV antibody, and, second, none of the proteins corresponded to the size predicted by the class IV derived amino acid sequence. The immunoprecipitated polypeptide that migrates at 34 kD was identified as the product of the class IV gene by transcription/translation experiments and by hybrid select-translation using the class IV cDNA. The reason for the retarded migration of this polypeptide has not been directly determined, but protein conformation and lowered SDS binding have been implicated in the altered migration of proline-rich collagen chains on Laemmli gels (Freytag, Noelken, and Hudson, 1979; Noelken, Wisdom, and Hudson, 1981).

The cross-reactivity of the antibody with three other zygote-specific proteins suggested that all share one or more common epitopes. Of the zygote-specific cDNA sequences that we have determined to date, the class VI sequence presented a possible common epitope in the form of two repetitive (Ser-Pro)_x domains. Hybrid select-translation and immunoprecipitation confirmed that the 66-kD band seen by the fusion protein antibody corresponded to the class VI gene. None of the class VI cDNAs we have isolated is full-length, so we do not have the complete derived amino acid sequence for this protein. It is clear, however, from our DNA sequencing data, that other than the two small regions of (Ser-Pro)_x, the protein bears little resemblance to higher plant extensins.

Despite homology at the amino acid level, the class IV and class VI DNA sequences are dissimilar in the (Ser-Pro)_x regions. The lack of homology at the nucleotide level presumably explains how the antibody can detect several

proteins homologous to the class IV product and yet the class IV cDNA hybridizes to only a single mRNA species on RNA gel blots and appears to be a single-copy gene on genomic DNA gel blots (Ferris and Goodenough, 1987). On the other hand, our studies suggest that the class IV mRNA shares some homology with the mRNA encoding the 36-kD protein: at the low stringency of hybridization used in the hybrid select-translation experiments, the class IV cDNA was able to anneal to both transcripts (Figure 5). The homology is apparently not high since the quantity of mRNA selected and the corresponding abundance of the 36-kD protein are lower than that of the class IV mRNA and protein. The 36 kD-protein is also less abundant in the hybrid select-translation than in the immunoprecipitates of the 30-min and 180-min zygote RNA in vitro translations (Figure 4) and of the in vivo cell lysate (Figure 8).

The in vivo labeling studies confirmed the results obtained from in vitro translations: the fusion protein antibody immunoprecipitated from cell lysates the same four bands detected in vitro. The stoichiometry of the four bands also parallels that found in vitro, with the 66-kD protein being the most abundant, followed by the 34-kD and 36-kD proteins, and then the 46-kD protein. The abundance of the four proteins is probably related, at least in part, to the quantity of each mRNA. In the case of the class IV and class VI genes, we know that the class VI mRNA is more abundant than that of class IV as deduced by RNA gel blot analysis (Ferris and Goodenough, 1987).

We anticipated that the fusion protein antibody might not immunoprecipitate any secreted zygote wall proteins because these proteins are highly glycosylated. Carbohydrate represents 40% to 60% of the weight of higher plant extensins and 60% of the zygote wall (Grief, O'Neill, and Shaw, 1987; Showalter and Varner, 1989). Presumably, the epitopes that dominate the nonglycosylated fusion protein are subsequently masked by sugar residues and/or by conformational changes induced by glycosylation. As a result, we are unable to identify, by immunolabeling, the class IV protein among the several secreted fibrous zygote wall proteins visualized by transmission electron microscopy (U.W. Goodenough, J.E. Heuser, and J.P. Woessner, unpublished data).

This report provides the first full-length sequence of an algal HRGP, and indicates that at least one of its prominent domains, (Ser-Pro)_x, will be shared by other HRGPs that constitute the wall (a prediction we will test by preparing antibodies against an oligopeptide of Ser-Pro repeats for isolation of other zygote HRGP genes). With identification of this zygote-specific wall gene, we are convinced that there are two sets of HRGP wall genes in *Chlamydomonas*: one set for the vegetative/gametic cell, and the other set for the zygote. The genes for some of the vegetative/gametic wall glycopolypeptides have now been isolated (Adair and Apt, 1989), and, in a collaborative effort, we anticipate a comparison of the two sets of wall proteins at the nucleotide and amino acid level. Long-term goals in-

clude the determination of the signals and upstream genomic sequences that regulate the switch in expression from one set of HRGP wall genes to the other.

METHODS

DNA Sequencing and Analysis

All DNAs to be sequenced were subcloned into the phagemids pUC118 or pUC119 (Vieira and Messing, 1987), and single-stranded DNA was generated. Both chemical cleavage (Maxam and Gilbert, 1980) and dideoxy sequencing (Sanger, Nicklen, and Coulson, 1977) with the Sequenase kit (United States Biochemical Corp.) were used. The sequences were compiled and analyzed using the Genetics Computer Group Sequence Analysis Software Package for VAX/VMS computers.

Isolation of a Genomic Clone for the Class IV cDNA

An unamplified EMBL3 λ library of total DNA from *Chlamydomonas reinhardtii* strain CC-620 (Ferris, 1989) was plated on *Escherichia coli* strain CES200 (Nader et al., 1985). Plating of phage, preparation of plaque lifts on nitrocellulose for screening by hybridization, and purification of phage DNA were performed as outlined in Maniatis, Fritsch, and Sambrook (1982).

Primer Extension

Primer extension was performed essentially as presented by Geliebter (1988) with the following changes: 120 μ g of total RNA were used in place of poly A⁺ RNA in the annealing reaction, which was done at 50°C. The extension reaction was loaded on a denaturing acrylamide gel adjacent to the corresponding sequence ladder generated by dideoxy sequencing with the appropriate single-stranded DNA annealed to the unlabeled primer. The 17-nucleotide primer was synthesized on an Applied Biosystems DNA Synthesizer.

Transcription and Translation with Bluescript

The entire cDNA was excised from pUC119 and ligated into the EcoRI and HindIII sites of the Bluescript KS (+) plasmid (Stratagene). RNA was transcribed using T7 RNA Polymerase (International Biotechnologies, Inc.) and capped using the Stratagene mRNA capping kit. The RNA transcripts were translated in a rabbit reticulocyte lysate system (Bethesda Research Laboratories) with ³⁵S-methionine (Du Pont-New England Nuclear). The products were analyzed by SDS-PAGE on a 5% to 15% Laemmli (1970) gel. After fixing 30 min in 25% isopropyl alcohol/10% acetic acid and shaking 30 min in ENLIGHTNING (Du Pont), the gel was dried down and exposed to Kodak XAR-5 film.

Construction of Fusion Protein and Antibody Preparation

A 925-bp HincII/PstI portion of the cDNA was cloned into the expression vector pWR590 (Guo et al., 1984). This puts the open

reading frame of the cDNA (with a portion of the putative signal sequence) in frame with and 3' to the first 590 amino acids of β -galactosidase. The recombinant plasmid was maintained in *E. coli* strain JM101 (Messing, Crea, and Sandburg, 1981).

Cells were grown overnight at 37°C in 20 mL of 2 \times YT (16 g of tryptone/10 g of yeast extract/10 g of NaCl per liter) with 50 μ g/mL ampicillin and 1 mM isopropylthiogalactopyranoside. The culture was made 1 mM phenylmethylsulfonyl fluoride and put on ice. Cells were pelleted at 4000g for 5 min, the supernatant was removed, and the pellet was resuspended in 1 mL of 2 \times SDS loading buffer (0.715 M β -mercaptoethanol/8 M urea/0.36 M sucrose/4% SDS/0.04% pyronin y/100 mM Na₂CO₃). After boiling for 5 min, DNA was sheared by drawing the solution up and down through a 21-gauge needle, and the entire sample was loaded onto a 140 \times 140 \times 3 mm 8% Laemmli (1970) gel. The gel was run at 60 V for 1 hr and then at 115 V for 3 hr.

After treating the gel with ice-cold 0.1 M KCl, the white band corresponding to the fusion protein was cut out and the protein extracted using an ISCO model 1750 electroeluter as described by Schmidt et al. (1985). The negative electrode buffer was 0.082 M Tris-HCl, pH 8.6/0.04 M boric acid/0.1% SDS, while the positive electrode buffer was 0.21 M Tris-HCl, pH 8.9. The eluter was run at 1 W constant power for 5 hr, and the eluted protein was collected and precipitated with 5 volumes of acetone at -20°C overnight. The protein was pelleted out of the acetone with a 16,000g spin for 10 min, lyophilized, resuspended in 1 mL of distilled H₂O (dH₂O), and reprecipitated with 5 mL of acetone. Again, the protein was pelleted, dried, and resuspended in 1 mL of dH₂O. A Lowry reaction was performed to determine protein concentration (Lowry et al., 1951). Yields were generally 0.5 mg of fusion protein per 20 mL of initial culture.

To prepare antibody against the eluted fusion protein, a rabbit received intradermal injections of approximately 100 μ g of protein in 1 mL of Freund's adjuvant at 4-week to 6-week intervals. Ear bleeds of 5 mL to 10 mL were taken 1 week after each booster injection. Each bleed was allowed to coagulate overnight at 4°C, the clot was removed by centrifugation at 4000g for 5 min, and the serum was respun at 27,000g for 10 min to pellet all remaining cells. The antibody was further purified by precipitation in 50% ammonium sulfate essentially as described by Stelos (1967).

The final step in purification was to run the antibody over a column of *E. coli* proteins and β -galactosidase coupled to Sepharose. A 3.5-mL column was prepared in a 10-mL syringe according to manufacturer's instructions using cyanogen bromide-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) and a lysate from a 50-mL overnight culture of *E. coli* strain JM101 containing the pWR590 plasmid with no insert. The antibody that flowed through the column was tested on protein gel blots following the procedure of Blake et al. (1984) for reaction against the fusion protein.

Immunoprecipitation from in Vitro Translation

By the method of Kirk and Kirk (1985), total RNA was isolated from gametes of *C. reinhardtii* strain CC-621 and from zygotes 30 min and 180 min after mating gametes of strain CC-621 with those from CC-620. Poly A⁺ RNA was prepared from total RNA by two passes of each RNA over an oligo(dT)-cellulose column (Maniatis, Fritsch, and Sambrook, 1982). Two micrograms of each poly A⁺ RNA were translated in a rabbit reticulocyte lysate system with ³⁵S-methionine. After 90 min at 30°C, the 30- μ L reaction was

terminated by adding 10 μ L of 4 \times SDS denaturation buffer (16% SDS/20 mM EDTA/160 mM Tris-HCl, pH 7.4) and boiling 5 min. Immunoprecipitation with preimmune serum and the fusion protein antibody was performed as outlined in Schmidt et al. (1984). The reaction mix received 500 μ L of immunoprecipitation buffer (IPB: 40 mM Tris-HCl, pH 7.4/2% Triton X-100/2 mM EDTA/154 mM NaCl) containing 50 μ g/mL aprotinin (Sigma) and 10 μ L of 50 \times protease inhibitors (93 mg/mL iodoacetamide and 10.4 mg/mL *p*-aminobenzamidine in dH₂O). The mixture was incubated for 30 min at room temperature with preimmune serum and then 20 μ L of a slurry of protein A-Sepharose CL-4B beads (Pharmacia) in IPB were added. The tube was rotated for 3 hr at 4°C, the beads were separated from the mix by a brief spin in a microcentrifuge, and the fusion antibody was added to a concentration of 50 μ g/mL. After 30 min at room temperature, 20 μ L of beads were added and the tube was rotated for 3 hr at 4°C. The beads were pelleted and washed five times in IPB, once in 1 M NaCl and twice in 0.85% NaCl. The antibody-antigen complexes were released from the beads in 0.6 mL of 1 M acetic acid, lyophilized, and resuspended in 2 \times SDS loading buffer for SDS-PAGE and autoradiography as described above. The autorads were scanned using a computing densitometer from Molecular Dynamics.

Hybrid Select-Translation and Immunoprecipitation

Twenty micrograms of plasmid DNA were digested with the appropriate enzymes to release the cDNA insert, electrophoresed on an agarose gel, and transferred to nitrocellulose (Southern, 1975). A small rectangle corresponding to the insert band was excised from the filter, washed 30 min in 10 \times SSC (1.5 M NaCl/0.15 M trisodium citrate), and baked 2 hr at 80°C in vacuo. Prehybridization and hybridization were performed as outlined by Miller et al. (1983). The filter was hybridized overnight at 37°C in a 100- μ L solution of 50% formamide/10 mM Pipes, pH 6.4/0.4 M NaCl/2 μ g of poly A⁺ RNA from 30-min zygotes. After 10 washes of 1 \times SSC/0.5% SDS at 60°C and three washes in 2 mM EDTA, the bound RNA was eluted from the filters by boiling 1 min in 300 μ L of 1 mM EDTA with 10 μ g of wheat germ tRNA. Immediately after boiling, the tube was quick-frozen in an ethanol-dry ice bath. After thawing at room temperature, the supernatant was removed and precipitated with 100% ethanol. The RNA was pelleted 15 min in a microcentrifuge, washed 2 \times with 70% ethanol, lyophilized, and resuspended in 10 μ L of dH₂O. Five microliters of this RNA were translated in a rabbit reticulocyte lysate system with ³⁵S-methionine. After 90 min at 30°C, 25 μ L were removed for immunoprecipitation with the preimmune and fusion protein antibodies as described above. The remaining 5 μ L were mixed with 5 μ L of 2 \times SDS loading buffer. Both samples were subjected to SDS-PAGE and autoradiography.

In Vivo Labeling

Gametes from *C. reinhardtii* strains CC-620 (*mt*⁺) and CC-621 (*mt*⁻) were produced on Tris/acetate/phosphate agar plates and suspended in nitrogen-free high salt medium (HSM) (Martin and Goodenough, 1975). One milliliter of cells from each mating type at 1.6 \times 10⁷ cells/mL were mixed together. After 15 min, the cells were twice pelleted and resuspended in fresh nitrogen-free HSM using brief spins (up to 2600g and back down), removing released gamete walls. The zygotes were left in 2 mL of nitrogen-free HSM

until 60 min after mating, when 20 μ C (in 20 μ L of nitrogen-free HSM) of universally labeled ¹⁴C-acetic acid (56.5 mCi/mmol, Du Pont-New England Nuclear) were added. The cells were shaken for 30 min during labeling. The supernatant and the cells were separately collected after a 5-min 16,000g spin. Five volumes of acetone were added to the supernatant and it was precipitated overnight at -20°C. The pelleted cells were resuspended and washed one time in nitrogen-free HSM. Finally, the cells were brought up in 100 μ L of nitrogen-free HSM and squirted into 1 mL of acetone for lysis. This tube was mixed and incubated overnight at -20°C. After both samples were centrifuged for 10 min at 16,000g, their supernatants were removed and the pellets lyophilized. Each pellet was resuspended in 40 μ L of 1 \times SDS denaturation buffer and boiled 5 min. Immunoprecipitation, SDS-PAGE, and autoradiography were performed as above. The autorads were scanned with a Molecular Dynamics computing densitometer.

ACKNOWLEDGMENTS

We are indebted to Bimei Hong, who supplied the Bluescript mRNA transcription kit. We also thank Drs. Patrick Ferris and Gail Matters for helpful comments on the manuscript and invaluable assistance with various experiments. This research was supported by a postdoctoral fellowship from the American Cancer Society (PF-3154) and by a grant from the U.S. Department of Agriculture (88-37261-3725).

Received June 12, 1989; revised July 13, 1989.

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Molecular characterization of a zygote wall protein: an extensin-like molecule in *Chlamydomonas reinhardtii*.

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Plant Cell 1989;1;901-911

DOI 10.1105/tpc.1.9.901

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