The Plant Cell, Vol. 4, 425–433, April 1992 © 1992 American Society of Plant Physiologists

A Carrot Somatic Embryo Mutant Is Rescued by Chitinase

Anke J. De Jong,[a] Jan Cordewener,[a,1] Fiorella Lo Schiavo,[b] Mario Terzi,[b] Joël Vandekerckhove,[c] Ab Van Kammen,[a] and Sacco C. De Vries[a,2]

[a] Department of Molecular Biology, Agricultural University of Wageningen, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

[b] Department of Biology, University of Padova, Via Trieste 75, 35121 Padova, Italy

[c] Laboratory of Physiological Chemistry, University of Gent, K. L. Ledeganckstraat 35, 9000 Gent, Belgium

At the nonpermissive temperature, somatic embryogenesis of the temperature-sensitive (ts) carrot cell mutant ts11 does not proceed beyond the globular stage. This developmental arrest can be lifted by the addition of proteins secreted by wild-type cells to the culture medium. From this mixture of secreted proteins, a 32-kD glycoprotein, designated extracellular protein 3 (EP3), that allows completion of somatic embryo development in ts11 at the nonpermissive temperature was purified. On the basis of peptide sequences and biochemical characterization, EP3 was identified as a glycosylated acidic endochitinase. The addition of the 32-kD endochitinase to ts11 embryo cultures at the nonpermissive temperature appeared to promote the formation of a correctly formed embryo protoderm. These results imply that a glycosylated acidic endochitinase has an important function in early plant somatic embryo development.

INTRODUCTION

In carrot cell cultures, somatic embryos develop from single embryogenic cells or from small clusters of embryogenic cells designated proembryogenic masses (Halperin, 1966; Nomura and Komamine, 1985). Several reports have indicated that proteins secreted into the medium of carrot cell cultures accompany the formation of embryogenic cells and somatic embryos. In one instance, an extracellular protein (EP1) that is only secreted by nonembryogenic cells has been identified (Van Engelen et al., 1991). Sterk et al. (1991) reported that another extracellular protein (EP2), identified as a lipid transfer protein, was only synthesized by embryogenic cells and somatic embryos. From these and other extracellular proteins described (Satoh and Fujii, 1988), it emerges that the developmental state of carrot suspension cells is reflected in the type of secreted proteins synthesized by these cells.

To answer whether these and other secreted proteins are directly involved in somatic embryo development, two different assay systems have been developed. The first of these made use of the fungal antibiotic tunicamycin, which prevents N-glycosylation of proteins. Tunicamycin was found to inhibit somatic embryo development at an early, preglobular stage. This inhibition could be overcome by the simultaneous addition of correctly glycosylated proteins to the culture medium (De Vries et al., 1988a). The responsible glycoprotein was purified and identified as a cationic peroxidase (Cordewener et al., 1991). Based on the observed expansion of small embryogenic cells in the presence of tunicamycin and the identification of a peroxidase activity that prevents this expansion, a model has been presented that identifies the peroxidase-mediated restriction of cell size as an important prerequisite for successful somatic embryogenesis to occur (Van Engelen and De Vries, 1992). A second assay system was based on the observation that the phenocritical period in temperature-sensitive (ts) arrest at globular stage in the carrot cell mutant ts11 coincided with the period of sensitivity to replacement of the conditioned medium by fresh growth medium. When medium conditioned by a wild-type cell line was added to the ts11 culture medium, arrest at the globular stage under nonpermissive temperatures was lifted and embryo development in ts11 was completed up to torpedo stage, resulting in the formation of plantlets. This effect was found to be protease sensitive, suggesting that secreted proteins were the causative component of the conditioned medium (Lo Schiavo et al., 1990).

In this study, we present the purification of the secreted protein that is responsible for the observed rescue of ts11 embryos arrested at the globular stage. Partial protein sequences obtained from the purified protein, as well as biochemical characterization, identified this extracellular protein, designated EP3, as a 32-kD glycosylated acidic endochitinase. These results indicate that, apart from their postulated role in the plant defense response, at least one member of the family of plant proteins with chitinase activity has a function in somatic embryo development.

[1] Current address: Centre for Plant Breeding and Reproduction Research, Mansholtlaan 15, 6708 PA Wageningen, The Netherlands.

[2] To whom correspondence should be addressed.

RESULTS

Arrested ts11 Embryos Exhibit Aberrant Protoderm Formation and Are Rescued by a Single Secreted Glycoprotein of 32 kD

Cell-free conditioned medium obtained from a 10-day-old embryo culture of the wild-type line A^+ was reported to enable completion of ts11 embryo development (Lo Schiavo et al., 1990). This effect was observed when globular ts11 embryos, developed at 24°C, were transferred to the nonpermissive temperature of 32°C in medium conditioned by an A^+ embryo culture. Prior treatment with trypsin abolished the effect of medium conditioned by an A^+ embryo culture. To confirm this observation, ts11 embryo cultures were grown at 24°C and globular embryos were manually isolated from the embryo culture. Thirty individual ts11 embryos were collected and incubated at 32°C in 2 mL of fresh basal medium alone or in the presence of concentrated medium proteins isolated from the medium of a 10-day-old embryo culture of another wild-type line, 10 (De Vries et al., 1988b). This preparation of secreted proteins allowed 21 of the 30 individual ts11 globular embryos to develop into torpedo stage embryos at 32°C, resulting in an embryo progression coefficient of 0.7 as shown in Figure 1A, lane 1. This value is ~75% of the value observed with medium conditioned by an A^+ embryo culture (Lo Schiavo et al., 1990). No development of ts11 embryos beyond the globular stage was observed with unsupplemented basal medium (Figure 1A, lane 2).

Concentrated medium proteins from a 10-day-old embryo culture of line 10 were applied to a cation exchange column. The bound fraction contained 30% of the amount of protein applied and could be completely eluted with a linear gradient of 0 to 0.1 M KCl. None of the eluted fractions, including those that contained peroxidase activity (Cordewener et al., 1991), had a positive effect on ts11 embryo development. The embryo rescue activity remained present in the protein fraction not bound to the cation exchange column (data not shown). These proteins were then applied to a DEAE anion exchange column. Elution of the bound proteins with a linear gradient of 0 to 0.25 M KCl yielded seven major A_{280} peaks. All of the seven anion exchange column peak fractions were tested for ts11 embryo rescue activity. Only one protein fraction eluting at ~0.12 M KCl exhibited a rescue effect comparable to the effect of unfractionated medium proteins. This peak fraction contained several proteins (Figure 1A, lane 3). Separation of these proteins by Mono Q anion exchange chromatography yielded eight different fractions (Figure 1A, lanes 4 to 11), each of which was analyzed for ts11 embryo rescue activity. Only one fraction, eluting at 0.145 M KCl, promoted the transition of globular into torpedo-shaped embryos with an efficiency comparable to the unfractionated mixture (Figure 1A, lane 7). SDS-PAGE of this fraction showed two protein bands with apparent molecular masses of 32 and of 35 kD. The adjacent fraction (Figure 1A, lane 8) contained predominantly the 35-kD protein and only a low amount of the 32-kD protein. The

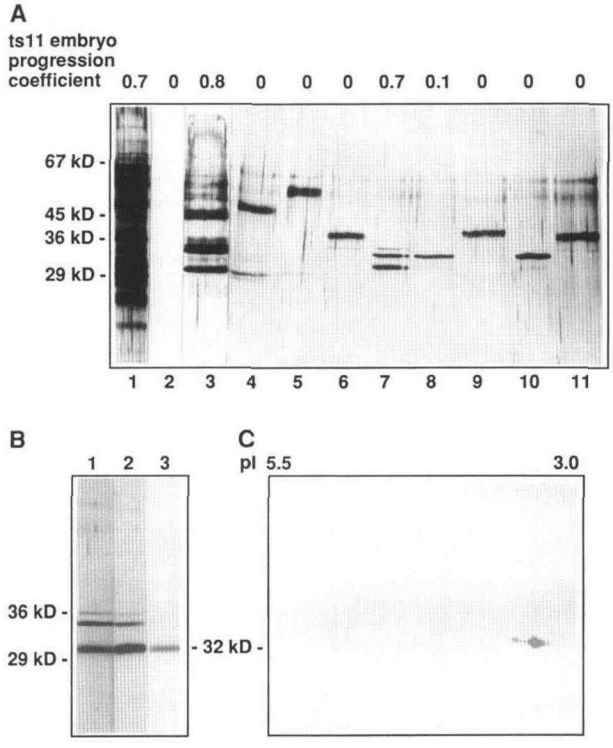

Figure 1. Purification of Secreted Medium Proteins Able to Rescue Arrested Somatic Embryos of the Mutant ts11.

(A) The effect of secreted medium proteins on the development of arrested ts11 globular embryos. Embryo rescue is expressed as a progression coefficient, which is the ratio of ts11 embryos developed past the globular stage (heart, torpedo, and plantlet) to the total number of embryos (Lo Schiavo et al., 1990). Embryo rescue assays have been performed as described in Methods. Proteins present in the different fractions analyzed in the course of the purification are visualized on silver-stained polyacrylamide gels. Lane 1, unfractionated conditioned medium; lane 2, basal B5 medium; lane 3, DEAE-Sepharose column fraction with ts11 embryo rescue activity; lanes 4 to 11, fast-protein liquid chromatography Mono Q column peak fractions derived from the DEAE-Sepharose column fraction shown in lane 3.

(B) The 32- and the 35-kD proteins analyzed for the presence of sugar side chains. Lane 1, silver-stained pattern of fraction shown in **(A)**, lane 7; lane 2, proteins reacting with concanavalin A lectin; lane 3, proteins reacting with *Ulex europaeus* agglutinin I, a fucose-specific lectin.

(C) The purified 32-kD glycoprotein analyzed by silver staining after two-dimensional SDS-PAGE.

latter fraction had a marginal effect on ts11 embryo progression, which suggested that the 32-kD protein is mainly responsible for the embryo rescue effect. This was further supported by the fact that, whereas both the 32- and 35-kD proteins reacted with the glucose- and mannose-specific lectin concanavalin A, only the 32-kD protein reacted with a fucose-specific lectin (Figure 1B). The involvement of the 32-kD protein in the ts11 embryo rescue would therefore be in line

with the observation of Lo Schiavo et al. (1990) that, whereas core glycosylation of secreted proteins in ts11 is not affected, glycoprotein processing steps that result in the addition of fucose are affected in ts11 at 32°C. Media supplemented only with the appropriate amounts of column buffer did not have an effect on ts11 embryo development.

So far we had only looked at the effect of the protein fractions upon the transition from globular to heart stage embryos. When the 32-kD glycoprotein-containing fractions were added directly to a newly initiated ts11 embryo culture, a quite dramatic effect on the development of globular ts11 embryos was observed as shown in Figure 2. In a ts11 embryo culture grown at 24°C, normal globular stage embryos developed, whereas at 32°C irregular shaped globular embryos developed (Figure 2A). But when the 32-kD glycoprotein-containing fractions were added, normal globular embryos that were able to develop into heart (Figure 2B) and later into torpedo stage embryos were formed. This result indicated that the rescue activity of the added proteins may also be necessary before the late globular embryo stage.

To confirm that the same proteins able to rescue ts11 embryos are also present in the embryogenic carrot cell line A^+, we have fractionated and tested secreted proteins from this line. Presumably due to the presence in reduced amounts of the 35-kD glycoprotein in media of the line A^+, we could use the basic purification scheme as described above to obtain a protein fraction that contained only the 32-kD glycoprotein as seen on a silver-stained SDS-polyacrylamide gel (data not shown). The purity of this 32-kD glycoprotein preparation was further analyzed by two-dimensional PAGE and showed the presence of two protein spots (Figure 1C). V8-protease digestion of the individual protein spots cut from a two-dimensional gel showed identical digestion profiles, which indicated that both glycoproteins share the same protein core and represent two closely related variants that only differ in their isoelectric points. Both glycoproteins were fucosylated as revealed by appropriate lectin staining (data not shown).

Both the 32- and the 35-kD glycoproteins were added separately to newly initiated ts11 embryo cultures at day 0. The results, as presented in Figures 2C and 2D, clearly showed that only in the presence of the 32-kD glycoprotein correctly formed ts11 globular embryos developed. These globular embryos were subsequently able to develop into the heart and torpedo stages. This result confirmed the previous assumption (cf. Figure 1A, lane 7) that only the 32-kD glycoprotein is required for embryo rescue. Optimal rescue activity of the 32-kD glycoprotein was observed as a concentration-dependent optimum in a broad range between 0.035 and 0.25 μg/mL or from 1 to 9 nM. Heat treatment (10 min at 80°C) abolished the rescue activity. The addition of neither the 32- nor the 35-kD glycoprotein to manually isolated globular embryos from wild-type lines had any visible effect on embryo development in this line.

The main morphological effect of the 32-kD glycoprotein on the ts11 globular embryo appeared to be a restoration of the embryo protoderm, as opposed to the lack of proper protoderm formation in ts11 cultures supplemented with only the 35-kD glycoprotein (cf. Figure 2C). Apart from the aberrant protoderm, accumulation and oxidation of probably phenolic compounds are also features of arrested ts11 embryos. This phenomenon, visible as dark brown ts11 embryos, did not occur in ts11 embryos supplemented with the pure 32-kD glycoprotein and might be a secondary effect of the lack of a proper protoderm.

We conclude from these results that rescue of ts11 embryos at the nonpermissive temperature of 32°C can be achieved by the addition of a single 32-kD glycoprotein secreted into the culture medium. Because the rescued embryos are able to develop into the heart stage, we propose that the lack of correct protoderm formation at the preglobular stage is the cause of the inability of ts11 embryos to develop beyond the globular stage.

The Secreted 32-kD Glycoprotein Is an Acidic Endochitinase

To identify the 32-kD glycoprotein, we tried to determine its amino acid sequence. Because it was blocked at the N terminus, the protein was blotted onto a polyvinylidene difluoride (PVDF) membrane after two-dimensional PAGE. Both spots (cf. Figure 1C) were cut together from the membrane, pooled from several two-dimensional gels, and digested with trypsin. The resulting tryptic fragments were eluted from the membranes, separated by HPLC, and subjected to N-terminal sequencing according to Bauw et al. (1987). No evidence was found for more than one protein core, which supports the results after V8-protease digestion of the individual spots. The amino acid sequences of the two 32-kD peptides analyzed were found to share homology with several plant endochitinases as shown in Figure 3.

To determine whether the 32-kD glycoprotein exhibits chitinase activity, two different enzyme assays were performed. After electrophoresis of the purified 32-kD glycoprotein in a native polyacrylamide gel, the gel was overlaid with a glycol chitin gel. Clearing of the glycol chitin substrate coincided with the single silver-stained band of the 32-kD glycoprotein, which indicates that the 32-kD glycoprotein indeed has chitinase activity as shown in Figure 4A, lanes 1 and 2. The second assay, employing ^{3}H-labeled chitin, showed the release of ^{3}H-*N*-acetylglucosamine with a specific activity of 60 nkat/mg for the 32-kD chitinase (Figure 4B). Analysis by thin-layer chromatography of the soluble products, which were derived from the ^{3}H-labeled, high-molecular mass chitin after prolonged incubation with the 32-kD chitinase, showed the presence of dimers, trimers, and tetramers but no monomers of ^{3}H-*N*-acetylglucosamine (Figure 4C). This indicates that the 32-kD glycoprotein only has endochitinase activity. Isoelectric focusing of the purified 32-kD endochitinase revealed a pI of 3.6; therefore, it belongs to the secreted acidic endochitinases. The 32-kD endochitinase had the ability to bind high molecular weight chitin, but a lysozyme-like activity could not be detected (A. J. De Jong, K. M. Kragh, and S. C. De Vries,

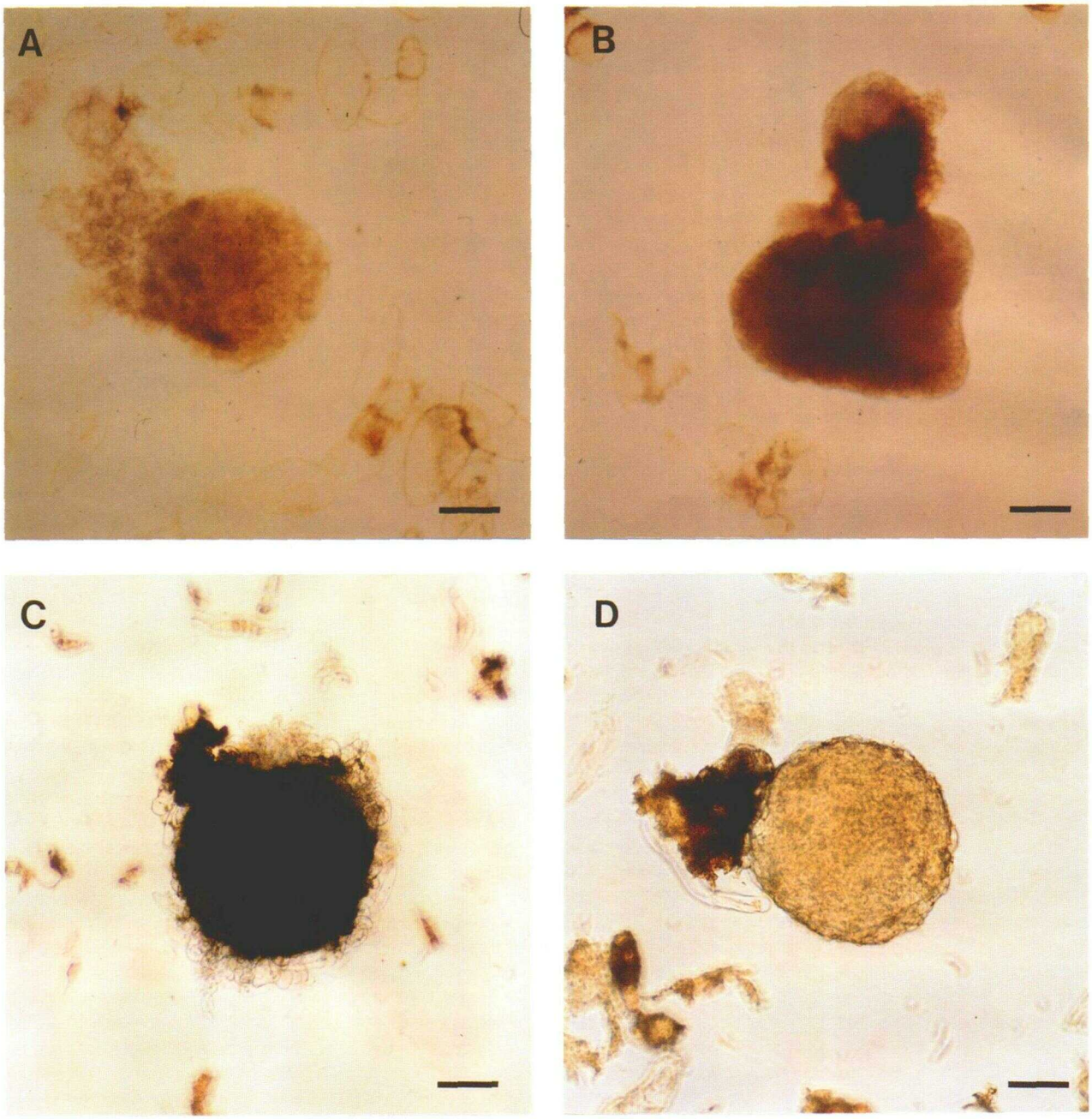

Figure 2. Effect of Secreted Proteins Purified from Wild-Type Embryo Culture Medium on the Development of ts11 Embryos at the Nonpermissive Temperature of 32°C.

(A) Aberrant globular embryo in a 4-week-old ts11 embryo culture. Bar = 180 μm.

(B) Globular and heart stage embryos in a 4-week-old embryo culture to which 0.1 μg each of the 32- and 35-kD glycoproteins had been added per milliliter of culture medium. Bar = 200 μm.

(C) Globular embryo in a 14-day-old embryo culture to which 0.1 μg of pure 35-kD glycoprotein had been added per milliliter of culture medium. Bar = 180 μm.

(D) Globular embryo in a 14-day-old embryo culture to which 0.1 μg of pure 32-kD glycoprotein had been added per milliliter of culture medium. Bar = 180 μm.

```
                 165          175          185
               PEPTIDE I          PEPTIDE II
CARROT      PLQLTFNYNYIDAGKSNQFDGLNNPDIVASDA
BEAN        .I.ISW....GQC.RAIGV.L..K..L..T.S
TOBACCO     .I...NQN..EK..NAIRQ.LV....L..T..
RICE        .I..SY.F..GP..QAIGA.L.GD..L.....
SUGAR BEET  ...I.W.....P..R.IG.....A.ET..NN.
```

Figure 3. Amino Acid Sequences of Two Adjacent 32-kD Peptides.

Regions of homology between several plant chitinases and two peptides of the 32-kD glycoprotein are shown. The homology on the amino acid level is 47% with a basic bean endochitinase (Broglie et al., 1986), 56% with an acidic tobacco chitinase (Linthorst et al., 1990), 53% with a basic rice chitinase (Zhu and Lamb, 1991), and 69% with a basic sugar beet chitinase (Mikkelsen et al., 1992).

unpublished results). An unusual feature of the 32-kD endochitinase is the presence of complex glycans, as indicated by staining with several lectins (cf. Figure 1B). All chitinases thus far described are devoid of complex-type N-linked carbohydrates.

Comparison of the unfractionated mixture of secreted glycoproteins in the medium conditioned by an A^+ wild-type embryo culture with the purified 32-kD endochitinase showed the presence of at least five other acidic chitinases (Figure 4A, lanes 3 and 4). In that the 32-kD endochitinase activity was not visible in the unfractionated protein preparation, it was evident that the 32-kD endochitinase activity is only minor compared with the total chitinase activity in the conditioned medium. None of these other carrot chitinases has been found to have rescue activity when added to arrested ts11 embryos. In addition, a bacterial exochitinase from *Streptomyces griseus*, a 36-kD basic endochitinase from barley suspension cultures (Kragh et al., 1991), a 28-kD basic endochitinase from sugar beet (Mikkelsen et al., 1992), and a 26-kD acidic endochitinase from tomato leaves (Joosten and De Wit, 1989) were tested, but no effect was observed on the development of ts11 embryos comparable to the effect of the 32-kD carrot endochitinase (data not shown). Taken together, we conclude that the 32-kD endochitinase has a specific function in carrot somatic embryo development that cannot be substituted by other members of the plant endochitinase family.

DISCUSSION

Previously, it was shown that the temperature-sensitive arrest in the carrot cell mutant ts11 can be overcome by the addition to the culture medium of a mixture of proteins secreted by wild-type embryo cultures (Lo Schiavo et al., 1990). In this study, we have shown that a single secreted glycoprotein is responsible for this effect. This protein was then identified as an acidic endochitinase of 32 kD, bearing complex-type carbohydrate side chains.

In many studies, it has been reported that plant-produced hydrolases like chitinases and β-1,3-glucanases are part of a defense mechanism against attack by pathogenic organisms (Schlumbaum et al., 1986; Mauch et al., 1988). Chitinase activity is increased upon wounding and by fungal elicitors (Hedrick et al., 1988; Kurosaki et al., 1990) and is part of the hypersensitive response to pathogen attack (Metraux and Boller, 1986). The biochemical basis of this function is believed to reside in the ability of some plant-produced chitinases and β-1,3-glucanases to partially degrade fungal cell walls (Broekaert et al., 1988). However, several of the isolated plant chitinases do not possess antifungal activity in vitro (Woloshuk et al., 1991). Chitinase genes were also shown to be expressed in the absence of pathogens (Shinshi et al., 1987; Lotan et al., 1989; Kragh et al., 1990), a finding that is usually explained by assuming that they are part of a continuously present defense mechanism. Some of the chitinase genes appear to be expressed in a tissue-specific manner. A basic chitinase gene is expressed in the epidermis cells of healthy tobacco leaves (Keefe et al., 1990), whereas another basic chitinase gene is expressed in transmitting tissue of the style in tomato flowers (Harikrishna et al., 1991). In addition, chitinase genes are among the first genes whose expression is enhanced after treatment of tobacco epidermal peels with auxin and cytokinin (Meeks-Wagner et al., 1989; Neale et al., 1990). Therefore, it appears reasonable to assume that plant-produced chitinases

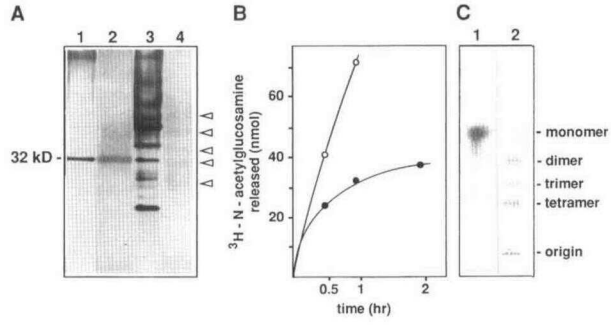

Figure 4. Chitinase Activity of the 32-kD Glycoprotein.

(A) Chitinase activity in a gel containing 0.01% (w/v) glycol chitin as substrate. Open arrowheads indicate degradation of high molecular mass chitin visualized by the reduction of calcofluor white fluorescence. Lanes 1 and 2 each contain 0.1 μg of the purified 32-kD glycoprotein; lanes 3 and 4 each contain 10 μg of concentrated secreted medium proteins of a wild-type embryo culture. Lanes 1 and 3 are silver-stained gel lanes, and lanes 2 and 4 show glycol chitin patterns.

(B) Formation of ^{3}H-*N*-acetylglucosamine residues after incubation of ^{3}H-labeled chitin with 0.3 μg of carrot 32-kD glycoprotein (●) or with 0.2 μg of exochitinase from *Serratia marcescens* (○) as a function of time. ^{3}H-*N*-acetylglucosamine residues released in controls with no protein added were <1% of the input of ^{3}H-labeled chitin and have been subtracted.

(C) Separation by thin-layer chromatography of water-soluble ^{3}H-*N*-acetylglucosamine residues released after prolonged incubation of ^{3}H-labeled chitin with the carrot 32-kD endochitinase. Lane 1, monomer ^{3}H-*N*-acetylglucosamine; lane 2, released products after incubation of ^{3}H-labeled chitin with 2 μg of the carrot 32-kD endochitinase for 4 hr at 30°C.

may have other functions apart from their role in a defense mechanism.

In this study, we have not been able to demonstrate directly that the catalytic properties of the 32-kD endochitinase are required for the observed positive effect on the development of ts11 embryos. If this is indeed the case, then what is the possible substrate for this enzyme in the plant cell wall? The presumed natural substrate for chitinases, high molecular mass chitin that consists of β-(1,4)-linked polymers of *N*-acetylglucosamine, is not considered to be a constituent of higher plant cell walls. Employing cytochemical labeling, Benhamou and Asselin (1989) were able to show binding of chitinases and wheat germ agglutinin, a plant lectin that is specific for oligomers of *N*-acetylglucosamine, to secondary cell walls of a variety of Solanaceae in the absence of pathogens. Although no biochemical identification of this material was performed, their results imply that in plant secondary cell walls, oligomers of *N*-acetylglucosamine are present. If oligomers of at least three *N*-acetylglucosamines are present, they might be putative substrates for plant endochitinases because three residues are the minimum sugar chain length required to serve as a substrate for endochitinases (Molano et al., 1979; Usui et al., 1990). Despite the fact that the substrate specificity of a wheat germ endochitinase is restricted to β-(1,4)-linked *N*-acetylglucosamines (Molano et al., 1979), it cannot be excluded that other β-(1,4)-linked sugars or combinations of different sugars might serve as substrate for other members of the chitinase family. If for instance the two *N*-acetylglucosamines, flanked by asparagine and mannose, which are present in all the inner cores of N-linked carbohydrate chains of glycoproteins, could be cleaved, then this would considerably broaden the range of potential substrates for the 32-kD endochitinase described in this work.

Our morphological observations indicate that ts11 globular embryos that developed after the addition of the 32-kD endochitinase have a properly formed protoderm, whereas ts11 globular embryos that developed in the absence of the 32-kD endochitinase have an aberrantly formed protoderm. This implies that the putative substrate of the 32-kD endochitinase in the plant cell wall has to be accessible, which points to a location at the cell walls of the peripheral cells of the embryo, and that this substrate is required for the formation of a proper protoderm. As was previously noted for the ability of a 38-kD peroxidase to restore somatic embryogenesis in tunicamycin-inhibited cultures (Cordewener et al., 1991), a concentration-dependent optimum of the rescue activity was found. Although, at present, we lack a clear explanation for these findings, it may point to a dual activity of these proteins, either in terms of their substrate specificity or in their temporal requirement during embryo development. Further evidence for the disturbed protoderm formation in ts11 globular embryos was obtained when the presence of glycoproteins reacting with concanavalin A was analyzed in sections of arrested ts11 embryos (Lo Schiavo et al., 1990). In that work, it was argued that concanavalin A–stainable glycoproteins were distributed in a diffuse manner throughout the arrested ts11 globular embryo, instead of being restricted to the protoderm, as was found in wild-type globular embryos. A similar result was obtained by in situ localization of the epidermis-specific EP2 mRNA. In wild-type embryos, the EP2 mRNA was restricted to the embryo protoderm, but appeared more diffuse in several subprotodermal cell layers in arrested ts11 globular embryos (Sterk et al., 1991). Taken together, we conclude that protoderm formation is an essential step in the formation of globular somatic embryos and a prerequisite for development to subsequent embryonic stages.

METHODS

Plant Material and Culture Conditions

The embryogenic wild-type carrot suspension cultures 10 and A^+ as well as the temperature-sensitive mutant ts11 were maintained as described previously (Giuliano et al., 1984; De Vries et al., 1988a).

ts11 Embryo Rescue Assays

Rescue assays were performed with ts11 globular embryos or with newly initiated ts11 embryo cultures. ts11 embryo cultures were initiated by resuspending the 70- to 170-μm fraction, enriched for proembryogenic masses, of a 6-day-old ts11 suspension culture. Globular embryos were manually isolated from ts11 embryo cultures grown at 24°C. Unfractionated (50 to 100 μg/mL) and fractionated (1 to 10 μg/mL) extracellular proteins were mixed in three different amounts (10, 20, and 40 μL) with 1 mL of basal B5 medium (Gamborg, 1970), filter sterilized (0.2 μm, type FF030/3; Schleicher & Schuell), and added to 35-mm-diameter Petri dishes (tissue culture quality; Greiner, Alphen a/d Rijn, The Netherlands) in duplicate. Subsequently, 1 mL of basal B5 medium with 30 isolated ts11 globular embryos or 1 mL of a diluted proembryogenic mass-enriched fraction at 6000 cell clusters per mL (Lo Schiavo et al., 1990) was added. Each series of experiments included negative controls of unsupplemented basal B5 medium and basal B5 medium to which an appropriate column buffer had been added. In assays with *Streptomyces griseus* exochitinase (Sigma) and heterologous chitinases, the proteins were added to ts11 embryo cultures in the same concentration range as the 32-kD carrot endochitinase. All assays were then incubated at the nonpermissive temperature of 32°C. Positive control cultures consisted of ts11 globular embryos in unsupplemented basal B5 medium or unsupplemented newly initiated ts11 embryo cultures incubated at 24°C. The newly initiated ts11 embryo cultures were examined under a microscope after 14 days for the formation of proper globular embryos. All cultures were followed at regular intervals for up to 2 months after initiation. Only the results obtained from at least three independent experiments with a protein concentration that gave optimal rescue activity are presented here.

Protein Purification

Conditioned medium was obtained from a 9-day-old embryo culture by filtration through Whatman 1 MM paper followed by a 0.2-μm Durapore (polyvinylidene difluoride [PVDF]) filter using a Minitan pressure filtrator (Millipore, Etten-Leur, The Netherlands). The resulting cell-free

conditioned medium was concentrated ~200 times by pressure dialysis employing a YM5 filter (5-kD cutoff; Amicon, Oosterhout, The Netherlands) and equilibrated with the appropriate starting buffer for column fractionation. The extracellular proteins were then applied to a cation exchange column (S-Sepharose FF; Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) and equilibrated in 50 mM Mes buffer, pH 5.8, and the absorbed proteins were eluted with a continuous linear salt gradient from 0 to 0.1 M KCl (total volume, 100 mL; flow rate, 40 mL/hr). Fractions of 4 mL were collected and monitored at 280 nm. Unabsorbed proteins were applied to a DEAE-Sepharose FF (Pharmacia LKB Biotechnology Inc.) anion exchange column equilibrated in 25 mM Tris buffer, pH 8.5, and eluted with a linear salt gradient from 0 to 0.25 M KCl with a total volume of 100 mL. Fractions of 4 mL were collected at a flow rate of 40 mL/hr. Final purification was performed by fast-protein liquid chromatography (Pharmacia LKB Biotechnology Inc.) employing anion exchange chromatography with a Mono Q column (Pharmacia LKB Biotechnology Inc.) equilibrated in the same buffer as was used for the DEAE-Sepharose column. For large-scale purification of the 32-kD endochitinase, batch-wise absorption to DEAE-Sepharose FF resin was used. The cell-free conditioned medium was filtrated as described above and then adjusted to pH 8.6 with solid Tris. Fifty milliliters of DEAE-Sepharose FF suspension, equilibrated in 25 mM Tris buffer, pH 8.5, was added to 3 L of cell-free conditioned medium and incubated for 1 hr at 4°C on a rotary shaker. The DEAE-Sepharose FF resin was separated from the medium by filtration through a 10-μm mesh filter and incubated for 15 min in 30 mL of 25 mM Tris buffer, pH 8.5, 0.2 M KCl. The eluate was then diluted with 3 volumes of 25 mM Tris buffer, pH 8.5, and loaded on a DEAE-Sepharose FF column. Further purification was then performed as described above.

Amino Acid Sequence Determination

The 32-kD glycoprotein containing fast-protein liquid chromatography fractions was concentrated by centrifugation using Centricon tubes (10-kD cutoff; Amicon). Concentrated proteins were subsequently separated on two-dimensional polyacrylamide gels as described by De Vries et al. (1982). Isoelectric focusing was performed within the pH range of 3 to 6. Two-dimensional gels were blotted overnight at 4°C on PVDF membranes with 25 mM Tris, 150 mM glycine, and 20% ethanol as transfer buffer. After blotting, the PVDF membranes were washed twice in PBS (6.7 mM NaH_2PO_4, 150 mM NaCl, pH 7.2) to remove residual glycinate. Protein spots were visualized after amido black staining, cut out, and stored at −20°C in Eppendorf tubes. In situ membrane tryptic digestions, separation of peptides, and amino acid sequence determination were done as described by Bauw et al. (1987).

Biochemical Characterization

SDS-PAGE was carried out according to Laemmli (1970). After electrophoresis, the gels were silver stained according to Blum et al. (1987). Protein determination was done by comparing the intensity of the Coomassie Brilliant Blue R 250-stained protein band in a polyacrylamide gel with a concentration range of bovine serum albumin in the same gel.

Lectin staining of the proteins on blots was performed according to Clegg (1982). Biotinylated lectins (concanavalin A, specific for glucose and mannose, and *Ulex europaeus* agglutinin I, specific for fucose; both from Sigma) were used in a concentration of 5 μg/mL in PBS followed by an incubation with 1 μg/mL avidin-labeled peroxidase (Sigma) in PBS. The blots were subsequently stained for peroxidase activity using 0.2 mg/mL 3-amino-9-ethylcarbazole in 50 mM NaAc, pH 5.0, 0.01% H_2O_2.

To detect chitinase activity of the 32-kD glycoprotein in the gel, the protein was run on a native polyacrylamide gel (Laemmli, 1970, without SDS and β-mercaptoethanol). A glycol chitin gel was overlaid on the native gel and incubated at 30°C for 2 hr. The glycol chitin was stained with calcofluor white M2R, and chitinase activity was visible as a nonfluorescent dark band in contrast to the fluorescent intact glycol chitin (Trudel and Asselin, 1989).

The radiometric chitinase activity assay employed regenerated ^{3}H-labeled chitin as a substrate (Molano et al., 1977). The specific radioactivity of the ^{3}H-labeled chitin used was 338 cpm/nmol *N*-acetylglucosamine equivalents or 1.7×10^6 cpm/mg ^{3}H-labeled chitin. The reaction mixture consisted of 50 μL of ^{3}H-labeled chitin suspension containing 200,000 cpm, 50 mM potassium phosphate, pH 6.3, and protein in a final volume of 300 μL. After incubation at 30°C for 0.5 to 2 hr, 300 μL of 10% trichloroacetic acid was added. After centrifugation for 10 min at 16,000*g*, the radioactivity of 300 μL of the supernatant was used for scintillation counting. The exochitinase from *Serratia marcescens* (Sigma) was used as a control. The products of the chitinase reaction were analyzed by thin-layer chromatography according to Boller et al. (1983).

Chitin binding activity was determined by using a column of regenerated chitin according to Molano et al. (1979). To detect possible lysozyme activity of the 32-kD endochitinase, a native polyacrylamide gel was overlaid with a polyacrylamide gel containing lyophilized *Micrococcus luteus* cells. Lysozyme activity was then visible as clear or opalescent bands within the opaque greyish substrate (Audy et al., 1988).

ACKNOWLEDGMENTS

We thank Magda Puyte and Jozef Van Damme for protein sequencing, Karsten Kragh for providing barley and sugar beet chitinase, Matthieu Joosten for providing tomato chitinase, Theo Hendriks for critical reading of the manuscript, and Peter van Druten for photography. These investigations were supported by the Foundation for Biological Research, which is subsidized by the Netherlands Organization for Scientific Research, and partially by a grant of the European Community to S.C.De V.

Received December 23, 1991; accepted February 14, 1992.

REFERENCES

Audy, P., Trudel, J., and Asselin, A. (1988). Purification and characterization of a lysozyme from wheat germ. Plant Sci. **58,** 43–50.

Bauw, G., De Loose, M., Inzé, D., Van Montagu, M., and Vandekerckhove, J. (1987). Alterations in the phenotype of plant cells studied by NH_2-terminal amino acid sequence analysis of proteins electroblotted from two-dimensional gel-separated total extracts. Proc. Natl. Acad. Sci. USA **84,** 4806–4810.

Benhamou, N., and Asselin, A. (1989). Attempted localization of a substrate for chitinases in plant cells reveals abundant *N*-acetyl-D-glucosamine residues in secondary walls. Biol. Cell **67,** 341–350.

Blum, H., Beier, H., and Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8,** 93–99.

Boller, T., Gehri, A., Mauch, F., and Vogeli, U. (1983). Chitinase in bean leaves: Induction by ethylene, purification, properties and possible function. Planta **157,** 22–31.

Broekaert, W.F., Van Parijs, J., Allen, A.K., and Peumans, W.J. (1988). Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. Physiol. Mol. Plant Pathol. **33,** 319–331.

Broglie, K.E., Gaynor, J.J., and Broglie, R.M. (1986). Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris.* Proc. Natl. Acad. Sci. USA **83,** 6820–6824.

Clegg, J.C.S. (1982). Glycoprotein detection on nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: Application to arenavirus and flavivirus proteins. Anal. Biochem. **127,** 389–394.

Cordewener, J., Booij, H., Van Der Zandt, H., Van Engelen, F., Van Kammen, A., and De Vries, S. (1991). Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes. Planta **184,** 478–486.

De Vries, S.C., Springer, J., and Wessels, J.G.H. (1982). Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. Planta **156,** 129–135.

De Vries, S.C., Booij, H., Janssens, R., Vogels, R., Saris, L., Lo Schiavo, F., Terzi, M., and Van Kammen, A. (1988a). Carrot somatic embryogenesis depends on the phytohormone-controlled expression of correctly glycosylated extracellular proteins. Genes Dev. **2,** 462–476.

De Vries, S.C., Booij, H., Meyerink, P., Huisman, G., Wilde, H.D., Thomas, T.L., and Van Kammen, A. (1988b). Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta **176,** 196–204.

Gamborg, O.L. (1970). The effects of amino acids and ammonium on the growth of plant cells in suspension culture. Plant Physiol. **45,** 372–375.

Giuliano, G., Lo Schiavo, F., and Terzi, M. (1984). Isolation and developmental characterization of temperature sensitive carrot cell variants. Theor. Appl. Genet. **67,** 179–183.

Halperin, W. (1966). Alternative morphogenetic events in cell suspensions. Am. J. Bot. **53,** 443–453.

Harikrishna, K., Jampates-Beale, R., and Charles, S. (1991). A basic chitinase is expressed at high levels in the transmitting tissue of tomatoes. In Molecular Biology of Plant Growth and Development, Program and Abstracts, R.B. Hallick, ed (Tucson: Department of Biochemistry, University of Arizona), abstr. 511.

Hedrick, S.A., Bell, J.N., Boller, T., and Lamb, C.J. (1988). Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. Plant Physiol. **86,** 182–186.

Joosten, M.H.A.J., and De Wit, P.J.G.M. (1989). Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium vulvum* (syn. *Fulvia fulva*) as 1,3-β-glucanases and chitinases. Plant Physiol. **89,** 945–951.

Keefe, K., Hinz, U., and Meins, F., Jr. (1990). The effect of ethylene on the cell-type–specific and intracellular localization of (1-3) β-glucanase and chitinase in tobacco leaves. Planta **182,** 43–51.

Kragh, K.M., Jacobsen, S., Mikkelsen, J.D., and Nielsen, K.A. (1990). Tissue-specific expression and differential induction of chitinase isoforms in barley (*Hordeum vulgare* L.). Ph.D. Dissertation, for K.M. Kragh (Frederiksberg, Denmark: Royal Veterinary and Agricultural University), pp. 65–78.

Kragh, K.M., Jacobsen, S., Mikkelsen, J.D., and Nielsen, K.A. (1991). Purification and characterization of three chitinases and one β-1,3-glucanase accumulating in the medium of cell suspension cultures of barley (*Hordeum vulgare* L.). Plant Sci. **76,** 65–77.

Kurosaki, F., Tashiro, N., and Nishi, A. (1990). Chitinase induction in carrot cell cultures treated with various fungal compounds. Biochem. Int. **20,** 99–106.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Linthorst, H.J.M., Van Loon, L.C., Van Rossum, C.M.A., Mayer, A., Bol, J.F., Van Roekel, J.S.C., Meulenhoff, E.J.S., and Cornelissen, B.J.C. (1990). Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. Mol. Plant-Microbe Interact. **3,** 252–258.

Lo Schiavo, F., Giuliano, G., De Vries, S.C., Genga, A., Bollini, R., Pitto, L., Cozzani, G., Nuti-Ronchi, V., and Terzi, M. (1990). A carrot cell variant temperature sensitive for somatic embryogenesis reveals a defect in the glycosylation of extracellular proteins. Mol. Gen. Genet. **223,** 385–393.

Lotan, T., Ori, N., and Fluhr, R. (1989). Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell **1,** 881–887.

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue. Plant Physiol. **88,** 936–942.

Meeks-Wagner, D.R., Dennis, E.S., Tran Thanh Van, K., and Peacock, W.J. (1989). Tobacco genes expressed during in vitro floral initiation and their expression during normal plant development. Plant Cell **1,** 25–35.

Metraux, J.P., and Boller, T. (1986). Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial and fungal infections. Physiol. Mol. Plant Pathol. **28,** 161–169.

Mikkelsen, J.D., Berglund, L., Nielsen, K.K., Christiansen, H., and Bojsen, K. (1992). Structure of endochitinase genes from sugar beets. In Proceedings of the 5th International Conference on Chitin and Chitosan, C.J. Brine, ed (Amsterdam: Elsevier), in press.

Molano, J., Duran, A., and Cabib, A. (1977). A rapid and sensitive assay for chitinase using tritiated chitin. Anal. Biochem. **83,** 648–656.

Molano, J., Polacheck, I., Duran, A., and Cabib, E. (1979). An endochitinase from wheat germ. Activity on nascent and preformed chitin. J. Biol. Chem. **254,** 4901–4907.

Neale, A.D., Wahleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meeks-Wagner, D.R., Peacock, W.L., and Dennis, E.S. (1990). Chitinase, β-1,3-glucanase, osmotin and extensin are expressed in tobacco explants during flower formation. Plant Cell **2,** 673–684.

Nomura, K., and Komamine, A. (1985). Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. Plant Physiol. **79,** 988–991.

Satoh, S., and Fujii, T. (1988). Purification of GP57, an auxin-regulated extracellular glycoprotein of carrots and its immunocytochemical localization in dermal tissues. Planta **175,** 364–373.

Schlumbaum, A., Mauch, F., Vogeli, U., and Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. Nature **324,** 365–367.

Shinshi, H., Mohnen, D., and Meins, F., Jr. (1987). Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissue by auxin and cytokinin. Proc. Natl. Acad. Sci. USA **84,** 89–93.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell **3,** 907–921.

Trudel, J., and Asselin, A. (1989). Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal. Biochem. **178,** 362–366.

Usui, T., Matsui, H., and Isobe, K. (1990). Enzymic synthesis of useful chitooligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate. Carbohydr. Res. **203,** 65–77.

Van Engelen, F.A., and De Vries, S.C. (1992). Extracellular proteins in plant embryogenesis. Trends Genet. **8,** 66–70.

Van Engelen, F.A., Sterk, P., Booij, H., Cordewener, J.H.G., Rook, W., Van Kammen, A., and De Vries, S.C. (1991). Heterogeneity and cell type–specific localization of a cell wall glycoprotein from carrot suspension cells. Plant Physiol. **96,** 705–712.

Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlage, M., van den Elzen, P.J.M., and Cornelissen, B.J.C. (1991). Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans.* Plant Cell **3,** 619–628.

Zhu, Q., and Lamb, C.J. (1991). Isolation and characterization of a rice gene encoding a basic chitinase. Mol. Gen. Genet. **226,** 289–296.