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Analyses of a genic male-sterile mutant in soybean

by

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A dissertation submitted to the graduate faculty

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For my grandparents, parents, uncle, mother-in-law, and friends who have never failed to support me, encourage me and believe in me even when I did not. And for my husband, Patrick, and my daughter, Jane, who have sometimes patiently but always lovingly put up with me for over four and half years and are teaching me what is important in life.

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CHAPTER 1. INTRODUCTION

Dissertation Organization

This dissertation consists of a general introduction (Chapter 1), four individual manuscripts (Chapters 2, 3, 4, and 5) and general conclusions (Chapter 6). Chapter 1 outlines and introduces the project goals and literature review. Chapter 2 documents the genetics and development of a new genic male-sterile soybean mutant, and examines the possible mechanism of male sterility. This mutant is controlled by a single recessive gene, which is not allelic to any of the known male-sterile genes. Male sterility is caused by the retention of callose during microsporogenesis. This manuscript has been published in *Sexual Plant Reproduction* 10: 13-21, 1997. Permission has been granted from Springer-Verlag to include this copyrighted material in the dissertation. The manuscript presented as Chapter 3 involves molecular mapping of the male-sterility gene and will be submitted to *Crop Science*. The manuscript presented as Chapter 4 involves a detailed cloning, classification, sequencing, and mapping of β-1,3-glucanase gene families in soybean. This manuscript will be submitted to *The Proceedings of National Academy of Science USA*. The manuscript presented as Chapter 5 deals with molecular mapping of the *Fr1* (root fluorescence) locus by bulked segregant analysis and will be submitted to *Theoretical and Applied Genetics*. Chapter 6 summarizes the entire male sterility project.

Project Goals

The male-sterile mutant was obtained from Midwest Oilseeds, Inc., through a grant to H. T. Horner and R. G. Palmer. The mutant was considered to be cytoplasmic male sterile. The mutant has high seed set compared to other known genic male-sterile soybean mutants. High seed set is a very important feature in considering male-sterile systems for hybrid seed production.

Therefore, goals of this dissertation were: 1) to study this male-sterile mutant genetically, i.e. to determine if this mutant was a cytoplasmic male sterile or a genic male sterile; 2) to study the biological mechanism of male sterility; 3) to determine the genomic location of the male-sterile gene; 4) to understand the molecular basis for male sterility; and 5) to isolate the candidate gene for male sterility. Results obtained from these studies might lead to the identification of the ms gene and its biochemical process that is responsible for male sterility.

Literature Review

Male Sterility

Male sterility in plants is an important agronomic phenomenon of particular value when breeding for hybrid vigor (heterosis). In higher plants, normal pollen development can be disrupted in a variety of ways that cause male sterility. Nuclear gene mutations affecting male cell development are designated as genic male sterile (gms). Most of them are recessive, and expressed in sporophytic tissues. A large number of independent nuclear gene mutations which result in male sterility have

been identified in many plant species. For example, there are about 60 nuclear genes that affect male fertility for maize, 55 for tomato, 48 for barley, 24 for pea, 12 for pepper, 10 for cotton, 6 for soybean, and 4 for rice (for a review, see Horner and Palmer, 1995). The other form of male sterility is called cytoplasmic male sterility (cms), which is caused by cytoplasmic gene mutations (Laser and Lersten, 1972; Levings, 1993, Williams and Levings, 1992). This type of sterility is maternally inherited and generally leads to complete sterility under normal environmental conditions.

Mechanisms of Male Sterility

General mechanisms

Nuclear male-sterile mutants have been observed in more than 175 plant species. Even more have been obtained by using mutagens (Kaul, 1988). The many loci identified and their phenotypic diversity indicate that male fertility is a complex developmental process involving many types of tissue and the coordination of a large number of genes. These include the formation of stamens from the meristem; the development of pollen in the anther locules; the timely release of pollen; and finally, the interaction of the male and female gametes to attain fertilization. Mutations can be obtained that disrupt any of these processes, giving rise to male-sterile plants. For example, stamens that are either not formed or formed abnormally will result in male sterility (structural ms, Johns et al., 1981); the filament and anther development are normal, but the abnormalities may occur during microsporogenesis or microgametogenesis, which result in male sterility (sporogenous ms, Kaul, 1988); or pollen is produced but is either not released from

the anther or is unable to reach the stigma and initiate fertilization (functional ms, Kaul, 1988). In this dissertation, the emphasis is on the sporogenous ms.

Successful pollen development comprises three major developmental stages; sporogenesis, or the differentiation of the sporogenous cells and meiosis, the postmeiotic development of free microspores, and microspore mitosis (McCormick, 1993). Any abnormality in these processes can lead to male sterility. Sporogenesis takes place inside anther locules. The tapetum is the innermost layer of the anther wall. The tapetal cells are closely associated with the cells of the sporogenous layer (Mariani et al., 1990). The tapetum plays important roles in microspore development (Echlin, 1971): it provides nutrients for the developing microspores and pollen; it produces sporopollenin which is deposited on the outer walls of the released microspores (Mascarenhas, 1990); and it produces the enzyme callase which degrades the callose walls of the microspore tetrads that are the products of meiosis. After the microspores are released from the tetrads, sporopollenin produced by the tapetum is deposited on the thin walls of the released microspores. Tapetaly derived material, such as flavonols also are deposited on the walls of the microspores and pollen, and are thought to be important for microspore/pollen wall development, pollen germination, and pollen-stigma interactions (Mo et al., 1992). Many authors suggest that disruption in one or more of these process could interfere with microspore and pollen development, and release of the pollen from the anthers (Shivanna et al., 1997) that would lead to male sterility. The sporogenous male-sterile mutants are likely to define genes that trigger the archesporial cells to become sporogenous; some of these genes are likely to define members of

transduction chains that initiate sporogenesis. Genes required for the function of tapetum and male-specific meiosis are likely to constitute the vast majority of the sporogenous male-fertility genes. A large number of tapetum- and anther-specific genes already have been isolated based on their tissue-specific expression in tapetum or pollen (Goldberg et al., 1993; McCormick, 1993; Twell et al., 1990). By making a construct of the promoters of these tissue-specific genes with reporter genes such as GUS, an in vivo system for testing the function of these male-fertility genes can be constructed.

In several crops including tomato (Stevens and Rick, 1986), corn (Coe et al., 1987), and soybean (Graybosch and Palmer, 1988) and experimental systems [e.g., *Arabidopsis* (Chaudhury, 1993; Dawson et al., 1993)], a large number of ms mutants with defects at various stages of microsporogenesis have been identified. In most ms systems, microspore abortion occurs between meiosis and the final maturation stages; however, breakdown at premeiotic stages also is known to occur (Bhandari, 1984; Kaul, 1988; Laser and Lersten, 1972).

Soybean male-sterile mutants

Genic male sterility has been used in some soybean breeding studies (Brim and Stuber, 1973; Lewers et al; 1996; Lewers and Palmer, 1997), but so far it has not been used for commercial production of hybrid seed because large quantities of hybrid seed cannot be produced. Six genic male-sterile mutations (*ms*1, *ms*2, *ms*3, *ms*4, *ms*5, and *ms*6) have been reported in soybean (Palmer et al., 1992). All of these are nuclear gene mutations and inherited as monogenic recessive traits. All genic-male sterile mutations, except *ms*5, arose spontaneously in field plantings or

breeding populations. The *ms5* mutation was induced through mutagensis (Buss, 1983). A cytoplasmic male-sterile mutation has been reported but not confirmed in soybean (Sun et al., 1994).

Studies on the genetics and developmental reproductive biology of most of the soybean mutants have been reviewed (Graybosch and Palmer, 1988; Palmer et al., 1992). In soybean, male sterility may result from dysfunction of either the tapetum or the reproductive cells, or both. For the *ms1 ms1* mutant, male sterility is caused by the absence of cytokinesis resulting in the production of quadrinucleate microspores. These quadrinucleate cells produce walls of sporopollenin and engorge with reserve materials in the same fashion as viable pollen grains. However, these modified pollen grains are not released readily from the anther (Albertsen and Palmer, 1979).

The process of postmeiotic cytokinesis also is influenced by the *ms4* mutation, but pollen wall differentiation is abnormal as well (Delannay and Palmer, 1982; Graybosch and Palmer, 1985b). In soybean mutants *ms2* (Graybosch and Palmer, 1985a), *ms3* (Palmer et al., 1980; Buntman and Horner, 1983), and ms (Jin et al., 1997), male sterility is due to abortion of microspores caused by failure of callose dissolution at the tetrad stage. There are several studies in other species which indicate that male sterility was due to failure of callose dissolution at the proper stage (Eschrich, 1961; Frankel et al., 1969; Izhar and Frankel, 1971; Mepham and Lane, 1969; Stieglitz and Stern, 1973; Tsuchiya et al., 1995; Warmke and Overman, 1972; Worrall et al., 1992). Therefore, the association between callose dissolution and tapetum is an important factor causing male sterility. Mutation at the soybean

ms6 locus affected the proper differentiation and function of the primary tapetal layer. In male-sterile *ms6 ms6* plants, all layers of the anther wall including the epidermis, endothecium, and the two parietal layers, expressed cell hypertrophy, and degenerated tapetal cells were still present in mature anthers. Subsequently, tapetum malfunction leads to microspore degeneration at the tetrad stage (Skorupska and Palmer, 1989).

Cloning of Male-Sterility Genes

Although some aspects of the function of the ms gene products can be inferred from genetic studies, a molecular understanding of their biochemical functions must await molecular cloning. Two different strategies have been used to clone the male-fertility gene, map-based cloning and gene tagging. Four genes have been isolated that produce a male-sterile phenotype when mutated, but their gene products do not provide obvious clues to the roles that these genes play during pollen development. For example, Moffatt and Somerville (1988) isolated an adenine phosphoribosyl transferase (aprt-) *Arabidopsis* mutant, but how a lesion in a nucleic acid salvage pathway disrupts pollen development is unclear. The *ms2* gene (isolated by gene tagging) from *Arabidopsis* contains a small region homologous to a wheat mitochrondrial ORF (Aarts et al., 1993), but no function has been ascribed to this mitochondrial region or to the gene product of the *ms2* gene. Later study (Aarts et al., 1997) showed that it is also similar to a protein of 493 amino acids encoded by the jojoba (*Simmondsia chinensis*) fatty acyl reductase (JJFAR) gene (Metz et al., 1994). The enzyme is involved in reduction of fatty acyl groups to fatty alcohol groups, this may be one of the steps in the formation of sporopollenin. In maize, a

male-sterile gene designated *Ms45* was cloned (transposon tagging) (Albertsen et al., 1996) that showed 33% homology, over 256 amino acids, with strictosidine synthase. Although the action of strictosidine synthase in the indole alkaloid pathway is understood, it is not clear whether or how indole sythesis might be involved in pollen development. Gorman et al. (1996) identified a YAC clone with an insert size of ~610 Kb that contains the *ms*14 gene in tomato. All of these examples serve to illustrate the complexities of male gametophyte development. Therefore, it probably will be necessary to isolate and characterize a large number of male-sterile gene products in order to develop a comprehensive understanding of the sporophytic contribution(s) to pollen development, and the functional roles of male-fertility genes.

Map-based cloning is a reasonable strategy for isolation of male-sterile genes in soybean since the technique requires only that the target gene has a clear phenotype and that its position on a genetic map be known. Map-based cloning has been used successfully to isolate target genes from *Arabidopsis* (Arondel et al., 1992; Bent et al., 1994; Giraudat et al., 1992; Putterill et al., 1995) and tomato (Gorman et al., 1996; Martin et al., 1993). This technique involves identifying markers closely linked to the target locus, determining the physical genetic relationship (Kb/cM) in the target region, isolating clones that contain the locus from a large insert library and confirming the locus by complementation of the recessive phenotype with the dominant allele. Thus identification of markers closely linked to the gene is the starting point for map-based cloning.

Molecular Mapping

Molecular markers

Many types of DNA markers have been developed and applied in genetic studies. RFLP (Restriction Fragment Length Polymorphisms) uses restriction enzymes to cut DNA into small fragments of different lengths. The different sizes of fragments can be separated by gel electrophoresis (Southern, 1975). This size difference is called a polymorphism (Kochert, 1994). Variation in the sizes of allelic DNA fragment(s) results from loss or gain of a restriction site, due to point mutations, deletions, insertions, translocations, unequal crossing over, or replication slippage (Kochert, 1994; Lavi et al., 1994). There are advantages to RFLP markers; for example, they are codominant markers, and detectable in all tissues and at all developmental stages, but analysis requires considerable labor-intensive isolation of DNA suitable for DNA blot analysis.

RAPD (Random Amplified Polymorphic DNA) employs random DNA primers in polymerase chain reactions (PCR) to rapidly generate polymorphic markers that can be used to create genetic linkage maps. RAPDs are scored for the presence or absence of a single allele, and are usually dominant markers. The advantages of RAPD markers are technically simple, quick to perform, require only a small amount of DNA, and involve no radioactivity (Waugh and Powell, 1992). But RAPD is not as dependable as other markers because of poor reproducibility.

SCARs (Sequence Characterized Amplified Regions) are generated by cloning and sequencing RAPD fragments or other DNA fragments of interest, and designing specific primers that are complementary to the ends of the original RAPD or other

DNA fragments (Paran et al.,1993). SCARs may be dominant polymorphisms (one fragment amplified) or may show codominant polymorphisms.

SSR (Single Sequence Repeat), or microsatellite DNAs, are short segments of DNA consisting of a small number of repeated nucleotide sequences such as (CA)n, (AAT)n, and (AGAT)n (Tautz and Renz, 1984). Polymorphisms result from different numbers of tandem repeats of a core DNA sequence. SSR combines the advantages of the other markers, highly polymorphic, single locus markers, which are very abundant (Wang et al., 1994), reproducible, and easily amenable to genetic analysis (Lavi et al., 1994; Morgante and Olivieri, 1993).

The technique of AFLPs (Amplified Fragment Length Polymorphisms) is similar to that used in RFLP analysis (Lin and Kuo, 1995; Zabeau and Voss, 1992) but it is PCR based. Because each locus is represented by the presence or absence of one band, AFLP is a dominant marker.

The technical difficulty, reliability, level of polymorphism, marker inheritance, and amount of DNA required of the molecular markers have been compared (Andersen and Fairbanks, 1990; Beckmann and Soller, 1986; Rafalski and Tingey, 1993a; Walton, 1993; Waugh and Powell, 1992). Different types of markers complement each other. Therefore, the combined use of various types of markers is helpful.

Importance of mapping a gene

Genetic mapping allows study of any morphological, physiological, or developmental process in which genetic variants exist (Paterson and Wing, 1993). Genetic mapping provides information about the number of genes influencing a trait, the locations of genes on the chromosomes, and the effects of variation in dosage of

these genes. The availability of molecular markers tightly linked to the ms gene should provide plant breeders with an efficient method for rapid transfer of the ms gene to elite soybean germplasm. Molecular marker-assisted selection (MAS) may be especially useful for the traits that are difficult to detect, such as male sterility. Male-sterile genes have been mapped in rice (Subudhi et al., 1997; Wang et al., 1995; Zhang et al., 1994) and tomato (Gorman et al., 1996). Finally, mapping a gene is the first step toward "map-based cloning" of genes responsible for specific phenotypes.

Mapping simply-inherited traits

The chromosomal location of a mutation is determined by identifying nearby genetic markers which are co-transmitted from parent to progeny with the mutated gene. Mapping genes is very time consuming and labor-intensive. In recent years, several approaches have been developed to simplify and accelerate the mapping of discrete mutations with molecular markers. One early approach, was the use of "near-isogenic lines" (NIL), which were created by plant breeders, often for the purpose of introducing a valuable gene from a wild species to a crop plant. NILs are developed by backcrossing, e.g. crossing a "donor genotype" carrying a specific trait of interest to a "recipient" with generally desirable attributes. By recurrently selecting for the trait of interest, and repeatedly crossing to the recipient, donor chromatin is progressively eliminated except for a small amount which is closely-linked to the trait under selection. By comparing the backcross-derived stock to the original recurrent parent, one can determine the likely location of the target gene simply by identifying

DNA markers that reveal the donor allele in the backcross-derived stock (Young et al., 1988).

Although near-isogenic lines are efficient mapping tools, they require many breeding cycles to produce. A molecular approach called "Bulked Segregant Analysis" (BSA) provides a faster alternative (Michelmore et al., 1991; Giovannoni et al., 1991). The principle of DNA pooling strategies is the grouping of informative individuals together so that a particular genomic region can be studied against a randomized genetic background of unlinked loci. The first example of this approach involved genes for resistance to lettuce downy mildew (Michelmore et al., 1991). Use of BSA to map genes minimizes the number of independent DNA samples which must be analyzed, and simultaneously provides information on polymorphisms between the parents and possible linkage between a marker and targeted gene. This process can reduce cost by several-fold, particularly when used with PCR-based markers.

DNA pooling can be very useful even when the location of the gene already is known. Map-based cloning of a gene is aided by having a large number of different DNA markers near the gene, to pinpoint it to as small a region as possible. One can use DNA markers flanking the gene to develop alternative DNA pools with different genotypes near the gene, thereby targeting a search of additional DNA markers to a very specific chromosomal region.

Soybean molecular mapping

Several molecular genetic maps have been constructed in soybean (Apuya et al.,1988; Lark et al., 1993; Rafalski and Tingey, 1993b; Shoemaker et al., 1997).

Many traits have been identified and mapped with molecular markers since then. Examples are phytophthora resistance (Diers et al., 1992a; Polzin et al., 1994b), root fluorescence (*Fr2*) (Devine et al., 1993), cyst nematode resistance (Concibido et al., 1994; Webb et al., 1995; Weisemann et al., 1992), mosaic virus resistance (Yu et al., 1994), nodulation (Landau-Ellis et al., 1991; Polzin et al., 1994a), mildew resistance (Polzin et al., 1994a), linolenic acid content (Brummer et al., 1995), palmitic acid content (Nickell et al., 1994), hard seediness (Keim et al., 1990), seed protein and oil contents (Diers et al., 1992b; Lark et al., 1994; Mansur et al., 1993), fatty acid content (Diers and Shoemaker, 1992), and iron-deficiency chlorosis (Diers et al., 1992c). However, none of the male-sterile genes have been mapped in soybean.

β-1,3-Glucanase

β-1,3-glucanases are involved in the breakdown and turnover of β-linked glucans which are very important from both basic and applied research perspectives. They are involved in various important physiological processes, such as microspore development, seed germination, cell growth, and defense against pathogens (Abeles et al., 1971; Ballance et al., 1976; Bucciaglia and Smith, 1994; Chang et al., 1992; Cote et al., 1991; Fry, 1989). They are expressed in many tissues in both spatial- and temporal-specific manners. The wide-spread, complex, and varied expression patterns suggest that β-1,3-glucanases are involved in other unidentified physiological processes. As β-1,3-glucanases are major components of plant cell walls, it is likely that β-1,3-glucanases are involved in many physiological roles, since

any process that might involve altering the structure and function of cell walls could involve β-1,3-glucanases.

The β-1,3-glucanases are regulated by numerous growth regulators (e.g., gibberellin, auxin, cytokinin, ethylene, and abscisic acid) (Felix and Meins, 1986; Mohnen et al., 1985) and pathogen attack (fungal, bacterial, and viral), biotic elicitors (e.g., pathogen extracts, lipids, β-glucans, and salicylic acid), abiotic elicitors (e.g., mercury ions and ozone), and wounding. These diverse forms of regulation apparently reflect the diversity of roles in which β-1,3-glucanases are involved.

The β-1,3-glucanases belong to large gene families, some of which have been well characterized in tobacco and several other species. The gene families can be subdivided into five broad categories based upon protein isoelectric point, expression pattern, and sequence similarity (Bucciaglia and Smith, 1994; Linthorst et al., 1990; Ori et al., 1990; Payne et al., 1990; Shinshi et al., 1988; Van den Bulcke et al., 1989). The best characterized class containing the basic isoforms, is induced by either pathogen infection or ethylene treatment, exhibits developmental regulation (Felix and Meins 1986), and appears to be localized primarily in the vacuole of the cell (Van den Bulcke et al., 1989; Koltunow et al., 1990). RNAs of this class accumulate in mature leaves and roots and in response to pathogen invasion (reviewed by Linthorst, 1991). The role of these genes in the plant defense response or development has not been determined, although the gene products can inhibit the growth of fungi (Mauch et al., 1989).

The second class of β-1,3-glucanases, which includes the pathogenesis-related proteins PR-2, PR-N, PR-O, and PR-35 (Kaufmann et al., 1987; Van den Bulcke et al., 1989), also is induced by pathogens and appears to be localized in the extracellular space (Ward et al., 1991). These gene products do not accumulate in healthy leaves but are induced in response to pathogen invasion.

The third class of β-1,3-glucanases, which includes the pathogenesis-related protein PR-Q' (Payne et al., 1990), also is induced by pathogens and appears to be localized in the extracellular compartment. The fourth class of β-1.3-glucanases is acidic, sp41a and sp41b, accumulating to high levels in the transmitting tract of the style and is not pathogen inducible (Ori et al., 1990).

The fifth class of β-1,3-glucanase, is a secreted, acidic anther β-1,3-glucanase and seems to be expressed in the tapetum (Bucciaglia and Smith, 1994) and is involved in dissolution of callose around the tetrads.

A related gene family of monocots, the β-1,3; 1,4-glucanases, encodes proteins similar to β-1,3-glucanases. In barley, these proteins are produced in the scutellum and aleurone of germinating seeds, where they catalyze the hydrolysis of a β-1,3; 1,4-glucan, a major component of endosperm cell walls (Fincher et al., 1986).

In addition to the previous classification system, β-1,3-glucanases also are classified into two groups depending on the nature of their enzymatic action on β-1,3-glucans. Endoglucanases cleave the substrate into short-chain, reducing sugars whereas exoglucanases hydrolysis release single glucose units. These different end

products are the basis of assays, which can distinguish between endo- and exoglucanase activities.

β-1,3-glucans

As β-1,3-glucanases hydrolyze β-1,3-glucans, it is obvious that information about β-glucans is highly relevant. The β-1,3-glucans are found in various forms and major components of plant cell walls. The β-1,3-glucans are macromolecular carbohydrates composed principally of glucosyl residues linked by β-glycosidic bonds. β-glucan, sometimes called callose, has only β-1,3-linkages. β-glucan accumulation is developmentally, spatially, and environmentally regulated, and it functions in a variety of physiological processes (reviewed by Stone, 1984).

β-1,3-glucanases involved in flowering

β-1,3-glucanases are expressed in various floral tissues. Several of them which are involved in flowering, originally were identified as stress related due to their structural and enzymatic similarities to stress-related β-1,3-glucanases (Neale et al., 1990). Some β-1,3-glucanases are expressed specifically in the flowers, such as the one expressed in anthers and it is involved in release of microspore tetrads (Bucciaglia and Smith, 1994), and sp41, which is expressed in the pistil (Lotan et al., 1989; Ori et al., 1990).

β-1,3-glucanases and microspore development

During microspore development, microsporocytes undergo meiosis to form tetrads of haploid microspores. In all higher plants the tetrad of microspores is surrounded by a callose wall, a β-1,3-glucan polymer. As meiosis occurs, callose

also accumulates along the cellular plates until each individual microspore of the tetrad is enclosed in a thick callose wall. The proposed functions of the callose walls are: 1) chemical isolation of the developing gametes from sporophytic tissue (Heslop-Harrison and Mackenzie, 1967); 2) mechanical isolation of the meiocytes and tetrads (Waterkeyn and Bienfait, 1970); 3) protection from environmental and osmotic stress (Bhandari, 1984); and 4) providing a surface upon which the outerwall, or exine, is assembled (Waterkeyn and Bienfait, 1970). Degraded callose also could provide a source of glucose for postmeiotic development. At a critical developmental stage, the callose is degraded by the β-1,3-glucanases (Frankel et al., 1969) which are secreted by the tapetal cells (Stieglitz and Stern, 1973). The microspores then are released into the anther locule and eventually develop into pollen grains (for review, see McCormick, 1993). The build-up of callose around the microsporocytes and tetrads and its subsequent rapid dissolution is one of the most cytologically dramatic, and clearly distinguishable events in microsporogenesis. The developmental importance of correct expression of β-1,3-glucanase is underscored by the occurrence of mutants where premature or delayed callase activity results in microspore abortion and male sterility. The timing of β-1,3-glucanase activity seems to be critical for the normal development of microspores (Frankel et al., 1969). Premature breakdown of callose was observed in male-sterile sorghum (Warmke and Overman, 1972), in cms petunia (Izhar and Frankel, 1971), and transgenic tobacco (Tsuchiya et al., 1995; Worrall et al., 1992). Absent or delayed callose degradation was reported in *ms*2 soybean (Graybosch and Palmer, 1985a), *ms*3 soybean (Buntman and Horner, 1983), ms soybean (Jin et al., 1997), cms *Capsicum*

(Horner and Rogers, 1974), cms *Helianthus* (Horner, 1977), *Lolium* (Hayward and Manthiratna, 1972), and *Pisum* (Gottschalk and Kaul, 1974). Recently, putative genes encoding an anther-specific β-1,3-glucanase (*A6*) were reported in *Brassica napus* (Hird et al., 1993), *Arabidopsis thaliana* (*A6, A9*) (Hird et al., 1993; Paul et al., 1992), and *Nicotiana tobacum* (*Tag1*) (Bucciaglia and Smith 1994). But no glucanase activity has been associated with *A6* and *A9* proteins in *Brassica* and *Arabidopsis* (Hird et al., 1993).

β-1,3-glucanases in other floral tissues

A 41-kDa β-1,3-glucanase called sp41 is expressed in tobacco pistils (stigmas/styles) (Lotan et al., 1989; Ori et al., 1990). Sp41 expression did not seem to be a result of a stress response. Its expression can be detected even before the flower buds were opened. The sp41 β-1,3-glucanase is structurally distinct from previously characterized β-1,3-glucanases. Sp41 is glycosylated with a very large (~9 kDa) glycos moiety. The glycosylation has no apparent effect on enzymatic activity (Ori et al., 1990). The specific high-level expression of sp41 in the transmitting tract strongly suggests that it plays a significant role. Two possible functions of sp41 have been proposed: the first is that it is involved in facilitating or regulating pollen-tube growth (Richards, 1986); the second role may be as a constitutive defense against pathogen attack, because the pistil is open and vulnerable to pathogens that may exploit this nutrient-rich channel to access the plant, in particular the developing seeds.

β-1,3-glucanases involved in germination

The β-1,3-glucanases associated with germination have been studied most extensively in barley and rye (Ballance et al., 1976; Manners and Marshall, 1969). It has been proposed that the β-1,3-glucanases expressed during germination are involved in defense of the seed against pathogen attack, especially fungal pathogens (Hoj et al., 1989). Evidence that the barley β-1,3-glucanase is expressed in a defense-related fashion in leaves gives support to this proposal (Jutidamrongphan et al., 1991).

β-1,3-glucanases involved in growth

The β-1,3-glucanases are thought to be involved in plant growth, playing a role in altering the composition and viscoelastic properties of the cell wall matrix and thereby causing or making cell expansion and growth (Goldberg, 1977; 1980; Hoson and Nevins, 1989; Nevins et al., 1977; Wong and Maclachlan, 1979; Zarra and Masuda, 1979). A model for the role of β-1,3-glucanases in promoting growth has been described by Fry (1989). The β-1,3-glucan is part of young and actively growing plant cell walls. This β-1,3-glucan forms hydrogen bonds both to itself and to other cell wall components. This bonding stabilizes the wall, resulting in a physical hindrance to turgor-mediated cell expansion. The β-1,3-glucanases act to cleave the β-1,3-glucan and thereby reduce the cell wall rigidity. Cell expansion and growth driven by turgor pressure results; however, to date, no specific β-1,3-glucanases involved in growth have been identified. It may be because their identification is inherently difficult.

β-1,3-glucanases and phloem transport, tissue ripening and abscission

The β-1,3-glucan has been proposed to regulate phloem transport by plugging the sieve plates (Currier, 1957; Fulcher and McCully, 1976). Also β-1,3-glucan often is a transient component of cell walls during development and cell division (Fulcher and McCully, 1976). How β-1,3-glucanases may be involved in phloem transport is not clear. The β-1,3-glucanase activity is associated with fruit ripening, but how it is regulated is unknown (Hinton and Pressey, 1980). The β-1,3-glucanase expression is induced in bean leaf abscission zones, stems, and petioles upon treatment with ethylene (Del Campillo and Lewis, 1992a). This expression could be related to defense against pathogens at the site of abscission because a number of other pathogenesis-related proteins such as PR1 and chitinase also were expressed (Del Campillo and Lewis, 1992b). However, β-1,3-glucanases might also play a role in facilitating abscission by degrading β-1,3-glucans in the abscission zone.

Pathogenesis-related β-1,3-glucanases

Plants have evolved various ways for defending themselves against hostile environments that include pathogens such as viruses, fungi, bacteria, and insects. Production of pathogenesis-related proteins, such as β-1,3-glucanases and chitinases is one of the ways that plants respond to pathogen attack. β-1,3-glucanases were proposed to be involved in plant defense as early as 1971 (Abeles et al., 1971). There are a number of reports of the coordinate expression of β-1,3-glucanases at the level of transcription in response to viral, bacterial, and fungal infection (Beffa et al., 1993; Beffa et al., 1996; Bol et al., 1990; Dong et al., 1991;

Edington et al., 1991; Keen and Yoshikawa, 1983; Linthorst et al., 1990; Meins and Ahl, 1989; Moore and Stone, 1972; Shinshi et al., 1987; Takeuchi et al., 1990; Tuzun et al., 1989; Van den Bulcke et al., 1989; Van Loon, 1983; Vogeli et al., 1988; Vogeli-Lange et al., 1988). These β-1,3-glucanases are divided into acidic PI and basic PI groups (Legrand et al., 1987). The acidic and basic β-1,3-glucanases show differences in their regulation in response to pathogen attack, and tissue-specific and developmental expression patterns (Memelink et al., 1990). The acidic enzymes are secreted and are thought to play an important role in encountering and destroying fungal pathogens. The basic enzymes are located in the vacuole and also are thought to play a role in defense.

The level and onset of β-1,3-glucanase expression often is positively correlated with the level of pathogen resistance. Muskmelon and tomatoes infected with *Fusarium oxysporum* showed a higher and more rapid expression of β-1,3-glucanase in resistant than in susceptible varieties (Ferraris et al., 1987; Netzer and Kritzman, 1979). Tomato plants resistant to the fungal pathogen *Cladosporium fulvum* produced β-1,3-glucanase earlier than susceptible varieties (Joosten and Dewit, 1989). Maize β-1,3-glucanase expression is induced by the fungus *Exserohilum trucicum* , and the level of expression is correlated positively with the resistance levels of near-isogenic lines (Jondle et al., 1989). These correlations suggest that β-1,3-glucanases play an important role in achieving defense, but the mechanisms involved are not clear. There is no evidence to suggest that β-1,3-glucanase genes represent plant resistance genes of the type described by the

gene-for-gene model of plant-pathogen interactions (Godiard et al., 1994; Jones, 1994; Staskawicz et al., 1995). A general model states that upon pathogen attack, the β-1,3-glucanases release small β-1,3-glucans. These β-1,3-glucans elicit increased β-1,3-glucanase and other defense-related gene expression by an unknown signal transduction pathway (Kombrink et al., 1988; Lamb et al., 1989; Mauch and Staehelin, 1989; Takeuchi et al., 1990).

Over 37 plant β-1,3-glucanase gene sequences have been published. The published sequences reveal several highly conserved amino acids that are possibly important for enzyme function. Plant β-1,3-glucanases are typically encoded by moderately sized gene families. There are about 12 β-1,3-glucanase genes in tobacco (Linthorst et al., 1990). The β-1,3-glucanase sequences are being used to study genome and gene family evolution (Sperisen et al., 1991). As more sequences become available, the amino acids involved in the catalytic site, the functions of the genes, and gene family evolution should become even more apparent. The functions of β-1,3-glucanases are just speculations. It is difficult to determine their functions because no mutations in β-1,3-glucanases have been found. Possibly this is due in part because the gene families are relatively large, therefore, making loss of function mutations less apparent. There is little genetic analysis of these genes, either to determine map position or function because mutants have not been identified.

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CHAPTER 2. GENETICS AND CYTOLOGY OF A NEW GENIC MALE-STERILE SOYBEAN [*GLYCINE MAX* (L.) MERR.]

A paper published in the journal Sexual Plant Reproduction[1]

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Abstract

Genetic and cytological studies were conducted with a new male-sterile, female-fertile soybean [*Glycine max* (L.) Merr.] mutant. This mutant was completely male sterile and was inherited as a single-recessive gene. No differences in female or male gamete transmission of the recessive allele were observed between reciprocal cross-pollinations in the F_1 or F_2 generations. This mutant was not allelic to any previously identified soybean genic male-sterile mutants: *ms1*, *ms2*, *ms3*,

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Key words Soybean, Male sterility, Genetics, Callase, Callose

ms4, *ms5*, or *ms6*. No linkage was detected between sterility and flower color (*W1* locus), or between sterility and pubescence color (*T1* locus). Light microscopic and cytological observations of microsporogenesis in fertile and sterile anthers were conducted. The structure of microspore mother cells (MMC) in male-sterile plants was identical to the MMCs in male-fertile plants. Enzyme extraction analyses showed that there was no callase activity in male-sterile anthers, and this suggests that sterility was caused by retention of the callose walls, which normally are degraded around tetrads at the late tetrad stage. The tapetum from male-sterile anthers also showed abnormalities at the tetrad stage and later stages, which were expressed by an unusual formation of vacuoles, and by accumulation of densely staining material. At maturity, anthers from sterile plants were devoid of pollen grains.

Introduction

Male sterility is a condition in plants in which male gametophytic function is prevented, but the potential for female reproduction remains. Based on inheritance patterns, there are two general types of male sterility: genic (nuclear) male sterility (**gms**) and cytoplasmic male sterility (**cms**). Male-sterile mutations provide source material for studies in plant breeding, genetics, reproductive biology, and molecular biology. Male sterility has been used in soybean breeding studies (Brim and Stuber 1973; Lewers et al. 1996) but so far it has not been used for commercial production of hybrid seed because large quantities of hybrid seed can not be produced at the present time. During the past two decades, six genic male-sterile mutations (*ms1*,

ms2, *ms3*, *ms4*, *ms5*, and *ms6*) have been reported in soybean (Palmer et al. 1992). All of these are nuclear mutations inherited as monogenic recessive traits. **Cms** has not been confirmed in soybean.

Observations on the genetics and developmental reproductive biology of most of the soybean mutants have been summarized (Graybosch and Palmer 1988; Palmer et al. 1992). In soybean mutants, *ms*2 and *ms*3, male sterility is due to abortion of microspores caused by failure of callose dissolution at the tetrad stage. In the present study, we have observed a similar phenomenon in a potentially new male-sterile line leading to microspore abortion. Therefore, the objectives of this study were to determine whether this soybean mutant was a cytoplasmic male sterile or a new genic male-sterile line with phenotypic characteristics different from *ms1*, *ms4*, *ms6*, but similar to *ms2* and *ms3*, and to determine the extent of callase activity in anthers from male-fertile and male-sterile plants (see table1).

Materials and Methods

Genetics

Seeds of the male-sterile line were obtained from Midwest Oilseeds, Adel, Iowa 50003 (see Acknowledgements). This line has unusually high seed set in the field. To test the completeness of male sterility, 300 plants, which were progeny of known heterozygotes, were grown in a glasshouse at Iowa State University, Ames, in the summer of 1995, in the absence of insect pollinators. At anthesis, plants were

Table 1. Phenotypic expression of genic male-sterile, female-fertile mutants in soybean[+]

Mutant	Meiocyte	Tetrad	Microspore	Pollen
ms1 ms1	——	failure cytokinesis, tapetum degeneration	——	——
ms2 ms2	——	callose retention, no microspore wall formed, tapetum degeneration	——	——
ms3 ms3	——	callose retention, microspore wall initiated, tapetum degeneration	——	——
ms4 ms4	——	failure cytokinesis, tapetum degeneration	——	——
ms6 ms6	——	tapetum degeneration	——	——
msp msp	inconsistent, abortion occurs between premeiocyte and pollen stages			
ms ms[++]	——	callose retention, microspore wall initiated	——	——

[+] Mutant *ms5 ms5* has not been studied cytologically

[++]Mutant described in this study; seeds from Midwest Oilseeds, Inc.

classified for fertility and sterility based on the presence or absence of pollen. Fertile plants were rogued. The male-sterile plants were saved and checked for seed set a month after the plants had completed flowering.

Inheritance studies were conducted to determine whether the mutation was a cytoplasmic male-sterile mutant or whether the mutation was nuclear. Allelism tests also were conducted to determine whether the new mutation arose at a new locus, or represented an independent mutation at one of the previously described loci (*ms1*, *ms2*, *ms3*, *ms4*, *ms5*, or *ms6*) (Table 1). Crosses were conducted in 1993 by using a known recessive sterile homozygote as the female parent and the F_1 hybrid (heterozygote) from Midwest Oilseeds as male parent. F_1 seeds were planted either in a glasshouse at Iowa State University, Ames, or at the University of Puerto Rico Soybean Breeding Nursery, at the Isabela Substation, Isabela, Puerto Rico. F_1 plants were single-plant threshed, and the F_2 seeds were planted at the Bruner Farm near Ames, Iowa. For certain cross combinations, fertile F_2 plants were single-plant threshed; F_3 seeds were planted in Puerto Rico. All F_1 ,F_2, and F_3 plants were classified for male sterility / fertility at maturity.

Male gamete transmission tests were conducted with F_1 hybrids from Midwest Oilseeds as the male parent. Cross pollinations were made by using five Plant Introduction (PI) lines (PI 91167, PI 261474, PI 427099, PI 297544, PI 227333) and a cultivar A. K. Harrow as the female parents.

Linkage tests were conducted between the male-sterile mutant and the flower color (*W1*) locus, and between the male-sterile mutant and the pubescence color (*T1*) locus. Linkage determinations are presented by using the general relationship

a = XY, b = Xy, c = xY, and d = xy (Skorupska and Palmer 1989) for gene pairs listed as Xx and Yy. Plants were classified as having either purple or white flowers at flowering, and having tawny or gray pubescence at maturity.

Microscopy

Cytological observations of anther and pollen development were obtained by collecting reproductive buds of various sizes from both male-fertile and male-sterile plants. Male-fertile and male-sterile plants were identified by squashing late stage anthers in an aqueous solution of I_2KI; anthers from male-fertile plants displayed densely staining pollen grains, whereas anthers from male-sterile plants were almost empty, with only a few stained bodies. Buds from both lines were dissected, and individual anthers were fixed in 3% glutaraldehyde in 0.1M phosphate buffer, pH 7.24, for 14-16 hr at 4^0C. After three buffer washes, anthers were postfixed in 1% osmium tetroxide for 1 hr at room temperature in the same buffer, washed again with the buffer, dehydrated in a graded acetone series, embedded in Spurr's resin (hard recipe), and polymerized at 70^0C for 24 hr.

Specimen blocks were sectioned on a Reichert Ultracut S microtome. For light microscopic observations, 1 μm thick sections were stained with methylene blue-azure II and basic fuchsin (Humphrey and Pittman 1974). Specimens were observed and photographed on a Leitz orthoplan microscope.

For fluorescence microscopy, buds were fixed in a 3:1 mixture of ethanol : glacial acetic acid. Anthers were removed and squashed in a 0.005% solution of aniline blue in 0.15 M phosphate buffer, pH 8.2 (Jensen 1962), to detect the presence or absence of callose.

In vitro enzyme activity assay

For *in vitro* callase activity assay, flowers of known developmental stages from fertile and sterile plants were chosen. Crude callase was extracted from male-fertile and male-sterile anthers at the tetrad stage according to Frankel et al. (1969), with modifications. The anthers were removed from the flower buds and placed in 1.5 ml microfuge tubes chilled on dry ice. The anthers were ground and treated with extraction buffer consisting of 0.08 M acetate and 0.08 M NaCl, pH 4.8, and then allowed to sit for 30 min at room temperature, the tubes were microfuged for 15 min at 4^0C. The supernatant contained the crude enzyme extract. Isolated tetrads from fertile and sterile anthers were placed on separate glass slides, the enzyme extracts were added, and the preparations were coverslipped. The four combinations were: 1) fertile anther extract to fertile tetrads; 2) fertile anther extract to sterile tetrads; 3) sterile anther extract to fertile tetrads; and 4) sterile anther extract to sterile tetrads. The reaction mixtures were incubated for about 18 hr at 37 ^{0}C in a moist chamber. After incubation, lacmoid (0.1% resorcin blue in absolute ethanol) was added to stop the reaction and to stain the undigested callose (Frankel et al. 1969). The color intensity and presence of the tetrad callose walls were determined and recorded photographically on a Leitz orthoplan microscope.

Results

Genetics

In the glasshouse experiment without the presence of insect pollinators, there were no pods formed on the male-sterile plants, which indicated that this was a

completely male-sterile line and that the summer glasshouse environment (June to August, 1995) did not influence the expression of the male-sterility gene. Classifications of F_2 plants obtained from cross pollination in the male gamete transmission test are given in Tables 2 and 3.

Table 2 shows that the ratio of nonsegregating and segregating families is 1 : 1, indicating that the two male gametes transmitted equally. A ratio of 3 fertile plants : 1 sterile plants in segregating F_2 families was observed (Table 3), which indicates that male sterility is conditioned by a single-recessive gene. Therefore, this genic male-sterile mutant will be designated as ms ms and its fertile heterozygote as Ms ms.

In linkage tests between the ms and *W1* (flower color) loci, $X^2 = 2.65$ was calculated (P = 0.45) (Table 4), and for independent assortment between the ms and *T1* (pubescence color) loci, $X^2 = 0.30$ (P = 0.96) (Table 5). In both tests, the observed values fit the expected ratios of 9:3:3:1. There was no linkage between the ms and *W1* loci, or between the ms and *T1* loci.

Classifications of F_2 families obtained from allelism tests are given in Tables 6 and 7. If ms was a mutation at a different locus than the one tested, the F_1 populations would contain only male-fertile plants. In the F_2, 50 percent of F_1-derived families would segregate in a ratio of 3 male-fertile plants : 1 male-sterile plants, and 50 percent would produce a population consisting of 9 male-fertile plants : 7 male-sterile plants. No male-sterile plants were observed in any of the F_1 generations. (data not shown). This indicates that the allele for male sterility in this genic male-sterile mutant was at a locus different from the six known genic male-

Table 2. Male gametophyte transmission test: F_2 data

Cross combinations	No. families		$X^2(1:1)$	P (df = 1)
	Nonsegregating	Segregating		
PI 91167 x M2[+]	9	17	2.46	0.12
PI 261474 x M2	10	8	0.22	0.64
PI 427099 x M2	7	6	0.08	0.78
PI 297544 x M2	12	6	2.00	0.16
PI 227333 x M2	8	9	0.06	0.81
A. K. Harrow x M2	6	5	0.09	0.76
Total	52	51	0.01	0.92

[+]Midwest Oilseeds M2 = 502-1 x 71005-7; F_1 plants are heterozygous, Ms ms

Table 3. Segregation for fertility / sterility in segregating F_2 families

Cross combinations	No. plants		$X^2(3:1)$	P (df = 1)
	Fertile	Sterile		
PI 91167 x M2[+]	978	306	0.93	0.33
PI 261474 x M2	505	161	0.24	0.62
PI 427099 x M2	379	136	0.55	0.46
PI 297544 x M2	479	159	0.00	1.00
PI 227333 x M2	747	249	0.00	1.00
A.K. Harrow x M2	449	129	2.21	0.14
Total	3537	1140	0.97	0.32

[+] Midwest Oilseeds M2 = 502-1 x 71005-7; F_1 plants are heterozygous, Ms ms

Table 4. Linkage test between ms and *W1* loci in soybean: F_2 data

Cross combinations	Frequency of phenotype[+]				Total	X^2 (9:3:3:1)	P(df = 3)
	a	b	c	d			
PI 91167 x M3[++]	140	44	46	17	247	0.24	0.97
PI 261474 x M3	42	18	12	7	79	0.32	0.96
PI 427099 x M1[+++]	97	28	40	10	175	0.12	0.99
PI 297544 x M1	210	82	60	35	387	2.61	0.46
PI 227333 x M3	182	52	48	19	301	0.92	0.82
A.K. Harrow x M3	188	54	52	16	310	0.05	1.00
Total	859	278	258	104	1499	2.65	0.45

[+] Linkage determinations: a = XY, b = Xy, c = xY, d = xy

[++] Midwest Oilseeds M3 = 527-8 x 91133; F_1 plants are heterozygous, Ms ms

[+++] Midwest Oilseeds M1 = 502-19 x 82854; F_1 plants are heterozygous, Ms ms

Table 5. Linkage test between ms and *T1* locus in soybean: F_2 data

Cross combinations	Frequency of phenotype*				Total	X^2 (9:3:3:1)	P (df = 3)
	a	b	c	d			
PI 91167 x M3**	97	30	27	6	160	0.44	0.93
PI 261474 x M3	127	48	35	10	220	0.50	0.92
PI 227333 x M3	143	49	50	23	265	0.96	0.81
A.K. Harrow x M3	88	17	18	8	131	2.87	0.41
PI 261474 x M1***	47	12	19	7	85	0.44	0.93
PI 427099 x M1	101	43	34	8	186	1.91	0.59
PI 297544 x M1	41	18	13	10	82	0.94	0.82
Total	644	217	196	72	1129	0.30	0.96

* Linkage determinations: a = XY, b = Xy, c = xY, d = xy

** Midwest Oilseeds M3 = 527-8 x 91133; F_1 plants are heterozygous, Ms ms

*** Midwest Oilseeds M1 = 502-19 x 82854; F_1 plants are heterozygous, Ms ms

Table 6. F_2 segregation for fertility / sterility from crosses between known mutations at *ms1*, ms2, *ms3*, *ms4*, *ms5*, and *ms6* loci with ms soybean

Cross combinations	No. of families[+] (3:1)	(9:7)	X^2(1:1)	P (df = 1)
ms1 ms1 x M3[++]	6	9	0.60	0.44
ms2 ms2 x M3	4	4	0.00	1.00
ms3 ms3 x M3	11	2	6.20	0.01
ms4 ms4 x M3	1	7	4.50	0.03
ms5 ms5 x M3	6	3	1.00	0.32
ms6 ms6 x M3	10	11	0.05	0.82

[+] 3 : 1 = 3 fertile : 1 sterile; 9 : 7 = 9 fertile : 7 sterile

[++] Midwest Oilseeds M3 = 527-8 x 91133; F_1 plants are heterozygous, Ms ms

Table 7. F_2 segregation for fertility / sterility from crosses between known mutations at *ms1*, *ms2*, *ms3*, *ms4*, *ms5*, and *ms6* loci with ms soybean

Cross combinations	No. plants Fertile	Sterile	X^2(3:1)	df	P	No. plants Fertile	Sterile	X^2(9:7)	df	P
ms1 ms1 x M3[+]										
total	561	200	1.3	5	0.93	651	530	2.16	8	0.98
pooled			0.66	1	0.42			0.61	1	0.43
homogeneity			0.64	4	0.96			1.55	7	0.98
ms2 ms2 x M3										
total	257	100	1.73	3	0.63	222	182	2.12	3	0.55
pooled			1.72	1	0.19			0.28	1	0.60
homogeneity			0.01	2	1.00			1.84	2	0.40
ms3 ms3 x M3										
total	728	255	2.77	10	0.99	75	56	0.06	1	0.81
pooled			0.46	1	0.50			0.07	1	0.80
homogeneity			2.31	9	0.99				0	
ms4 ms4 x M3										
total	28	6		0		261	217	1.65	6	0.95
pooled				0				0.53	1	0.80
homogeneity				0				1.12	5	0.95
ms5 ms5 x M3										
total	260	94	1.64	5	0.90	69	49	0.53	2	0.77
pooled			0.46	1	0.50			0.23	1	0.63
homogeneity			1.18	4	0.88			0.30	1	0.58
ms6 ms6 x M3										
total	441	141	2.94	9	0.97	543	445	4.05	10	0.95
pooled			0.19	1	0.66			0.67	1	0.41
homogeneity			2.75	8	0.95			3.38	9	0.95

[+] Midwest Oilseeds M3 = 527-8 x 91133; F_1 plants are heterozygous, Ms ms

sterile loci. In the F_2 generation, there were two kinds of families with almost equal frequency, except for *ms3* and *ms4*. These families segregated in 3:1 or 9:7 ratios (Tables 6 and 7). These data indicate that the male sterility of ms was controlled monogenically by a single recessive allele and was, therefore, different from the six known soybean male-sterile loci. Regarding *ms3* and *ms4*, there were two segregation patterns in the F_2 generation. With *ms3*, 11 families segregated in a 3:1 ratio and two families in a 9:7 ratio. Classification of F_3 plants descended from fertile F_2 (9:7) families of *ms3* cross combination confirmed the F_2 (9:7) ratio (Table 8). With *ms4*, one family segregated in a 3:1 ratio and 7 families segregated in a 9:7 ratio. Similarly, classification of F_3 plants descended from the single F_2 (3:1) family of the *ms4* cross combination confirmed the F_2 (3:1) ratio (Table 8).

Cytology

Anther and pollen development in male-fertile soybean has been described by Carlson and Lersten (1987), Albertsen and Palmer (1979), and Buntman and Horner (1983). Observations of male-fertile development are therefore presented only where pertinent. Anthers from male-sterile plants were white, instead of the yellow color typical of fertile anthers. Also, anthers of male-sterile plants were slightly smaller at maturity than the anthers of fertile plants. When mature anthers were squashed in I_2KI solution, male-sterile anthers consisted mostly of degenerated microspores (Fig. 1), whereas densely staining pollen grains were observed in fertile anthers (Fig. 2). Aniline blue staining indicated that callose was retained around degenerated microspores in male-sterile anthers (Figure 3).

Table 8. F_3 segregation for fertility / sterility from crossed between known mutations at *ms3* and *ms4* loci with ms soybean. Male-fertile F_2 plants single-plant threshed from *ms3* families segregating 9:7 and from *ms*4 family segregating 3:1

	No. plants					No. plants				
Cross combinations	Fertile	Sterile	X^2(9:7)	df	P	Fertile	Sterile	X^2(3:1)	df	P
ms3 ms3 x M3[+]	448	353								
total			2.90	4	0.57					
pooled			0.03	1	0.86					
homogeneity			2.87	3	0.41					
ms4 ms4 x M3						1213	395			
total								5.16	9	0.82
pooled								0.16	1	0.69
homogeneity								5.00	8	0.76

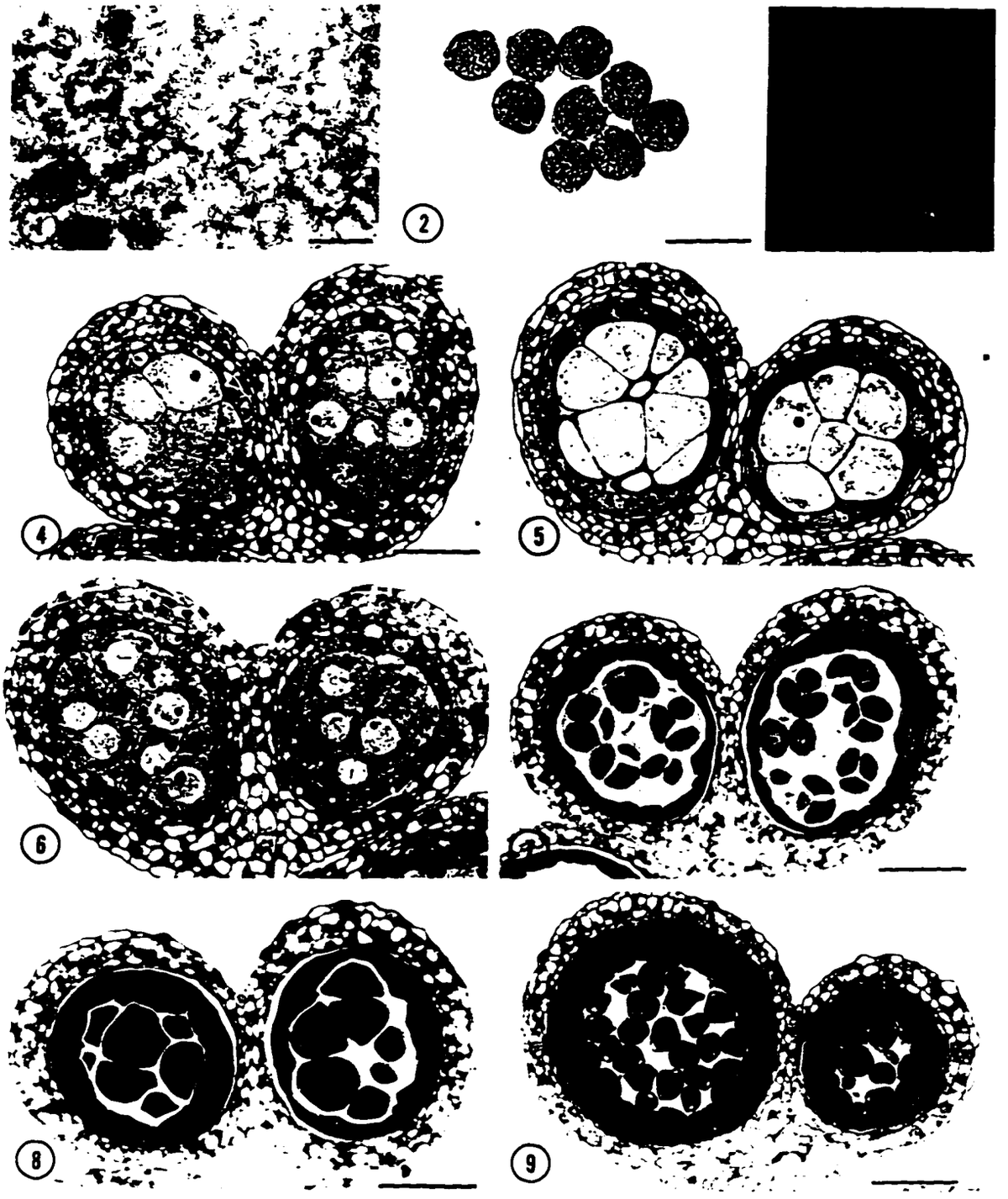
[+] Midwest Oilseeds M3 = 527-8 x 91133; F_1 plants are heterozygous, Ms ms

Anther development in male-sterile plants appeared normal during the earliest stages of microsporogenesis. Each of the four young locules contained sporogenous mass cells (SMC) which were surrounded by an epidermis, endothelium, and up to two parietal layers. The innermost layer, the tapetum, separated the SMCs from the parietal layers (Fig. 4). Microspore mother cells (meiocytes) differentiated from SMCs.

During prophase I, small cytoplasmic vacuoles appeared in the tapetal cells of both male-sterile (Fig. 5, arrows) and male-fertile anthers (Fig. 6, arrows). As meiosis progressed, meiocytes divided to form dyads, and then tetrads of microspores in both male-fertile (Fig. 7) and male-sterile anthers (Fig. 8). Microspores within tetrads became isolated by callose (Figs. 7,8). The tapetal cells in male-sterile anthers enlarged, and became densely stained (Figs. 8,9), tapetal cells of male-fertile anthers remained cytoplasmically dense. The sheath of callose surrounding the tetrads from male-sterile anthers remained (Figs. 9, arrows, 11-13, arrows), and each microspore cytoplasm became highly vacuolate (Figs. 11-13). In male-fertile anthers, callose was degraded, and individual microspores were released (Fig. 10).

The behavior of the tapetum in male-sterile anthers was variable. In some locules, male cells degenerated, whereas the tapetum retained its cytoplasm and seemed functional (Figs. 8,9); or the tapetal cells became highly vacuolate (Fig. 12) or enlarged (Fig. 9). Some tapetal cells accumulated a densely staining material before collapsing (Fig. 11, arrows). In later stages of development, the tapetum collapsed into a mass of darkly staining material (Fig. 13).

Figures **1-9**. Squashes and cross sectional images of male-fertile and male-sterile soybean anthers. All bars equal 10 μm. **1**. Squash of mature anther from male-sterile plant in I_2KI showing degenerated microspores. **2**. Squash of mature male-fertile anther in I_2KI showing engorged pollen grains. **3**. Squash of male-sterile anther in aniline blue at late microspore or early pollen stage showing microspores still encased in callose. **4**. Fertile sporogenous mass stage; epidermis (E), wall layers (W), parietal layers (P), tapetum (T), and male cells (M). **5.** Male-sterile meiocytes anther in prophase of meiosis. **6**. Male-fertile meiocytes in prophase of meiosis. **7**. Male-fertile microspore tetrads. **8**. Male-sterile microspore tetrads. **9**. Late microspore tetrads from male-sterile plant, tapetal cells enlarged, tetrads appear normal.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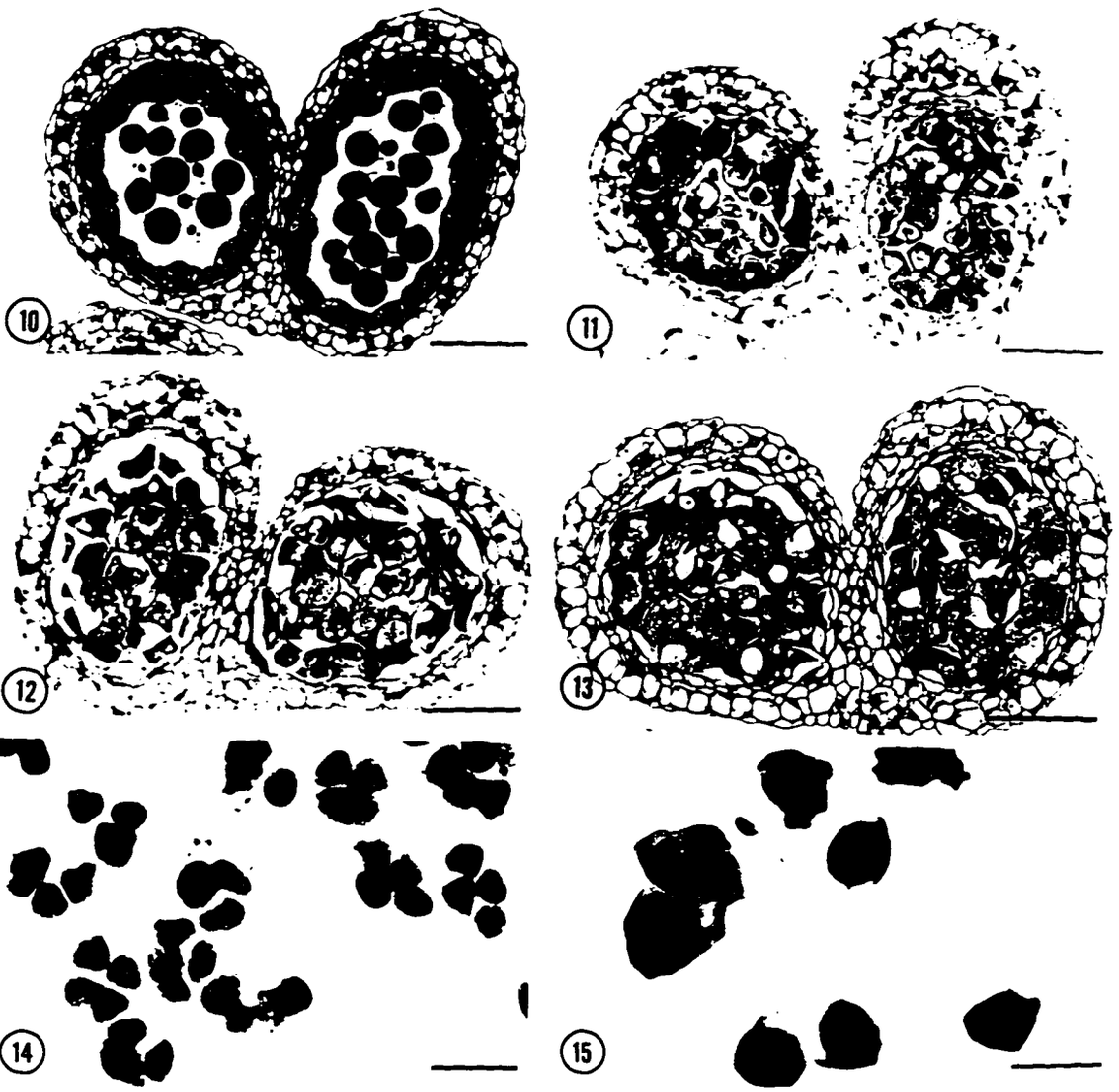
Microsporogenesis in male-sterile anthers did not progress beyond the tetrad stage. While still enclosed in callose, many tetrads shriveled and collapsed (Fig. 11). The tapetal cell walls of both the male-fertile and male-sterile anthers remained intact (Figs. 10, 11), but there was little cytoplasm remaining in the male-sterile tapetal cells. When anthers were mature, there were only degenerated cells within the locules of male-sterile anthers (Fig. 12), whereas engorged pollen grains were present in the locules of male-fertile anthers (Fig. 2). Even at or near anthesis. microspores from male-sterile plants remained encased in callose (Fig. 13).
The results of tests for callase activity in both male-fertile and male-sterile anthers are shown in Figs. 14 and 15. Callose around isolated fertile and sterile tetrads (Fig. 14) was digested after treatment with fertile anther enzyme extracts but not with sterile anther extracts (Fig. 15; fertile tetrads). The results indicate there was no callase activity in male-sterile anthers but callase was active in the male-fertile anthers.

Discussion

The genetic data indicate that the male-sterile soybean (ms) in this study is **gms** and is controlled monogenically by a single recessive allele. The mutation occurs at a locus that differs from the already characterized *ms1*, *ms2*, *ms3*, *ms4*, *ms5*, and *ms6* soybean lines. A skewed ratio was observed in cross combinations with *ms3* and *ms4*. This was the result of the small number of F_2 families tested because F_3 data confirmed F_2 data, rather than gamete interaction between ms and *ms3* and *ms4*. Skorupska and Palmer (1990), however, observed a skewed ratio with *ms4* in

Figures **10-15**. Cross sectional images of male-fertile and male-sterile soybean anthers. All bars equal 10 μm. **10**. Male-fertile young microspores after dissolution of callose. **11-13**. Male-sterile post-tetrad stages showing degenerated tetrads still surrounded by callose (arrows). Tapetum has degenerated and contains unidentified densely-stained material. **14**. Isolated sterile tetrads treated with crude extract from fertile anthers. Callose is absent; stain is lacmoid. **15**. Isolated normal tetrads treated with crude extract from sterile anthers. Callose still surrounds the tetrads; stain is lacmoid.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testcrosses (excess of fertile F_1 plants). The authors had no plausible explanation for the skewed ratio.

The co-segregation of a closely linked marker locus *(W1)* with a male-sterility locus (*ms*6) was used to produce large quantities of F_1 hybrid soybean seed (Lewers et al. 1996). The *W1* and *T1* loci were independent of the ms locus in the present study which precludes their use in any soybean breeding program using the present ms line.

Based on results of the glasshouse experiment, this is a completely male-sterile line. Marrewijk (1969) reported that the phenotypic effect of partial male-sterility systems was subject to environmental modification. Temperature has more influence than any other environmental factor; however, water stress, photoperiod, nutrient supply, and hormone applications also influence male-sterile phenotypes (Edwardson 1970; Heslop-Harrison 1957). In soybean the *msp* mutant is affected by temperature (Stelly and Palmer 1980; Carlson and Williams III 1985). The expression of the male-sterile gene in this study is at least not affected by the summer glasshouse environment.

Compared to known male-sterile soybean mutants, this new **gms** mutant is similar to *ms2*, and more similar to *ms3* phenotypically but genetically controlled by different genes. All three mutants result in a degeneration of tetrads because release of microspores from their encasing callose walls is prevented, a phenomenon also described in other non-legume species. For example, the failure of callose to breakdown at the proper time in **cms** petunia anthers resulted in sterility (Frankel et al. 1969). The retention of callose seemingly blocks developmental

metabolic processes (physical constraints imposed by the callose wall) and intercellular communication between male cells and locular fluids and between male cells and surrounding tissues.

Abnormal behavior of callase has been observed in several male-sterile systems. Previous studies indicate that the enzyme callase is synthesized in the tapetum, then secreted into the locules, and degrades the callose walls surrounding the microspore tetrads. The timing of production and release of callase by the tapetum, therefore, seems to be critical for normal pollen development (Eschrich 1961; Mepham and Lane 1969; Stieglitz and Stern 1973; Frankel et al. 1969; Izhar and Frankel 1971; Worrall et al. 1992; Tsuchiya et al. 1995). Premature breakdown of callose was observed in male-sterile sorghum (Warmke and Overman 1972) and in **cms** petunia (Izhar and Frankel 1971). Worrall et al. (1992) and Tsuchiya et al. (1995) reported that premature breakdown of callose caused male-sterility in transgenic tobacco. Absent or delayed callose degradation was reported in *ms2* (Graybosch and Palmer 1985) and *ms3* soybean (Buntman and Horner 1983), in **cms** *Capsicum* (Horner and Rogers 1974), and in **cms** *Helianthus* (Horner 1977), and is similar to what is reported here for the ms mutant. Therefore, the timing of callase activity is critical for normal development of microspores.

As in many of the male-sterile mutations of angiosperms, abnormal tapetum activity or premature degeneration is associated with the abortion of microspores (Gottschalk and Kaul 1974; Laser and Lersten 1972; Koltunow et al. 1990). The most obvious abnormalities of tapetal cells in this soybean male-sterile mutant were cell enlargement, the accumulation of an unidentified, densely-staining material, and

premature degeneration. This accumulated material, based on its staining, is suspected to be sporopollenin or its precursors. The tapetum is regarded as the site for synthesis for precursors of sporopollenin (Echlin 1971, Horner and Pearson 1978; Nakashima et al. 1984).

Based on the results presented in this study, there are four possibilities for the underlying cause of the sterility in this new genic male-sterile line: 1) callase is not produced, or is produced but below the threshold level to digest the existing callose; 2) the callase is molecularly defective; 3) the callose is molecularly defective and is not sensitive to the callase; and 4) the callase is not active in the environment of the locular fluid (i.e., sub-optimal pH). One of these possibilites has been tested using the crude anther enzyme extracts, and is discounted as the cause of the sterility; namely, that callose is molecularly defective. Both the fertile and sterile tetrad callose walls were digested with the crude extracts from male-fertile anthers. This leaves the other three possibilities that are presently being tested by molecular approaches.

Genic male-sterile mutants have been proposed for many crop species breeding programs (Horner and Palmer 1995). Controlled production of hybrid seed is necessary for breeding programs and genetic studies. The most feasible methods should utilize close genetic linkage between a male-sterility locus and a seedling marker locus. In soybean, the close genetic linkage (2 to 4 cM, Skorupska and Palmer 1989) between a male-sterility locus and a seedling marker locus (*W1*) is known as the Co-segregation Method to produce F_1 seeds (Lewers et al. 1996). Development and use of this method will assist in the elucidation of the genetic

control of complex traits, the identification of lines to improve these traits, the improvement of populations for these traits through the use of recurrent selection, and determination of the agronomic potential of commercial hybrid soybean. The identification of additional soybean genic male steriles linked to a seedling marker locus will reduce the genetic vulnerability of soybean production of a single genic male sterile. The new genic male-sterile line described in this study, because of its high seed set, would be valuable for breeding programs if a useful linked genetic marker or a molecular marker can be identified.

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CHAPTER 3 MOLECULAR MAPPING OF A MALE-STERILE GENE IN SOYBEAN

A paper to be submitted to the journal Crop Science

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Abstract

A newly identified genic male-sterile mutant in soybean has high seed set under natural field conditions and is potentially useful in hybrid seed production. Sterility in this mutant is caused by failure of callose dissolution at the tetrad stage, which results in microspore abortion; however, little is known about the male-sterile gene at the molecular level. The objective of this study was to identify molecular markers linked with the male-sterile gene (ms) and to place the ms gene onto the soybean molecular genetic map. An F2 population of 111 individuals was constructed from a cross between the mutant msMOS (ms ms) and the cultivar Minsoy (Ms Ms). Two hundred and seventy markers, including 219 RFLP and 51 SSRs, were evaluated. Of these, 102 RFLP probes and 31 SSR markers detected polymorphisms between the parents. The F2 population was screened for segregation of these polymorphic

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Abbreviation: cM, centiMorgan; cms, cytoplasmic male sterility; LOD, logarithm of odds ratio; ms, male sterility; MAS, marker-assisted selection; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat.

molecular markers. Analyses revealed that the male-sterile locus, designated 'ms', was located on linkage group D1b of the USDA/ARS/ISU soybean molecular genetic map. The availability of linked DNA markers will facilitate the genetic analysis of this male-sterility gene in relation to soybean breeding programs, and will be a starting point for the isolation of the ms gene by map-based cloning.

Introduction

A major obstacle to F1 hybrid soybean seed production is the intensive hand-labor requirement for large numbers of pollinations. Male sterility can be exploited to take advantage of insect pollination (Kaul, 1988). Cytoplasmic male sterility (cms) is the ideal system for hybrid seed production (Kaul, 1988); however, cms has been reported, but not confirmed, in soybean (Sun et al., 1997). Thus, nuclear male sterility, determined by nuclear genes, is currently the only available system. At least seven nuclear male-sterile mutants, including the mutant in this study have been documented in soybean (Jin et al., 1997; Palmer et al., 1992).

There are several limitations to using nuclear male sterility for hybrid seed production. Because this type of sterility is predominantly determined by recessive alleles, only the homozygous recessive can be identified in segregating populations. As a result, when using the classical backcross method for interline transfer of male sterility, fertile homozygotes and fertile heterozygotes are not distinguishable in the segregating backcross generations. To solve this problem, a breeder must identify

lines carrying the male-sterile allele by self pollination. A second concern is that recessive nuclear male sterility must be propagated via the heterozygotes. Therefore, the yield of male-sterile progeny is limited to 50% in a backcross and 25% in an F2 generation.

Tight linkage between a male-sterile locus and a selectable marker locus will allow for the transfer of the male-sterile allele among breeding lines via uninterrupted backcrossing (for review see Horner and Palmer, 1995). If the marker could be identified in early growth stages, it would be possible to eliminate most if not all of the undesirable fertile plants before flowering. For example, the close genetic linkage (2 to 4% recombination) between the phenotype markers purple hypocotyl and flower (*W1* locus), and male fertility (*Ms6* locus) has been used in hybrid soybean seed production (Lewers et al., 1996). The *W1* _ seedlings have purple hypocotyls, and *w1* *w1* seedlings have green hypocotyls. The *ms6* *ms6* plants are male sterile and female fertile. This closely-linked marker system has the benefit of allowing elimination, shortly after emergence of nearly all male-fertile individuals from evaluation plots, and allowing greater control of parental contributions during intermating. Therefore, half-sib recurrent-selection methods in soybean are compatible with this method (Lewers and Palmer, 1997).

The ms mutation described in this study is recessive, segregates as a single Mendelian locus, and is not linked to either of the two morphological markers; flower color (*W1* locus) and pubescence color (*T1* locus) (Jin et al., 1997). This ms mutation is non-allelic to ms1 through ms6 (Jin et al., 1997). The simple and

recessive inheritance of this male-sterile gene, as well as knowledge from previous extensive genetic and developmental studies (Jin et al., 1997), make this ms mutation an excellent system for further genetic and molecular studies. Molecular markers have been identified for a number of male-sterile genes in plants, such as rice photoperiod-sensitive genic male-sterile genes (Subudhi et al., 1997; B Wang et al., 1995; J Wang et al., 1995; Zhang et al., 1994) and *ms*14 from tomato (Gorman et al., 1996). There are no reports of molecular markers linked to male-sterile genes in soybean.

The objectives of our research were a) to identify molecular markers linked to the male-sterile gene, ms and b) to map the locus within the soybean genome. The results will facilitate the transfer of the ms allele in soybean breeding programs, and will help to characterize the behavior of the ms gene in various genetic backgrounds. We anticipate that as the soybean molecular map becomes more saturated and additional markers are identified that are even more closely linked to ms, these markers will be useful in isolating the ms gene by map-based cloning.

Material and Methods

Plant Material

Four cultivars [Williams 82, Harosoy, Noir I (PI 290136), and Minsoy (PI 27890)] and the male-sterile mutant (msMOS) were screened with 219 mapped RFLP probes and 51 SSRs to survey for DNA polymorphisms between msMOS and other cultivars. This process demonstrated that the mutant msMOS and Minsoy

possessed a relatively high level of polymorphism (49%). A cross between msMOS and Minsoy was subsequently made during the summer of 1995. The F1 seeds were planted at the University of Puerto Rico Soybean Breeding Nursery, at the Isabela Substation, Isabela, Puerto Rico. F2 plants were grown in the growth chamber at photoperiods of 16 hr for the first 4 weeks, 14 hr for 2 weeks, and 13 hr until mature, and at 30°C daytime, and 24°C nighttime temperatures. Fertile F2 plants were single-plant threshed and F3 seeds were planted in Puerto Rico for classifying male sterility / fertility at maturity based on the successful seed set.

Male-fertile and male-sterile plants were identified at flowering by squashing late-stage anthers in an aqueous solution of I_2KI (Jensen, 1962). Anthers from male-fertile plants displayed densely staining rounded pollen grains, whereas anthers from male-sterile plants were void of densely staining rounded pollen grains (Jin et al., 1997). Two to three flowers per plant were evaluated on different days. Chi-square tests were performed to determine the goodness of fit of the phenotype of the F2 generation to a 3:1 ratio and of the F3 generation to a 1:2:1 ratio to identify the F2 genotype.

RFLP and SSR Analyses

Soybean DNA was isolated from freeze-dried leaf tissue of parental, F1, and F2 plants according to Keim et al. (1988), and digested with five restriction endonucleases (*Hind* III, *Eco* RI, *Eco* RV, *Dra* I, and *Taq* I). Digested DNA was separated by agarose gel electrophoresis (10 μg/lane, 0.8% agarose), and transferred onto Zeta Probe Nylon membrane (BioRad) according to Sambrook et

al. (1989). Blots were hybridized with randomly primed ^{32}P-dCTP-labeled probes. Hybridizations and washes were performed at 65°C and 60°C, respectively, according to Zeta Probe recommendations (BioRad). Preliminary screening of parental DNA identified polymorphic clones used to collect RFLP data from the F2 progeny. The segregation of alleles at each locus was tested by chi-square analysis to determine the fit to expected ratios. Segregation data were collected for 102 clones, including 90 from soybean (Shoemaker and Olson, 1993; Shoemaker et al., 1997) and 12 from common bean (Vallejos et al., 1992) or mung bean (Menancio-Hautea et al., 1993).

Simple-sequence repeat (SSR) markers (Akkaya et al., 1995) also were evaluated, thus bringing the number of markers evaluated to 133. For SSR analysis, PCR reaction mixtures contained 60 ng of soybean genomic DNA, 1.5 mM Mg^{2+}, 0.3 μM of sense and antisense primers, 200 μM of each nucleotide, and 1x PCR buffer in a total volume of 20 μL. Cycling consisted of 30 sec at 94°C, 30 sec at 47°C, and 30 sec at 68°C for 45 cycles on a Perkin-Elmer 960 Thermal Cycler. PCR products were run on 2.5% - 3.5% (depending on the sizes of the polymorphic fragments of the two bands) Metaphor (FMC) agarose gel in TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) buffer with ethidium bromide incorporated in the gel.

Linkage Analysis

The Mapmaker program (Lander et al., 1987) was used to construct a linkage map. A LOD score of 3 was used as the lower limit for accepting linkage between

two markers. Recombination frequencies were converted to map distances in centiMorgans (cM) using the Kosambi (Kosambi, 1944) function. Based on two-point analyses, Mapmaker generated log-likelihood values for the most probable order.

Results

Parental Survey

The four cultivars were screened with 219 mapped RFLP probes. Restriction patterns were compared with those obtained from the same digestion of msMOS with five restriction endonucleases (*Hind* III, *EcoR* I, *EcoR* V, *Dra* I, and *Taq* I). The msMOS and Minsoy combination demonstrated the highest level of polymorphism (49%, data not shown). Therefore, Minsoy was chosen as the male parent to cross with msMOS for constructing an F2 population.

Segregation of ms in the F2 Generation

The F1 plants were fertile. The segregation of fertile to sterile plants in the F2 followed a 3:1 ratio (data not shown). This confirmed the observation of Jin et al. (1997) that ms is a recessive gene. Following progeny testing the data demonstrated that the population of 111 F2 individuals showed 1:2:1 genotypic segregation ($\chi^2 = 0.16$, Table 1).

Identification of RFLP and SSR Markers Linked to ms

Initial screening of the F2 population was conducted by selecting several RFLP and SSR markers from each linkage group (Shoemaker et al., 1997). The markers were chosen to divide each linkage group into segments of less than 20 cM. Two-

point analyses indicated that the ms gene was linked to SSR marker Satt005 (LOD 38.9) on linkage group D1b (Cregan et al., unpublished). Additional markers from the linkage group were screened against the F2 population (Satt157, Satt296, Satt412, Satt266, A605, A747, Bng47, Mng137, Satt141, Satt189, Satt290, B194, L161, and K411). Based on LOD scores generated from the Mapmaker program, we found that the ms locus was linked to RFLP marker, Bng047, and SSR markers Satt157, Satt412, Satt005 and Satt290 with the LOD score of 31.5, 7.9, 10.7, 38.9 and 48.7 respectively. The most likely order of these markers is shown in Fig. 1. No polymorphisms were detected at loci: A605, A747, Mng137, and B194 using the restriction endonucleases *Hind* III, *EcoR* I, *EcoR* V, *Dra* I, *Taq* I, *Acc* I, *Alu* I, *Hha* I, *Hae* III, *Ssp* I, and *BamH* I, nor were polymorphisms observed using the SSR markers: Satt296, Satt266, Satt141, and Satt189. Although markers K411 and L161 were polymorphic in this cross, they segregated independently of ms. Segregation ratios of all RFLP and SSR markers provided good fits to the 1:2:1 ratio or the 3:1 ratio. Only those markers linked with the ms gene are shown in Table 1.

Discussion

In this study, we mapped the nuclear male-sterile gene, ms, to linkage group D1b. The gene was flanked by SSR markers, Satt157, and Satt412. Male-sterile genes have been mapped in several different species (Subudhi et al.,1997; Gorman et al., 1996; J Wang . et al., 1995; Zhang et al., 1994); however , this is the first

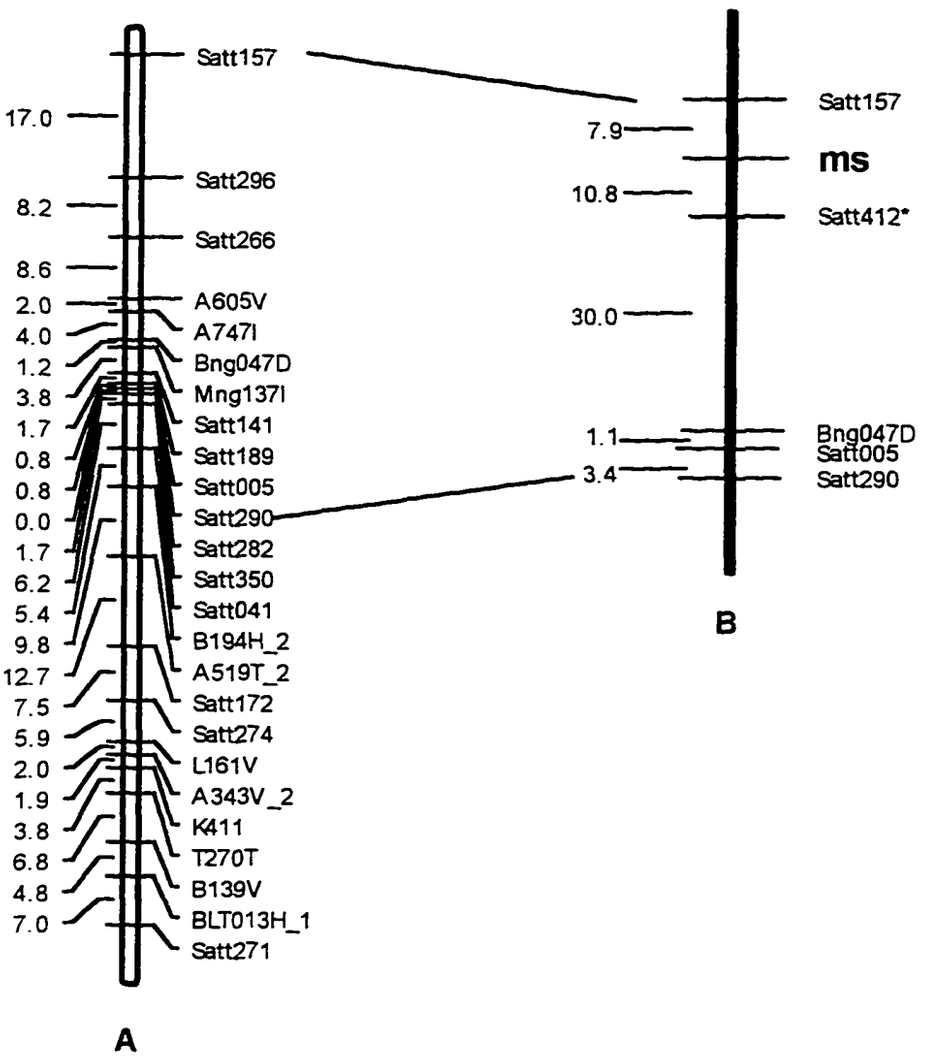

Fig. 1. Linkage map of linkage group D1b of the USDA/ARS/ISU molecular genetic map. Distances are shown in centiMorgans. A) A linkage map of linkage group D1b constructed from the *Glycine max* x *G. soja* population, where ms was placed between SSR markers Satt157 and Satt412. B) Location of the ms locus, from the cross msMOS x Minsoy, on linkage group D1b. *Satt412 has not been mapped in *G. max* x *G. soja* population.

Table 1. Segregation of the ms (male sterility) locus, and linked restriction fragment length polymorphism and simple sequence repeat markers in an F2 soybean population from msMOS X Minsoy

Traits or markers	No. of F2 plants	Observed no.							X^2	
		AA‡	HH‡	BB‡	BB‡	DD‡	AA‡	CC‡	(1:2:1)	(3:1)
ms	109	25	55	27					0.16	
Satt157	101	24	56	21					1.81	
Satt412	106	26	57	23					0.29	
Bng047	108						25	83		0.30
Satt005	111	24	61	26					1.20	
Satt290	111	25	60	26					0.76	

‡ Genotypes: AA = msMOS; HH = heterozygous; BB = Minsoy; DD = not BB; CC = not AA

report on the molecular mapping of a soybean male-sterile gene.

In an independent population the distance between Satt157 to Bng047 has been shown to be 39.8 cM (Cregan et al., in review) whereas in our population we calculated the distance to be 48.7 cM. These differences may arise from the relatively low sample number or may be complicated by different recombination frequencies in this region in the specific genetic material used for our crosses (Mock, 1972). Additionally, recombination distortions may have occurred because

of different environment/genotype interactions at the time of F1 meiosis (Stephens, 1950; Williams et al., 1995).

Soybean nuclear male sterility is controlled predominately by single recessive genes and the presence of male-sterile genes (heterozygotes) in breeding lines can only be detected by progeny testing. Presently, breeders use self-pollination alternating with backcrossing to identify lines carrying the male-sterile allele (Palmer et al., accepted). DNA markers linked to the ms locus provide a useful approach for early and accurate identification of lines carrying a male-sterile allele and they eliminates the necessity of several seasons of self-pollination. These markers should provide plant breeders with an efficient method for rapid transfer of the ms gene to elite soybean germplasm and may facilitate the development of hybrid soybean.

SSRs are excellent genetic markers in that they are highly abundant and highly polymorphic (Akkaya et al., 1992; Tautz, 1989). They are evenly distributed throughout the genome (Weber, 1990), generally detect only a single genetic locus, and are disseminated easily among laboratories by publishing primer sequences. In this study SSR markers were scored with agarose electrophoresis without the use of radioisotopes. Such ease in screening for SSRs should further facilitate their use in practical plant breeding.

Pollen development in higher plants is a complex process (reviewed in McCormick, 1993). Four male-sterile genes have been cloned (Aarts et al., 1993; Albertsen et al., 1996; Gorman et al., 1996; Moffatt and Somerville, 1988), but

because of the complexities of male gametophyte development, the mechanism(s) of nuclear-encoded male sterility is (are) not clear. It will be essential to clone and characterize a large number of male-sterile gene products from a number of species to understand the myriad functions of male-sterile genes. Map-based cloning is a reasonable strategy for isolation of male-sterile genes in soybean because the technique requires only that the target gene has a clear phenotype and that its position on a genetic map be known. The placement of the ms phenotype onto the soybean genetic map is a first step in this process.

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CHAPTER 4. ANALYSIS AND MAPPING OF GENE FAMILIES ENCODING β-1,3-GLUCANASES OF SOYBEAN

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Abstract

Oligonucleotide primers designed for conserved sequences from coding regions of β-1,3-glucanase genes from a number of different species were used to amplify related sequences from soybean [*Glycine max* (L.) Merr.]. Sequencing and cross hybridization of amplification products indicated that at least 12 classes of β-1,3-glucanase genes exist in soybean. Members of classes mapped to 34 loci on 5 different linkage groups using an F2 population of 56 individuals. We found that β-1,3-glucanase genes are clustered onto regions of five linkage groups. Northern blot analyses performed on total RNA from root, stem, leaf, pod, flower bud, and hypocotyl, using the different classes of β-1,3-glucanases as probes, revealed that

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Key words: Soybean [*Glycine max* (L.) Merr.]; β-1,3-glucanase gene families; sequence; genomic mapping

Abbreviations: SGlu, soybean β-1,3-glucanase; BAC, bacterial artificial chromosome.

accumulation patterns of β-1,3-glucanase mRNA in these organ. Data suggest that the mRNA accumulation of β-1,3-glucanase genes may be correlated with their levels of sequence divergence and their chromosomal locations.

Introduction

β-1,3-glucanases (EC 3.2.1.39) are hormonally and developmentally regulated plant hydrolytic enzymes, found during anther and coleoptile development, pollen tube growth, in endosperm cell walls and in the end walls of sieve elements. They also are induced upon pathogen infection or environmental stresses (Abeles et al., 1971; Beerhues and Kombrink, 1994; Beffa et al., 1996; Boller, 1987; Brederode et al., 1991; Castresana et al., 1990; Dong et al., 1991; Leah et al., 1991; Mauch et al., 1988; Schroder et al., 1992; Takeuchi et al., 1990; Ward et al., 1991). Five distinct classes and a total of 12 genes of β-1,3-glucanase have been identified in tobacco (Bucciaglia and Smith, 1994; Felix and Meins, 1986; Linthorst et al., 1990; Ori et al., 1990; Payne et al., 1990; Van den Bulcke et al., 1989). The best characterized are the basic isoforms which are induced by either pathogen infection or ethylene treatment, exhibit developmental regulation (Felix and Meins, 1986), and seem to be localized primarily in cell vacuoles (Van den Bulcke et al., 1989). Members of the second class of β-1,3-glucanases, which include the pathogenesis-related proteins PR-2, PR-N, PR-O and PR-35 (Kaufmann et al., 1987; Van den Bulcke et al., 1989), also are induced by pathogens and seems to be localized in the extracellular spaces. The third class of β-1,3-glucanases, which include the pathogenesis-

related protein PR-Q' (Payne et al., 1990), also are induced by pathogens and seems to be localized in the extracellular space. The fourth class of β-1,3-glucanases are acidic, sp41a and sp41b, accumulate to high levels in the transmitting tract of the style and are not pathogen inducible (Ori et al., 1990). The fifth class is a secreted, acidic anther β-1,3-glucanase that seems to be expressed in the anther tapetum (Bucciaglia and Smith, 1994) and is involved in tetrad dissolution. Related monocot β-1,3;1,4-glucanases encode proteins similar to β-1,3-glucanases. The function of these proteins is to catalyze the hydrolysis of β-1,3;1,4-glucans, major components of endosperm cell walls (Fincher et al., 1986).

Although many physiological studies have been conducted, little has been done toward genetic analysis of β-1,3-glucanase genes. Li et al. (1996) mapped seven members of a β-1,3-glucanase gene family in barley. The seven genes were all located on the long arm of chromosome 3. Six of the seven genes were clustered in a region less than 20 cM in length. The regulation of these genes is unknown. Genes on the same chromosome seemed to be closely related to one another but substantially different from those on other chromosomes (Muthukrishnan et al., 1984).

The isolation, characterization, and localization of β-1,3-glucanase genes will provide information about soybean β-1,3-glucanase gene families and the evolution of the gene families. In this paper we present results related to the cloning of β-1,3-glucanase genes, the expression patterns of these genes, and the chromosomal localization of β-1,3-glucanases in the soybean genome. The linkage group

distributions of β-1,3-glucanase genes expressed in different organs, sequence relationships among β-1,3-glucanase genes found at various loci, and the genomic organization of β-1,3-glucanase loci have implications for the mechanisms underlying β-1,3-glucanase gene regulation, evolution, and possible functions of multiple β-1,3-glucanase gene loci.

Materials and Methods

Plant Material and Nucleic Acid Manipulations

Plant tissues for nucleic acid isolation were collected from the soybean cultivar Minsoy (PI 27890) grown in the greenhouse or growth chamber. Material for RNA extraction was immediately frozen in liquid nitrogen and stored at -80°C until used. Soybean genomic DNA was extracted from freeze-dried young leaves, following the protocol of Keim et al. (1988). Total DNA (10 μg) was digested and subjected to Southern blotting as described by Sambrook et al. (1989). Total RNA was extracted using guanidinium isothiocyanate extraction (Chomczynski and Sacchi, 1987), electrophoresed on formaldehyde-agarose gels, and blotted onto Zeta Probe Nylon membrane (BioRad) as described by Sambrook et al.(1989). Filters were prehybridized 3 hrs at 65°C in 5x SSC, 2% SDS, 5x Denhardt's solution, 0.1 mg/ml herring sperm DNA. Hybridization was carried out overnight at 65°C in the same solution. Filters were washed at 60°C with: 2X SSC+0.4% SDS, and 1XSSC+0.4% SDS at 60°C before exposure for autoradiography.

Genetic Mapping of β-1,3-glucanase Genes

β-1,3-glucanase genes were mapped in a *Glycine max* (L.) Merr. X *G. soja* Zieb. & Zucc. population containing 56 individuals (Keim et al., 1990). Parental DNA was digested with 14 restriction endonucleases (*Acc* I, *Alu* I, *Bcl* I, *BamH* I, EcoR I, *EcoR* V , *Dra* I, *Hae* III, *Hha* I, *Hind III*, *Hinf* I, *Rsa* I, Ssp I, *Taq* I). Each polymorphism was mapped using MapMaker (Lander et al., 1987). The Kosambi (Kosambi, 1944) mapping function was selected and a minimum logarithm of odds (LOD) score of 3 required for a two-point linkage.

PCR Amplification, Cloning, Sequence Analysis

Coding region segments encoding β-1,3-glucanase were amplified from soybean genomic DNA and flower bud cDNA using primers that matched conserved sequences of the β-1,3-glucanases from other species (Simmons, 1994). To obtain cDNA, mRNA was prepared from flower buds, the mRNA was incubated with 5μM random hexamers, 1mM each of dATP, dCTP, dGTP, and dTTP, and 2 U/μl RNase inhibitor (Promega) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$ for 30 min at 37°C, and then for a further 45 min at 37°C following the addition of 20 U/μl superscript reverse transcriptase (BRL). After heating at 65°C for 3 min, cDNA prepared from 0.1μg of mRNA was used in the PCR experiments. Each 25 μl PCR reaction contained 60 ng of soybean genomic DNA or 20 ng of flower cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 2.5 U of Taq polymerase (BRL Life Technology), and 2 μM (each) PCR primer. PCR amplifications were performed according to 96°C for 1 min, 44°C for 1

min, 72°C for 1 min for 4 cycles, followed by 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 30 cycles. The 5' primer was 5'CGCGGNGTNTGYTAYGG 3'; the 3' primer was 5'CGCGGCCANCCNSWYTC 3' (where N = A, C, G, T; R = A, G; Y = C, T; S = C, G and W = A, T). The regions used correspond to amino acids 37-41 and 276-282 (Simmons, 1994). An aliquot of each PCR product was analyzed by agarose gel electrophoresis. A 700 bp fragment was cut from the gel and subjected to another round of PCR using identical conditions. Aliquots of these PCR products were digested with restriction enzymes recognizing 4-bp sites and the digestion products were compared with undigested DNA on agarose gels. The PCR products were cloned into the pGEM-T vector (Promega), and about 280 clones were tested by dot blot analysis (Sambrook et al., 1989) to determine if they cross hybridized with one another.

Clones were sequenced using an automated sequencer. Two to five sequences were obtained for each class. DNA sequence analysis was carried out with the DNAsis (Hitachi), GCG (University of Wisconsin Genetics Computer Group, Madison) sequence analysis packages. Alignment of sequences was done using CLUSTAL (Thompson et al., 1994) Phylogenetic analysis of amino acid sequences was performed using PAUP version 3.0 (Swofford, 1991).

BAC Library Screening

The soybean BAC library (Marek and Shoemaker, 1996; 1997) was replicated onto nylon membrane (Zeta-Probe GT; Bio-Rad). The membranes were screened using two β-1,3-glucanase probes, SGlu2 and SGlu5, and BACs were identified that contained these sequences.

Results

Isolation of Soybean β-1,3-glucanase Genes and Sequence Analysis

PCR amplification of soybean genomic DNA using degenerate primers designed from conserved regions of other β-1,3-glucanases resulted in production of a ~700 bp fragment. The fragment was excised from the gel and subjected to a further round of PCR (Fig. 1). We then asked whether the PCR product we obtained consisted of a mixture of DNA sequences, consistent with the amplification of multigene families. The PCR product was digested with *Hae* III, *Alu* I, *Rsa* I, *Mse* I and *Sau3A* I. The restriction digestion yielded a series of fragments whose molecular weights summed to a value greater than that of the original PCR product (data not shown). The presence of a heterogeneous PCR product suggested the involvement of multigene families.

The PCR products were cloned and ~280 clones were analyzed. The clones were grouped into 12 classes based upon cross-hybridization results under stringent hybridization conditions (0.1x standard saline citrate (SSC) / 0.1x SDS /60°C wash).

PCR experiments using cDNA as a template also generated a 700 bp band (Fig. 1) indicating that the targeted genomic coding regions of the members of the multigene families may not be interrupted by introns.

BAC library screening identified 15 BACs representing SGlu 5, and 5 BACs representing SGlu2. Copy number of β-1,3-glucanase sequences within each BAC was estimated by digesting the BACs with restriction enzymes that did not have recognition sites within the β-1,3-glucanase probe sequences and by hybridizing with each class-specific probe (results not shown). SGlu2 BACs each contained two copies of the sequence. This agreed with the prediction of two to four copies based on genomic Southern hybridization patterns (results not shown). The class 5 BACs each appeared to have one to two copies of the class 5 β-1,3-glucanase sequence. This agreed with the prediction of two copies based upon genomic Southern hybridization.

Three to five clones from each class were sequenced from both strands, and the deduced amino acid sequences of representative clones from each class are presented in Fig. 2. Among sequenced clones, classes 2 and 7 showed heterogeneity. The deduced protein contains 30 amino acids that are identical among all 12 classes, and classes I, II, III, IV, and V of tobacco β-1,3-glucanases (Fig. 2). These include the acidic amino acids glutamic acid, residues 96 and 245, and tryptophans residues 74 and 248. Glutamic acid 245 and tryptophan 248 are surrounded by highly conserved amino acids, which are similarly conserved in plant and yeast glucanases (MacGregor and Balance, 1991).

SGlu9 is probably a pseudogene because it contains multiple stop codons; however, it did show strong similarity with SGlu8. SGlu8 and SGlu9 of soybean β-1,3-glucanase clones differ at only 6 positions in 700 bases of overlapping sequence. Pair-wise comparisons between different classes revealed that amino acid identities ranged from 11.1% to 81.8%; similarities range from 35.2% to 99.9%. Class 7 also contained two subclasses, 7a and 7b, which differ at only 6 positions at DNA sequences and show 98.3% amino acid identity. These subclasses had different banding patterns on Southern blots (results not shown).

Phylogenetic Analysis of β-1,3-glucanase-coding Regions

To determine the relationships of the 12 classes of soybean β-1,3-glucanases to the five classes of β-1,3-glucanase genes from tobacco, a parsimony analysis was conducted using five classes from previously described tobacco β-1,3-glucanase genes. Amino acid sequences were aligned using CLUSTAL (Thompson et al., 1994) and subsequently analyzed using PAUP version 3.0 (Swofford, 1991). Fig. 3 shows the unrooted consensus tree (from 100 bootstrap replicates) which groups four soybean classes (SGlu1, SGlu8, SGlu4, and SGlu12) as a branch with tobacco Class I; five soybean classes, SGlu3, SGlu5, SGlu6, SGlu10, and SGlu11 are grouped closely to acidic β-1,3-glucanases (Class II) and stylar acidic glucanase (Class IV). The structurally similar, pathogen induced, acidic glucanase (Class II), and stylar acidic glucanases (Class IV) are grouped on one branch. This agrees with a previous report (Bucciaglia and Smith, 1994). SGlu2 is grouped close to

Class III. SGlu7a and 7b and Tag1 (Class V) are grouped together, suggesting that SGlu7 could be an anther specific β-1,3-glucanase.

Mapping of 12 Classes of β-1,3-glucanase Genes

An F2 population derived from an interspecific cross was used to determine the genomic location of 12 classes of β-1,3-glucanases. Clones representing separate classes were hybridized to Southern blots of soybean genomic DNA digested with 14 restriction enzymes to identify polymorphisms and to estimate gene family copy number. Most of the genes hybridized to multiple fragments indicating that most β-1,3-glucanase classes consisted of multigene families. Two to 7 bands were detected by ^{32}P-labeled DNA fragments of the coding region of β-1,3-glucanase genes (results not shown). This indicated that the soybean β-1,3-glucanase gene families consisted of at least 1 to 7 members. We detected a total of about 42-46 individual fragments in this analysis, which could represent the presence of at least 42 different genes. Most of the probes identified a number of different restriction fragments in parental DNAs. Most of these fragments are expected to represent different genes that belong to the same β-1,3-glucanase class.

The segregation analysis of 56 individuals of the F2 population placed the different classes on 5 of 26 linkage groups (Fig. 4): linkage groups B1, J, K, L, and N1. All of these linkage groups contained more than two loci of β-1,3-glucanases. Two classes, SGlu4 and SGlu12, cosegregrated with the pea β-1,3-glucanase, involved with fungal resistance (Chang et al., 1992).

Some class probes (SGlu2, SGlu3, SGlu5, and SGlu9) detected multiple polymorphic fragments. Not all of the fragments mapped to the same chromosomal locus. Therefore, not all of the β-1,3-glucanase genes belonging to the same class are located at the same locus and members of different classes can map to the same locus. SGlu7a and b were not mapped because no polymorphisms were detected between parental DNA digested with 14 restriction enzymes.

In soybean, large domains of different linkage groups seem to have been derived from the same ancestral linkage group through duplication (Shoemaker et al., 1996). The different domains contain homologous members of the same gene families. Some of the β-1,3-glucanase gene loci (e. g. SGlu2) lie within paralogous regions (Fig. 4). For example, two markers, pB162 and PEG488, detected loci on linkage groups L and N1. On both linkage groups these markers are also linked to SGlu2 loci (Fig. 4). Therefore, these linkages establish evolutionary relationships among these β-1,3-glucanase gene loci. These further suggests that these multiple β-1,3-glucanase gene loci have arisen, during evolution, via duplications of large chromosomal regions in which linkage relationships between β-1,3-glucanase genes and the other genes have been maintained.

Nucleotide Sequences, Phylogenetic Relationships and Chromosomal Locations

Twelve β-1,3-glucanase gene segments used in the mapping project were sequenced. and the extent to which β-1,3-glucanase genes mapped to individual locus (loci) is (are) related was analyzed (Fig. 4). The sequenced region covers

~60% of the coding region and includes regions proposed to be the catalytic sites. The 12 β-1,3-glucanase sequences show an average nucleotide sequence identity of 64.8% (range 21.4% to 95%), an average amino acid sequence identity of 36.2% (range 11.1% to 81.8%) and an average amino acid sequence similarity of 70.2% (range 35.2% to 99.9%). Those β-1,3-glucanases that mapped to the same locus always showed>88% nucleotide sequence identity. For example, SGlu5 and SGlu11 both mapped to a locus on linkage B (Fig. 4). As Figs. 3 and 4 show, sequence similar genes are clustered on one linkage group or on duplicated regions of linkage groups; e.g. SGlu1, 4, 8, and 12 all group into tobacco class I and are all mapped on linkage group K.

mRNA Accumulation Patterns of Different Classes

In order to study gene-specific mRNA accumulation patterns, the presence of mRNAs corresponding to each class was analyzed by RNA blot analyses using the gene-specific probes (Fig. 6). The expression levels of all classes were quite low in young leaves. SGlu2, SGlu4, SGlu7, and SGlu12 mRNA were highly accumulated in young roots and hypocotyls. SGlu7 also was expressed in the pod and flower bud. Interestingly, we were unable to detect mRNA from SGlu 1, SGlu3, SGlu8, and SGlu9 (pseudogene) genes on RNA gel blots. The lack of detectable mRNA accumulation from these four classes suggested that these genes were nonexpressed, expressed at very low level or were inducible genes.

Discussion

The primary goal of this study was a thorough analysis of β-1.3-glucanase gene families in soybean. Hybridization of β-1,3-glucanase probes to soybean genomic DNA blots revealed gene families of moderate size. Through exhaustive cross hybridizations and sequence analyses, we classified clones into 12 groups. We then analyzed sequence relationships among these genes, and determined their genomic locations.

To our knowledge this is the first instance of direct mapping of β-1,3-glucanase genes in plant. Using an F2 population, 45 restriction fragment length polymorphisms were mapped with 14 restriction enzymes and 12 β-1,3-glucanase class-specific probes. We identified 34 distinct β-1,3-glucanase gene loci on 5 different linkage groups. Multiple genes were found to map to five of the loci, indicating that some of the loci contain clusters of β-1,3-glucanase genes. Because of the small population size, it was not possible to distinguish very tightly linked loci from a single locus. Many polymorphisms were probably due to sequence variations among class members. It is likely that at least some genes will be tandemly arranged in a cluster as is observed with other multigene families (Kanazin et al., 1996; Sullivan et al., 1996; Sutliff et al., 1991; Yamaguchi-Shinozakik et al., 1989). A tandem array of this sort provides a template for recombination events, including unequal crossing over and gene conversion, which can lead to expansion and further diversification of the sort apparent among the classes we have cloned (for review see Maeda and Smithies, 1986; Clegg et al., 1997).

The wide distribution of β-1,3-glucanase genes in the genome may protect against catastrophic losses of β-1,3-glucanase genes by unequal crossing over or gene slippage that might occur if β-1,3-glucanase genes were clustered at a single locus (Dover, 1993). The existence of multiple β-1,3-glucanase genes might also promote the development and maintenance of structural and functional diversity in the β-1,3-glucanase gene families.

Our data suggest that some β-1,3-glucanase genes are organized into duplicated regions in the genome. In these regions, each β-1,3-glucanase gene locus seems to be related to other β-1,3-glucanase gene loci by linkage to other markers. These β-1,3-glucanase gene loci are probably evolutionarily related. These β-1,3-glucanase gene loci have arisen during evolution via duplications of large chromosomal regions. However, retrotransposition and duplication of individual genes also occur during the evolution of β-1,3-glucanase gene families since SGlu9 mapped as a single locus both on linkage group B1 and J.

In higher plants, β-1,3-glucanases are encoded by gene families of considerable complexity (Linthorst et al., 1990; Xu et al., 1992). The significance of the gene multiplicity is unclear. It could provide more flexibility for spatial and temporal regulation of β-1,3-glucanase diverse functions: microsporogenesis, pollen-tube growth, senescence, and disease resistance described previously, or "pathogenesis-related" β-1,3-glucanases might be expressed either constitutively or inducibly, and there may be requirements for different responses in different tissues. It also reflects functional differences between closely related proteins. Multiplicity of

β-1,3-glucanase functions might confer advantages to the plants by providing several lines of defense against invading microorganisms. Also the diversity of β-1,3-glucanases, as well as their organ specificity and developmental and differential expression patterns, may indicate that this group of enzymes has additional, as yet, unidentified biological functions in plant growth and development.

The deduced β-1,3-glucanase protein is very similar to other plant β-1,3-glucanases at residues thought to be important in binding and glucan hydrolysis (Fig. 2, residues 245 to 248), suggesting that these proteins have β-1,3-glucanase activity. Chen et al. (1993) reported that barley endo-β-1,3- and β-1,3-;1,4 glucanase activities were abolished by modification of glutamic acid residues 275 and 335. Based on sequence conservation and amino acid experiments, this region is assumed to be a catalytic site (MacGregor and Balance, 1991). All 12 classes of soybean β-1,3-glucanases contain this site. There is an additional conserved glutamic acid surrounded by identical or functionally conserved amino acids (Fig. 2, residue 96) which is present in soybean glucanases.

In an effort to group 12 classes of soybean β-1,3-glucanases into previously described glucanase classes (Bucciaglia and Smith, 1994; Linthorst et al., 1990; Ori et al., 1990; Payne et al., 1990; Shinshi et al., 1988), we used parsimony analysis to determine the relationship of these β-1,3-glucanases (Fig. 3). Our results are consistent with previously reported classification systems.

Sequence and phylogenetic analyses of the β-1,3-glucanase genes showed that members of the same β-1,3-glucanase class, as well as closely related classes,

often mapped to the same linkage group or to duplicated regions of linkage groups. All class I β-1,3-glucanases mapped on linkage group K, all class III β-1,3-glucanases mapped on duplicated regions on linkage groups L and N1, and all class II and IV β-1,3-glucanases mapped on linkage groups B1 and J. Other examples of genes with similar function and expression patterns that reside on the same linkage group can be found among gene families involved in the regulation of floral identity and cell differentiation (Pickett and Meeks-Wagner, 1995), and disease resistance genes (Kanazin et al., 1996).

The multigene families encoding the β-1,3-glucanases are large; all of the member genes clearly have a common ancestral origin but have undergone considerable divergence such that individual genes encoding proteins share 11.1%-81.5% amino acid identity. Subclasses are apparent, with groups of genes sharing greater homology among themselves than with members of other subclasses. Three classes of tobacco β-1,3-glucanase cDNA clones differ at only 18 positions in 1055 bases of overlapping sequence (Shinshi et al., 1988). The high homology level of the tobacco β-1,3-glucanases is maintained by intergenomic DNA exchange between genes (Sperisen et al., 1991). In soybean, two classes of β-1,3-glucanase genes (SGlu8 and SGlu9) differ at only 6 positions in 700 bases. How this high level of homology is maintained in soybean is unknown.

Our results indicate that in soybean, pathogen invasion is not a prerequisite for expression of some of the β-1,3-glucanase genes. SGlu2, SGlu4, SGlu7 and SGlu12 were constitutively expressed in roots and hypocotyl. These could

represent a permanent form of defense against possible threats from rhizosphere microbes (Memelink et al., 1990). These results do not preclude a role of three β-1,3-glucanases in defense against microorganism infection because pre-emptive or 'proactive' expression of the enzymes (independent of microbial attack) could provide some measure of insurance against infection during crucial phases of the life cycle in which plant tissues are susceptible to pathogen attack (Knogge et al., 1987).

In soybean β-1,3-glucanase gene families, expression patterns are associated with both the degree of sequence variation and the chromosomal location of a gene. Most eucaryotic genes are regulated by *Cis* elements in their promoters. However, it is known that chromosomal location can influence gene expression (Ohshima et al., 1990). It will be interesting to learn more about the expression pattern of other genes that are located near the β-1,3-glucanase genes we have isolated.

The 12 classes of β-1,3-glucanase genes exist in clusters, and two of them are associated with known fungal resistance genes. Our findings demonstrate that β-1,3-glucanase genes with similar sequence and similar temporal expression patterns are clustered on the same linkage group or duplicated regions of the linkage groups. Both duplications of large chromosomal domains followed by extensive gene duplication and divergence, and duplication of individual genes are involved in the evolution of the β-1,3-glucanase gene families.

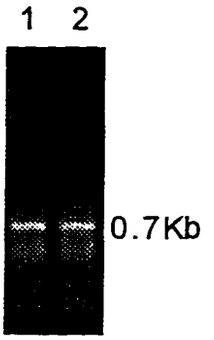

Fig. 1. Ethidium bromide-stained 1% agarose gel of the second round PCR products. Lane 1, PCR using soybean genomic DNA as a template; Lane 2,PCR using soybean flower bud cDNA as a template. Molecular size marker is in Kb.

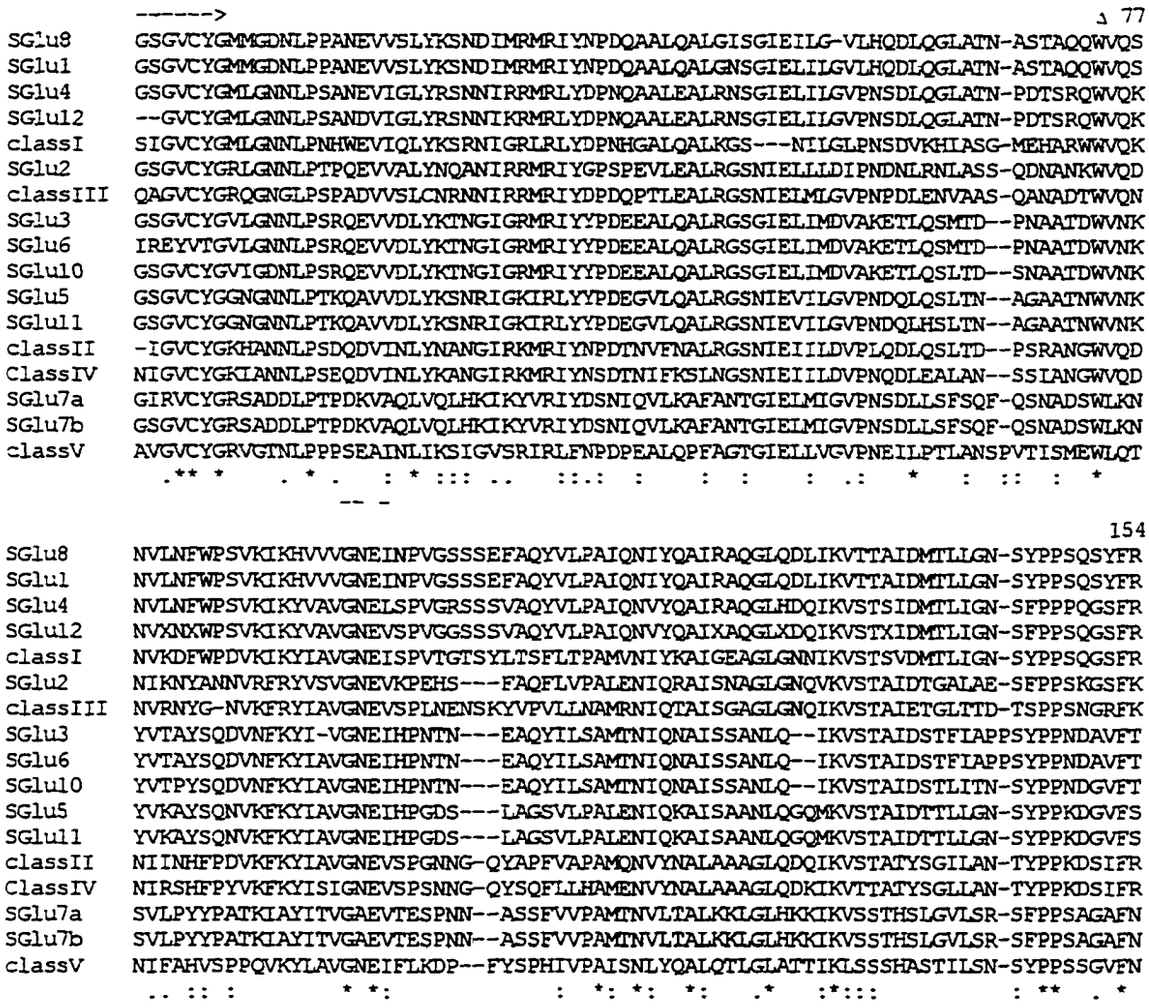

Fig. 2. Alignment of deduced amino acid sequences of ß-1,3-glucanases from soybean. Arrows indicate locations of PCR primers used to amplify ß-1,3-glucanase sequences. Dotted regions indicate gaps in sequences introduced to maximize alignment. Identical residues in all 17 sequences are marked by an asterisk. Conserved tryptophans are marked by a Δ. Identical or functionally conserved amino acids surrounding acidic residues are marked with a hyphen. Arrows indicate primer region. The tobacco class I, class II, class III, class IV and class V amino acid sequences were included for comparison. Database accession numbers for these proteins are: class I, accession M20619; class II, accession M60462; class III, accession X54456; class IV, accession X54430; class V, accession Z28697.

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                                                                           231
SGlu8       TDVR-SYLDPIIGYLVYANAPLLANVLPYFSYSDNP-IDISLSYALFNSTNVVV-WDGQYGYQNLFDAMLDAVHVAI
SGlu1       TDVR-SYLDPIIGYLVYANAPLLANVLPYFSYSNNP-IDISLSYALFNSTNVVV-WDGQYGYQNLFDAMLDAVHVAI
SGlu4       GDV--SYLDPIIGYLVYANAPLLVNVYPYFSYTGNP-RDISLPYALFTAPNVVV-WDGQYGYQNLFDAMLDSVHAAI
SGlu12      GDVR-SYIDPIIGYLVYANAPLLVNVYPYFSYTGNP-RDISLPYALFTAPNVVV-WDGQYGYQNLFDAMLDSVHAAI
classI      NDAR-WFTDPIVGFLRDTRAPLLVNIYPYFSYSGNP-GQISLPYSLFTAPNVVV-QDGSRQYRNLFDAMLDSVYAAL
SGlu2       SDYRGAYLDGVIRFLVNNNAPLMVNVYSYFAYTANP-KDISLDYALFRSPSVVV-QDGSLGYRNLFDASVDAVYAAL
classIII    DDVR-QFIEPIINFLVTNRAPLLVNLYPYFAIANN--ADIKLEYALFTSSEVVV-NDNGRGYRNLFDAILDATYSAL
SGlu3       SDAE-PYVKPIIDFLVRNEAPLLANVYPYFAYANDQQNSIPLAYALFTQQ-----GNNDAGYQNLFDAMLDSIYAAV
SGlu6       SDAE-PYVKPIIDFLVRNEAPLLANVYPYFAYANDQQNSIPLAYALYTQQ-----GNNDAGYQNLFDAMLDSIYAAV
SGlu10      SDAE-PYIKPIINFLVSNGAPILANVYPYFAYANDQ--SIPLAYALFTQQ-----GNNDVGYQNLFDAMLDSIYAAL
SGlu5       SSAS-SYIRPIVNFLARNGAPLLANVYPYFAYVNNQQ-SIGLDYALFTKH-----GNNEVGYQNLFDALLDSLYAAL
SGlu11      SSAS-SYIRPIVNFLARNGAPLLANVYPYFAYVNNQQ-SIGLDYALFTKH-----GNNEVGYQNLFDALLDSLYAAL
classII     GEFN-SFINPIIQFLVQHNLPLLANVYPYFGHIFNT-ADVPLSYALFTQQ-----EANPAGYQNLFDALLDSMYFAV
ClassIV     EEFK-SFINPIIEFLARNNLPLLANIYPYFGHIYNT-VDVPLSYALFNQQ-----GTNSTGYQNLFDALLDSIYFAV
SGlu7a      SSHA-HFLKPMLEFLAENQSPFMIDIYPYYAHRDSR-SKVSLDYALFDASSEVIDPNTGLLYTNMFDAQIDAIYFAL
SGlu7b      SSHA-HFLKPMLEFLAENQSPFMIDIYPYYAYRDSR-SKVSLDYALFDASSEVIDPNTGLLYTNMFDAQIDAIYFAL
classV      STIR-PFLLPFLQFLRHTSSPLMVNVYPFFAYINNP-QYVSLDHAVFRSS--YVEYDQNLAYDNMFDASIDAFVYAM
                 :   .:  :*     *:: :: .::.   .    : * ::::              * *:*** :*:   *:

                            Δ251
                         -  --
SGlu8       DNTGIGYVEVVVSEWGWPNS
SGlu1       DNTGIGYVEVVVSERGWPNS
SGlu4       DNTKIGYVEVVVSESGWPN-
SGlu12      DNTKIGYVEVVVSESGWPNS
classI      ERSGGASVGIVVSESGWPSA
SGlu2       EKAGGGSLNIVVSEWGWPNS
classIII    EKASGSSLEIVVSESGWPSA
SGlu3       EKVGASNLQIVVSESGWPNS
SGlu6       EKVGASQFADSGFEKRWPNS
SGlu10      EKVGASNLQIVVSEG-WPNS
SGlu5       EKVGAPNVKVVVSECGWPNS
SGlu11      EKVGAPNVKVVVSECGWPNS
classII     EKAGGQNVEIIVSESGWPSE
ClassIV     EKAGGPNVEIIVSESGWPSE
SGlu7a      MALDFRTIKVMVTECGWPNS
SGlu7b      MALNFRTIKVMVTECGWPNS
classV      EKEGFEGIPVMVTETGWPTA
                   .     *  **.
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Fig. 2 (continued)

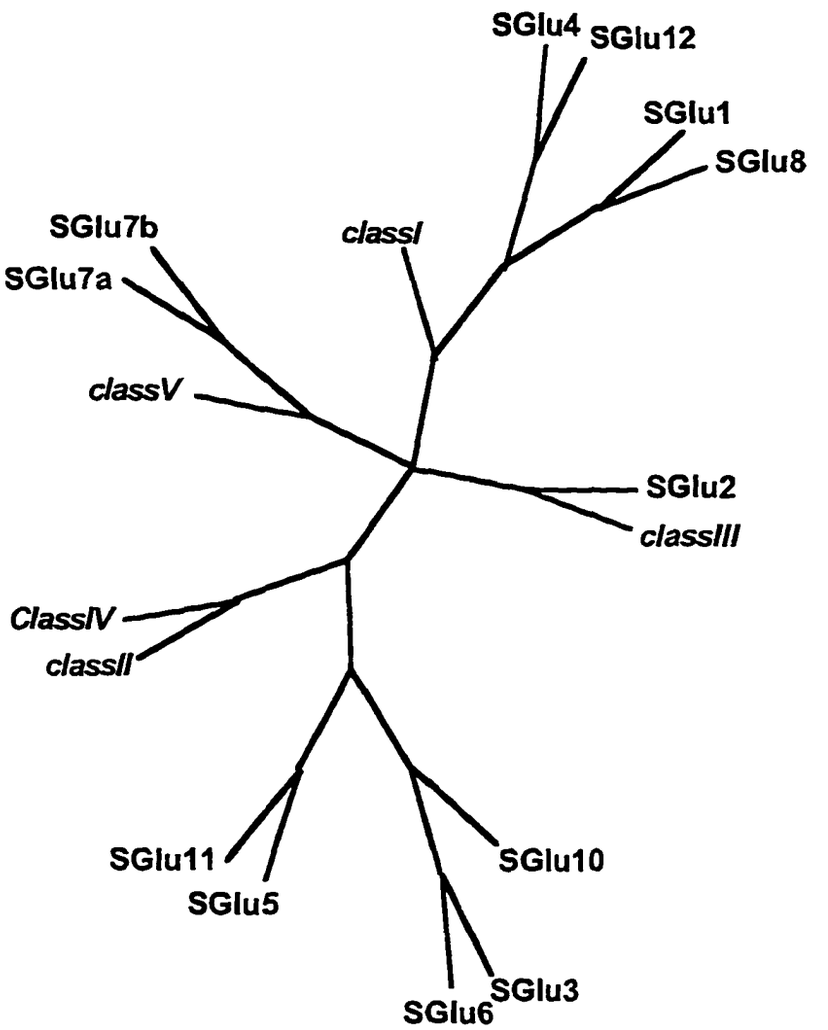

Fig. 3. Phylogenetic analysis of selected plant β-1,3-glucanases. Deduced amino acid sequences were aligned and an unrooted tree was established using parsimony analysis.

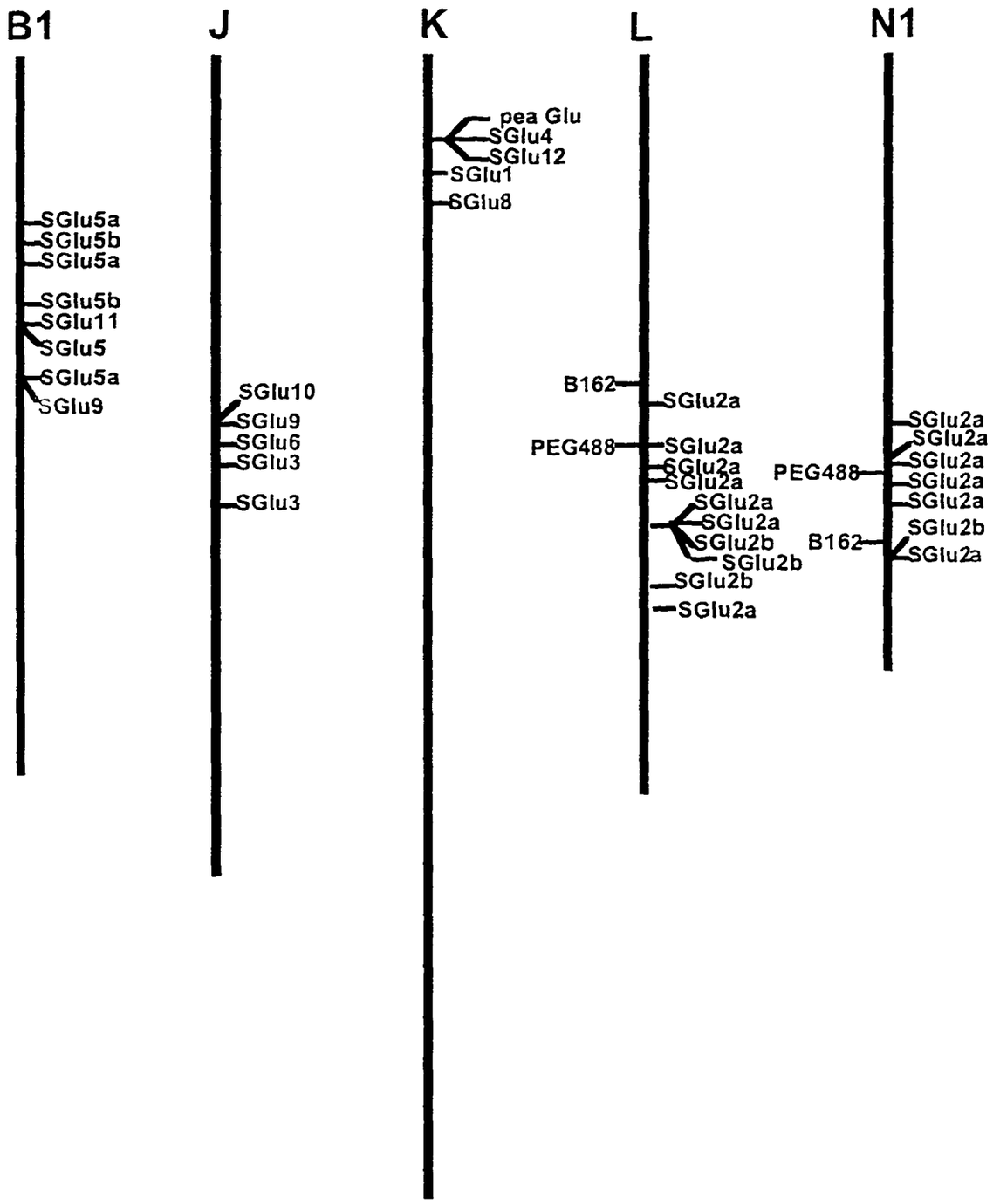

Fig. 4. Distribution of β-1,3-glucanases on the soybean genetic map. Loci are shown on the right of each linkage group. B162 and PEG488 are RFLP markers.

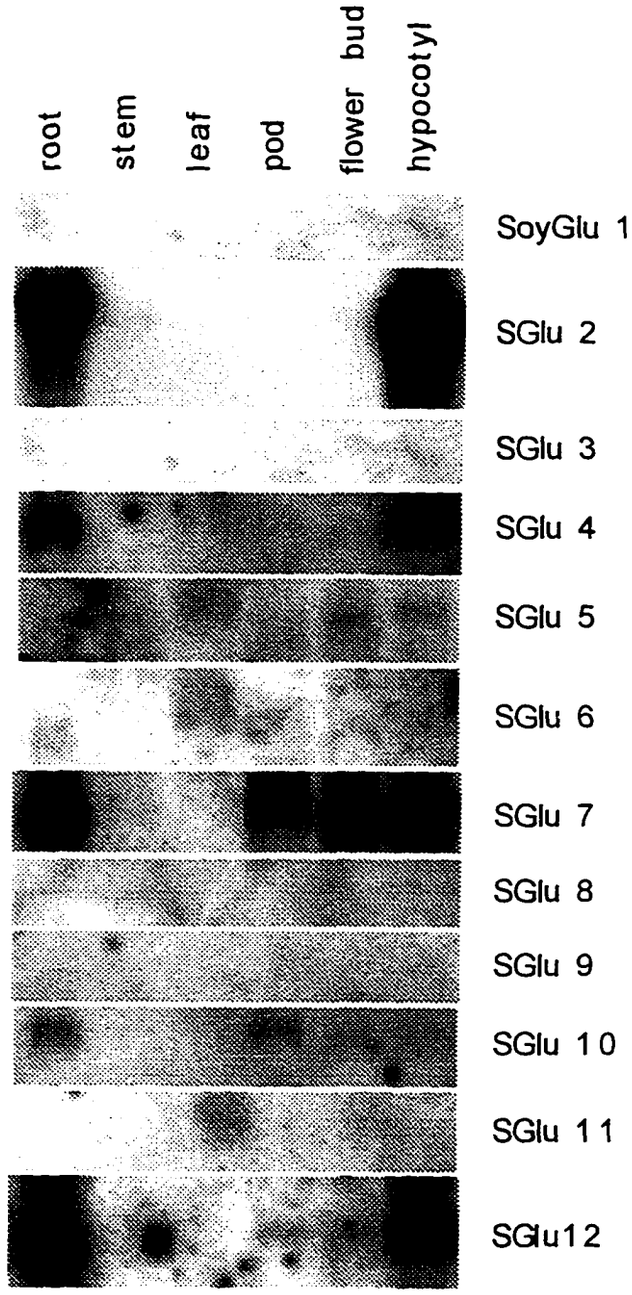

Fig. 5. Organ specific expression of 12 β-1,3-glucanase class transcripts. RNA gel blot hybridization analysis illustrates that β-1,3-glucanase classes mRNA accumulates in root, stem, leaf, pod, flower bud and hypocotyl. The mRNA transcripts of all β-1,3-glucanase genes are about 1.2 Kb.

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CHAPTER 5. GENETIC MAPPING OF THE *FR1* (ROOT FLUORESCENCE) LOCUS IN SOYBEAN [*GLYCINE MAX* (L.) MERR.] USING SSR MARKERS AND BULKED SEGREGANT ANALYSIS

A paper to be submitted to the journal Theoretical and Applied Genetics

W. Jin, R. G. Palmer, H. T. Horner, and R. C. Shoemaker

Abstract

We report the use of bulked segregant SSR analysis for rapid identification of DNA markers linked to the *Fr1* locus in soybean. Pooled DNA extracts from 10 homozygous *Fr1 Fr1* and 10 *fr1 fr1* F2 plants, derived from a msMOS X Minsoy cross, were analyzed using 65 SSR markers. Five SSRs produced repeatable polymorphisms between paired bulks. Linkage with the *Fr1* locus then was tested using these five SSR primers and DNA from individual plants of each bulk. DNA polymorphisms generated by these five primers were linked to the *Fr1* gene. Linkage of SSR loci with the *Fr1* locus was verified by using an F2 population segregating for *Fr1*. The five SSR markers and *Fr1* are on linkage group K of the USDA/ARS/ISU molecular genetic map. The markers flanking *Fr1* are Satt337 (11.0 cM) and Sat_044 (0.6 cM). *Fr1* previously was mapped on linkage group 12 of the

classical genetic map. Thus, classical genetic linkage group 12 has been correlated to linkage group K of the molecular genetic map. Six SSR markers were chosen on linkage group K to test the segregation ratios. All six SSRs consistently showed distorted segregation ratios, three of them were significantly skewed. This suggested that a gametophyte factor may reside in the region close to *Fr1* and most likely close to Satt046.

Introduction

Soybean root fluorescent mutants are important in characterizing germplasm diversity (Delannay and Palmer, 1982), in tissue culture (Roth et al., 1982), and in genetic linkage studies (Devine et al., 1993; Griffin et al., 1989; Palmer and Chen accepted). Five distinct loci controlling root fluorescence have been reported (Delannay and Palmer 1982; Sawada and Palmer 1987) and the genomic locations of several loci controlling root fluorescence have been defined. *Fr2* was placed on a molecular genetic map approximately 6.5 cM from pBLT 73 and 6 cM from pBLT 42 (Devine et al., 1993). *Fr1* and *Fr3* have been located on the classical genetic map. *Fr1* is 41 cM distant from the *Ep* (seed coat peroxidase level) locus on linkage group 12 (Griffin et al., 1989). *Fr3* is mapped on classical linkage group 9 (Palmer and Chen, accepted). The linkage relationships of the *Fr1*, *Fr3*, *Fr4*, and *Fr5* loci on molecular genetic maps are unknown.

A classical genetic map of soybean has been developed with great effort over many years (Palmer and Hedges, 1993). Integration of this map with the soybean

molecular map would be useful to help design breeding strategies for soybean improvement and to use the molecular map as a bridge to obtain a comprehensive map of important genes (Shoemaker and Specht, 1995). In soybean, diversity at the DNA sequence level is low (Keim et al., 1989). This has made the construction of a RFLP-based genetic map difficult and tedious.

Recently, simple sequence repeat (SSR) markers have been developed (Weber and May, 1989; Akkaya et al., 1995). Simple sequence repeats are composed of tandemly repeated 2 to 5 nucleotide DNA core sequences. The sequences flanking the SSR are conserved, thus allowing the selection of PCR primers useful for selectively amplifying the repeated sequence. Variation in the number of nucleotide repeats results in PCR product length differences (Tautz, 1989). Because SSRs are highly polymorphic, stable and simple, they have great potential for molecular mapping. The combination of SSR and bulked segregant analysis (Michelmore et al., 1991) will facilitate the identification of markers that are tightly linked to the gene of interest.

In the present study, we describe the application of bulked segregant analysis as a method for rapidly identifying SSR markers linked with the *Fr1* gene. Our goals were to place *Fr1* on the molecular genetic map and, thus, to associate classical linkage group 12 with a molecular linkage group.

Material and Methods

Plant material

msMOS (a male-sterile line obtained from Midwest Oilseeds, Adel, IA; *Fr1 Fr1*) as a female parent, was crossed with Minsoy (*fr1 fr1*) in Ames, Iowa, in the summer of 1995 to form an F2 population for testing linkage between SSR markers and the *Fr1* locus. The resulting F1 seeds were planted at the University of Puerto Rico Soybean Nursery, at the Isabela Substation, Isabela, Puerto Rico. F2 seeds were planted on germination paper for scoring root fluorescence. Fluorescence was observed by irradiating roots with ultraviolet light (Delannay and Palmer, 1982). After scoring, F2 seedlings were transfered to a growth chamber, leaf material was harvested, and DNA was isolated. F2 plants were single-plant threshed, and F2 seeds were planted in Puerto Rico to generate the F3 generation. F3 seeds were planted on germination paper for scoring root fluorescence to determine F2 genotypes (*Fr1 Fr1*, *Fr1 fr1*, *fr1 fr1*).

DNA extraction and bulk DNA preparation

DNA isolation was conducted as described by Keim et al. (1988). Equal quantities of DNA were combined from each of 10 homozygous F2 *Fr1 Fr1* plants and from each of 10 homozygous F2 *fr1 fr1* plants to obtain two DNA bulks.

SSR/BSA (Bulked Segregation Analyses)

For SSR/BSA analyses, PCR amplifications were carried out in a total volume of 20 μl containing 60 ng of soybean genomic DNA, 1.5 mM Mg^{2+}, 0.3 μM of sense and antisense primers, 200 μM of dNTPs, 1X PCR buffer, and 2.5 Units of Taq DNA

polymerase. Cycling consisted of 30 sec. at 94°C, 30 sec. at 47°C, and 30 sec. at 68°C for 40 cycles on a Perkin-Elmer 960 thermal cycler. PCR products were run on 2.5% Metaphor (FMC) agarose gel in TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002M EDTA) buffer with ethidium bromide incorporated into the gel. Initial screening was performed on samples of *Fr1 Fr1* (msMOS), *fr1 fr1* (Minsoy), pooled DNA from 10 *Fr1 Fr1* (pool *Fr1*) genotypes and pooled DNA from 10 *fr1 fr1* (pool *fr1*) genotypes.

Linkage analysis

The segregation ratios of *Fr1* and each molecular marker in the F2 population were tested for goodness of fit to a 1:2:1 genotypic ratio by Chi-square test. After finding that three SSRs tested showed skewed segregation ratios, six more SSR markers (Satt137, Satt247, Satt046, Sat_044, Satt326, Satt001) from linkage group K were chosen to investigate segregation ratios further. Three (Satt137, Satt046, Satt326) out of six markers showed polymorphisms and were analyzed further.

The Mapmaker program (Lander et al., 1987) was used to construct a linkage map by using F2 segregation data. A logarithm of odds ratio (LOD) score of 3 was used as the lower limit for accepting linkage between two markers. Distances between markers were calculated in centiMorgans derived with the Kosambi function (Kosambi, 1944).

Results

The root fluorescence test on the F3 progenies, derived from 80 F2 plants, yielded 14 homozygous *Fr1 Fr1*, 42 segregating and 24 homozygous *fr1 fr1* families (Table 1). This segregation fit the expected ratio of 1: 2 :1 ($\chi^2 = 2.7$). Ten homozygous *Fr1 Fr1* and 10 homozygous *fr1 fr1* F2 plants then were selected for preparation of the two DNA bulks. Sixty-five SSR primers were screened for polymorphisms against the two bulks and the parental lines, msMOS and Minsoy. Primers for loci that are polymorphic and linked to the gene detected clear differences between the bulks (Fig. 1). In contrast, unlinked loci appeared heterozygous with approximately equal band intensities in each bulk. SSR analyses of positive primers with the individual genotypes from the F2 population were performed. Polymorphic markers were screened against the 80 F2 DNAs including those used for bulk preparation. SSR markers segregated codominantly in the F2 population, and all six SSR markers that tested on linkage group K consistently gave distorted segregation ratios. Only those markers that are on linkage group K are shown in Table 1.

In total, 65 SSR markers distributed on the 23 linkage groups of the soybean molecular genetic map (Cregan et al., in review) were tested with DNA from *Fr1 Fr1* (msMOS) parent; *fr1 fr1* (Minsoy) parent, a pool of 10 *Fr1 Fr1* genotypes (pool *Fr1*), and a pool of 10 *fr1 fr1* genotypes (pool *fr1*) (Fig. 1). Of these, 4 (6%) did not produce any amplification product and were not analyzed further. The pool size of 10 genotypes for BSA analyses was determined on the basis of detection limit, desired interval of the genome to be covered (Giovannoni et al., 1991; Michelmore

et al., 1991), and our population size. Six of the remaining 65 SSRs detected polymorphisms between pooled *Fr1* and *fr1* DNAs. These polymorphisms were

Table 1. Segregation of the *Fr1* (root fluorescence) locus, and six SSR markers in an F2 soybean population from msMOS x Minsoy. Five SSRs (Satt055, Satt337, Satt046, Sat_044 and Satt326) linked to *Fr1*; Satt137 did not link to *Fr1* locus.

Trait or markers	No. of F2 plants	Observed No. AA-	HH-	BB-	X^2 (1:2:1)
Satt137	78	13	40	25	3.75
Satt055	80	12	42	26	4.10*
Satt337	80	12	41	27	5.67*
Satt046	79	10	41	28	8.30**
Fr1	80	14	42	24	2.70
Sat_044	80	13	43	24	3.70
Satt326	78	14	43	21	2.07

- genotypes: AA = msMOS; HH = heterozygous; BB = Minsoy

*, ** significant deviation from 1:2:1 ratio at 5% and 1% levels, respectively

repeated to verify reproducibility. Primers for these six SSRs were used to amplify DNA from each of the 80 F2 progeny. All six markers tested showed skewed segregation ratios, three of them were statistically significant skewed (Table 1). In all cases fewer msMOS gametes were transmitted than expected. Because three SSRs showed statistically significant distorted segregation ratios, these three loci have an inconsistant sequence with soybean genetic map. But the segregation ratios for two markers flanking *Fr1* locus were Sat_044 and Satt337 were not

significant skewed. The sequence of the two loci agreed with soybean molecular genetic map. The two markers flanking *Fr1* locus were Sat_044 and Satt337. The fragment amplified by Sat_044 in the *Fr1 Fr1* bulk and *Fr1 Fr1* parent, was present in all 14 *Fr1* plants and was not present in any *fr1* individuals. The fragment amplified in the *fr1* bulk and *fr1* parent was present in all 24 homozygous *fr1 fr1* plants. For Satt337, the fragment amplified in the *Fr1 Fr1* bulk and the *Fr1 Fr1* parent was present in 13 of 14 plants scored as *Fr1 Fr1* and was present in three of 24 plants scored as *fr1 fr1*. The fragment amplified in the *fr1 fr1* bulk and the *fr1 fr1* parent was present in 23 of 24 plants scored as *fr1 fr1*. Segregation analysis with 80 F2 progenies determined that these two SSRs (Sat_044 and Satt337) were linked to the *Fr1* locus (Fig. 2) with LOD scores of 30 and 16, respectively. The closest linkage (0.6 cM) was found with SSR marker Sat_044. The SSR profiles of some of the individuals amplified with Sat_044 primers are shown in Fig. 3. The two SSR markers flanking the *Fr1* locus are on linkage group K in USDA/ARS/ISU map (Cregan et al., in review).

Discussion

Mapping genes with molecular markers can be laborious and costly. One of the most time-consuming aspects of mapping, the screening of the entire mapping population with every probe or primer to be tested, can be eliminated by the application of bulked segregant analysis (Michelmore et al., 1991). Bulked segregant analysis simultaneously provides information on polymorphisms between

the parents and possible linkage between a marker and a targeted gene. This process can reduce cost by several-fold, particularly when used with PCR-based techniques such as SSR and RAPD. Bulked segregant analysis combined with RAPD also has been used to detect markers linked to many traits including disease resistance genes (Mouzeyar et al., 1995; Yaghoobi et al., 1995), a male-sterile gene in rice (Zhang et al., 1994), and a fertility restoration gene in rapeseed (Delourme et al., 1994). To our knowledge, this is the first report on using bulked segregant analysis with SSR for molecular mapping in plants. SSR/BSA efficiently identified markers linked to the gene of interest, and allowed their rapid placement on a genetic map.

In this study, the combination of SSR markers and bulked segregant analysis proved to be faster and less expensive than using RFLPs to find markers flanking the *Fr1* locus. We tested 61 SSR polymorphic markers with pools of DNA from 10 *Fr1 Fr1* and 10 *fr1 fr1* genotypes and found that the *Fr1* locus mapped to linkage group K of the USDA/ARS/ISU map, and was flanked by two SSR markers, Satt337 and Sat_044. This identification is a contribution toward the construction of a comprehensive genetic map of soybean.

Segregation distortion has been reported in wide crosses of rice (Nakagahra, 1986, Maekawa et al., 1981; Maekawa and Kita, 1985; Lin et al., 1992), maize (Rashid and Peterson, 1992), and soybean (Honeycutt et al., 1988, Honeycutt et al., 1990). Many instances of segregation distortion have been reported through studies of isozymes (Ishikawa et al., 1987a, b; Wu et al., 1988) and RFLP alleles

(McCouch et al., 1988; Saito et al., 1991). The mechanisms of segregation distortion are not well understood. The segregation ratio may be affected by gametophytic factor or the abortion of male or female gemetes. Studies by Nakagahra (1972, 1986) localized gametophyte gene loci on chromosome 3 based upon a clear locus that was responsible for the partial or total elimination of gametes carrying one of the alleles at that locus.

There were consistently skewed segregations within an interval on linkage group K (Table 1). Based on Chi-square analysis of SSR segregation data, Chi-square values peak in the interval between Satt046 and Satt337 (Fig. 4). This suggests the presence of a gametophytic factor (Gonella and Peterson, 1975; Rashid and Peterson, 1992) in the interval affecting transmission of the msMOS gametes. The tighter the linkage between a marker locus and the gametophytic factor gene, the more extream the segregation distortion is expected.

Development of the classical genetic map has proceeded slowly in soybean because of the difficulty in performing crosses and generating large numbers of hybrid seed, the lack of detailed cytogenetic markers, and low genetic variation in the germplasm (Keim et al., 1989). To exploit fully the potential of a molecular genetic map, it is essential to integrate molecular and conventional markers into one linkage map. Integration of the maps can also be pursued by screening near-isogenic lines (NIL) (Young et al., 1988). According to Shoemaker and Specht (1995) about half of the 19 soybean classic linkage groups have been associated with their corresponding molecular genetic linkage groups. But classical linkage

group 12 had not been associated with any of the molecular genetic linkage groups. *Fr1* is known to reside on classical linkage group 12 (Griffin et al., 1989). Our results have shown that the *Fr1* locus is located on molecular genetic linkage group K. Therefore, classical linkage group 12 is now integrated into molecular genetic linkage group K.

Acknowledgements

We gratefully acknowledge Drs. David Grant and Arla Bush for helpful discussions during the course of this project, and Drs. David Grant, John Imsande, and Joane Labate for critically reviewing the manuscript.

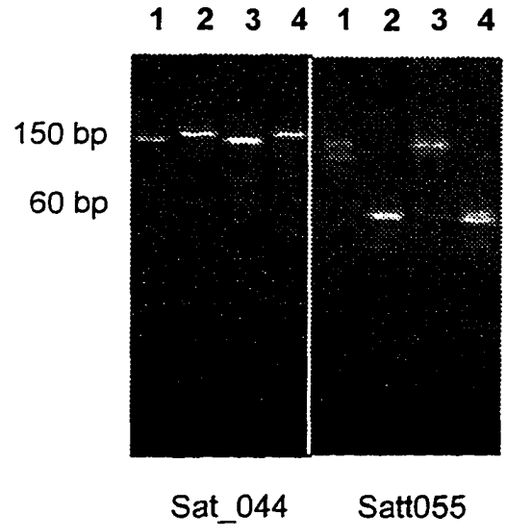

Fig. 1. SSR markers detecting polymorphisms between DNA bulks of genotypes *Fr1 Fr1* and *fr1 fr1*. The first two lanes contain parental DNA from msMOS (*Fr1 Fr1*) and Minsoy (*fr1 fr1*). The third lane contains bulked DNA from homozygous *Fr1 Fr1* individuals, and the fourth lane contains bulked DNA from homozygous *fr1 fr1* individuals.

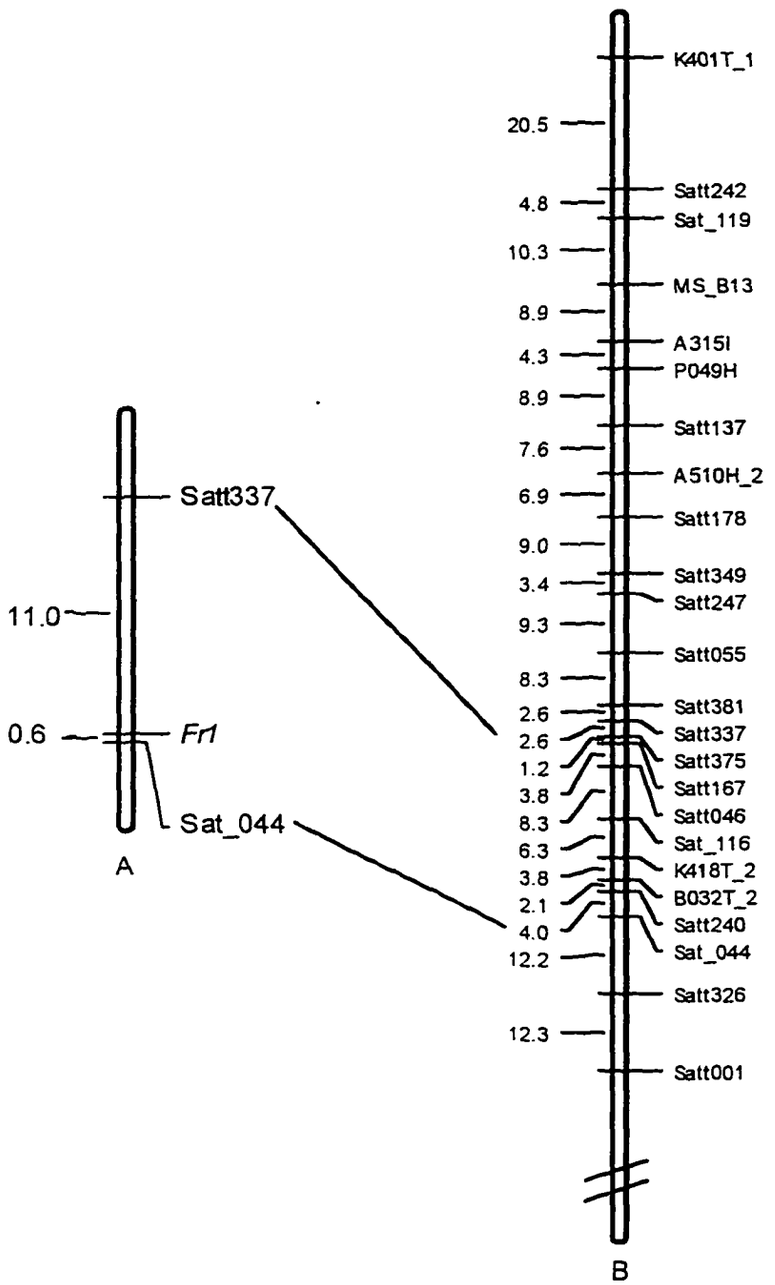

Fig. 2. Position of the *Fr1* locus on molecular genetic linkage group of soybean. A. A part of the soybean genetic group K showing the linkage map around the *Fr1* locus obtained from analysis of 80 F2 plants derived from the cross between msMOS (*Fr1 Fr1*) and Minsoy (*fr1 fr1*). B. A linkage map of linkage group K constructed from the *Glycine max* x *G. soja* population (Shoemaker et al., 1997). Linkage group K extends beyond hash marks.

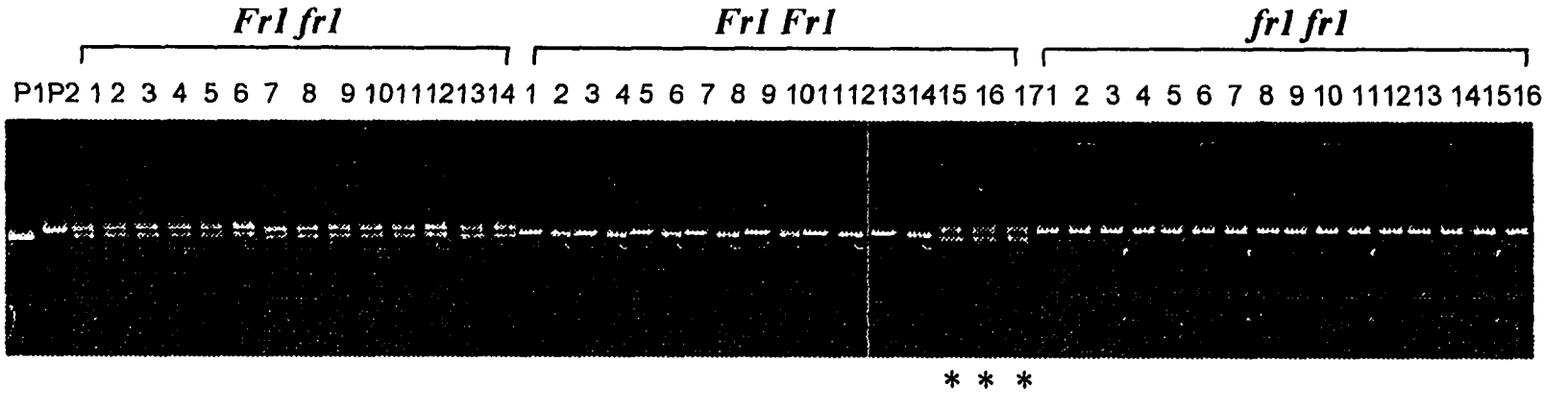

Fig. 3. SSR profiles of some of the F2 individual genotypes of the pools with Sat_044, * indicates recombinants. P1 = parent1 (msMOS), P2 = parent2 (Minsoy).

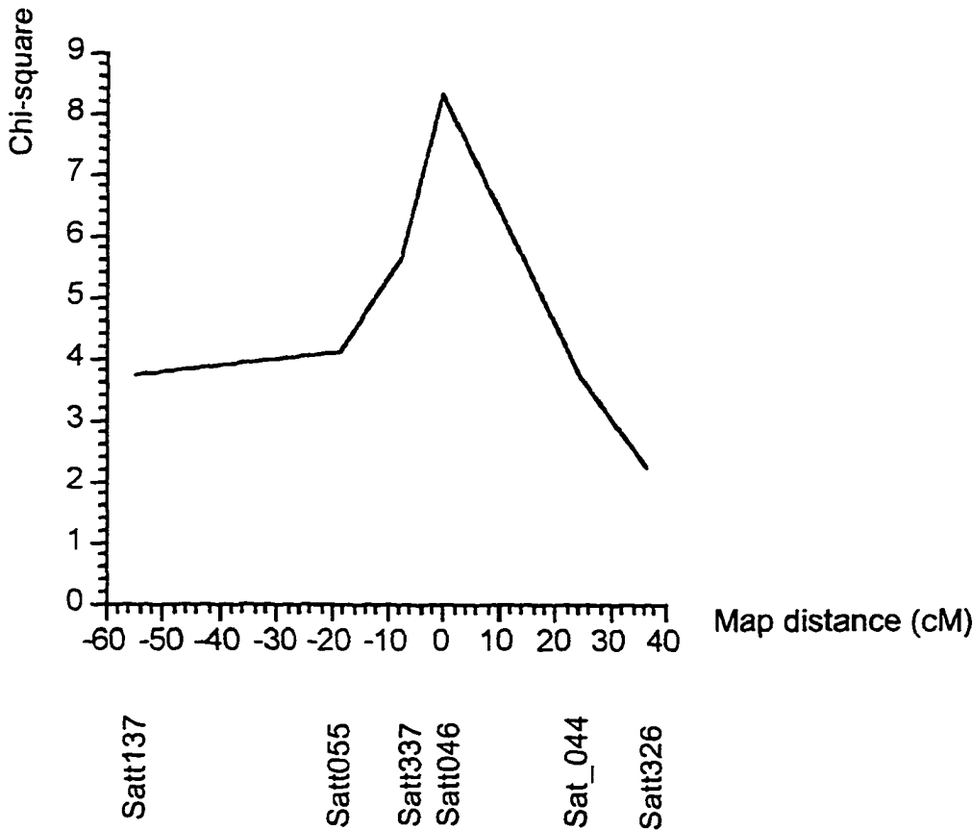

Fig. 4. Effect of map distance (cM) from Satt046 locus on segregation ratios (chi-square)

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CHAPTER 6. SUMMARY AND CONCLUSIONS

I have demonstrated that novel mutant is a nuclear male sterile and is inherited as a single-recessive gene. No differences in female or male gamete transmission of the recessive allele were observed between reciprocal cross-pollinations in the F1 or F2 generations. This mutant is not allelic to any previously identified soybean genic male-sterile mutants: *ms1, ms2, ms3, ms4, ms5*, or *ms6*. No linkage was detected between the male-sterile gene and flower color (*W1 locus*), or between the male-sterile gene and pubescence color (*T1 locus*).

Developmental studies and enzyme extraction analyses showed that there was no β-1,3-glucanase (callase) activity in male-sterile anthers, and this suggests that the biological mechanism of male sterility is the retention of callose walls, which normally are degraded around tetrads at the late tetrad stage. The tapetum from male-sterile anthers also showed abnormalities at the tetrad and later stages, which were expressed by an unusual formation of vacuoles, and by accumulation of densely staining material.

In the second manuscript of this dissertation, the male-sterile gene was mapped on linkage group D1b of the USDA/ARS/ISU soybean molecular genetic map. An F2 population of 111 individuals constructed from a cross between the mutant msMOS (ms ms) and the cultivar Minsoy (MS MS) was used for the mapping project. Two hundred and seventy probes, including 219 RFLP and 51 SSRs were evaluated. Of these, 102 RFLPs and 31 SSR probes detected polymorphisms. The

F2 population was screened for segregation of these polymorphic molecular markers. Analyses revealed that the male-sterile locus was flanked by Satt157 and Satt412 on linkage group D1b. This is a contribution toward the construction of a comprehensive genetic map of soybean. The availability of linked DNA markers will facilitate the genetic analysis of this male-sterile gene in relation to soybean breeding programs, and will be a starting point for the isolation of the ms gene by map-based cloning.

In the third manuscript of this dissertation, 12 classes of β-1,3-glucanase genes were cloned, analyzed, and mapped. Oligonucleotide primers designed for conserved sequences from the coding region of β-1,3-glucanase genes from a number of different species were used to amplify related β-1,3-glucanase genes from soybean [*Glycine max* (L.) Merr.]. Sequencing and cross hybridization of amplified products indicated that at least 12 classes of β-1,3-glucanase genes exist in soybean. Northern blot analyses performed on total RNA from root, leaf, flower bud, stem, and hypocotyl, using different classes of β-1,3-glucanases as probes, indicated organ specific mRNA accumulation patterns. Members of these classes were mapped to 34 loci on 5 different linkage groups using an F2 population of 56 individuals previously characterized. We found that β-1,3-glucanase genes are broadly distributed in the genome, some of them are clustered within multiple loci. Two pathways seem to be involved in the evolution of the gene families: duplications of large chromosomal domains followed by extensive gene duplication and divergence; and retrotransposition and duplication of individual β-1,3-glucanase

genes. These features suggest that the expression patterns of β-1,3-glucanase genes may be correlated with their levels of sequence divergence and their chromosomal locations. Phylogenetic analysis and expression studies indicated that SoyGlu7 may be the candidate gene involved in male sterility in the mutant.

In the fourth manuscript of this dissertation, the *Fr1* locus was mapped by bulked segregant analysis using the same F2 mapping population as the ms mapping. The reason we mapped this gene was mainly as follows: it segregated in the F2 population constructed for ms mapping; and soybean root fluorescence is an very important marker in genetic linkage studies and in the characterization of germplasm diversity. Pooled DNA extracts from 10 homozygous *Fr1 Fr1* and 10 *fr1 fr1* F2 plants, derived from a msMOS (*Fr1 Fr1*) x Minsoy (*fr1 fr1*) cross, were analyzed using 61 SSR markers. Five SSRs produced repeatable polymorphisms between paired bulks. Linkage with the *Fr1* locus then was tested using these five SSR primers and DNA from 80 individual F2 plants. Linkage of SSR loci with the *Fr1* locus was verified by using the F2 plants. The five SSR markers and *Fr1* are on linkage group K of the USDA/ARS/ISU molecular genetic map, markers flanking *Fr1* are Satt337 (11 cM) and Sat_044 (0.6). More importantly, *Fr1* was previously mapped on linkage group 12 of the classical genetic map, thus, classical genetic linkage group 12 has been correlated to linkage group K of the molecular genetic map.

APPENDIX

Application for

United States Letters Patent

of

Wei Jin

Harry T. Horner

Reid G. Palmer

Randy C. Shoemaker

for

Soybean Glucanases,

Compounds Which Encode Therefor and Related Methods

Soybean Glucanases,

Compounds Which Encode Therefor and Related Methods

BACKGROUND OF THE INVENTION

The present inventors recently characterized a genic male-sterile mutant of soybean: 83(6) *Am. J. Bot.* 42, abstract 121 (1996); Jin et al., 10 *Sex Plant Reprod.* 13 (1997). Microscopic analysis of the mutant showed the callose encasement of the microspores was retained at an inappropriate developmental stage. The persistent callose encasement resulted in no release.and subsequent degeneration of the microspores. Other results in these publications showed that callase, the enzyme which is responsible for digestion of the callose wall, was inactive. The reason for the inactivity was not elucidated at that time.

Other publications by the present inventors identified the callase as a ß-1,3-glucanase. Abstract P14. *Genetics Society of Canada* (June 1997); 114(3) *Plant Physiology* 172, abstract 842 (June 1997); 84(6) *Am. J. Bot.* 63, abstract 182 (June 1997).

Soybean ß-1,3 glucanases have been cloned in the past. For example, US Patent Number 5,477,001 discloses cloned ß-1,3- glucanases from soybean. The gene products are implicated in disease resistance. No disclosure of the involvement of ß-1,3- glucanases as a callase is described in the patent. Non-soybean glucanases, such as those described in US Patent 5,554,743 describe ß-1,3-glucanases involved in cell wall polysaccharide degradation.

A search of the National Institutes of Health BLAST database disclosed a line of soybean "*Glycine max* cv. Century 84" with cloned ß-1,3-glucanases. None of the sequences found were identical to the presently-claimed sequences. The following is a table of the results of the search:

SEQ ID NO	CLOSEST HOMOLOGY	REFERENCE
1	85% (soybean glucanase)	US Patent 5477001, Seq 8: same as WO9413790, Seq 15
2	98% (soybean endoglucanase)	none available
3	91% (Century 84)	26 *Crop Science* 199 (1986)
4	97% (soybean glucanase)	US Patent 5477001, Seq 8: same as WO9413790, Seq 15
5	76% (Century 84)	26 *Crop Science* 199 (1986)
6	91% (Century 84)	26 *Crop Science* 199 (1986)
7	54% (unknown)	US Patent 5614395, Seq 13; same as WO9413790, Seq 15
8	85% (soybean glucanase)	US Patent 5477001, Seq Id 8: same as WO9413790, Seq 15
9	75% (Century 84)	26 *Crop Science* 199 (1986)
10	97% (Century 84)	26 *Crop Science* 199 (1986)
11	76% (Century 84)	26 *Crop Science* 199 (1986)
12	96% (soybean glucanase)	US Patent 5477001, Seq Id 8: same as WO9413790, Seq 15

The 5,477,001 patent does not disclose or suggest the present invention, because the sequences described herein are not disclosed. Moreover, the emphasis in the 5,477,001 patent is the use of a particular soybean glucanase to resist plant diseases. In certain aspects of the present invention, the focus is on the elimination of active soybean glucanases; the 5,477,001 patent therefore teaches away from those aspects of the present invention.

Century 84 is a line of soybean which was released jointly by the Ohio Agricultural Research and Development Center, The Ohio State University and the USDA which contained over-expressed glucanase for the purpose of disease resistance. Registration Number 188, 26 *Crop Science* 199 (1989).

Citation of the above documents is not intended as an admission that any of the foregoing is pertinent prior art. For example, in some instances above, the publication was less than one year before the filing date of this patent application. All statements as to the date or representation as to the contents of these documents is based on subjective characterization of information available to the applicant at the time of filing, and does not constitute an admission as to the accuracy of the dates or contents of these documents.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide sequences useful to engineer male-sterile soybean lines.

It is a further object to provide methods to engineer male-sterile soybean lines.

It is yet another object to provide male-sterile soybean seeds.

It is yet another object to provide male-sterile soybean plants.

In all of the above embodiments, it is an object to provide seed mixes with male-sterile and female-fertile seeds.

It is also an object of the invention to provide materials such as vectors for genetic engineering male-sterile soybean lines.

It is an additional object to provide a method to improve seed germination using the materials herein disclosed.

Lastly, it is therefore an object of the present invention to provide sequences useful to engineer disease-resistant soybean lines.

Definitions:

For the purposes of the present application, the following terms have the following meanings. All other terms have the meaning as generally recognized in the art.

"Knockout construct" means a DNA sequence which has been altered via any known means, for example, deletion, insertion, point mutation or rearrangement, so as to alter the function of the naturally-occurring gene product, but not so as to alter the ability of the DNA sequence to recombine with the naturally-occurring sequence.

"Knockout mutants" are cells, embryos or plants in which a naturally-occurring ß-1,3-glucanase gene has been replaced through genetic engineering with a knockout construct, so as to result in a male-sterile phenotype.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides twelve different sequences for soybean ß-1.3 glucanases and the proteins for which they encode. The DNA sequences are as follows:

SEQ ID NO	SEQUENCE
1	CGGGATCCGGGGTGTGTTACGGCATGATGGGCGACAATCTACCACCGGCAAATGAAGTTGTAAGTCTTTACAAA TCCAACGACATAATGAGAATGAGAATCTATAATCCTGATCAAGCTGCTTTACAAGCACTGGGAAATTCGGGCAT TGAGCTTATTCTTGGGGTGCTCCACCAAGACCTTCAAGGCCTTGCCACCAATGCTAGCACTGCTCAACAATGGG TGCAAAGTAACGTGTTGAACTTTTGGCCTAGTGTCAAAATCAAGCACGTGGTAGTTGGCAACGAAATCAATCCT GTGGGAAGCTCTTCTGAGTTTGCCCAATATGTTCTACCTGCAATCCAAAACATATACCAAGCTATAAGAGCTCA AGGCCTTCAAGATCTAATCAAGGTTACAACAGCTATTGACATGACCCTGTTAGGAAACTCCTACCCCCCATCAC AAAGCTACTTCAGGACTGATGTGAGATCATACTTAGACCCCATAATTGGGTACTTGGTATATGCAAATGCACCT TTACTAGCCAATGTGTTGCCTTATTTTAGTTACTCCAATAACCCGATTGACATATCACTTTCCTATGCTCTTTT TAACTCAACAAATGTTGTGGTTTGGGATGGTCAATATGGGTACCAAAATTTGTTTGATGCTATGTTGGATGCGG TGCATGTTGCAATTGATAACACAGGGATTGGTTATGTGGAGGTTGTTGTATCCGAGAGAGGTTGGCCGAATTCC G
2	CGGGATCCGGCGTGTGTTATGGAAGACTTGGCAACAACTTACCAACCCCTCAAGAAGTTGTGGCCCTCTACAAT CAAGCCAACATTCGCAGGATGCGAATCTACGGTCCAAGCCCAGAAGTCCTCGAAGCACTAAGAGGTTCCAACAT TGAGCTTTGCTAGACATTCCAAATGACAACCTCAGAAACCTAGCATCTAGCCAAGACAATGCAAACAAATGGG TGCAAGACAACATCAAAAACTATGCCAACAATGTCAGATTCAGATACGTTTCAGTGGGAAATGAAGTGAAACCC GAACACTCATTTGCACAATTTCTAGTGCCTGCATTGGAAAACATTCAGAGGGCCATTTCTAATGCTGGCCTTGG AAACCAAGTAAAAGTTTCCACTGCCATTGATACTGGTGCCTTGGCAGAATCATTCCCACCATCAAAGGGTTCCT TCAAATCTGATTATAGAGGAGCATATCTTGATGGTGTCATCAGATTTCTAGTGAACAATAATGCCCCATTAAT GGTTAATGTGTACTCTTACTTCGCTTACACTGCAAACCCTAAGGACATTAGTCTTGACTATGCACTTTTTAGGT CTCCTTCGGTGGTAGTGCAAGATGGTTCACTTGGTTACCGTAACCTCTTTGATGCTTCGGTTGATGCTGTTTAT GCTGCATTGGAGAAAGCAGGAGGAGGGTCATTGAACATAGTTGTGTCTGAGTGAGGATGGCCGAATTCCG

3	CGGGATCCGGAGTTTGCTATGGAGTACTCGGTAATAATCTACCATCAAGGCAAGAAGTTGTGGACTTGTATAAA ACAAATGGGATAGGTAGAATGCGTATATACTATCCAGATGAAGAAGCGCTCCAAGCCCTTAGAGGTTCAGGCAT TGAGTTGATTATGGACGTGGCTAAGGAAACCCTTCAATCAATGACAGACCCCAATGCTGCTACAGATTGGGTCA ATAAGTATGTTACAGCCTACTCGCAAGACGTCAATTTCAAGTACATCGCTGTTGGAAATGAAATTCACCCCAAT ACCAATGAGGCACAGTACATTCTATCTGCCATGACCAACATTCAGAATGCAATTTCATCAGCCAATTTACAAAT CAAGGTGTCAACAGCAATAGACTCTACTTTCATTGCTCCGCCCTCCTATCCACCCAATGATGCTGTTTTCACTA GCGATGCAGAGCCATATGTAAAACCCATAATAGACTTCCTAGTGAGAAATGAGGCGCCACTTCTTGCCAATGTG TACCCTTACTTTGCTTATGCGAATGATCAACAAAACAGTATTCCTCTTGCCTATGCTCTTTTTACCCAACAAGG AAACAACGACGCTGGGTACCAAAACCTCTTCGATGCTATGTTGGATTCAATATACGCTGCAGTGGAGAAAGTGG GAGCATCCAATTTGCAGATAGTGGTTTCTGAATCTGGTTGGCCGAATTCCG
4	CGGGATCCGG GGTATGTTAT GGCATGCTGG GCAACAATCT ACCATCAGCA AACGAAGTTA TAGGTCTTTA TAGATCAAAT AACATAAGGA GAATGAGACT CTATGATCCT AATCAAGCTG CTCTAGAAGC ACTTAGAAAT TCTGGCATTG AACTCATTCT TGGGGTGCCA AACTCTGACC TTCAAGGCCT TGCCACCAAT CCTGACACTT CTCGTCAATG GGTGCAAAAA AACGTGTTGA ACTTTTGGCC TAGTGTCAAA ATCAAGTACG TGGCAGTTGG AAATGAACTG AGTCCCGTTG GACGCTCTTC TTCGGTAGCC CAATATGTTC TACCTGCCAT CCAAAATGTA TACCAAGCAA TAAGAGCTCA AGGCCTTCAT GATCAAATCA AGGTTTCAAC ATCTATTGAC ATGACCCTAA TAGGAAACTC TTTCCCTCCA CCGCAAGGTT CCTTCAGGGG TGATGTGTGA TCATACCTAG ATCCCATAAT TGGGTACTTG GTATATGCAA ATGCACCATT ACTAGTCAAT GTGTACCCTT ATTTTAGTTA CACTGGTAAC CCCCGTGACA TATCACTTCC CTATGCTCTT TTCACAGCAC CAAATGTTGT GGTATGGGAT GGTCAATATG GGTACCAAAA TTTGTTTGAT GCTATGTTGG ATTCAGTACA TGCAGCCATT GATAACACTA AGATTGGTTA TGTGGAGGTT GTTGTATCCG AAAGCGGATG GA
5	CGGGCATCCGGTGTCTGTTACGGAGGAAATGGAAACAATCTACCAACAAAGCAAGCAGTGGTGGATCTTTACAA ATCAAACAGAATAGGCAAAATCCGTTTATACTATCCAGACGAAGGAGTCCTTCAAGCCCTCAGAGGTTCAAACA TAGAGGTGATCCTCGGTGTCCCTAATGACCAACTTCAATCTCTCACCAACGCTGGAGCTGCCACAAATTGGGTC AACAAGTACGTGAAAGCATACTCACAAAACGTGAAATTCAAGTACATTGCAGTTGGTAACGAAATTCACCCTGG TGACTCTTTAGCAGGGTCTGTACTTCCAGCACTTGAAACCATTCAGAAAGCAATTTCTGCCGCCAATTTACAAG GCCAAATGAAGGTGTCAACAGCAATAGACACCACTTTACTTGGCAACTCTTACCCACCAAAAGATGGCGTTTTC AGCAGTAGTGCAAGTTCATACATAAGACCAATTGTAAACTTTTTAGCAAGAAATGGAGCCCCACTTCTCGCAAA CGTGTACCCTTACTTCGCCTATGTTAACAACCAACAAAGCACTGGTCTTGACTATGCCTTGTTTACTAAACATG GTAACAACGAGGTTGGGTACCAAAACCTGTTTGATGCAT TGTTGGATTCTCTATACGCTGCTCTTGAGAAAGTAGGGGCACCAAATGTGAAGGTTGTTGTGTCTGAAAGAGGT TGGCCGAATTCCG

6	CGGGATCCGGGAGTATGTTACGGGAGTACTCGGTAATAATCTACCATCAAGGCAAGAAGTTGTGGACTTGTATA AAACAAATGGGATAGGTAGAATGCGTATATACTATCCAGATGAAGAAGCACTCCAAGCCCTTAGAGGTTCAGGC ATTGAGTTGATTATGGACGTGGCTAAGGAAACCCTTCAGTCAATGACAGACCCCAATGCTGCTACAGATTGGGT CAATAAGTATGTTACAGCCTACTCGCAAGACGTCAATTTCAAGTACATCGCTGTTGGAAATGAAATTCACCCCA ATACCAATGAGGCACAGTACATTCTATCTGCCATGACCAACATTCAGAATGCAATTTCATCAGCCAATTTACAA ATCAAGGTGTCAACAGCAATAGACTCTACTTTCATTGCTCCGCCCTCCTATCCACCCAATGATGCTGTTTTCAC TAGCGATGCAGAGCCATATGTAAAACCCATAATAGACTTCCTAGTGAGAAATGAGGCGCCACTTCTTGCCAA TGTGTACCCTTACTTTGCTTATGCGAATGATCAACAAAACAGTATTCCTCTTGCCTATGCTCTTTATACCCAAC AAGGAAACAACGACGCTGGGTACCAAAACCTCTTCGATGCTATGTTGGATTCAATATACGCTGCAGTGGAGAAA GTGGGAGCATCCCAATTTGCAGATAGTGGTTTCTGAAAGAGATGGCCGAATTCCG
7	CGGGATCCGAGTTTGTTATGGAAGAAGTGCTGATGACCTCCCTACACCTGACAAGGTGGCACAGTTGGTTCAAC TTCATAAAATCAAATATGTCAGGATTTATGATTCTAATATACAGGTTCTGAAGGCCTTTGCAAACACTGGAATT GAGCTTATGATTGGGGTTCCAAATTCGGACTTGCTTTCATTCTCTCAGTTCCAATCTAATGCAGACTCTTGGCT GAAAAACAGCGTGCTTCCTTACTATCCGGCTACAAAGATCGCATACATCACTGTCGGCGCCGAAGTCACTGAGA GTCCTAACAATGCATCTTCATTTGTAGTGCCTGCCATGACCAATGTGCTTACAGCACTCAAGAAACTTGGGCTG CACAAAAAGATTAAAGTTTCCAGCACCCATTCCCTTGGGGTTTTGTCGCGATCCTTCCCGCCTTCTGCTGGGGC TTTCAATAGCAGCCATGCACATTTCCTGAAGCCAATGCTAGAATTTCTTGCTGAAAATCAGTCACCTTTTATGA TTGATATATATCCTTATTATGCCCACCGTGATTCCCGGAGTAAAGTGTCTTTAGACTATGCCCTGTTTGATGCA TCCTCTGAAGTAATTGATCCAAACACAGGCTTGCTGTACACAAACATGTTTGATGCCCAGATTGATGCTATTTA CTTTGCACTGATGGCCTTGGACTTCAGAACAATTAAGGTCATGGTCACTGAGTGCGGATGGCCGAATTCCG
8	CGGGATCCGGGGTGTGTTACGGCATGATGGGCGACAATCTACCACCGGCAAATGAAGTTGTAAGTCTTTACAAA TCCAACGACATAATGAGAATGAGAATCTATAATCCTGATCAAGCTGCTTTACAAGCACTGGGAATTTCGGGCAT TGAGCTTATTCTTGGGGTGCTCCACCAAGACCTTCAAGGCCTTGCCACCAATGCTAGCACTGCTCAACAATGGG TGCAAAGTAACGTGTTGAACTTTTGGCCTAGTGTCAAAATCAAGCACGTGGTAGTTGGCAACGAAATCAATCCT GTGGGAAGCTCTTCTGAGTTTGCCCAATATGTTCTACCTGCAATCCAAAACATATACCAAGCTATAAGAGCTCA AGGCCTTCAAGATCTAATCAAGGTTACAACAGCTATTGACATGACCCTGTTAGGAAACTCCTACCCCCCATCAC AAAGCTACTTCAGGACTGATGTGAGATCATACTTAGACCCCATAATTGGGTACTTGGTATATGCAAATGCACCT TTACTAGCCAATGTGTTGCCTTATTTTAGTTACTCCGATAACCCGATTGACATATCACTTTCCTATGCTCTTTT TAACTCAACAAATGTTGTGGTTTGGGATGGTCAATATGGGTACCAAAATTTGTTTGATGCTATGTTGGATGCGG TGCATGTTGCAATTGATAACACAGGGATTGGTTATGTGGAGGTTGTTGTATCCGAATGGGGTTGGCCGAATTCC G

9	CGGGATCCGGGGTTTGTTACGGAGGAAAAGGAAACAACCTACCAAAAATGCAAGCAGTGGTGGATTTATACAAA TCAAACCGAATTGACAAAATCCGTTTATACCATCCAGACGAAGGAGCCCTTCAAGCCCTCAGAGGTTCAAACAT AGAGGTGGTCCTCGGTGTCCCTAATGACCAACTTCAATCTCTCATCAATGTTGCAAATGCCACAAATTGGGTCA ACAAGTACGTGAAAGCATACTCACAAAACGTGAAATTCAAGTACATTGCAGTCGGTAACGAATTCTTTAGCAGG GTCTGTACTTCCAGCACTTGAAAACATTCAGAACGCAACTTCTGCCGCCAATTTACAAGGCCAAATGAAGGTGT CAACAGCAATAGACACCACTTTACTTGGCAACTCTTACCCACCAAAAGATGGCGTTTTCAGCAGTAGTGCAAGT TCATACATAAGACCAATTGTAAACTTTTGAGCTAGAAATGGAGCTCCACTTCTCGCAAACGTGTACCCTTACTT CGCCTATGTTAACGACCAACAAAGCATTAGTCTCGACTATGCCTTGTTTACTGAACATGGTAACAACGAGGCTG GGTACCAAAACCTGTTTGATGCATTGTTGGATTCTCTATACGCTGCTCTTGAGAAAGTAGGGGCACCCAATGTG ACGGTTGTTGTGTCTGAAACGGGCTGGCCG
10	CGGGATCCGGTGTTTGTTATGGAGTGATTGGTGATAATCTACCATCAAGGCAAGAAGTTGTGGACTTATATAAA ACAAATGGCATTGGTAGAATGCGTATATACTACCCAGATGAAGAAGCACTCCAAGCCCTTAGAGGTTCAGGCAT TGAGTTGATTATGGACGTGGCTAAGGAAACCCTTCAATCATTGACAGACTCCAATGCTGCTACAGATTGGGTCA ATAAATATGTTACACCTTACTCGCAAGACGTCAATTTCAAGTACATCGCTGTTGGAAATGAAATTCATCCCAAT ACCAATGAGGCACAATATATTCTATCTGCCATGACCAACATTCAGAATGCAATTTCATCAGCAAATTTACAAAT TAAGGTGTCAACAGCTATAGACTCTACTTTGATTACTAACTCTTACCCTCCCAATGATGGCGTTTTCACTAGCG ATGCGGAGCCATACATAAAACCCATAATCAACTTCCTAGTGAGCAATGGGGCCCCAATTCTTGCCAACGTGTA CCCTTACTTTGCTTATGCAAATGATCAAAGCATTCCTCTTGCCTATGCTCTTTTTACCCAACAAGGAAACAACG ACGTTGGGTACCAAAACCTCTTTGATGCTATGTTGGATTCAATATATGCTGCTTTGGAGAAAGTGGGAGCGTCC AATTTGCAGATAGTGGTTTCTGAGTGAGGATGGCCGAATTCCG
11	CGGGATCCGGTGTGTGTTACGGAGGAAATGGAAACAATCTACCAACAAAGCAAGCAGTGGTGGATCTTTACAAA TCAAACAGAATAGGCAAAATCCGTTTATACTATCCAGACGAAGGAGTCCTTCAAGCCCTCAGAGGTTCAAACAT AGAGGTGATCCTCGGTGTCCCTAATGACCAACTTCATTCTCTCACCAACGCTGGAGCTGCCACAAATTGGGTCA ACAAGTACGTGAAAGCATACTCACAAAACGTGAAATTCAAGTACATTGCAGTTGGTAACGAAATTCACCCTGGT GACTCTTTAGCAGGGTCTGTACTTCCAGCACTTGAAAACATTCAGAAAGCAATTTCTGCCGCCAATTTACAAGG CCAAATGAAGGTGTCAACAGCAATAGACACCACTTTACTTGGCAACTCTTACCCACCAAAAGATGGCGTTTTCA GCAGTAGTGCAAGTTCATACATAAGACCAATTGTAAACTTTTTAGCAAGAAATGGAGCCCCACTTCTCGCAA ACGTGTACCCTTACTTCGCCTATGTTAACAACCAACAAAGCATTGGTCTTGACTATGCCTTGTTTACTAAACAT GGTAACAACGAGGTTGGGTACCAAAACCTGTTTGATGCATTGCTGGATTCCCTATACGCTGCTCTTGAGAAAGT AGGGGCACCAAATGTGAAGGTTGTTGTGTCTGAGTGCGGATGGCCGAATTCCG

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12    CGGAATTCGGCCATCCACTTTCGGATACAACAACCTCCACATAACCAATCTTAGTGTTATCAATGGCTGCATGT
      ACTGAATCCAACATAGCATCAAACAAATTTTGGTACCCATATTGACCATCCCATACCACAACATTTGGTGCTGT
      GAAAAGAGCATAGGGAAGTGATATGTCACGGGGGTTACCAGTGTAACTAAAATAAGGGTACACATTGACTAGTA
      ATGGTGCATTTGCATATACCAAGTACCCAATTATGGGATCTATGTATGATCTCACATCACCCCTGAAGGAACCT
      TGCGATGGAGGGAAAGATTTCCTATTAGGGTCATGTCAATATATGTTGAAACCTTGATTTGATCNTGAAGGCCT
      TGAGCTCTTATTGCTTGGTATACATTTTGGATGGCAGGTAGAACATATTGGGCTACCGAAGAAGAGCCTCCAAC
      GGGACTCACTTCATTTCCAACTGCCACGTACTTGATTTTGACACTAGGCCAGAAGTTCTACACGTTTTTTTGCA
      CCCATTGACGAGAAGTGTCAGGATTGGTGGCAAGGCCTTGAAGGTCAGAGTTTGGCACCCCAAGAATGAGTTCA
      ATGCCAGAATTTCTAAGTGCTTCTAGAGCAGCTTGATTAGGATCATAGAGTCTCATTCTCTTTATGTTATTTGA
      TCTATAAAGACCTATAACATCGTTTGCTGACGGTAGATTGTTGCCCAGCATGCCGTA
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Vectors which comprise the above sequences are within the scope of the present invention, as are plants transformed with the above sequences. Vectors may be obtained from various commercial sources, including Clontech Laboratories, Inc. (Palo Alto, CA), Stratagene (La Jolla, CA), Invitrogen (Carlsbad, CA), New England Biolabs (Beverly, MA) and Promega (Madison, WI).

Preferred vectors are those which are capable of tranferring the sequences disclosed herein into plant cells or plant parts.

Methods to inhibit plant diseases, including use of a compound herein disclosed, according to the procedures described in US 5,477,001, which patent is expressly incorporated by reference, are also part of the present invention.

Moreover, the most commercially significant use of the present invention is in the construction of "knockout mutants" using the above sequences, or known soybean sequences, for design and construction of male-sterile mutants. In other words, the present invention is informative to those skilled in the art as to their usefulness in making the naturally-occuring gene inactive. For example, the above sequences can be mutated by any means, i.e., deletion, insertion, point mutation, rearrangement, etc. so long as the mutated version retains the ability to recombine. The mutated version of the gene is then introduced into cells of a preferred soybean line via routine methods (ie. biolistic processes, lambda phage transformation, etc.).

Male-sterile mutants of the preferred line would then be selected and propagated. These "knockout" mutant embryos, seeds and plants are within the scope of the present invention, as are the knockout constructs, ie. sequences and vectors.

In particular, sequences near the active site of enzyme function, and the site itself, would be preferred targets. For example, the codons for amino acid residues 240 through 250 would be a preferred knockout, but most preferred would be a construct wherein the codons encoding 245 through 248 were deleted.

For example, the following seeds, embryos or plants transformed with knockout constructs are considered within the present invention: soybean, maize, beet, tobacco, wheat, barley, poppy, rape, sunflower, alfalfa, sorghum, rose, carnation, gerbera, carrot, tomato, lettuce, chicory, pepper, melon and cabbage. Particularly preferred are: soybean, tobacco and maize. However, any seed, embryo or plant which gives rise to a plant which has a callose encasement of the microspores is within the scope of the present invention. Of course, those in the art recognize that any seed, embryo or plant transformed with knockout constructs which are useful for producing plants for biomass are within the scope of the present invention.

Transformation of cells with the compounds of the present invention can be accomplished according to known procedures. For example, infective, vector-containing bacterial strains (such as *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens*) may be used for transformation. Zambryski, 43 *Ann. Rev. Pl. Physiol. Pl. Mol. Biol.* 465 (1992). The following procedures are also well-known: Pollen-tube transformation [Zhon-xun *et al.*, 6 *Plant Molec. Bio.* 165 (1988)]; direct transformation of germinating seeds [Toepfer *et al.*, 1 *Plant Cell* 133 (1989)]; polyethylene glycol or electroporation tranformation [Christou *et al.*, 84 *Proc. Nat. Acad. Sci.* 3662 (1987)]; and biolistic processes [Yang & Cristou, *Particle Bombardment Technology for Gene Transfer* (1994)]. The transformed cells are also within the scope of the present invention.

The transformed cells may be induced to form transformed plants via organogenesis or embryogenesis, according to the procedures of Dixon *Plant Cell Culture: A Practical Approach* (IRL Press, Oxford 1987).

Therefore, also provided are methods for constructing sequences with the ability to knockout the above sequences, comprising one of the following techniques: inserting a foreign piece of DNA into one of the disclosed sequences; deleting a piece of DNA from one of the disclosed sequences; or creating a mutation such that the ß-1,3-glucanase activity is eliminated.

Also provided are antisense constructs and methods to inhibit mRNA transcripts of the disclosed sequences, so as to either eliminate or reduce the amount of gene product. The procedures for antisense inhibition for mRNA are described in US Patent 5,554,743, which patent is expressly incorporated by reference into this application.

Also provided in the present invention are methods to improve seed germination, comprising expressing or overexpressing the ß-1,3-glucanase(s) described herein, and using the glucanases to digest the callose wall of mature seeds [i.e., *Cucumis* Speices; Yim and Bradford, 114(3) Plant Physiology 289, abstract 1506 (1997)]. The seeds can then be germinated according to traditional methods. Overexpression can be as skill of the art, in particular, according to the procedures described in US Patent 5,477,001.

Lastly, the present invention includes methods to alter the naturally-occurring expression pattern of the ß-1,3-glucanase genes so as to either delay or prematurely digest the callose wall. In other words, in a non-male-sterile plant, a glucanase gene is expressed (and the callose encasement digested) at a critical point in microsporogenesis called "the late tetrad stage." If the genes which digest the callose encasement are not active at the late tetrad stage, or if they are active before the late tetrad stage, the potential for pollen formation is lost. In the instance where the ß-1,3- glucanase genes are expressed earlier than the late tetrad stage, the microspores would be too immature. In the case where the ß-1,3-glucanase genes are expressed later than the late tetrad stage, the microspores would be arrested in development.

Since disease resistance is one characteristic conferred to a plant by the expression of ß-1,3-glucanase, an ideal method would be to activate the sequences disclosed herein (or other glucanase sequences), and have the gene constitutively expressed thereafter. However, another embodiment of this invention is to alter the expression pattern so that the ß-1,3-glucanase genes turn on later than the late tetrad stage. Constitutive expression thereafter may also be engineered. A preferred method in this regard is alteration of the regulatory regions of SEQ ID NO 7 so as to affect the expression of a ß-1,3-glucanase either earlier or later than the late tetrad stage.

In particular, in order to practice the altered expression pattern aspect of the present invention, one would have to construct a vector which provided for either an early or late promoter in conjunction with the present sequences. For instance, the following promoters would be useful in early expression of the present sequences:

Ogs4B (Tsuchiya *et al.*, 36 *Plant Cell Physiology* 487 (1994)
TA29 (Koltunow *et al.*, 2 *Plant Cell* 1201 (1990)
A3 & A9 (Paul *et al.*, 19 *Plant Molecular Biology* 611 (1992)

In order to then constitutively express the sequences described above, the construct optionally contains, for example, a 35S promoter.

Therefore, the present invention provides methods to express the ß-1,3-glucanase genes described herein in a plant which has a callose encasement of microspores, at a time other than the late tetrad stage, comprising growing a plant transformed with a vector which allows expression at a time other than the late tetrad stage of a sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11 and SEQ ID NO 12.

A method as above, wherein the ß-1,3-glucanase genes are then consititutively expressed is preferred.

Transformation of plants with these sequences would be according to known procedures as described above. Plants can be grown according to known procedures.

In addition, there are provided male-sterile soybean seeds, embryos and plants comprising a knockout construct of a soybean ß-1,3-glucanase gene, in particular, soybean seeds, embryos and plant wherein the soybean ß-1,3-glucanase gene knocked out is SEQ ID NO 7 are provided.

Examples

Example 1: Nucleic Acid Manipulations

Plant tissues for nucleic acid isolation were collected from the soybean cultivar Minsoy (PI 27890) grown in the greenhouse or growth chamber. Material for RNA extraction was immediately frozen in liquid nitrogen and stored at -80%C until used. Soybean genomic DNA was extracted from freeze-dried young leaves, following the protocol by Keim *et al.*, 15 *Soybean Genet. Newsl.* 150 (1988). Total DNA (10 &g) was digested and subjected to Southern blotting as described by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor 1989). Total RNA was extracted using guanidinum isothiocyanate extraction according to Chamczynski & Sacchi, 162 *Anal. Biochem.* 156 (1987), electrophoresed on formaldehyde-agarose gels, and blotted onto Zeta Probe Nylon membrane (Biorad) as described by Sambrook *et al.* (above). Filters were prehybridized 3 hours at 65%C in 5x SSC, 2% SDS, 5x Denhardt's solution, 0.1 mg/ml herring sperm DNA. Hybridization was carried out overnight at 65%C with 2x SSC+0.4% SDS and 1x SSC+0.4% SDS at 60%C before exposure for autoradiography.

Example 2: PCR Amplification, Cloning and Sequence Analysis

Coding region segments encoding ß-1,3-glucanase were amplified from soybean genomic DNA and flower bud cDNA using primers that matched conserved sequences within the ß-1,3-glucanases of other species as described by Simmons, 13 *Critical Rev. Plant Sci.* 325 (1994). To obtain cDNA, mRNA was prepared from flower buds, the mRNA was incubated with 5 &M random hexamers, 1 mM each of dATP, dCTP, dGTP and dTTP and 2 U/&l RNase inhibitor (Promega) in 10 mM Tris-HCl (pH 8.3), 50 M KCl, 2.5 M $MgCl^2$ for 30 min at 37%C, and then for a further 45 minutes at 37%C following the addition of 20 U/&l superscript reverse transcriptase (BRL). After heating at 65%C for 3 minutes, cDNA prepared from 0.1 &g of mRNA was used in the PCR experiments. Each 25 &l PCR contained 60 ng of soybean genomic DNA or 20 ng of flower cDNA, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl^2$, 200 &M (each dATP, dCTP, dGTP, and dTTP, 2.5 U of Taq polymerase (BRL Life Technology), and 2 &M (each) PCR primer. PCR amplifications were performed according to the follwing schedule: 96%C for 1 minute, 44%C for 1 minute, 72%C for 1 minute for 4 cycles, followed by 94%C for 1 minute, 55%C for 1 minute, 72%C for 1 minute for 30 cycles. The 5' primer was 5'-CGCGGNGTNTGYTAYGG-3'; the 3' primer was 5'-CGCGGCCANCCNSWYTC-3' (where N = A, C, G, T; R = A, G; Y = C, T; S= C, G and W = A, T). The regions used correspond to amino acids 37-41 and 276-282 according to Simmons (see above). An aliquot of each PCR product was analyzed by agarose gel electrophoresis. A 700 bp fragment was cut from the gel and subjected to another round of PCR using identical conditions. Aliquots of these PCR products were digested with restriction enzymes recognixing 4 bp sites and the digestion products were compared with undigested DNA on agarose gels. The PCR products were cloned into the pGEM-T vector (Promega) and about 280 clones were tested by dot blot analysis (Sambrook et al, see above) to determine if they cross-hybridized with one another.

Clones were sequenced using Applied Biosystems Model 337 PRISM automated sequencer. DNA sequence analysis was carried out with the DNAsis (Hitachi), GCG (University of Wisconsin Genetics Computer Group, Madison) sequence analysis packages. Alignment of sequences was done using CLUSTAL W according to Thompson *et al.*, 22 *Nucl. Acids Res.* 4673 (1994).

Example 3: Expression Patterns of Glucanases

In order to study gene-specific glucanase expression patterns, the presence of mRNAs corresponding to each sequence disclosed herein was analyzed by RNA blot analyses using the gene-specific probes. The expression levels of all classes were quite low in young leaves. SEQ ID NOs 2, 4, 7 and 12 were highly expressed in young roots and hypocotyls. SEQ ID NO 7 was also expressed in the pod and flower bud. The mRNA transcripts of all ß-1,3-glucanase genes were shown to be about 1.2 kb. mRNA from SEQ ID Nos 1, 3, 8 and 9 was undetectable, suggesting that these genes are either unexpressed, expressed at low level, or inducible.

Although the present invention has been fully described herein, it is to be noted that various changes and modifications are apparent to those skilled in the art. Such changes and modifications are to be understood as included within the scope of the present invention as defined by the appended claims.

ABSTRACT OF THE DISCLOSURE

The present invention provides 12 different sequences for soybean ß-1,3-glucanases and the proteins for which they encode. Also provided are methods for the utilization of knockout mutants of the sequences which are useful for engineering genic male-sterile plants. Other methods and materials related to these sequences are also provided.

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