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Premature Dissolution of the Microsporocyte Callose Wall Causes Male Sterility in Transgenic Tobacco

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Male sterility in a petunia cytoplasmic male sterile line has been attributed to the early appearance of active callase, a β-1,3-glucanase, in the anther locule. This leads to premature dissolution of the callose walls surrounding the microsporogenous cells. We have mimicked this aspect of the petunia line in transgenic tobacco by engineering the secretion of a modified pathogenesis-related vacuolar β-1,3-glucanase from the tapetum prior to the appearance of callase activity in the locule. Plants expressing the modified glucanase from tapetum-specific promoters exhibited reduced male fertility, ranging from complete to partial male sterility. Callose appearance and distribution are normal in the male sterile transgenic plants up to prophase I, whereupon callose is prematurely degraded. Meiosis and cell division occur normally. The resultant microspores have an abnormally thin cell wall that lacks sculpturing. The tapetum shows hypertrophy. Male sterility is probably caused by bursting of the aberrant microspores at a time corresponding to microspore release. These results demonstrate that premature callose degradation is sufficient to cause male sterility and suggest that callose is essential for the formation of a normal microspore cell wall.

INTRODUCTION

Before meiosis in angiosperms, microsporocytes synthesize a special cell wall consisting of callose, a β-1,3–linked glucan, between the cellulose cell wall and plasma membrane. Callose deposition continues through meiosis so that each of the products of meiosis, the tetrad of microspores, is also surrounded by callose. After the completion of meiosis and the initiation of microspore exine wall formation, the callose wall is broken down by callase, a tapetally secreted β-1,3-glucanase activity (Steiglitz, 1977), releasing free microspores into the locular space. In anthers of petunia and lily, callase activity follows a tight pattern of developmental regulation (Frankel et al., 1969; Steiglitz and Stern, 1973; Steiglitz, 1977). A relatively low level of activity is present in anthers during the first meiotic division, but once the second meiotic division starts, this rapidly increases and peaks at the time of microspore release.

In anther locules of several cytoplasmic male sterile (cms) petunia lines, callose wall dissolution occurs earlier (Rosy Morn [RM] cms) or later (partially restored cms) than normal due to the premature or delayed appearance of callase activity (Izhar and Frankel, 1971). It has been suggested that mistiming of callose wall degradation may be a primary cause of male sterility in these lines (Izhar and Frankel, 1971) and also in male sterile sorghum lines (Warmke and Overman, 1972). However, it is not known whether this is the only factor that contributes to male sterility. Changes in the abundances of amino acids in the locule (Izhar and Frankel, 1973) and alterations in tapetal morphology (Bino, 1985a, 1985b) precede callose wall degradation in the RM cms line. Because the molecular basis of the phenotype appears to be the expression of a novel mitochondrial protein (Nivison and Hanson, 1989), which may affect mitochondrial energy production (Connett and Hanson, 1990), there may be a general breakdown of normal tapetal or microsporogenous cell function leading to male sterility. These male sterile plants, therefore, provide only circumstantial evidence that the callose wall has a vital function in microsporogenesis.

Callose is not part of the normal cell wall; however, when produced in response to wounding (Goodman et al., 1986), it participates in the formation of a physical barrier against pathogen invasion. Delmer (1987) termed this callose "useful" and suggested that callose produced in other contexts, for example, in the cell plate and pollen tube wall, is an "accidental," nonfunctional product of callose synthase activity induced by elevated Ca^{2+} concentrations associated with other cellular processes. Thus, in the absence of clear evidence of an essential role, microsporocyte callose could be considered "accidental." However, several theories on the biological functions of this special callose wall have been advanced. Waterkeyn (1962) suggested that callose fulfills an important biological role in acting as a temporary wall that both isolates the products of meiosis to prevent cell cohesion and fusion and, upon its dissolution, results in the release of free cells. Heslop-Harrison (1964) proposed that the callose wall functions as a molecular filter isolating the developing microspores from the influence of the surrounding diploid tissue or sister spores (Heslop-Harrison and Mackenzie, 1967). These authors also

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suggested that the wall may prevent premature swelling of the microspores. Finally, Waterkeyn and Beinfait (1970) suggested that the callose wall provides a template or mold for the formation of the species-specific exine sculpturing patterns seen on mature pollen grains.

We have sought to mimic the natural RM cms phenotype by creating transgenic tobacco plants that secrete a modified pathogenesis-related (PR) vacuolar β-1,3-glucanase from the tapetum before the appearance of callase activity in the locule. As well as being attractive for plant breeding purposes to facilitate the production of hybrid seed (Mariani et al., 1990), the resulting phenocopies, it was envisaged, would give a better understanding of the role of callose in microsporogenesis. The results obtained demonstrate that premature callose dissolution is sufficient to cause male sterility in transgenic tobacco and that tapetal hypertrophy can be a consequence of a disruption in microspore development. Aberrant microspore cell wall formation observed in the male sterile plants also suggests that the callose wall is required for the correct formation and surface patterning of the microspore exine wall.

RESULTS

Construction of Chimeric Genes for the Expression of a Modified Basic PR β-1,3-Glucanase in the Tapetum

Two components are required for the construction of a chimeric gene that will cause premature callose wall dissolution when expressed in plants. These are a β-1,3-glucanase that can be secreted from the tapetal cells into the anther locule and a promoter to drive the expression of this β-1,3-glucanase prior to the appearance of normal callase activity in the locule. Although the anther-specific β-1,3-glucanase enzyme (callase) responsible for microspore release has not been cloned, several PR glucanases have been characterized. We have demonstrated that intercellular fluid extracted from salicylic acid–treated tobacco leaves, containing PR glucanase enzymes, is capable of releasing microspores from tetrads in vitro (R. Scott, unpublished results). Therefore, the PR β-1,3-glucanases are able to hydrolyze callose and might substitute for callase if expressed appropriately in vivo. Shinshi et al. (1988) have reported the nucleotide sequence of a tobacco basic vacuolar PR glucanase. We hoped that expression of this gene under the control of tapetum-specific promoters that are transcriptionally active during meiosis would cause premature breakdown of the callose wall and therefore induce male sterility.

Figure 1A shows that vacuolar isoforms of PR β-1,3-glucanases of tobacco are initially synthesized as precursors with N- and C-terminal peptide extensions. The glycosylated C-terminal propeptide (CTPP) and the N-terminal signal peptide are removed upon processing to the mature protein (Shinshi et al., 1988). Removal of the CTPP of barley lectin showed that this extension is necessary for vacuolar targeting (Bednarek et al., 1990). Without the CTPP, lectin follows the default pathway and is secreted from the cell. van den Bulcke et al. (1989) suggested that the 22–amino acid basic vacuolar glucanase CTPP may be the signal that directs this protein to the vacuole. More recently, cDNAs have been isolated that encode acidic glucanases found in the intercellular compartment of

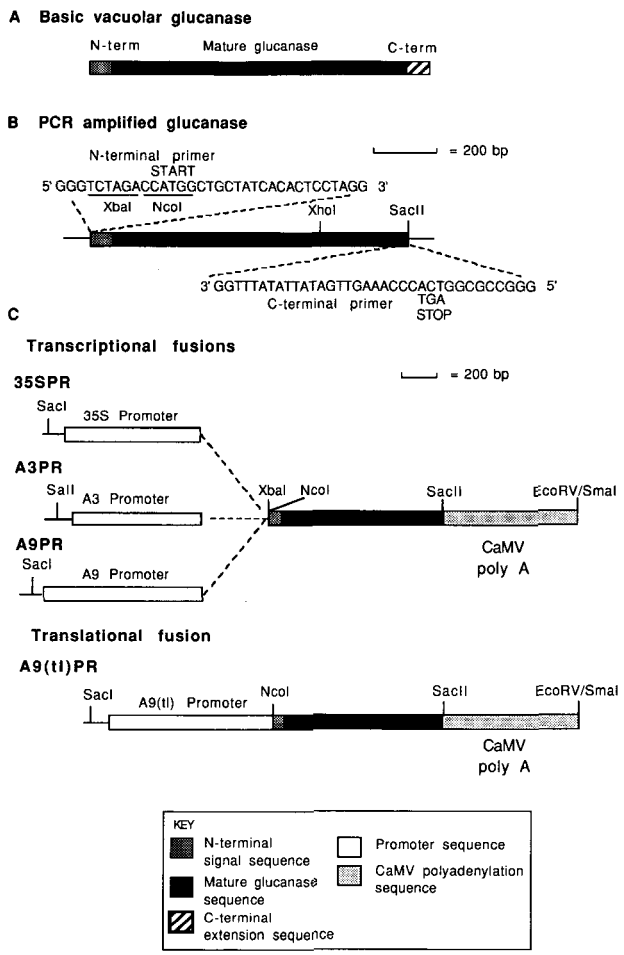

Figure 1. Construction of a Modified Basic PR β-1,3-Glucanase Gene.

(A) Schematic representation of the coding region of the tobacco basic vacuolar PR glucanase gene.

(B) Schematic representation of the modified PR glucanase showing the sequence of the primers used for PCR amplification and relevant restriction enzyme sites.

(C) Diagram showing the strategy for cloning each promoter-glucanase gene construct into pBin19 (Bevan, 1984). The transcriptional fusions, 35S PR and A9 PR, were both cloned as SacI-EcoRV fragments into SacI-SmaI-cut pBin19, and A3 PR was cloned as a SalI-EcoRV fragment into SalI-SmaI-cut pBin19. The translational fusion (A9(tl)PR) was cloned as a SacI-EcoRV fragment into SacI-SmaI-cut pBin19. The A9 translational fusion promoter, unlike that of the A9 transcriptional promoter, includes the entire untranslated A9 leader with the sequence around the initiating ATG of A9 mutated to an NcoI site (Paul et al., 1992).

leaves (Côté et al., 1991; Ward et al., 1991). These secreted forms of β-1,3-glucanases lack a CTPP. This indirect evidence supports the idea that the basic glucanase CTPP contains vacuolar targeting information.

On the basis of this information, we designed synthetic oligonucleotides complementary to the nucleotide sequence of the basic PR β-1,3-glucanase and used them as primers to amplify a modified glucanase that included the N-terminal signal peptide but lacked the CTPP (Figure 1B). This modification was presumed necessary to achieve secretion of the PR glucanase enzyme from the tapetum to the locule. The sequence of the final modified glucanase gene was identical to the equivalent region of the published basic glucanase (Shinshi et al., 1988), except for five nucleotide substitutions (see Methods).

This modified glucanase gene was then transcriptionally fused to a double cauliflower mosaic virus (CaMV) 35S promoter (Guerineau et al., 1988), forming the chimeric gene (35S PR) depicted in Figure 1C. The modified glucanase was also transcriptionally fused to the tapetum-specific promoters of the Arabidopsis A3 and A9 genes (Scott et al., 1991a, 1991b; Paul et al., 1992) (A3 PR and A9 PR constructs in Figure 1C) and translationally fused to the A9 promoter (A9(tl)PR, Figure 1C). Promoter–β-glucoronidase (promoter–GUS) fusions in transgenic tobacco have shown that the A3 and A9 promoters become active in tapetal cells of anthers containing microsporocytes in the early stages of meiosis. Promoter activity reaches a maximum during meiosis, and this level is sustained until shortly before the first microspore mitosis (Scott et al., 1991b; Paul et al., 1992). Thus, the CaMV 35S promoter and the tapetum-specific promoters should be suitable for the premature expression of glucanase in the anther. The chimeric genes were constructed and transformed into tobacco as described in Figure 1 and Methods.

Expression of the Modified Glucanase Gene from the Tapetum-Specific Promoters, but Not from the CaMV 35S Promoter, Causes Male Sterility in Transgenic Tobacco

DNA was extracted from kanamycin-resistant transgenic tobacco plants and analyzed for the presence of the modified glucanase gene by the polymerase chain reaction (PCR) utilizing the N- and C-terminal glucanase oligonucleotides as primers. The native PR glucanase was also amplified by the PCR reaction, but the presence of an intron within this gene resulted in the production of a larger fragment that was easily distinguishable from the transgene. Plants that produced only the larger native PR glucanase gene product upon PCR analysis were termed PCR negative.

Both PCR positive and negative plants were grown to maturity, and the flowers were analyzed. All transformants containing the modified glucanase gene under the control of the A3 or A9 anther-specific promoters were normal in appearance but displayed varying degrees of male fertility. In contrast, all of the PCR negative plants were completely fertile. Transformants with a severe phenotype had small recessed anthers that were brown and appeared to lack pollen grains. In these plants, only flowers that had been cross-pollinated with wild-type tobacco pollen developed normal seed pods. Only one of these plants, A9 PR 5, was completely male sterile; the rest formed a variable number of small pods that were either empty or contained few seeds in comparison to those of wild-type plants. Transformants containing the modified glucanase gene under the control of the double CaMV 35S promoter were completely normal in appearance and significantly more fertile than the plants expressing the modified glucanase gene from the tapetum-specific promoters. The fertility of 35S PR transformed plants was not appreciably different from that of wild-type plants. The bar graph in Figure 2 indicates the degree of fertility displayed by the transformants as determined by the average weight of seeds produced per pod. Plants containing A9 promoter fusions appeared less fertile than those containing the A3 promoter fusion, and the transcriptional fusion to the A9 promoter appeared more effective than the translational A9 fusion. These results are in accord with the relative strengths of these promoters in GUS fusion experiments, which show that the A9 transcriptional promoter is approximately four times more active than the A3 promoter (R. Scott, unpublished observations).

The transmission of the male sterile phenotype to progeny following cross-pollination with wild-type pollen was followed

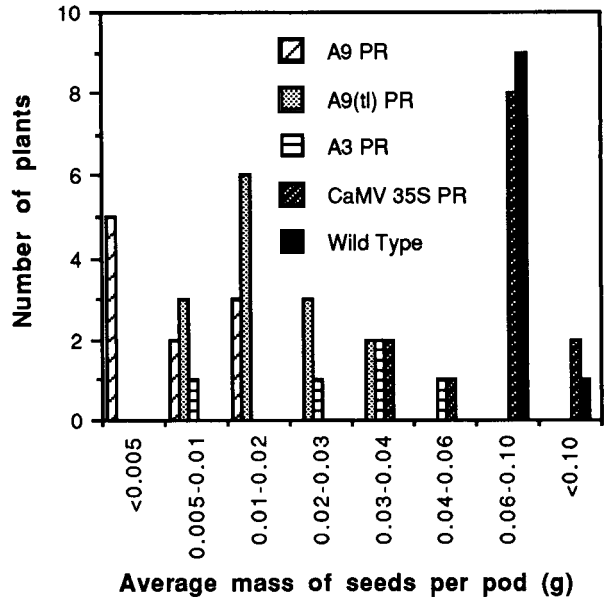

Figure 2. Fertility of Transgenic and Wild-Type Tobacco Plants.

Bar graph showing the average mass of seeds per pod in wild-type tobacco and each transgenic tobacco line (100 wild-type tobacco seeds weigh approximately 0.009 g).

for a number of transformants. For example, kanamycin-resistant seedlings of A9 PR5 × wild type (1 in 20 seedlings was kanamycin sensitive) were grown to maturity, and all exhibited the male sterile phenotype (data not shown).

Anthers from the PCR-positive plants were analyzed for the presence of the introduced glucanase protein by immunoblotting with an antiserum raised against an acidic PR glucanase from tomato that cross-reacts with the tobacco basic PR glucanase. Tobacco buds ranging in length from 8 to 14 mm encompass the period of development where the A3 and A9 promoters are most active, as shown by promoter GUS fusion data (Scott et al., 1991; Paul et al., 1992). The immunoblots shown in Figures 3A and 3B demonstrate that the modified glucanase protein is present in the anthers of transgenic plants. The introduced glucanase protein can be seen on the protein gel blots as an immunoreactive band with a molecular mass of approximately 35 kD that appears to have a slightly increased mobility in comparison to the immunoreactive glucanase enzymes in wild-type tobacco leaf; this is most noticeable in Figure 3C. The PR glucanase proteins present in leaf, represented by immunoreactive bands with molecular masses of approximately 35 to 37 kD, are not detectable in the anthers of control untransformed tobacco plants (Figure 3D). One transformant, A9(tl)PR 17 (Figure 3B, lane 4), which was PCR positive, did not contain a detectable 35-kD immunoreactive band and was significantly more fertile than the other plants containing the A9 and A3 promoters shown in Figure 3.

The presence of PR glucanase protein, as detected on immunoblots, correlates with the male sterile phenotype demonstrated by the transformed plants, except in the case of transformants containing the glucanase gene under the control of the CaMV 35S promoter (Figure 2). The glucanase protein detectable in the anthers of these plants is present at levels similar to those in transformants containing the other constructs, but the lack of a male sterile phenotype suggests that expression in the tapetal cells is either absent or too low to cause significant callose degradation. The glucanase accumulated in these anthers is likely to result from expression of the CaMV 35S promoter in other anther cell types.

The immunoblot in Figure 3C shows that the introduced glucanase protein accumulates in a temporal pattern consistent with the properties of the tapetum-specific promoter. The modified glucanase protein is first detectable during early meiosis, reaches a peak at the stage when microspore release occurs, and then declines in abundance again in anthers at a stage corresponding to microspore mitosis.

Male Sterility Is Associated with Premature Disappearance of the Callose Wall

A study of microsporogenesis was undertaken in transformants containing each of the promoter–glucanase constructs and untransformed control tobacco plants. Anthers at developmental stages between meiocyte and microspore release were dissected from buds and the locular contents stained for callose with aniline blue. The development and dissolution of the microsporocyte callose wall in all CaMV 35S PR plants examined were identical to that in wild-type plants. In contrast, the microsporocyte callose wall was prematurely degraded in the A9 PR, A9(tl)PR, and A3 PR plants that exhibited reduced male fertility. Preliminary studies found no significant difference in the extent and timing of callose degradation in these plants.

Figure 4 shows the appearance of the callose wall in anthers of untransformed tobacco and in male sterile tobacco anthers of an A9(tl)PR plant during the same developmental period. Microsporocytes of both sterile and fertile anthers appear normal before the initiation of meiosis. The meiocytes in both preparations have callose walls, seen as bright yellow fluorescence (Figure 4A). The first observable difference between fertile and sterile anthers occurs during prophase I, when the thick callose wall that previously surrounded the meiocytes disappears in the transformed material (Figure 4B). In wild-type tetrads, callose is deposited between the plasma membrane and the cell wall producing clear boundaries that separate the microspores (Figure 4E). The microspores of sterile anthers appear to lack a callose wall but still remain held together as tetrads by some other material that does not show aniline blue fluorescence. A second difference between tetrads of fertile and sterile anthers was that, whereas tetrads squeezed out from fertile anthers separate easily, presumably due to a lack of intercellular cytoplasmic connections, tetrads from sterile anthers adhered in amorphous clumps. Microspore release does occur in male sterile plants (Figure 4F), but very soon afterward, a large proportion of the deformed microspores burst. The relationship between anther length and developmental stage remains the same in both male fertile and sterile plants, and thus the timing of the individual events that together constitute microsporogenesis appears unaffected in the male sterile plants.

Meiosis Is Normal in the Absence of the Callose Wall

In addition to the microsporocyte cell wall, callose also appears transiently around the megasporocyte during megasporogenesis at early meiotic prophase (Bouman, 1984). Because the temporal and spatial distribution of callose is similar in both the male and female gametophyte, and callose is not a normal component of cell walls, it was considered possible that callose is involved in some aspect of meiosis in higher plants. To determine whether meiosis was normal in male sterile plants, meiotic cells were dissected from both male fertile and male sterile anthers and stained with aceto-orcein. In sterile anthers, despite premature dissolution of callose at early prophase I before the chromosomes have condensed, the sporocytes still undergo apparently normal meiosis. The four products of meiosis can be seen as separate entities held together within tetrads (Figure 4E). However, the four microspores within the tetrad are much more loosely associated than those seen in the untransformed control tetrad as often the nuclei of all four cells can be seen in the same plane. Aceto-orcein staining demonstrated that the microsporocytes had normal numbers of bivalents and chiasmata (results not shown).

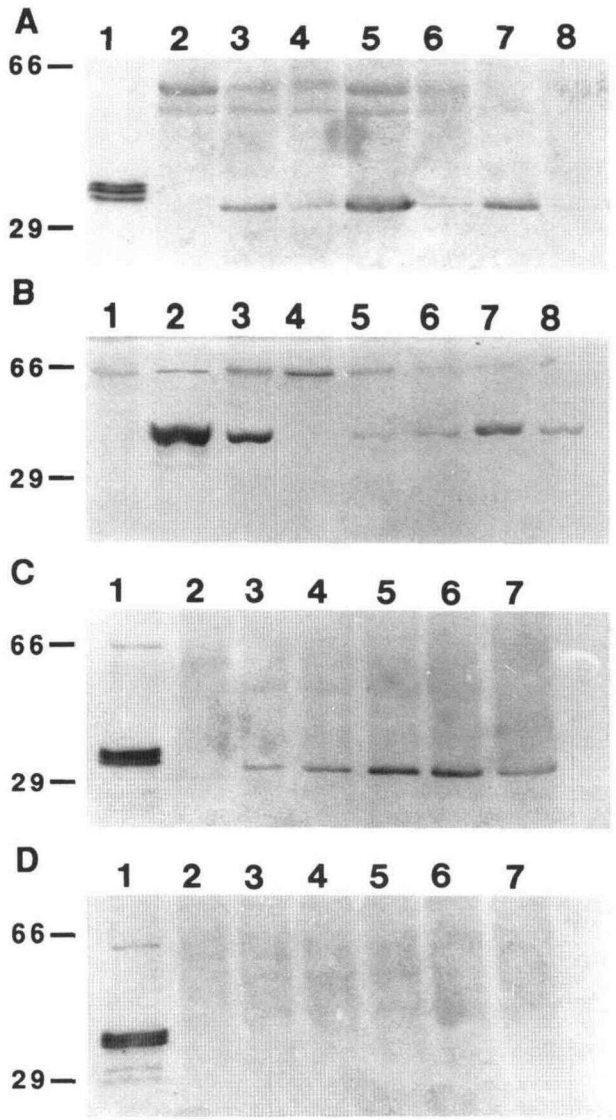

Figure 3. Modified Glucanase Protein Is Present in Anthers of Transgenic Tobacco Plants.

Protein gel blots of SDS–polyacrylamide gels were probed with an antibody raised against a tomato acidic PR glucanase.

(A) Protein gel blot of an 11% SDS–polyacrylamide gel. Lane 1 contains protein extracted from salicylic acid–induced wild-type tobacco leaves, and lanes 2 to 8 contain protein extracted from anthers dissected from buds ranging in length from 8 to 14 mm. Lane 2, wild-type plant; lane 3, A9 PR 1; lane 4, A9 PR 9; lane 5, A9(tl)PR 4; lane 6, A9(tl)PR 13; lane 7, A3 PR 2; lane 8, A3 PR 1.

(B) Protein gel blot of a 15% SDS–polyacrylamide gel. Lane 2 contains protein extracted from salicylic acid–induced wild-type tobacco leaves. Lane 1 and lanes 3 to 8 contain protein extracted from anthers dissected from buds ranging in length from 8 to 14 mm. Lane 1, wild-type plant; lane 3, A9(tl) PR 14; lane 4, A9(tl)PR 17; lane 5, A3 PR 1A; lane 6, 35S PR 2; lane 7, 35S PR 7; lane 8, 35S PR 14.

(C) Protein gel blot of an 11% SDS–polyacrylamide gel. Lane 1 contains protein extracted from salicylic acid–induced wild-type tobacco leaves, and lanes 3 to 7 contain protein extracted from the anthers of the transgenic tobacco plant A9(tl)PR 1. Protein was isolated from anthers at the following developmental stages: archesporial, lane 2; meiocyte, lane 3; tetrad, lane 4; microspore release, lane 5. Lane 6 contains anthers of buds between 12 and 20 mm in length, and lane 7 contains anthers from buds between 20 and 30 mm. Microspore release occurs at a bud length of 9 to 10 mm.

(D) Protein gel blot of an 11% SDS–polyacrylamide gel. Lane 1 contains protein extracted from salicylic acid–induced wild-type tobacco leaves. Lanes 2 to 7 contain protein extracted from wild-type anthers at the same developmental stages and between the same size ranges as described in **(C)**. Molecular mass markers are given at left in kilodaltons.

Microspores Exhibit Aberrant Wall Development in the Absence of Callose

The callose wall is thought to play an important role in the establishment of the first exine patterning seen on the surface of the microspore. Various hypotheses have been proposed to explain the involvement of callose in laying down the pattern of primary exine (primexine). Larson and Lewis (1962) proposed that the callose wall is a source of glucose for the development of cellulosic primexine, which provides the basic framework of the future exine. Waterkeyn and Bienfait (1970) suggested that the callose wall acts as a template or mold, which is filled by primexine. Because callose may play an essential role in the initial steps of microspore wall development, we examined the structure of the microspore wall of the A9(tl)PR transformants.

Electron micrographs revealed abnormal wall development in sterile microspores during the period when the microspores are still embedded in the callose wall of the tetrad in control fertile plants. Figures 5A to 5E show electron micrographs of developing microspores from fertile and sterile tobacco anthers. The thick callose wall surrounding each microspore of the tetrads from control fertile anthers (Figure 5A) is clearly absent in sterile microspores (Figure 5B). The microspores from fertile anthers also appear to have a more regular outline than those from sterile anthers. The characteristic vaulted structure of the exine of control microspores (Figure 5C) is replaced by lamellae of varying thicknesses overlain by irregular deposits of electron dense material (Figure 5D). This material is likely to be sporopollenin. Some binucleate microspores were observed in the sterile anthers that may result from the failure of cytokinesis or subsequent fusion of the microspores (Figure 5E).

The Tapetum Exhibits Hypertrophy in Male Sterile Anthers

In some types of male sterility, ultrastructural and histological examinations have revealed that the first signs of abnormality are found not in the microsporocytes or developing microspores, but in the tapetum (Horner and Rogers, 1974; Horner,

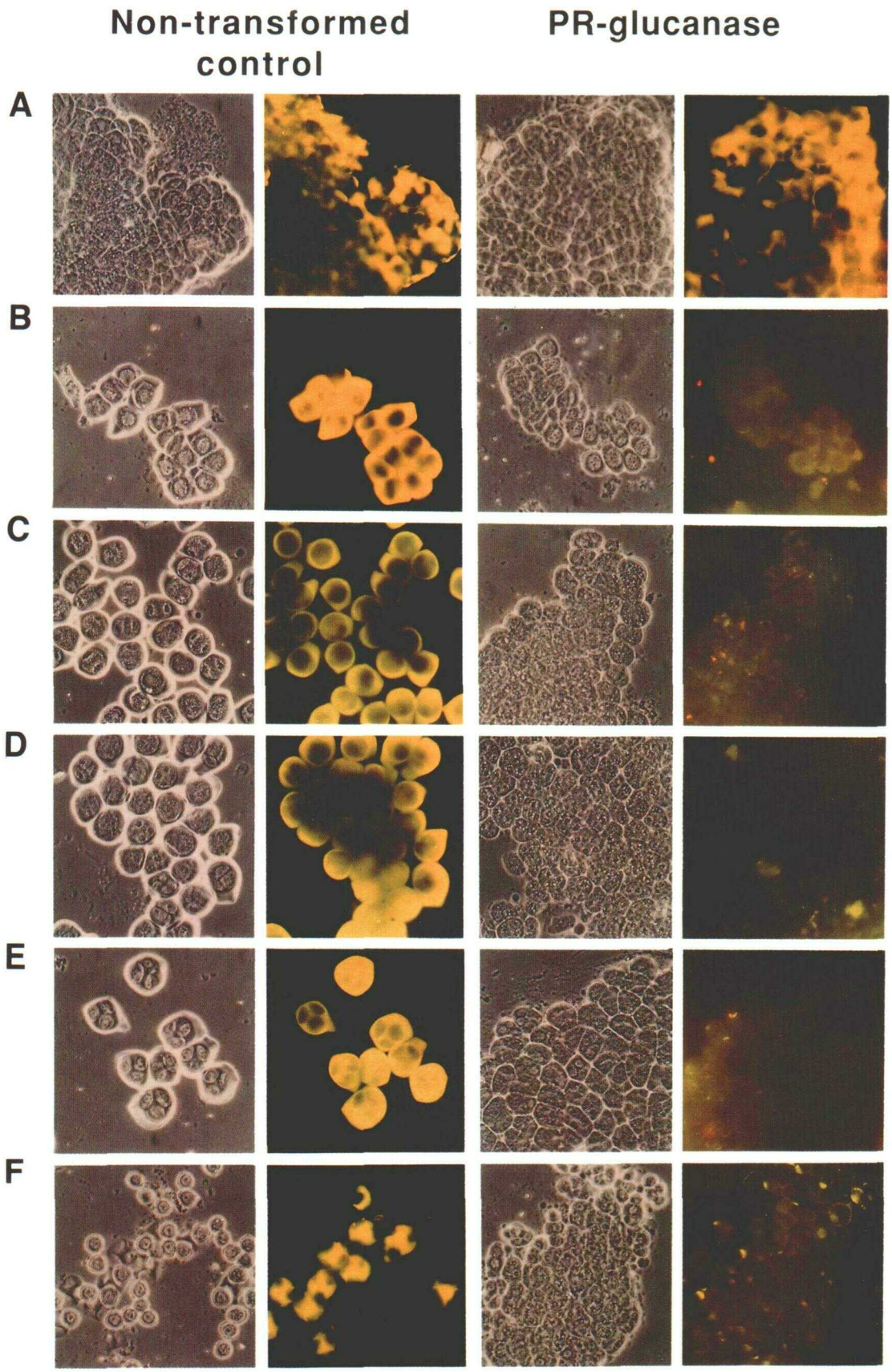

Figure 4. Comparison of the Callose Wall during Microsporogenesis in Wild-Type Tobacco and Those Transformed with Modified Glucanase A9(tl)PR.

1977; Warmke and Lee, 1977; Bino, 1985a, 1985b). However, degeneration of the developing microspores often occurs simultaneously with the proliferation of the tapetum, and therefore it is not clear whether the abortion of the developing microspores stimulates proliferation of the tapetum or whether the proliferating tapetum actually interferes with microspore development. A general comparison of tapetal morphology was therefore made between male fertile and sterile anthers to determine whether disruption of microspore development had any effect on the tapetum.

Sections were made from fertile anthers that contained tetrads and from male sterile anthers at an equivalent stage. These were stained with toluidine blue. Comparison of Figures 5H and 5I shows that the tapetal cells of the male sterile anther are larger and more vacuolate than those in the fertile anther. The locular side of the tapetal cells in the male sterile anther is lightly stained. The observed enlargement and apparent invasion of the locular space by the tapetal cells, a phenomenon termed hypertrophy, are often found in association with male sterility.

Electron micrographs of the tapetum also reveal differences in morphology between fertile (Figure 5F) and sterile (Figure 5G) anthers. The outer wall appears to be absent, and orbicules form irregular aggregations in the tapetum wall of sterile anthers. However, these features occur during normal tapetal development. A developmental sequence would therefore give a clearer indication of aberrant tapetal wall structure in the male sterile plants.

DISCUSSION

Secretion of a Modified Glucanase from the Tapetum Causes Male Sterility

The observation that male sterility appeared to be associated with mistiming of callase activity in cms lines of petunia suggested a novel route to the production of male sterile plants and a way to determine the role of callose in microsporogenesis. Ideally, phenocopies of the petunia mutants should express authentic callase activity in the anther locule either prematurely or late relative to the appearance of activity of the native callase. We chose to try and mimic the premature expression of callase because this avoids the need to suppress native callase activity. In addition, these mutants were more likely to yield information on the role of callose. In our laboratory, we have screened anther cDNA libraries with PR glucanase DNA probes and antibodies raised to PR glucanases in an attempt to clone the cDNA encoding callase. This approach proved unsuccessful, suggesting that callase is significantly diverged from other β-1,3-glucanases. However, the observation that PR glucanases could degrade callose surrounding tetrads in vitro suggested that these enzymes might substitute for callase in vivo. Therefore, a basic vacuolar β-1,3-glucanase was modified for secretion and expressed in tobacco from a CaMV 35S promoter and from tapetum-specific promoters. Transgenic plants expressing the modified glucanase gene from the tapetum-specific promoters exhibited moderate to complete reduction in male fertility. This phenotype was associated with the premature disappearance of callose from the microsporocyte walls, indicating that the modified glucanase is secreted from the tapetum and is active within the anther locule.

Removal of the C-Terminal Propeptide Directs Modified Glucanase to the Anther Locule

The secretion of proteins after entry into the endoplasmic reticulum is generally accepted to be a default pathway, whereas proteins directed to subcellular compartments contain specific targeting signals (Chrispeels, 1991). van den Bulcke et al. (1989) suggested that the CTPP of tobacco basic vacuolar glucanase may function to direct the protein to the vacuole. A basic glucanase lacking the CTPP has been expressed in the anther tapetum of transgenic tobacco plants, thereby indirectly examining the role of the CTPP in vacuolar targeting. The deletion of the CTPP resulted in secretion of the mature glucanase enzyme into the locular space. This experiment provided indirect evidence that the CTPP is required for vacuolar targeting. However, the CTPP may not be the complete signal because

Figure 4. (continued).

(A) to **(F)** The extruded locular contents of wild-type and transformed tobacco anthers at a series of developmental stages were stained for callose with aniline blue. In each case, the panels of the left-hand column show the material under phase contrast and the panels of the right-hand column display the same view under blue excitation to highlight callose (yellow fluorescence).

(A) Meiocyte.

(B) Prophase of meiosis I.

(C) Telophase of meiosis I.

(D) Telophase of meiosis II.

(E) Tetrad of microspores.

(F) Microspore release.

With the exception of the meiocyte preparation, photographic exposure time for aniline blue fluorescence in the A9(tl)PR material was three times that for the wild type.

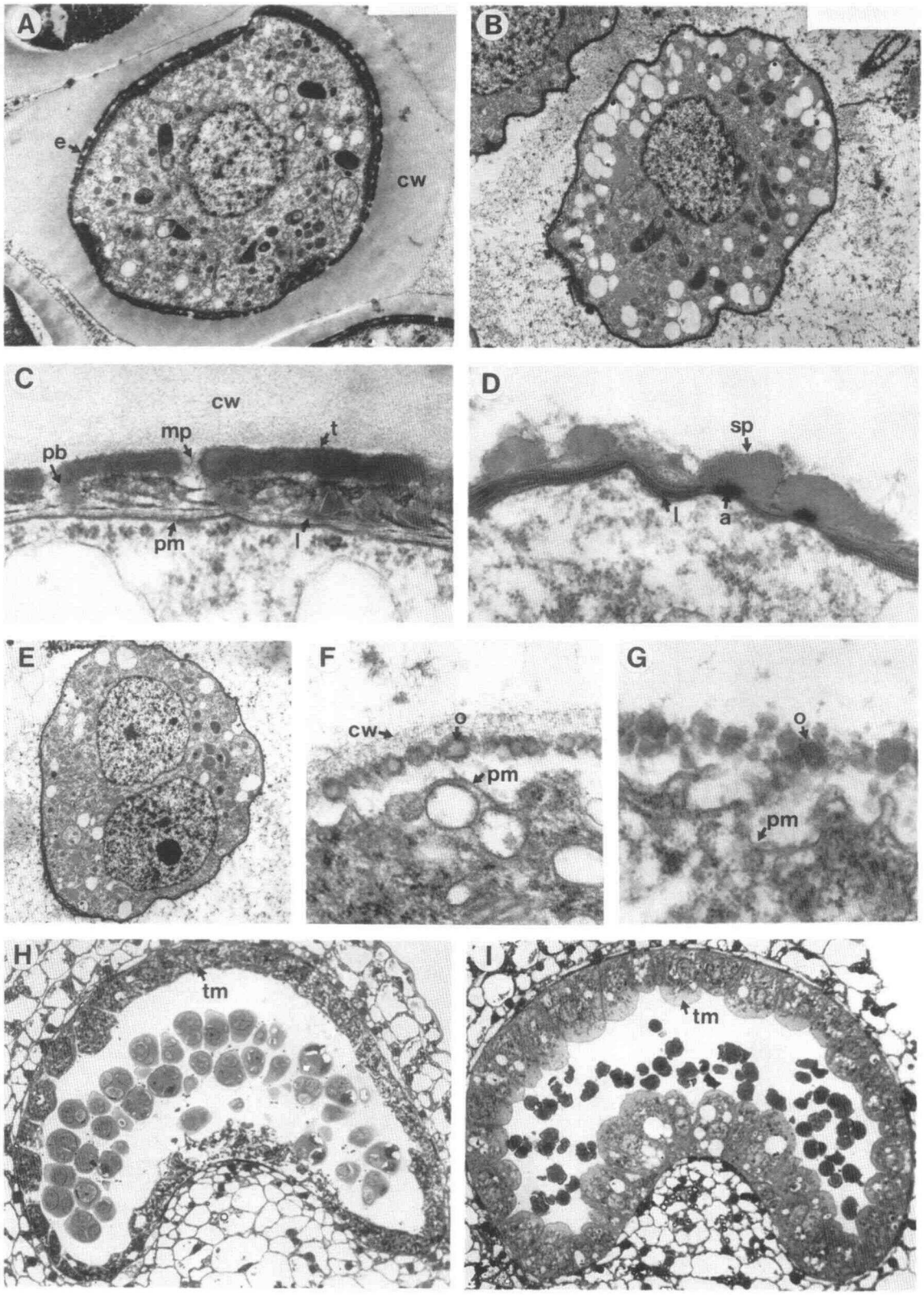

Figure 5. Premature Callose Dissolution Causes Aberrant Microspore and Tapetal Development.

further information could reside within the remaining glucanase sequence. Targeting a nonvacuolar reporter protein to the vacuole by the addition of the basic glucanase CTPP would confirm the suggested role.

Modified Glucanase Is Active in the Anther Locules of Transgenic Tobacco

Premature dissolution of callose requires that conditions in the anther locule, prior to normal callase activity, are conducive for glucanase activity. Studies in petunia (Izhar and Frankel, 1971) and lily (Linskens, 1956) have shown that the locular fluid pH falls during microsporogenesis. In petunia, the pH drops from 7.0 to 6.0 immediately preceding the onset of callase activity in the locule both in wild-type petunia and in the mutants with early or delayed callase activity (Izhar and Frankel, 1971). Because the optimum pH for callase activity is between 4.8 and 5.0, with activity undetectable above 6.3, Izhar and Frankel suggested that the timing of callase activity could be controlled by pH. Preliminary results suggest that a similar pH fall occurs during microsporogenesis in tobacco (D. Worrall, unpublished observation). Determination of the presence and timing of any fall in pH in the male sterile tobacco locules should determine whether the pH fall is independent of callase activation and callose degradation as predicted by Izhar and Frankel.

The pH activity curve of the basic vacuolar glucanase is significantly different from that of callase. At pH 7.0, where callase has no activity, the basic vacuolar glucanase retains 26% of its optimal activity, measured at pH 5.0 (Felix, 1984). This difference may have been crucial to the success of this work because in the absence of a coordinate pH drop, premature expression of callase may prove ineffective in causing premature callose degradation.

The transgenic plants expressing the modified glucanase gene from the tapetum-specific promoters displayed a range of male fertility. It is likely that complete male sterility requires high level accumulation of the modified glucanase in the locule because the pH of the locule is not optimum for enzyme activity. No clear correlation was observed on protein gel blots between the amount of modified glucanase in the anther and the degree of male sterility of the plant. This is probably due to the technical difficulty of collecting anthers that are precisely at the same developmental stage. However, there appeared to be a correlation between the known strength of the tapetum-specific promoters and the degree of male fertility.

Transgenic Plants Expressing the Modified Glucanase Gene from the CaMV 35S Promoter Do Not Show an Aberrant Phenotype and Are Male Fertile

Unlike the fusions to the tapetum-specific promoters, the fusion of the modified glucanase gene to the CaMV 35S promoter leads to the production of fertile plants. Plegt and Bino (1989) have shown with GUS fusions that the CaMV 35S promoter has strong activity in the vascular cylinder of the anther, but activity is undetectable in the tapetum and sporogenous cells during premeiotic, tetrad, and microspore release stages. This information supports the view that the modified glucanase protein accumulated in anthers of 35S PR plants is synthesized in cell types other than the tapetal or microsporogenous cells. Expression of the modified glucanase gene in the tapetum is either too low or occurs at a time in development inappropriate to cause male sterility.

Despite high levels of expression of the modified glucanase gene in the 35S PR plants, they appear to be phenotypically normal. It is possible that these plants will only display a phenotype when subjected to conditions that induce callose formation such as wounding or pathogen attack.

Figure 5. (continued).

Transverse sections through anthers from a wild-type and an A9(tl)PR tobacco plant, viewed by transmission electron microscopy (**A** to **G**) or by light microscopy (**H** to **I**). All anthers were at the tetrad stage of development.

(A) Microspore of a tetrad from a wild-type tobacco plant. The microspore exine (e) is well developed within the callose wall (cw) of the tetrad. ×3600.

(B) Microspore from an A9(tl)PR transformant. The callose wall is absent, and the microspore cell surface is electron dense and apparently lacks exine patterning. ×3600.

(C) Developing microspore wall in wild-type tobacco. Exine wall formation occurs between the callose wall (cw) and the plasma membrane (pm) of the microspore. Laminations (l) are present beneath the regular deposits of sporopollenin that constitute the young tectum (t) and probacula (pb) of the developing exine. Several micropores (mp) are present within the tectum. ×37,500.

(D) Aberrant microspore wall development in A9(tl)PR-transformed tobacco. Noncompressed laminations (l) are apparent at the cell surface. Globular deposits of a material with the appearance of sporopollenin (sp) lie on the outside of the laminations. Several of the globules contain a very electron dense region (a) that abuts the cell surface and interrupts the laminations. ×37,500.

(E) A binucleate microspore from an A9(tl)PR-transformed tobacco plant. ×3400.

(F) Portion of the luminal surface of a tapetal cell in a wild-type tobacco plant. A line of orbicules (o) can be seen between the cell wall (cw) and the plasma membrane (pm). ×34,500.

(G) Portion of the luminal surface of a tapetal cell in an A9(tl)PR-transformed tobacco plant. The cell wall is absent, and the orbicules (o) have an irregular distribution and appear less homogenous than in the wild type. pm, plasma membrane. ×34,500.

(H) Section through a wild-type anther showing the dimensions of the normal tapetum (tm). ×220.

(I) Section through an anther from an A9(tl)PR-transformed tobacco plant. The tapetum (tm) is highly vacuolated and shows signs of hypertrophy. ×220.

Callose Is Required for Normal Microspore Wall Formation in Tobacco

Although the RM cms line has been the subject of genetic (Nivison and Hanson, 1989), biochemical (Izhar and Frankel, 1971), and ultrastructural (Bino, 1985a, 1985b) studies, no detailed examination of the consequence of early callose dissolution has been undertaken. This may be due in part to the complex phenotype of this mutant; premature callose dissolution is probably only one manifestation of a general breakdown in microsporogenesis. A complicating aspect of this picture is that Bino (1985a), working with petunia lines apparently similar to those used by Izhar and Frankel (1971), could not detect premature callose wall dissolution. Therefore, the possibility exists that the ultrastructural studies of Bino (1985a, 1985b) may have been performed on a different petunia line.

The creation of transgenic tobacco plants that mimic only the premature callose dissolution observed by Izhar and Frankel (1971) has unambiguously determined the effect of premature callose dissolution on microsporogenesis. It appears that the callose cell wall is not required for meiosis or cytokinesis because four separate microspores are formed and held together in a tetradlike structure in the male sterile anthers. This suggests that material other than callose is capable of holding the microspores in a tetrad. Release of the microspores from the tetrad is also observed in the male sterile anthers, indicating that this process may normally require factors in addition to callase (Steiglitz, 1977; Sexton et al., 1990). One could speculate that the residual wall forming the tetrad may be cellulosic and that microspore release also requires the secretion of a cellulase. Callose is however required for the formation of a normal microspore cell wall. In its absence, the exine wall lacks regular sculpturing and is composed of an unusual multilaminate structure overlain by apparently random deposits of a material that is probably sporopollenin. The multilaminate wall appears to be an extreme form of the structures termed lamellae observed on the cell surface of developing microspores (Rowley and Southworth, 1967; Dickinson and Heslop-Harrison, 1968). Interestingly, this multilaminate structure is almost identical to those observed in early exine development in liverwort spores (Rowley and Southworth, 1967; Brown and Lemmon, 1990). In our material, sporopollenin is apparently deposited directly on the lamellae (Figure 5D) in accord with the model of Rowley and Southworth (1967).

The first step in the formation of the microspore exine wall appears to be the deposition of a cellulosic matrix between the cell plasma membrane and the callose wall. Dickinson and Heslop-Harrison (1968) suggest that the patterning of the exine is a consequence of the folding of the lamellae that appear after primexine formation. The lamellae lie either parallel to the microspore surface and subsequently form the tectum, or fold perpendicular to the cell surface forming radial protrusions that determine the position of the probacula. Sporopollenin, secreted by the microspore, then accumulates on the lamellae surface beneath the callose wall, the lamellae protrusions eventually forming the columnlike bacula of the exine. Following callose wall dissolution, the tapetum also contributes to sporopollenin deposition, leading to the completion of the intricate exine sculpturing of the mature pollen grain.

In the model of Waterkeyn and Beinfait (1970), the callose wall acts as a mold that is filled by the primexine. Subsequent sporopollenin deposition on the primexine leads to the final patterned exine wall. The internal surface pattern of the callose wall could arise by local differences in growth rate or callose degradation. In support of this model, the internal surface of the callose wall of *Ipomoea purpurea* was shown to have a regular hollow geometric pattern visible in phase contrast or fluorescence (Waterkeyn and Beinfait, 1970). Also, Vijayaragheven and Shukla (1977) showed by electron microscopy that microspores from *Pergularia daemia*, a species with no callose wall, develop a very sparse exine lacking typical surface patterning. In opposition to this model, a study of *Bougainvillea spectabilis* has shown that the plasma membrane rather than the callose wall is more likely to be the initiator of exine patterning (Takahashi and Skvarla, 1991). Thus, any model that proposes a role for callose in the formation of the microspore exine wall must explain why exine patterning is possible in the few species that do not produce callose, e.g., *Pananus odoratissimus* (Periasamy and Amalathas, 1991), or form exine after microspore release as in sorghum (Christensen et al., 1972).

In the transgenic sterile plants, the lamellae lie parallel to the microspore surface and do not fold to form probacula. This apparently explains the lack of surface sculpturing. However, at present our results do not help to determine whether callose forms a template for primexine patterning, provides a uniform surface against which primexine is deposited, or simply acts as a source of glucose for primexine formation. Further work is required to determine whether the primexine is actually formed in the mutants. Because the microspores burst shortly after release from the tetrad, perhaps due to an osmotic imbalance in the locular fluid, the possibility exists that microspores transferred to normal conditions would form unsculptured but viable pollen grains.

Tapetum Is Abnormal in Male Sterile Transgenic Tobacco

Apparently significant differences in tapetal morphology exist between fertile and male sterile anthers. The most obvious abnormality is the increase in volume and vacuolation of the male sterile tapetal layer. This may be caused by a retention in the tapetal cells of materials that would normally be utilized by the developing microspores or might be due to reabsorption of sugars arising from the premature degradation of the callose wall. Irrespective of the cause of the observed tapetal hypertrophy, this result indicates that tapetal hypertrophy observed in many male sterile plants may simply be due to microspore abortion rather than tapetal malfunction.

In conclusion, we have demonstrated that premature dissolution of the callose wall formed in microsporogenesis leads to male sterility in tobacco. This result supports the suggestion that the premature appearance of callase activity in RM cms petunia plants is sufficient to cause sterility in these lines. In the absence of the callose wall, an abnormal microspore wall is formed and microspores burst shortly after their release from tetradlike structures. In fertile plants, microspores rapidly increase in volume after microspore release. It is possible that this process is initiated in the male sterile plants, but because the microspore wall is abnormal the cells burst. Further work on these male sterile plants should clarify the role of callose in microsporogenesis.

METHODS

Plant Material

Material for nucleic acid isolation was obtained from *Nicotiana tabacum* SR1 plants grown under normal greenhouse conditions. To induce a wound response, tobacco plants were sprayed on two consecutive days with 5 mM salicylic acid (from a 0.25 M stock solution adjusted to pH 7.0 with KOH). One day after the final spraying, leaves were harvested, washed thoroughly with tap water, dried, frozen in liquid nitrogen, and stored at −80°C prior to nucleic acid extraction. Leaf material was ground to a fine powder with a pestle in a mortar that was cooled with liquid nitrogen, and RNA was isolated using a phenol-based method as described previously (Draper et al., 1988).

Production of Plants Containing a Modified Glucanase Gene

cDNA was synthesized from 5 μg of total tobacco leaf RNA using a cDNA synthesis kit (Amersham). Synthesis was according to the manufacturer's instructions with the following modifications. First-strand cDNA was primed with the pathogenesis-related (PR) glucanase C-terminal oligonucleotide (Figure 1B) and synthesized using murine reverse transcriptase. After second-strand synthesis, the cDNA was purified by phenol/chloroform extraction and ethanol precipitation. A linker, consisting of two complementary synthetic oligonucleotides, OG6 and OG7, was ligated to the cDNA. The sequences of the OG6 and OG7 primers are 5′-GGCCATGGAATTCATCTAGACC-3′ and 5′-GGTCTAGATGAATTC-3′, respectively. Because synthetic oligonucleotides lack a 5′ phosphate group, this linker ligates only to the 5′ end of the cDNA. Following linker ligation, the cDNA was diluted 1/50 and amplified by polymerase chain reaction (PCR) using the OG6 and C-terminal oligonucleotides as primers. Twenty-five amplification cycles were performed, each consisting of 1 min and 20 sec at 94°C, 2 min at 55°C, and 2 min at 72°C. A second round of amplification was then carried out using the N- and C-terminal oligonucleotide primers (Figure 1B) and under the conditions described above. All the primers were used at a concentration of 1 μM. Several different PCR products were cloned as XbaI and SacII fragments into XbaI, SacII-cut pBluescript KS- (Stratagene) and sequenced. The resulting PCR clone was found, by comparison to the published basic glucanase sequence (Shinshi et al., 1988), to have several frameshift mutations in the 3′ end that would have prevented the production of an active protein. Sequencing of several other PCR clones identified one that was free of mutations in this region. An XhoI site in the 3′ end enabled the construction of a composite gene from the two clones. Comparison with the published basic PR glucanase sequence (Shinshi et al., 1988) revealed five nucleotide substitutions that are as follows: the codon starting at position 282 is CGA instead of GCA, altering the amino acid to arginine from alanine; the codon starting at position 498 is AGC instead of AAC, altering the amino acid to serine from asparagine; the codon starting at position 864 is GTT instead of ATT, altering the amino acid to valine from isoleucine; the codon starting at position 996 is CTC instead of CCC, altering the amino acid to leucine from proline. Gene constructs were made as illustrated in Figure 1C, transferred to *Agrobacterium tumefaciens* pGV2260, and transformed into tobacco as described previously (Draper et al., 1988).

PCR Analysis of Kanamycin-Resistant Plants

A small piece of tobacco leaf was removed from each plant with the lid of a 1.5-mL microcentrifuge tube, and DNA was extracted by a rapid method described by Edwards et al. (1991). One one-hundredth of the resulting DNA solution was then used in a PCR reaction, using the N- and C-terminal oligonucleotides as primers, both at a concentration of 1 μM. Thirty amplification cycles were performed, each consisting of 1 min and 20 sec at 94°C, 2 min at 57°C, and 2 min at 72°C. The amplified DNA was then visualized by ethidium bromide staining after electrophoresis of samples on a 0.8% agarose gel.

Protein Extraction and Electrophoresis

Protein was extracted from eight anthers dissected from buds at the appropriate developmental stage. Anthers were frozen with dry ice and stored at −80°C prior to protein extraction. Frozen anther material was ground in an Eppendorf tube with a microhomogenizer, and 90 μL of cold extraction buffer (0.1 M Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM DTT, and 4 mM phenylmethylsulfonyl fluoride) was added. The samples were mixed, thawed, and then centrifuged for 3 min to remove the debris. The supernatant was combined with SDS sample buffer. After boiling for 3 min, a proportion of each sample was loaded onto a 11% or 15% SDS–polyacrylamide gel to give equal amounts of protein in each lane.

Total cell protein was extracted from salicylic acid–treated tobacco leaves by grinding tissue to a powder in a mortar cooled with liquid nitrogen. Four milliliters of buffer (0.1 M sodium citrate, pH 8.0, 10 mM sodium ascorbate, 1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol) was added per gram of tissue and ground with the frozen tissue. After thawing, the cell debris was pelleted from the homogenate by centrifugation at 10000 rpm for 20 min. Proteins in the supernatant were precipitated with ammonium sulphate to 95% saturation and finally resuspended in 10 mM Tris-HCl, pH 7.5. An aliquot of protein was combined with SDS sample buffer, and protein was loaded onto SDS–polyacrylamide gels to give the same concentration as that produced for the anther extracts.

Immunoblotting and Immunostaining

Protein extracts resolved by SDS–polyacrylamide gels were electroblotted onto a polyvinylidene fluoride membrane (Immobilon; Millipore,

Bedford, MA) using a Milliblot SDE system (Millipore) according to the manufacturer's instructions. The blot was blocked with 4% (w/v) Marvel milk powder in TBS (Tris-HCl, pH 7.4, 200 mM NaCl) containing 0.1% Tween 20 (TBS-Tween) for 30 min. After blocking, the blots were incubated for 1 to 2 hr with tomato acidic anti-β-1,3-glucanase antiserum (1/1000 dilution) in TBS containing 1% (w/v) Marvel milk powder. Blots were washed three times in TBS-Tween for 10 min and then incubated in secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG, whole molecule [Boehringer]) in TBS containing 1% Marvel milk powder (1/1000 dilution). After washing in TBS-Tween as before, the blots were developed by immersing in a solution of 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate and 0.3 mg/mL nitro blue tetrazolium in 0.1 M Tris-HCl, pH 9.5, 1 mM $MgCl_2$. The reaction was stopped by washing with distilled water. All treatments were carried out at room temperature.

Cytological Analysis of Callose

Anthers were dissected, and locular contents were squeezed directly into a 0.005% (w/v) aqueous solution of water-soluble aniline blue (Smith and McCully, 1978) made up in 0.15 M K_2HPO_4. Maximum staining was achieved after 10 to 15 min. Preparations were observed using a Zeiss standard microscope fitted with a fluorescence attachment 2FL and filter set 487709 (blue excitation). Anthers at a stage equivalent to microspore release were fixed and prepared for electron microscopy as described by Grant et al. (1986). Semi-thick (0.2 to 0.4 μm) sections were mounted on glass slides and stained with 1% toluidine blue in 1% Borax (disodium tetraborate).

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