**SOYBEAN MOSAIC VIRUS-SOYBEAN INTERACTIONS: MOLECULAR, BIOCHEMICAL, PHYSIOLOGICAL, AND IMMUNOLOGICAL ANALYSIS OF RESISTANCE RESPONSES OF SOYBEAN TO SOYBEAN MOSAIC VIRUS**

by

Chang Won Choi

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Approved:

Dr. Sue A. Tolin, Chairman

Dr. Robert C. Bates

Dr. Glenn R. Buss

Dr. Carole L. Cramer

Dr. Asim Esen

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# CHAPTER I

## INTRODUCTION

The soybean (*Glycine max*)-soybean mosaic virus (SMV) interaction represents a good model of a host-pathogen system with a range of responses and strain-specific resistance under single dominant gene control in the host. The most widely used approach to study the mechanism of resistance in a system is to measure the expression of host defense genes during development of the pathogenic response, with the hypersensitive response (HR) being the most widely studied. In soybeans, this response has been studied with both fungal and bacterial pathogens but apparently not with viruses (Ebel, 1986; Collinge and Slusarenko, 1987).

SMV is a potyvirus first observed in the early 1900s when soybeans were first introduced into the United States as a crop. It is seed-borne in soybeans and is transmitted by various species of aphids in a non-persistent manner. Worldwide, SMV occurs wherever soybeans are grown and causes reduction in yield and seed quality (Sinclair, 1982). Resistance is conditioned by a single dominant gene, but resistance-breaking virus strains exist. SMV isolates have been classed into seven strain groups, G1-G7, based on the differential reaction to certain soybean cultivars (Cho and Goodman, 1979).

The common or type strain of SMV is the least virulent

and resistance to it is controlled by a single dominant gene, *Rsv*. Resistant cultivars permit no detectable viral replication following leaf inoculation with the common strain, phenotypically show no symptoms and are operationally immune (R response). Cultivars that are susceptible (*rsv*) permit replication and systemic spread of the virus in the plant following inoculation (S response). The severity of resulting foliar symptoms varies from severe leaf distortion to mild mosaic, depending on the cultivar and virus strain. Another type of interaction is that in which necrosis symptoms are produced by necrotizing strains (N response).

Previous investigations indicate that five cultivars resistant to the G1 strain each possess a single dominant gene for resistance (Kiihl and Hartwig, 1979; Roane et al., 1983; Buzzell and Tu, 1984; Buss et al., 1989a; Chen et al., 1991). Genetic analysis of crosses from resistant X resistant cultivars has demonstrated the lack of segregation for susceptibility in $F_2$ and $F_3$ progenies and indicates with high probability that the resistance genes in these cultivars are alleles at the same locus (Chen et al., 1991).

There are numerous types of different necrotic responses in plants associated with viral infections. The typical type is the hypersensitive response (HR) in which the plant specifically recognizes the viruses at the onset of infection and activates an active defense response that

usually restricts further movement of the virus. However, sometimes the virus induces necrosis but is not limited to the initially infected area and is able to move to other parts of the plant. The soybean *Rsv* gene:SMV system differs from typical HR of the well-characterized tobacco *N* (Holmes, 1952) or *N'* (Weber, 1951) genes:TMV system in that (i) the necrotic reaction is not totally localized as a lesion but spreads systemically, and (ii) virus strains are available that specifically overcome the *Rsv* gene and result in phenotypically different responses. The heritibility of resistance, however, is similar and both segregate as single dominant genes (Weber, 1951; Holmes, 1952; Buss et al., 1989a).

However, the phenotypic symptoms of SMV-soybean interaction system appears similar to those of TMV-tomato system. Three tomato resistance genes, *Tm-1, Tm-2* and $Tm\text{-}2^2$ have been identified (Pelham, 1966) which prevent systemic mosaic symptoms, and respond with either localized necrosis resulting in resistance, or systemic necrosis resulting in severe disease depending upon the strain of inoculating virus, the genotype of the host, and temperature factors. Upon inoculation with certain strains of TMV, *Tm-2* and $Tm\text{-}2^2$ resistance genes appears to be allelic (Pelham, 1966). No strains of TMV have been found to overcome the resistance of the $Tm\text{-}2^2$ locus (Fraser, 1990). The *Tm-1* gene in tomato appears to inhibit virus replication

(Watanabe et al., 1987) and the *Tm-2* and *Tm-2*$^2$ genes prevent cell-to-cell spread (Meshi et al., 1987, 1989). The tomato resistance mechanism is regulated by three distinctive genes at different loci, but both the R and N responses in certain soybean cv. are regulated by a single dominant gene. At present nothing is known of the mechanism of the differential response of soybean to SMV leading to localized or systemic necrosis or mosaic.

The soybean-SMV system is a genetically well-defined system and its study should provide insight to the molecular basis for host-pathogen interactions, specifically because genotypes that respond as resistant and necrotic to specific strains of the virus are available. The overall goal of this research is to describe the molecular, biochemical, physiological and immunological analysis of interaction between soybean mosaic virus, a potyvirus, and its primary host, soybean, and to identify and characterize both the resistance factors from the soybean and the virulence or pathogenicity components in the virus. By analogy to other host-pathogen systems, we have generalized that host defense responses should be operative in soybean, and have focused on pathogenesis-related proteins and key biosynthetic enzymes of known defense-related pathways including phytoalexin and lignin synthesis, which are produced in the host-virus combinations involving necrosis (Collinge and Slusarenko, 1987). Ultimately, we may elucidate that the

mechanism of resistance is by regulation of expression of defense response genes. The specific objectives of this research were: 1) to demonstrate the accumulation of soybean pathogenesis-related proteins, to characterize their involvement in the process of necrogenesis in soybean infected with SMV, and to define their enzymatic functions; 2) to determine the involvement of the necrotizing strain G4 in inducing key enzymes of the phenylpropanoid, isoflavonoid, and lignin pathways; 3) to demonstrate the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase in soybean correlated with *Rsv* resistance to SMV-G1; and 4) to examine the coat protein (CP) heterogeneity of SMV and the proteolytic nature of this heterogeneity in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by comparing immunological properties, peptide maps by trypsin cleavage, and dephosphorylation of CP of SMV by alkaline phosphatase treatment.

## CHAPTER II

## LITERATURE REVIEW

### 2.1 Delineation of SMV strains.

Ross (1969) was the first to note variability of the virus by differing abilities to infect soybean cultivars. Cho and Goodman (1979) placed over 100 SMV isolates into seven strain groups by using the soybean cultivars Buffalo, Davis, Kwanggyo, Marshall, and Ogden selected because they were operationally immune to the type isolate of SMV. The reactions were either systemic necrosis (N), systemic mosaic (S), or resistance (R) in which no symptoms were evident and no viral replication could be detected. All of the strain groups, designated G1 through G7, were closely related serologically and produced typical mosaic symptoms on cultivars (Rampage, Clark) susceptible to the type isolate and local necrotic lesions on Topcrop bean (Cho and Goodman, 1979). Isolates of SMV from Virginia were characterized and placed into strain groups G1 and G3 (Hunst and Tolin, 1982).

### 2.2 Genetics of resistance to SMV.

Inheritance of reaction to SMV has been investigated by various groups using different soybean cultivars and SMV strains. Inheritance of resistance to SMV and its recent utilization by plant breeding programs have been reviewed by Buss et al. (1985, 1989a). Kiihl and Hartwig (1979) reported that resistance to SMV in PI96983 was governed by a single

dominant allele, *Rsv*, and that resistance in the cultivar Ogden was governed by $rsv^t$, another allele at the same locus, which is dominant to susceptibility but recessive to *Rsv*. They used Ross's (1969) strains of SMV later shown by Cho and Goodman (1979) to belong to G2 and G3 strain groups. Buzzell and Tu (1984) found that a dominant gene in the cultivar Raiden was at a different locus and labeled it $Rsv_2$. Lim (1985) found additional dominant genes for resistance at different loci from each other and from *Rsv*, but since the relation to $Rsv_2$ was not determined, it was not labeled.

In Virginia, studies have been focused on determining the relationship between the resistance genes in the group of cultivars used by Cho and Goodman (1979) to differentiate between strains of SMV. Results of inoculating F2 and F3 populations of a series of crosses between Lee68 or Essex and Marshall, Ogden, Kwanggyo, York and PI96983 with SMV-VA/G1 have shown that each cultivar has a single dominant gene for resistance and that all resistance genes are alleles at the same locus (Roane et al., 1983; Buss et al., 1987). Interestingly, in both field and greenhouse studies, as many as 10-25% of plants in a segregating population gave systemic necrosis in response to G1 inoculation. Analysis of data for best fit to genetic ratios confirms that these plants should be classed as resistant even though there is limited viral replication and systemic spread (Roane et al., 1983; Buss et al., 1987; Buss et al., 1989a,b; Chen et al.,

1991), as is expected in a hypersensitive response (Ponz and Bruening, 1986). Studies are in progress to inoculate the various crosses with selected strains to confirm the gene-for-gene relationship proposed for this system (Roane et al., 1986). The necrotic response was assumed to be heterozygous for the resistance gene which suggests an incomplete dominance (Kiihl and Harwig, 1979), and should be classified as resistant when evaluating segregating populations (Buss et al., 1989a,b).

2.3 **Characterization of SMV and potyvirus group.**

Ribonucleic acid (RNA) was demonstrated to be the nucleic acid of SMV (Hill and Benner, 1980). The molecular weight of SMV RNA was estimated to be 3.25 Md by polyacrylamide gel electrophoresis using formaldehyde-denatured RNA (Hill and Benner, 1980) and 3.3 Md using glyoxal-denatured RNA (Vance and Beachy, 1984a). Polyadenylation at the 3′ terminus of SMV RNA has been identified (Vance and Beachy, 1984a) and described as similar to eukaryotic mRNA's (Kates, 1970). Vance and Beachy (1984b) immunoprecipitated proteins from *in vitro* translation with antibodies to the coat, nuclear inclusion, and cytoplasmic inclusion proteins to determine the genome organization. A genomic map of SMV appears similar to the potyviral general genomic map (Dougherty and Hiebert, 1980).

Little work has been done to characterize the SMV strains other than by their reaction on soybean cultivars.

Hunst and Tolin (1983) showed that SMV-VA/G1, which induced a severe mosaic on the soybean cv. Essex, differed ultrastructurally from mild mosaic-inducing SMV-VA/G3 and Illinois G1 and G3 isolates in that virus particles were not amassed into cytoplasmic strands as they were with the three mild strains. They speculated that incorporation of virus into these strands was a localization mechanism leading to the tolerant reaction in Essex.

Until recently there was limited information available on the structure of the potyvirus genome and coat protein (CP). However with development of the improved biochemical and molecular tools, some progress has been made to understand the nature of the virus assembly (Shukla et al., 1988b), the location of antigenic regions of CP (Shukla et al., 1988b), and the biochemical and biological functions of potyviral gene products (Berger and Pirone, 1986; Carrington and Dougherty, 1987a,b; Domier et al., 1987). Complete genome sequences were first determined for two potyviruses in 1986 (Allison et al., 1986; Domier et al., 1986).

Earlier work done in our lab initiated the molecular characterization of SMV-VA/G1. A cDNA clone containing an insert of 1443 nucleotides representing the 3'terminus of the viral RNA genome was obtained (Gunyuzlu et al., 1987) and its sequence was determined by direct plasmid sequencing from deleted subclones (Hattori and Sakaki, 1986). Within this sequence was a single open reading frame of 1119

nucleotides terminating 224 nucleotides from a poly(A) tract of about 100 A residues. The amino(N)-terminus of the CP cistron was identified by a glutamine:serine dipeptide cleavage site 792 nucleotides upstream from the termination sequence, which would potentially code for a 30kD protein of 264 amino acid residues.

Comparison of this sequence with published sequences of CPs of other potyviruses, tobacco etch (Allison et al., 1985a,b), tobacco vein mottling (Domier et al., 1986), pepper mottle (Dougherty et al., 1985), sugarcane mosaic (Gough et al., 1987) and potato virus Y (Shukla et al., 1986), shows that there is a high degree of homology (>60%) in the central portion and carboxyterminal (C) end of the protein, but that the N-terminal region is highly variable. The recent findings of Shukla et al. (1988a,b,c,d) and of Dougherty and coworkers (Allison et al., 1985a; Dougherty et al., 1985) suggested that the N-terminal and C-terminal regions are located on the surface of the virus particles and are the most highly variable between potyviruses and among strains of the same potyvirus. Based on the SDS-PAGE, the molecular weights of the CPs of potyvirus group ranged from 28 to 34 kD depends on gel concentration used in the estimation (Hiebert and McDonald, 1973; Moghal and Francki, 1976; Gough and Shukla, 1981). There have been numerous results on the heterogeneity of the CP of potyviruses. The reason for the occurrence of multiple bands was a partial

degradation of the polypeptide by proteolytic enzymes of host or microbial origin (Moghal and Francki, 1976). Shukla et al. (1988b) found that mild trypsin digestion of several potyviruses removed the variable N-terminal region (29-67 amino acids depending on the virus) and 18-20 amino acids from the C-terminal region of the CP without destroying the rod-shaped structure of the virus or its infectivity. For example, storage at 4$^{o}$C of a purified preparation of tobacco etch virus (TEV) was found to change the CP ranging from 32 kD to 26 kD MW (Hiebert et al., 1984), amounting to complete removal of the surface exposed N and C termini (Shukla et al., 1988b). Since the N-terminal region contains the virus-specific epitopes, its gradual removal from particles *in situ* would render virus particles containing only nonspecific epitopes. Through the years various approaches have been applied for the identification and classification of viruses; serological properties would appear to offer the most practical tools for detecting viruses in infected plants (Hollings and Brunt, 1981; Moghal and Francki, 1981). However, the results reported among large potyvirus group have been unsatisfactory and inconsistent between related strains and distinct members. Recently, immunochemical analysis of native and trypsin-treated particles of potyviruses has revealed that proteolytic digestion removes the surface located epitopes that are recognized by the polyclonal antibodies raised against the whole-virus particles (Shukla

et al., 1988b). On the other hand, antibodies raised against the dissociated CP core recognized all 15 different definitive aphid-transmitted potyviruses tested (Shukla et al., 1988b, 1989). This indicated that core protein sequences of the CPs are highly conserved throughout the potyviruses.

In contrast to other gene products, the CP is the major and unique gene product, accounting for 95% of the potyvirus particle, characteristic amino acid composition, and an amino acid sequence that shows no significant homology between different groups of plant viruses (Fauquet et al., 1986a,b). Therefore, the structural properties of the CP may be better criteria than other properties for identification and classification of potyviruses.

The CP may play a role in pathogenesis since changes, either major or minor, have been detected between strains of the same virus. It is tempting to speculate that the role may involve induction of pathogen-related proteins. Fraser (1983) suggested that the properties of the virus CP may influence the interaction with the localization mechanism specified by the *N'* gene in tobacco after comparing strains of TMV with lower thermal stability than the common strain. The involvement of CP gene sequences in the induction of the *N'* gene HR was demonstated by substituting the CP gene of the HR-inducing TMV-L strain which does not induce the *N'* gene HR (Saito et al., 1987). It was also possible that the alteration of single nucleotide in the coat protein

would induce the HR (Knorr and Dawson, 1988). One possible assumption for the mechanism of induction of the HR in *N'* gene plants is that these plants recognize specific total three-dimensional structure of the TMV coat protein (Saito et al., 1989).

### 2.4 **Plant defense responses**

Pathogenesis-related (PR) proteins: One of the fruitful areas of plant virus research recently has been the study of PR proteins. PR proteins were first detected in tobacco leaf tissue responding hypersensitively to tobacco mosaic virus (TMV) to form necrotic lesions and localize the virus (van Loon, 1976). These proteins are characterized by being of low molecular weight (Antoniw et al., 1980; Redolfi and Cantisani, 1984; van Loon, 1985) and having charge differences which leads to large electrophoretic separations (Pierpoint, 1983). They are extractable at low pH, retain their solubility (Gianinazzi et al., 1977; Redolfi and Cantisani, 1984; Van Loon, 1976) and are highly resistant to proteases (Pierpoint, 1983; Van Loon, 1985). In tobacco and cowpea, PR proteins are located outside the cytoplasm in the intercellular spaces. Carr et al. (1987) localized tobacco PR1's extracellularly in Xanthi-nc near necrotic lesions. Parent & Asselin (1984) detected PR proteins in intracellular fluid of three species of tobacco and of Chenopodium responding hypersensitively to TMV.

PR proteins have been found in at least 16 plants in

response to various local lesion inducing viruses (Van Loon, 1985). The role of the PR proteins is not well understood but a number of hypotheses have been presented. They may serve to protect the plant from extensive damage by limiting the invading pathogen (Van Loon, 1985). The PR proteins have been proposed to limit multiplication and/or spread of the virus and to be associated with systemic acquired resistance (Fraser and Clay, 1983; Van Loon, 1985). Recent reports from several European laboratories have begun to clarify the nature of PR proteins and to associate them with plant defense responses previously identified with response to fungal- and bacterial-induced stress. In several of these reports, immunological or cDNA probes have been developed and have been used to separate PR proteins in a single species and compare PR proteins of similar size between species, as reviewed recently by van Loon (1985).

The tobacco PR proteins have been classified into five groups. Among the best characterized are the PR-1 group, PR-1a, -1b and -1c, which exist as acidic charge isomers based on serological assay. cDNA clones to tobacco mRNA encoding acidic PR-1 proteins have been isolated from TMV-infected tobacco tissue (Cornelissen et al., 1986; Pfitzner and Goodman, 1987; Cutt et al., 1988; Payne et al., 1988). The amino acid sequence homology among the three acidic proteins is more than 90 % in the protein-coding region (Pfitzner and Goodman, 1987; Payne et al., 1988). Immunologically

crossreacting homologues of tobacco PR-1 proteins have been found in tomato, potato, cowpea, maize and barley (Nassuth and Sänger, 1986; White et al., 1987), while another structurally unrelated PR-1 class has been found in parsley (Somssich et al., 1988).

Acidic PR-P and -Q and two basic proteins have chitinase activity (Legrand et al., 1987; Hooft van Huijsduijnen et al., 1987). Four other PR proteins, PR-O, -P, -2 and one basic protein have $\beta$-1,3-glucanase activity (Kauffmann et al., 1987). Both hydrolytic enzymes have an inhibitory effect on fungal growth in *in vitro* experiments (Mauch et al., 1988), coordinated induction in response to infection, increased enzyme activities in parallel to development of necrosis (Kauffmann et al., 1987; Legrand et al., 1987; Kombrink et al., 1988), and roles as elicitors of secondary metabolite synthesis (Keen and Yoshikawa, 1983). Another strutural class of $\beta$-1,3-glucanase, designated PR-Q', has been analysed as the basic form which is different from both the basic, vacuolar form and the acidic, extracellular forms. This is synthesized as a pre-pro-enzyme and upon maturation the 21 amino acid signal peptide and a 22 amino acid C-terminal peptide are removed (Payne et al., 1990). The previous studies led to the speculation that basic isoforms of $\beta$-1,3-glucanase and chitinase are predominantly localized in the vacuole; whereas, the structurally related acidic isoforms of both enzymes are secreted into extracellular

compartment (Kauffmann et al., 1987; Legrand et al., 1987; Boller, 1988; Van den Bulcke et al., 1989; Mauch and Staehelin, 1989). It has been proposed that the extracellular forms have an early defense involved in the recognition process, while the vacuolar forms function as the last line of the defense when cell lysis releases the vacuolar contents into the extracellular compartments (Mauch and Staehelin, 1989).

Studies with transgenic plants constitutively expressing PR-1 or -S have failed to show any direct involvement in the resistance to viruses or insects, or to present a necrotic symptom (Linthorst et al., 1989; Cutt et al., 1989). Another recent observation has shown other PR proteins, such as $\beta$-1,3-glucanase and chitinase, accumulated in tobacco leaves not undergoing necrosis as well as in leaves undergoing necrosis, suggesting these proteins were not sufficient to induce the formation of necrotic lesions associated with hypersensitivity (Godiard et al., 1990).

Phenylpropanoid, isoflavonoid and lignin pathway induction: In the incompatible interaction between soybean and fungal pathogens (*Phytophthora megasperma* f.sp. *glycinea*) in which active defense mechanisms are induced, the accumulation of phytoalexins (glyceollin) appears to play a definitive role in the expression of resistance (Hahn et al., 1985; Bonhoff et al., 1986 a,b; Bhattacharyya and Ward, 1987). Studies on the elicitation of post-infectional

defense mechanism in soybean have focused on the induction of phytoalexin accumulation upon inoculation with fungal spores or hyphae (Hahn et al., 1985; Bonhoff et al., 1986a,b; Bhattacharyya and Ward, 1986, 1987) or treatment with different types of elicitors (Keen et al., 1983). Phytoalexin accumulation was also elicited by a number of non-specific, abiotic stresses or wounding the plant (Kochs et al., 1987). The molecular mechanisms underlying operation in relation to phytoalexin induction in biologically stressed plant cells have been intensively reviewed (Dixon et al., 1983). The marked but transient increase in the rates of synthesis of phytoalexin biosynthetic enzymes concomitant with the onset of phytoalexin accumulation has been demonstrated (Börner and Grisebach, 1982; Lawton et al., 1983a,b; Bhattacharyya and Ward, 1987; Kochs et al., 1987; Latunde-Data et al., 1987). The transient increase in enzyme synthesis reflects increases in the levels of the corresponding mRNA activities (Ryder et al., 1984; Lawton et al., 1983a,b; Bell et al., 1984; Cramer et al., 1985a,b; Tepper et al., 1989). Phenylalanine ammonia lyase (PAL) is a key regulatory enzyme of plant metabolism catalyzing the first reaction in the biosynthesis from L-phenylalanine of several compounds including phytoalexins (antobiotics), anthocyanin pigments and lignins (Dixon et al., 1983; Jones and Porter, 1986). Chalcone synthase (CHS) is a key regulatory enzyme catalyzing the first reaction of a branch pathway specific to fla-

vonoid and isoflavonoid biosynthesis (Dixon et al., 1983). Chalcone isomerase (CHI) catalyses the stereospecific isomerization of chalcones to their corresponding (-)flavanones (Hahlbrock et al., 1970).

In contrast to phytoalexin accumulation, induced lignification of cell walls was not elicited by a range of abiotic treatments, but occurred in response to pathogens (Pearce and Ride, 1980). It has been proposed as a structural defense mechanism of disease resistance (Vance et al., 1980; Moerschbacher et al., 1988). Peroxidase has been implicated in diverse physiological processes such as wound healing (Espelie et al., 1986), pathogen defense (Hammerschmidt et al., 1982), and lignification (Grisebach, 1981; Lagrimini and Rothstein, 1987). The anionic isozyme has been shown to catalyze efficiently the polymerization of the cinnamyl alcohols into lignin *in vitro* (Mäder and Füssl, 1982).

In case of viral infections, it is not known how these compounds influence virus entry, replication, and cell-to-cell movement. Tobacco plants infected with TMV have been shown to have a marked increase of PAL activity in the hypersensitive response, however the induction of further pathways against viral infection is not well evidenced or is only partially characterized (Ponz and Bruening, 1986).

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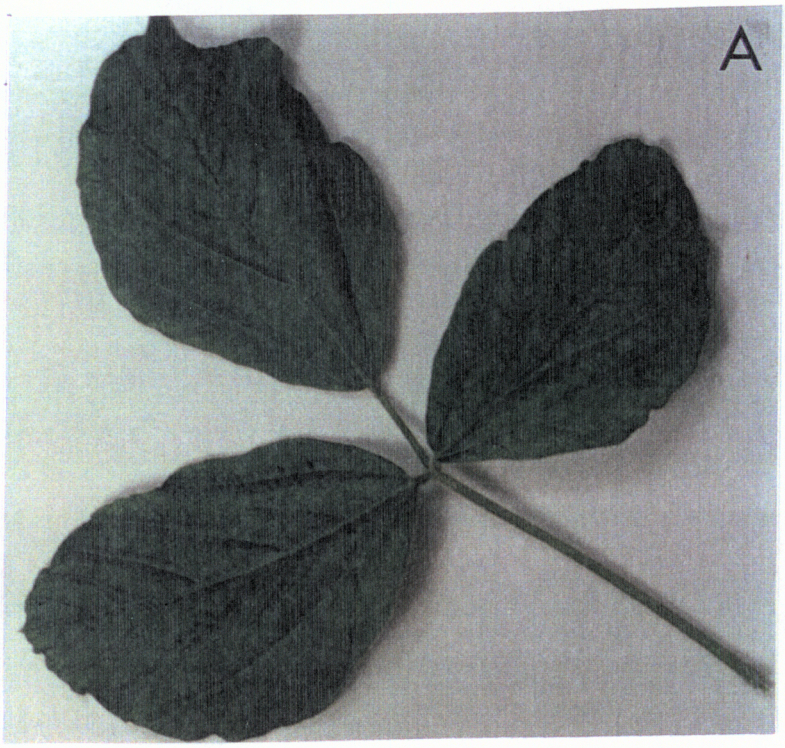
## CHAPTER III.

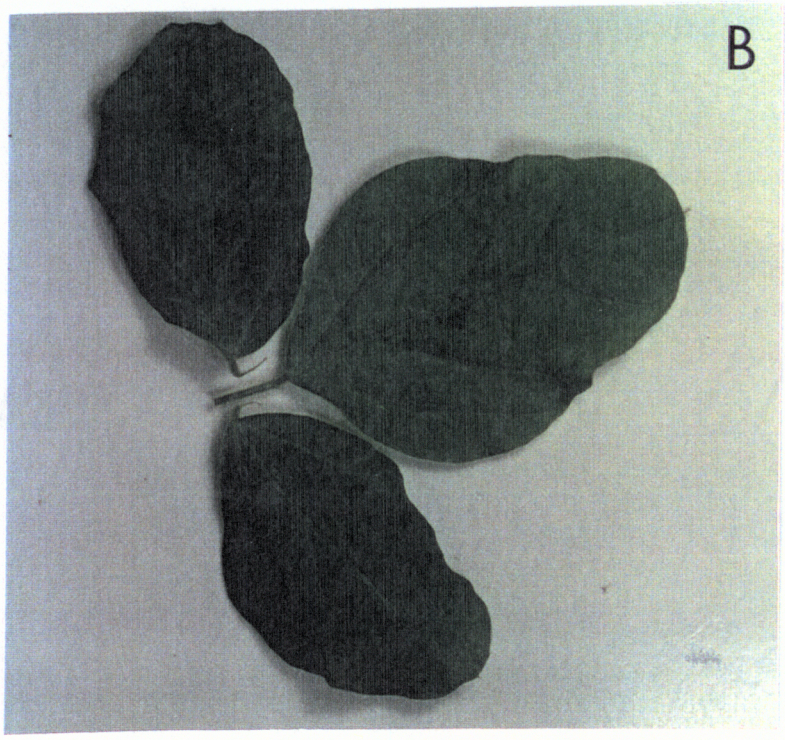
### SMV-Soybean Interaction II: Symptomatology

Symptomatology is still very important in plant virus research for identification and classification of virus, or determination of susceptibility and resistance of host plants in genetic studies. However, depending upon disease symptoms for understanding mechanisms of pathogenicity and resistance has led to much confusion, because generally unknown factors may have a marked effects on the disease produced by a given virus. In addition, certain viruses under suitable conditions may replicate and infect a plant without producing any visual symptoms. In this chapter, typical visual symptoms induced by soybean mosaic virus (SMV) are described and categorized as follows.

Systemic Mosaic (S): Generally, the first symptoms on plants after mechanical inoculation of the primary leaves is the appearance of yellowish vein-clearing along the small, branching veins of the first trifoliolate leaflets 4-5 days after inoculation (DAI) with systemically infecting strains. This symptom is transitory and occurs only in the first trifoliolate leaflets. Chlorotic spots appear in the young and not-fully expanded second trifoliolate, which then develops a mild mosaic pattern in fully expanded leaves 10-14 DAI or longer (Fig. 1A).

Fig. 1. Mild mosaic symptom with light and dark green areas along the veins appears in soybean cv. Lee plant at 2 weeks after inoculation with SMV-G1 (A). Vein clearing with formation of blisters on the surface of soybean cv. York caused by SMV-G5 at 2 weeks after inoculation (B).


A


B

Increasingly more severe and typical symptoms develop on subsequent leaves, which eventually show blisters with pattern of light and dark green areas giving a mosaic effect 2-3 weeks after inoculation (Fig. 1B). Blisters are scattered on either side of the veins to give a vein-banding pattern (Fig. 2). The most severe symptoms may be raised dark green areas to give a blistering effect, and the margins of the leaf may be irregular and twisted downward (Fig. 2). Severity varies with strains and cultivar.

Systemic mosaic necrosis (SMN): The systemic spread of viral-induced necrosis can result in severe disease. The expression of SMN displays necrotic lesions in the inoculated leaves and necrosis intermixed with mosaic symptoms in the upper leaves (Fig. 3).

Necrosis (N): There are three types of necrotic responses induced by SMV depending upon strain-cultivar interactions. These include limited necrotic lesions (Fig. 4A) which is similar to the hypersensitive response, local veinal necrosis (Fig. 5A), and lethal systemic necrosis (Fig. 5A,B). It is not clear whether these necrotic responses are distinctly different or only variations of the same process. Interestingly, the different tissues in the same plant respond differently to the same necrosis-inducing strain. For example, at the young seedling stage, initial pin-point lesions appear in the inoculated lower leaves at 4

Fig. 2. Leaf twist and vein-banding with scattered blisters in soybean cv. Lee at 3 weeks after inoculation with SMV-G2.

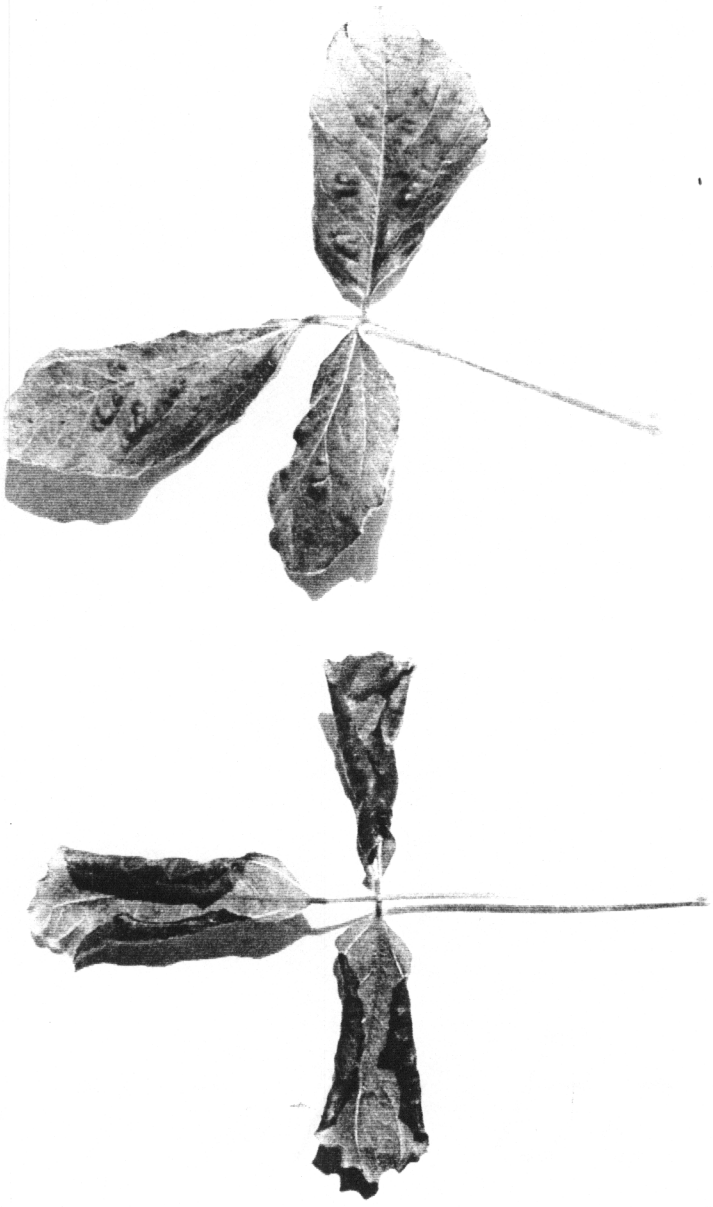


Fig. 3. Necrosis intermixed with mosaic symptom appeared in the upper leaves of soybean cv. Marshall plant at 2-3 weeks after inoculation with SMV-G2 .

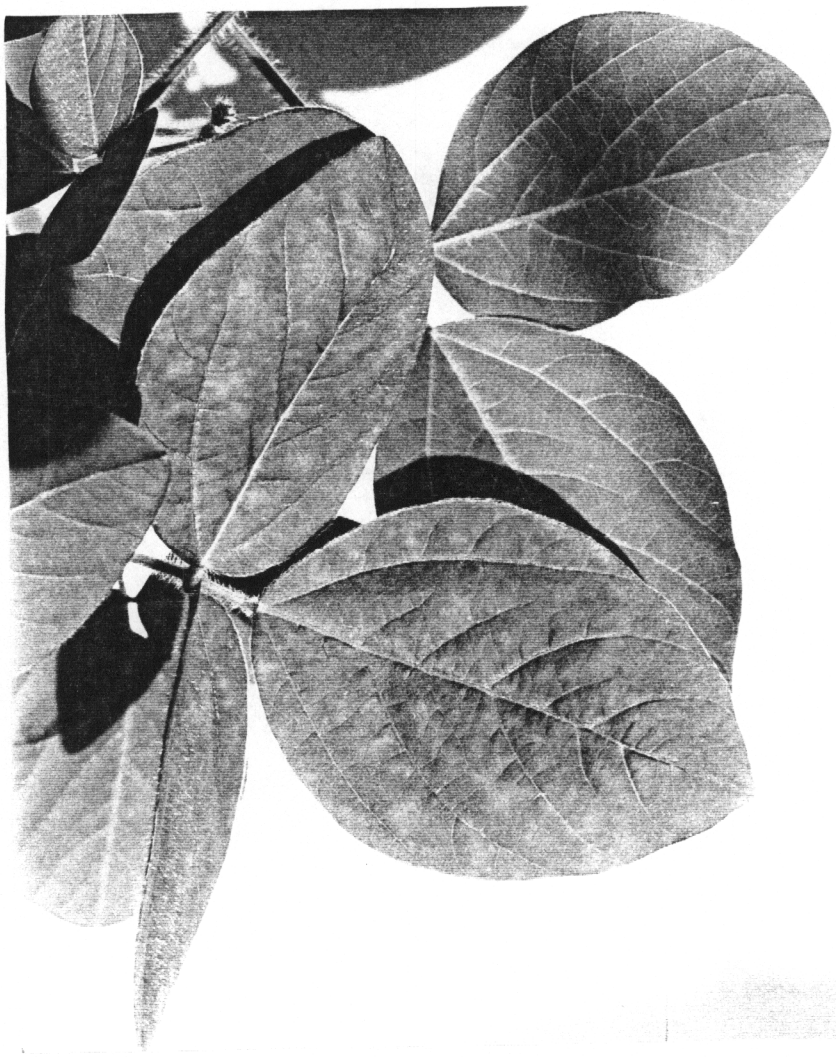
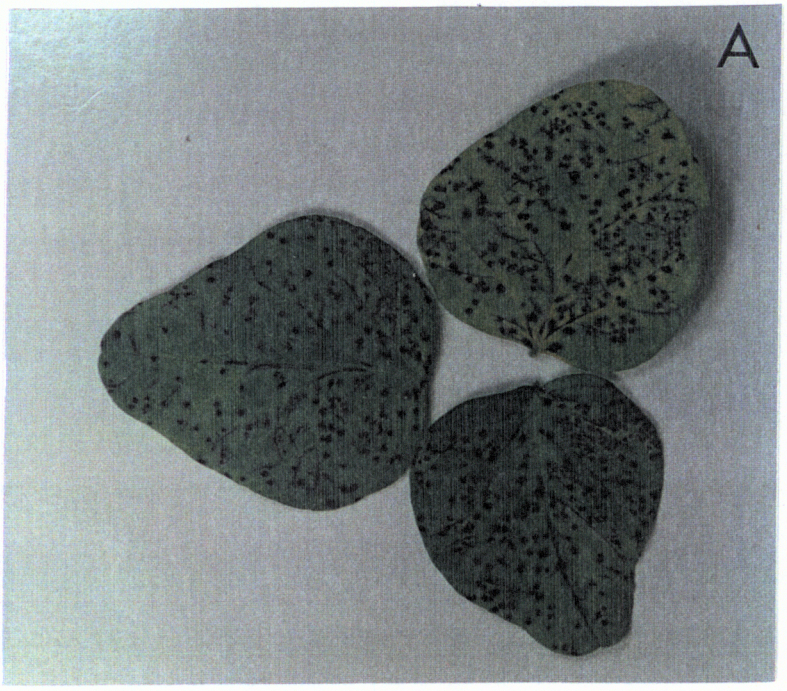


Fig. 4. Leaves of Soybean cv. York at the second trifoliolate stage inoculated with SMV-G4 showing limited necrotic lesions and local vein necrosis at 4 days after inoculation (A) and systemic necrosis appearing as tiny local necrotic lesions in the third trifoliolate at 6 days after inoculation (B).


A

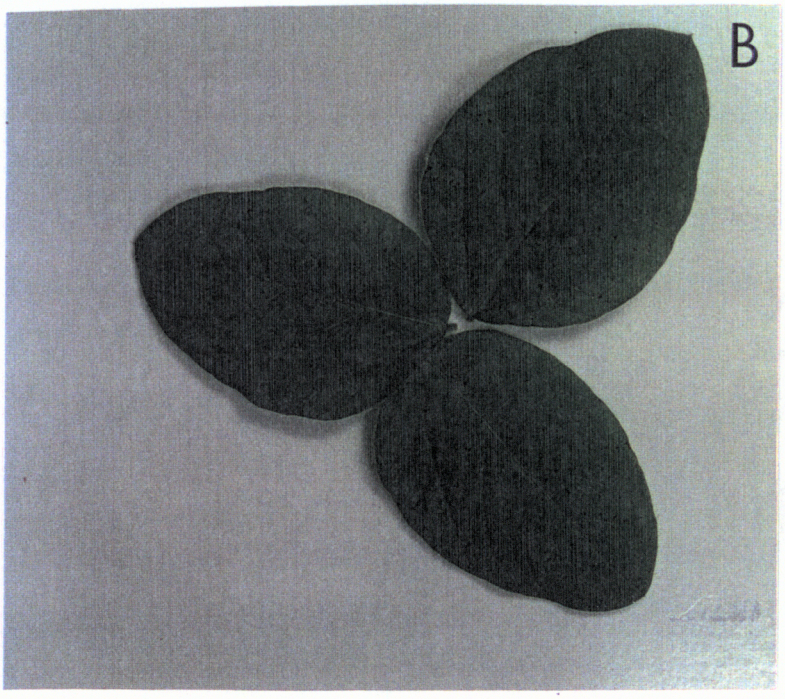
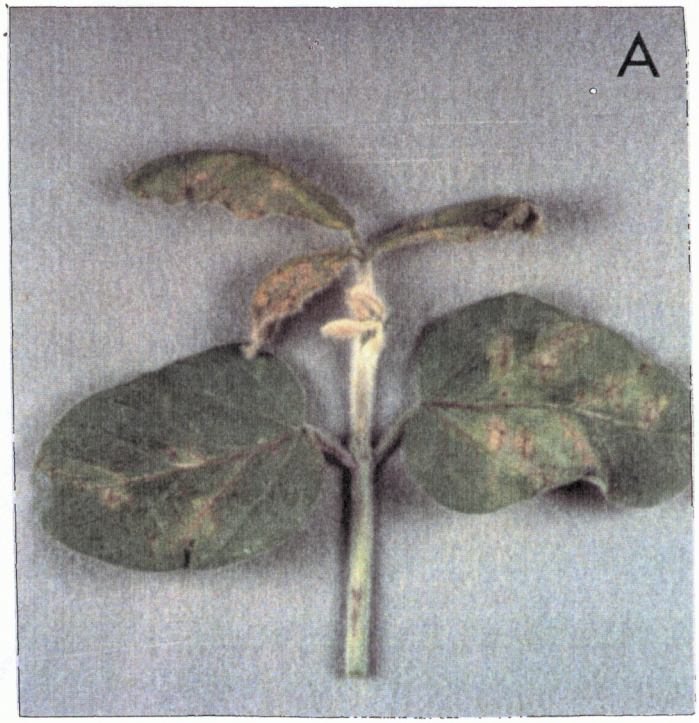
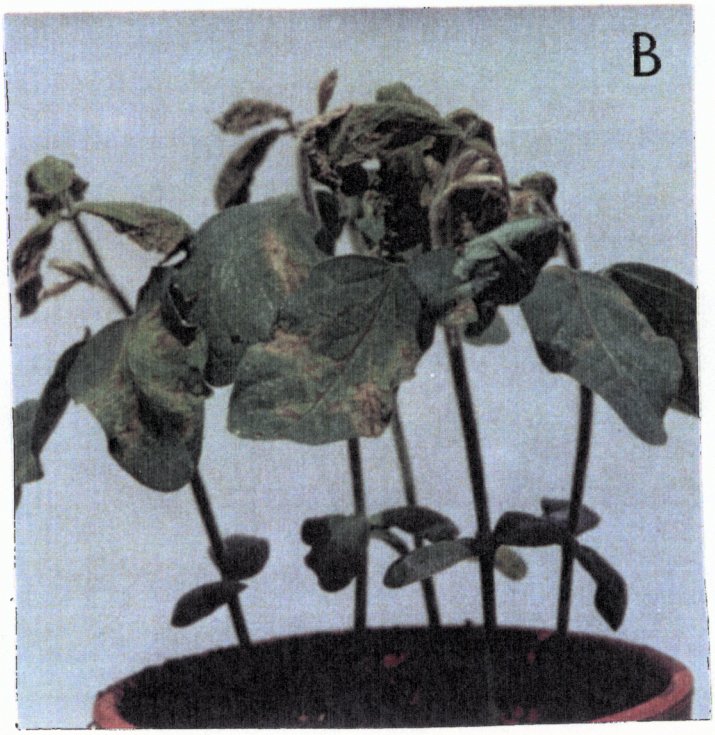

B

Fig. 5. SMV-G4 inoculated primary leaves show local vein necrosis at 4 days after inoculation followed by systemic necrosis in the upper immature leaves at 7-8 days after inoculation (A). Rapid necrosis spreads and leads to plant death (B).


A


B

DAI, extend rapidly into local vein necrosis at 5-6 DAI, and are followed by systemic necrosis in the upper immature leaves at 7-8 DAI (Fig. 5A). However, at the mature seedling (3-4 leaves) stage, inoculated leaflets develop limited necrotic lesions with partial necrosis and delayed systemic necrosis in the upper leaves (Fig. 4B). The symptoms associated with necrosis include a dark brown discoloration of leaf veins, yellowing of the leaves, necrotic lesions, stunting of the plants, browning of petioles, stem or stem tips, bud blight, and wilting of the necrotic areas. Necrosis spreads rapidly and leads to plant death eventually (Fig. 5B). This type of necrosis is often observed on soybean plants that have resistance genes or alleles following inoculation with G1 strain. It is quite prominent with the following combinations: G4/York, G5/Kwanggyo, and G7/PI96983. Necrosis is more limited, and rarely lethal, with G2 and G3 on soybean cvs. Marshall and Ogden (Buss et al., 1989; Chen et al., 1991). The development of stem-tip necrosis has been reported to be temperature-dependent (Tu and Buzzell, 1987). Temperature shift treatment has been reported to induce susceptibility of several soybean lines resistant to a strain of SMV (Mansky et al., 1991). Low temperature delayed the appearance and development of necrotic symptoms, and high temperature increased the severity. Our tests, however, indicate the systemic necrosis may be temperature-independent.

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## CHAPTER IV.

# SMV-Soybean Interaction II: Identification and Characterization of Pathogenesis-Related Proteins Associated with Differential Responses of Soybean Cultivars Infected with Soybean Mosaic Virus.

Chang W. Choi,[1] Glenn R. Buss,[2], David C. Bays[1] and Sue A. Tolin[1]

[1]Department of Plant Pathology, Physiology and Weed Science;[2]Department of Crop and Soil Environmental Sciences, Virginia Polytechnic Institute & State University, Blacksburg, VA 24061, USA

**ABSTRACT**

Strain-specific resistance conditioned by a single dominant gene in soybean cv. York inoculated with SMV-G1 was overcome by the resistance-breaking strain, SMV-G4, which induced lethal necrosis (N). Fifteen PR proteins extractable at pH 2.8 were found to accumulate in the N response and were named SPR (soybean pathogenesis-related) proteins. On the basis of major similarities in molecular weight characteristics and enzyme-substrate specificities, it is proposed that SPR proteins be classed into four groups: SPR1: 1a (17.2 kD), 1b (15.8 kD), 1c (14.5 kD) and 1d (17.5 kD); SPR2: 2a (33.5 kD), 2b (34 kD) and 2c (32 kD); SPR3: 3a (29 kD), 3a′(18.7 kD), 3b (29 kD), 3c (30 kD), 3d (31 kD) and 3e (29 kD); SPR4 (26 kD). The SPR1 and SPR4 groups accumulated predominantly in the N response, however their function has not yet been determined. The SPR2 group was identified as $\beta$-1,3-glucanases (GLN) and classified into two subgroups, SPR2a (GLN1) and -2b (GLN2) distinct from -2c (GLN3). The SPR3 group was identified as chitinases (CHN) and classified into three distinct subgroups based on the analysis of differential induction. The first class, SPR3a (CHN1) and -3b (CHN2), appeared to be constitutively expressed. The second class, SPR3c (CHN3), -3d (CHN4) and -3e (CHN5), appeared to be induced only in the N response. The unusual isoform SPR3a′ possesses different molecular weight and represents a third structural molecular class.

## INTRODUCTION

Certain cultivars of soybean (*Glycine max* (L.) Merr.) are known to react differentially to strains of soybean mosaic virus (SMV) (Cho and Goodman, 1979). The resistance of soybean to SMV is conditioned by a single dominant gene, *Rsv*, which has several alleles at the same locus that respond to resistance-breaking strains of SMV (Buss et al., 1989). In the York cultivar (cv.), $Rsv^y$ conditions complete resistance (R) to SMV-G1, an unrestricted necrotic hypersensitive resistance (N) to SMV-G4, and no resistance (S) to SMV-G5 (Buss et al., 1989). However, little is known about what viral and host factors are triggered in the *Rsv* resistance.

In a typical hypersensitive resistance such as tobacco inoculated with tobacco mosaic virus (TMV), small necrotic lesions are formed on the inoculated leaves within 4 days after inoculation (DAI), and spread of the virus to other parts of the plant is prevented by killing off the cells in the leaves which were inoculated. The necrotic reactions produced in the soybean/SMV system differ from those of the tobacco/TMV system, because SMV-induced necrosis is not localized in the inoculated leaves, but eventually becomes a systemic necrosis. Even though it might be regarded as expression of extreme sensitivity, the results of genetic studies indicate that necrotic plants should be classed as resistant when evaluating segregating populations (Buss et

al., 1989).

Pathogenesis-related (PR) proteins are well-known induced forms of resistance which are expressed in a variety of plants infected with various pathogens; viruses (Antoniw et al., 1980 ; Van Loon et al., 1987; Nasser et al., 1988), other necrotizing pathogens (Camacho-Henriquez and Sänger, 1982; Van Loon, 1989; Granell et al., 1987; Kombrink et al., 1988; Joosten and De Wit, 1989), or exposure to various chemicals (Antoniw and White, 1980; Hooft van Huijsduijnen et al., 1986; White et al., 1987). The concurrent induction of resistance and synthesis of PR proteins has led to speculation about mechanisms that cause the virus to be localized or that confer a certain degree of resistance to subsequent spread of virus in neighboring tissue (Fritig et al., 1989; Van Loon, 1989; Bol et al., 1990). In previous studies with tobacco cultivars reacting hypersensitively to infection with TMV, there is a close parallel in time between an increase in necrosis and the stimulation of enzyme activities, and between the phenomenon of acquired resistance and the accumulation of PR proteins (Kauffmann et al., 1987; Legrand et al., 1987).

Up to now, the work on PR proteins and genes encoding them has been mainly focused on the tobacco/TMV system. Five classes or groups of PR proteins have been established (Van Loon et al., 1987; Bol et al., 1990). In this group of proteins, charge isoforms of PR-1 group proteins have been most

investigated, however the functions of these proteins are still unknown. PR-2 group proteins (PR-2, N and O) have $\beta$-1,3-glucanase activity (Kauffman et al., 1987), whereas PR-3 group proteins (PR-P and Q) have chitinase activity (Legrand et al., 1987). These hydrolytic enzymes have been demonstrated to have an inhibitory effect on fungal growth in *in vitro* experiments (Mauch et al., 1988), to coordinate induction in response to infection (Legrand et al., 1987; Kauffmann et al., 1987; Kombrink et al., 1988), and to produce glycosidic fragments that are active as elicitors of secondary metabolite synthesis (Keen and Yoshikawa, 1983). Previous studies have led to the speculation that basic isoforms of $\beta$-1,3-glucanase and chitinase are predominantly localized in the vacuole; whereas, the structurally related acidic isoforms of both enzymes are secreted into the extracellular compartment (Kauffmann et al., 1987; Legrand et al., 1987; Boller, 1988; Van den Bulcke et al., 1989; Mauch and Staehelin, 1989). It has been proposed that the extracellular forms function as a frontline defense involved in the recognition process, while the vacuolar forms function as a last line of defense when cell lysis releases the vacuolar contents into the extracellular compartments (Mauch and Staehelin, 1989). Although these enzymes are also synthesized in response to virus infection, definite proof of an involvement in blocking virus movement as chemical barriers or inhibition of virus multiplication

is still lacking.

The broad objective of this research was to examine the possible role of soybean pathogenesis-related (SPR) proteins in the resistance mechanism of soybean cv. with different alleles of the *Rsv* gene to strains of SMV. The present paper is specifically aimed to demonstrate the accumulation of soybean pathogenesis-related (SPR) proteins, to characterize their involvement in the process of necrogenesis in soybean infected with SMV, and to define their enzymatic functions.

## Materials and Methods

**Inoculation of soybean with SMV-G1, -G4, and -G5.** The virus strains G1 (Hunst and Tolin, 1982), G4 and G5 (Cho and Goodman, 1979) were maintained in the greenhouse by regular passage in cv. Lee68 for G1 and G4, and cv. York for G5. Inoculum was prepared by homogenizing infected leaves of soybean maintenance cultivars in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% celite (Hunst and Tolin, 1982). A 1:10 sap dilution was rubbed with a pestle onto leaves either at the unifoliolate stage for detecting virus or at the 2-3 leaf stage for extracting acid-soluble proteins and enzymes. Mock-inoculated control was performed identically except buffer with celite alone was used.

**Virus detection by enzyme-linked immunosorbent assay (ELISA).** Antiserum against SMV-G1 (Hunst and Tolin, 1982) was used for the ELISA tests as previously described (Clark and Adams, 1977) with minor modifications to detect the presence of SMV. Selected six leaf disks were taken from three soybean plants at 6, 9, and 14 DAI with G1, G4, or G5. The crude antigen dilutions (1:50, 1:100 and 1:500) in phosphate-buffered saline solution (pH 7.4) containing 2% polyvinylpyrollidone (PVP) and 0.05% Tween-20, were added to the polystyrene microtiter plates (Dynatech Corp.) precoated with immunoglobulin G (IgG) (1 ug/ml) in 0.05 M sodium carbonate buffer (pH 9.6). After adding alkaline phosphatase (Sigma) conjugated IgG (1 ug/ml) and substrate (1 mg/ml in

10% diethanolamine buffer, pH 9.8), absorbance of the resulting color reactions was measured at 405 nm with the Bio-Tek EL 307 EIA Reader.

**Acid-soluble protein extraction.** Leaves were harvested 6 DAI and homogenized in acidic extraction buffer (pH 2.8) as described by Antoniw et al. (1980) for PR proteins. The homogenates were filtered and centrifuged at 10,000 g for 20 min, and ammonium sulfate was added to the supernatant to 60% saturation. After centrifugation, the pellet was resuspended in 0.05 M Tris-HCl (pH 7.5) containing 0.05% mercaptoethanol (Van Loon et al., 1987) and dialyzed overnight against 3 changes of electrophoresis buffer (0.025 M Tris, 0.192 M glycine, pH 8.3).

**Gel electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) in native conditions was performed at pH 8.3, according to Davis (1964), using 15% (w/v) polyacrylamide resolving gels and 4.5% (w/v) stacking gel (1.5 mm thickness). For denaturing conditions, SDS-PAGE was performed according to Laemmli (1970), using a 10% separation gel and Bio-Rad SDS-PAGE Low Molecular Weight Standards. After electrophoresis at constant 30 mA at room temperature, the gels were stained with Coomassie Brilliant Blue R-250 (Sigma) or subsequently double stained with silver (Mallinckrodt) according to Morrissey (1981).

**Two-dimensional (2-D) gel electrophoresis.** First PAGE was performed in the Davis system (1964) for separation of

native SPR proteins. The proteins in vertical strips were excised from native gels, denatured by heating for 5 min at 100°C in 125 mM Tris-HCl, pH 6.8, containing 2.5% SDS and 0.01% (w/v) bromophenol blue, and were transferred on top of a 1.5 mm thick 7.5-15% linear gradient polyacrylamide-SDS gel. Electrophoresis was for 4 hr at 20 mA at room temperature.

**Enzyme extraction.** Leaves (4 g) at 1-7 DAI with SMV-G4, and -G5, and mock-inoculated were homogenized in 0.1 M sodium citrate (pH 5.0) containing 4.8 mM 2-mercaptoethanol and 2.5% (w/v) insoluble PVP (5 ml of buffer per gram fresh weight). The homogenates were filtered through four layers of cheesecloth and centrifuged at 20,000 g for 20 min. The supernatant was adjusted to 20% saturation of ammonium sulfate and stirred for 30 min. The precipitate was removed by centrifugation. A second fraction between 20-75% saturation was obtained in the same manner by adjusting the supernatant to 75% ammonium sulfate. The latter precipitate was dissolved in 10 mM sodium acetate buffer (pH 5.2) and used for further assays.

**Assay for chitinase.** Colorimetric assay was carried out using a modification of Boller et al. (1983). For assay of exo- and endochitinase, the reaction mixture contained the enzyme extract solution, 0.1 ml of powdered chitin (5 mg/ml in 0.01 M Na Acetate, Sigma) and 0.1 ml of 0.01 M sodium acetate buffer (pH 5.2) in a total volume of 0.5 ml. It was

incubated at 37°C for 2 hr and then centrifuged at 10,000 g for 5 min. For the endochitinase assay, the 0.3 ml of supernatant was incubated at 37°C with 0.02 ml 3% (w/v) desalted snail gut enzyme (Boehringer Mannheim), and then added to 70 ul of sodium borate buffer (1M, pH 9.8). For the exochitinase assay, 100 ul of sodium borate buffer was added to the 0.3 ml of supernatant before heating for 3 min in a boiling water bath. In both exo- and endochitinase assays, 2 ml of the reagent was added and incubated at 37°C for 20 min. The reagent stock solution contained 8 g of 4-dimethylaminobenzaldehyde (Sigma) in 70 ml glacial acetic acid and 10 ml of concentrated HCl. The amount of liberated N-acetylglucosamine (GlcNAc) was determined according to Reissig et al. (1955). Enzyme and substrate blanks were included as well as internal standards. The activity was determined from a calibration according to Boller et al. (1983). One unit of chitinase is defined as the amount that catalyzes the release of soluble chitooligosaccharides containing 1 umol of GlcNAc in 1 min at 37°C.

**Assay for $\beta$-1,3-glucanase.** The activity of $\beta$-1,3-glucanase was assayed by measuring the rate of sugar production with laminarin (Sigma) as the substrate. The assay mixture contained in a total volume of 0.5 ml was: 100 ul enzyme extract, 250 ul of laminarin (2 mg/ml), and 10 uM of sodium acetate buffer (pH 5.2). After incubation at 37°C for 20 min, the reducing sugar content was determined with

the neocuproine method (Dygert et al., 1965). Standards of glucose and enzyme or substrate blanks were included. One unit was defined as the amount of enzyme which produces reducing groups equivalent to 1 mg of glucose per hour at 37°C.

**Protein determination.** Protein concentration was determined by the Bio-Rad protein microassay procedure, as described by Bradford (1976), with bovine serum albumin (Sigma) as a standard.

**Basic $\beta$-1,3-glucanases purification.** Soybean cv. York leaves (100 g) were harvested 7 DAI with SMV-G4 and homogenized in 0.1 M sodium citrate buffer (pH 5.0) containing 4.8 mM 2-mercaptoethanol and 2.5 % PVP. The homogenates were filtered through cheesecloth and centrifuged at 12,800 g for 20 min. The supernatant was heated to 50°C for 10 min, centrifuged at 12,800 g for 20 min, mixed with DEAE-cellulose (1 g/30 ml, Sigma) and filtered through filter paper (Whatman No. 1) on a Buchner funnel. The DEAE-cellulose filtrate was concentrated by adding ammonium sulfate to 60 % saturation and centrifuging at 12,800 g for 20 min. The pellet was resuspended with 0.05 M sodium phosphate buffer (pH 7.0), dialyzed against 3 changes of distilled water, and concentrated using freeze-dry vacuum (Speed Vac, Savant). The glucanase was then purified on a 30 X 1.5 cm hydroxyapatite (Sigma, type I) column at room temperature. The column was eluted with a linear gradient consisting of 120 ml of 0.005

M and 120 ml of 0.2 M sodium phosphate buffer (pH 6.8). After monitoring glucanase activity, the pooled fractions were separated on a 30 X 1.5 cm Sephadex G-100 (Sigma, 40-120 u bead size) column. The column was eluted with 0.05 M sodium phosphate buffer (pH 6.8) containing 0.1 M NaCl and 3 mM sodium azide. The fractions which had glucanase activities were pooled and concentrated with an Amicon centricon-10.

**Activity staining for detection of $\beta$-1,3-glucanase in native gels.** After 15% (w/v) PAGE in native conditions for separation of acid soluble proteins and purified $\beta$-1,3-glucanases, one-half gel (containing one set of treatments) was incubated with laminarin (2 mg/ml in 0.05 M Na acetate, pH 5.0) and then stained with 2,3,5-triphenyltetrazolium chloride (Sigma) to detect $\beta$-1,3-glucanase activity as previously described (Pan et al., 1989). The other half of the gel was incubated under the same conditions without laminarin to detect false bands. After staining, the gels were put into 7.5% acetic acid to reduce background, photographed, and then stained with Coomassie blue.

**Activity staining for detection of chitinase in gels.** Staining for chitinase activity after gel electrophoresis was performed according to Trudel and Asselin (1989). Gels were placed in close contact with a 7.5% (w/v) polyacrylamide overlay gel containing 0.01% (w/v) glycol chitin in 100

mM sodium acetate buffer (pH 5.0) and incubated at 37°C for 1 hr. The overlay gel was removed and stained by 0.01% (w/v) Calcofluor White M2R (Sigma) in 500 ml Tris-HCl (pH 8.9) for 10 min and destained by incubation for 1 hr at room temperature in distilled water. The lytic zones were marked by visualizing the gels on a UV-transilluminator and photographed with Polaroid type 55 film with UV-haze and orange filters. The resolving gel was then stained with Coomassie blue to identify protein bands corresponding to lytic zones in the overlay gel. The 15% (w/v) SDS-PAGE gels or 2-D gels were first incubated in 25 mM sodium acetate (pH 5.0) containing 1% (v/v) Triton X-100 for 18 hr at 37°C for renaturation of enzyme activity. Overlay gels without glycol chitin were processed in the same manner to detect false bands. Direct visualization of activity staining after 2-D gel electrophoresis was visualized by Calcofluor White M2R staining following Coomassie and aqueous silver staining (Trudel et al., 1990). The lytic zones appeared white as zones, since silver nitrate gives a uniform dark background, but does not stain where glycol chitin was lysed by chitinase.

## RESULTS

**Comparative concentration of SMV in differentially responded leaves.** SMV accumulation in the different areas from tissue at 9 DAI is shown in Table 1. The virus concentration in Lee and York cultivars varied depending upon inoculation with three different strains. No virus was detected by ELISA in the R response inoculated with SMV-G1 with York, consistent with the results of genetic studies (Roane et al., 1983). The virus concentrations were measured in both inoculated unifoliolate and uninoculated first trifoliolate leaves of plants exhibiting the S responses were higher than in all other responses. In the S responses more virus particles accumulated in upper uninoculated leaves than in lower inoculated ones at 9 DAI. The highest virus concentration was detected in upper leaves of the S response produced from G1/Lee combination. The selected necrotic leaf areas from the N response from G4/York combination were tested to detect the presence of SMV and resulted in negative values by ELISA.

**Identification of SPR proteins.** Extracts from the N response leaves contain several major bands of proteins visible after PAGE (Fig. 1A). We have chosen to call them "soybean pathogenesis-related (SPR)" proteins. There was a slightly greater accumulation of SPR proteins in the R response than in mock-inoculated leaves, but both responses had a much lower accumulation of SPR proteins than did the N

Table 1. Comparative concentrations of SMV in differential responses of soybean leaves.

| Strain /cultivar /response | DAI** | Dilution of virus sample* lower inoc. (veinal)[1] 1:50 | lower inoc. (interveinal)[2] 1:50 | upper inoc. (lateral)[3] 1:50 |
|---|---|---|---|---|
| G1/York R | 9 | 0.078±0.001[a] | 0.093±0.001 | 0.078±0.003 |
| G4/York N | 9 | 0.105±0.009 | 0.098±0.003 | 0.119±0.002 |
| G5/York S | 9 | 0.187±0.013 | 0.232±0.023 | 0.294±0.041 |
| G1/Lee S | 9 | 0.657±0.010 | 0.434±0.004 | 1.066±0.094 |
| G4/Lee S | 9 | 0.183±0.002 | 0.159±0.004 | 0.160±0.003 |
| G5/Lee S | 9 | 0.366±0.016 | 0.492±0.025 | 0.647±0.008 |
| Healthy/York | 9: | 0.094±0.008[b] | | |
| Healthy/Lee | 9: | 0.143±0.040 | | |

[a]Data are the mean and standard deviation $A_{405nm}$ values of duplicate readings.

[b]Data for healthy plants are the mean and standard deviation $A_{405nm}$ values of duplicate readings of 1:50, 1:100, and 1:500 dilution of crude sap

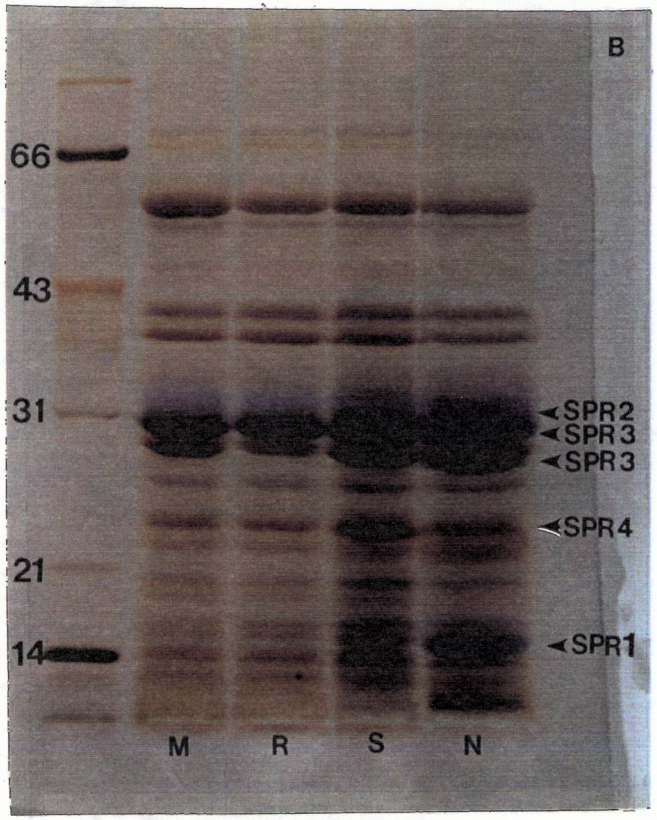
[1,2]Leaf disks were selected veinal and interveinal areas in lower inoculated leaves except G4/York (around local lesion & green tissue)

[3]Leaf disks were selected areas including both vein and intervein.

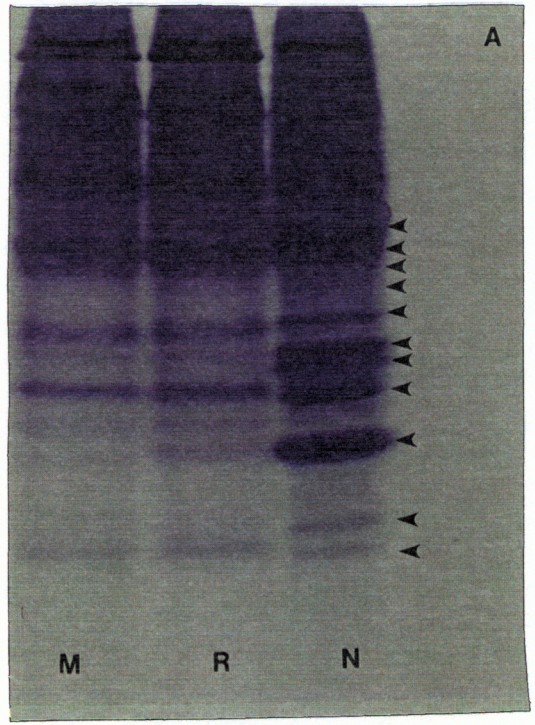
*Dilutions were made 1:50, 1:100, and 1:500 from crude sap. Values represent at 1:50 dilution only.

**Virus concentrations were measured at 6, 9 and 14 days after inoculation. Values represent at 9 DAI only.

**Fig. 1. A.** Electrophoretic patterns in 15% non-denaturing gel of acid soluble proteins extracted at pH 2.8 from soybean cv. York inoculated with buffer (Mock), SMV-G1 (R), and -G4 (N), respectively, 6 days after inoculation. All samples contained the same amount of protein (100 ug). The positions of SPR proteins are indicated by arrowheads. (Coomassie blue staining only) **B.** Electrophoretic patterns in 10% SDS-PAGE of acid soluble proteins extracted at pH 2.8 from soybean cv. York. Mock-inoculated (M), SMV-G1 inoculated (R), SMV-G5 inoculated (S), and SMV-G4 (N) inoculated. All samples contained the same amount of protein (100 ug). (Coomassie blue and silver staining)




10 % SDS-PAGE




15% PAGE

response.

Fig 1B shows denaturing separation of SPR proteins using the same extracts as applied into the non-denaturing gel (Fig. 1A). Leaves of host cultivar York, inoculated with SMV-G1 showed protein patterns similar to that of mock-inoculated leaves. However, major changes were noted in the N response induced by SMV-G4 inoculation. Similar to tobacco PR proteins, the additional and induced proteins in soybean were all of low molecular weights in the range of 15-33 kD. Although double staining (Coomassie blue & silver staining) revealed many additional bands (Fig. 2B), major SPR proteins were detected sufficiently by Coomassie blue staining alone (Fig. 2A).

The profiles of SPR proteins are generally similar to the profiles of tobacco PR proteins (Van Loon, 1987), which have been intensively studied using non-denaturing gel systems (Fig. 2A and B). After 2D-gel electrophoresis, the molecular weights of SPR proteins were estimated relative to molecular weight standard markers (Fig. 3A and 3B) and classified as to group and subgroup based on molecular and enzymatic characteristics (Table 2). We identified four major groups of SMV-induced SPR proteins in the N response, and designated them SPR1 (1a; 1b; 1c; 1d), SPR2 (2a; 2b; 2c), SPR3 (3a; 3a′; 3b; 3c; 3d; 3e), and SPR4, based on similarities in molecular weights and enzymatic functions. Two dimensional-gel electrophoresis showed clearly differences

**Fig. 2.** PR protein profiles under non-denaturing separation subjected to 15% PAGE gels. **A.** Coomasie blue staining. **B.** Coomassie and silver staining. lane 1; PR proteins induced by tobacco mosaic virus (TMV)/ tobacco Xanthi-nc (panel A and B). lane 2; SPR proteins induced by SMV-G4/ soybean York (panel A and B). All samples contained the same amount of protein (100 ug).

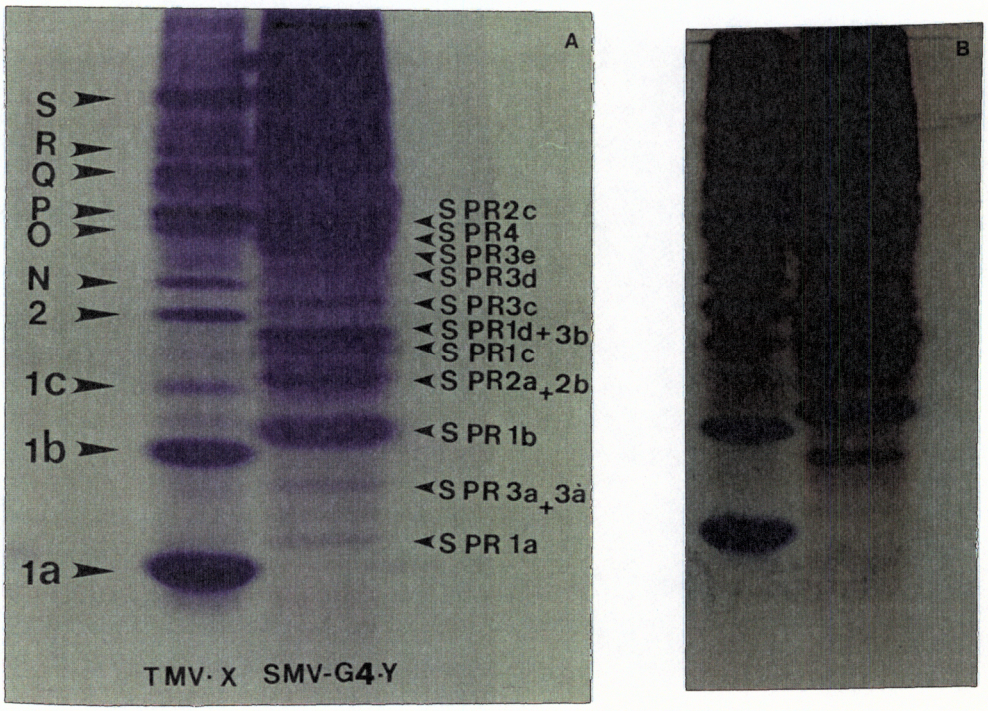

A
S
R
Q
P
O
N
2
1c
1b
1a
S PR2c
S PR4
S PR3e
S PR3d
S PR3c
S PR1d+3b
S PR1c
S PR2a+2b
S PR1b
S PR3a+3a
S PR1a
TMV·X SMV-G4·Y
B

Table 2. Classification of SPR proteins based on molecular weights and enzymatic characteristics

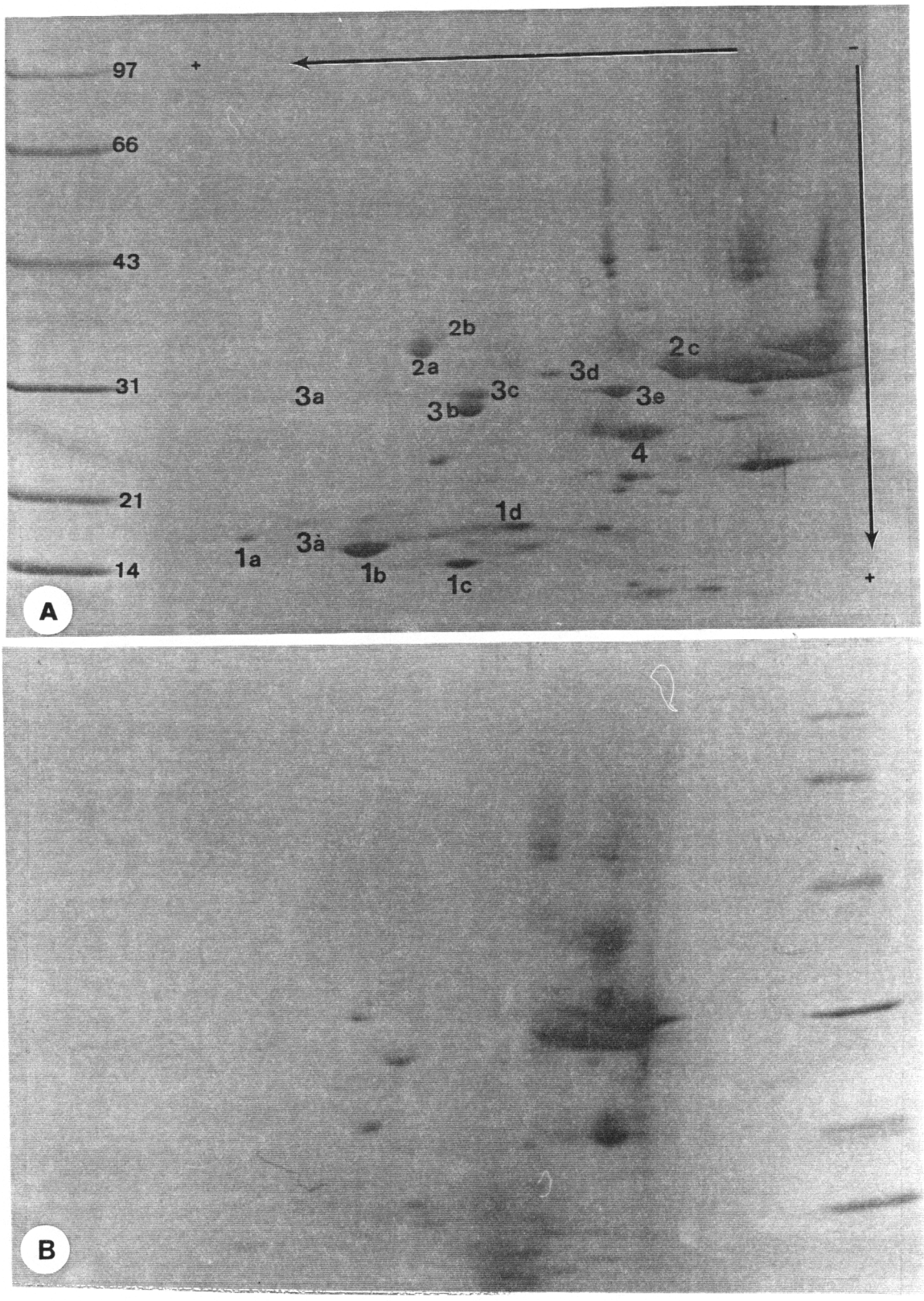
| Group | Name | Mol wt (kD) | characteristics | Function |
|---|---|---|---|---|
| SPR1 | 1a | 17.2 | N-enhanced | Unknown |
| | 1b | 15.8 | N-enhanced | |
| | 1c | 14.5 | N-enhanced | |
| | 1d | 17.5 | N-enhanced | |
| SPR2 | 2a (GLN1) | 33.5 | basic, N-enhanced | |
| | 2b (GLN2) | 34.0 | basic, N-enhanced | $\beta$-1,3- |
| | 2c (GLN3) | 32.0 | acidic,N-enhanced | glucanase |
| SPR3 | [a]3a (CHN1) | 29.0 | constitutive | |
| | 3b (CHN2) | 29.0 | constitutive | |
| | 3c (CHN3) | 30.0 | N-specific | chitinase |
| | 3d (CHN4) | 31.0 | N-specific | |
| | 3e (CHN5) | 29.0 | N-specific | |
| | [b]3a' | 18.7 | processed form | |
| SPR4 | | 26.0 | N-enhanced | Unknown |

[a,b]SPR3a and 3a' represent two polypeptide components of different molecular weights with similar net electric charges.

in accumulation of individual SPR proteins between N response and mock-inoculated leaves. In particular, 3 proteins that migrated as a single band after first-dimension electrophoresis and interpreted as a single protein in native gels (Fig. 2), were resolved to two proteins in the 2D-gel (SPR3a and -3a′; SPR2a and -2b; SPR1d and -3b) (Fig. 3A).

**Induction of Chitinase and β-1,3-glucanase activities during time course after SMV inoculation.** The time of initial appearance of necrotic lesions in the N response was about 4 days after inoculation. At this time chitinase activity increased markedly, while the activity was low in either the mock-inoculated leaves or the S response (Fig. 4A). There appeared to be a nearly three-fold increase in activity at 6 days after inoculation over that of mock-inoculated leaves. Exochitinase activities could not be detected in any responses, suggesting that these enzymes are endochitinases. Figure 4B shows a similar time course for β-1,3-glucanase accumulation. In the N response, β-1,3-glucanase activity increased at 4 days after inoculation. Enzyme activity during continuing development of necrosis increased by seven-fold over that of the S response at 7 days after inoculation. Buffer inoculation with abrasive-induced leaf surface wounding (mock-inoculated) did not induce an increase in the activity of either enzyme. An increase in the amount of 32 kD (SPR2), 30 kD (SPR3), and 29

**Fig. 3.** 2-D gel electrophoretic pattern of SPR proteins from (**A**) N response (100 ug) and (**B**) Mock-inoculated (100 ug); first dimension (horizontal arrow); electrophoresis in 15% non-denaturing gel; second dimension (vertical arrow); 7.5-15% SDS-containing gradient gel. Gels were stained with Coomassie blue. The identification of protein bands refers to the nomenclature of the bands after electrophoresis under native conditions (Fig. 2A). The positions of the mol. wt markers in kD are indicated. Arrows indicate the direction of migration.

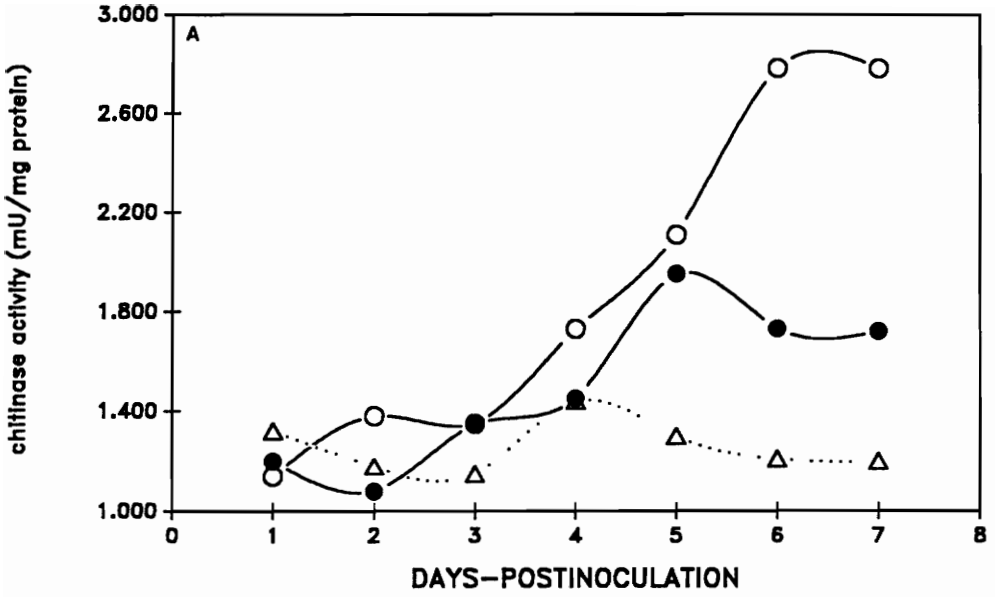

97
66
43
31
21
14
+
-
+
2b
2a
2c
3d
3a
3c
3e
3b
4
1d
3à
1a
1b
1c
A
B

kD (SPR3) proteins at 4-6 days post-inoculation in the N response, detected by 10% SDS-PAGE, correlated temporally with the increase in both β-1,3-glucanase and chitinase activity (Fig. 5).

**Purification of basic β-1,3-glucanases from the N response and determination of their molecular weights.** The basic forms of β-1,3-glucanases from extracts of the N response were separated by DEAE-cellulose adsorption, fractionated by ammonium sulfate precipitation, and further purified by hydroxyapatite chromatography and gel filtration chromatography. One major and three minor peaks were detected in the hydroxyapatite column (Fig. 6A), whereas a single major peak was detected in the Sephadex G-100 column (Fig. 6B). The protein recovered by purification, when analysed by 10% SDS-PAGE was a single protein band with a molecular weight of 32 kD, with very few contaminants (Fig. 7, lane 4,5). This band stained as a single isoform SPR2(a+b) of β-1,3-glucanase in native gel (Fig. 8B), however, it separated as dimeric forms corresponding to SPR2a and -2b in 2-D gel system (Fig. 3A) .

**Gel assay for detection of β-1,3-glucanase isoforms.** Before analyzing SPR proteins in gels for β-1,3-glucanase activity, we determined the reliability of the 2,3,5-triphenyltetrazolium chloride staining method (Pan et al., 1989), using tobacco PR proteins with known activities (Kauffmann et al., 1987) as a control (Fig. 8A). Activity

**Fig. 4.** Time course curve of SMV-induced (**A**) chitinase and (**B**) $\beta$-1,3-glucanase activities. Enzyme extracts were made at pH 5.0 after inoculation of soybean cv. York with buffer (△--△), with SMV-G5 (S, •-•), or with SMV-G4 (N, o-o). Values are the means of duplicate measurements.


A
chitinase activity (mU/mg protein)
3.000
2.600
2.200
1.800
1.400
1.000
0
1
2
3
4
5
6
7
8
DAYS–POSTINOCULATION

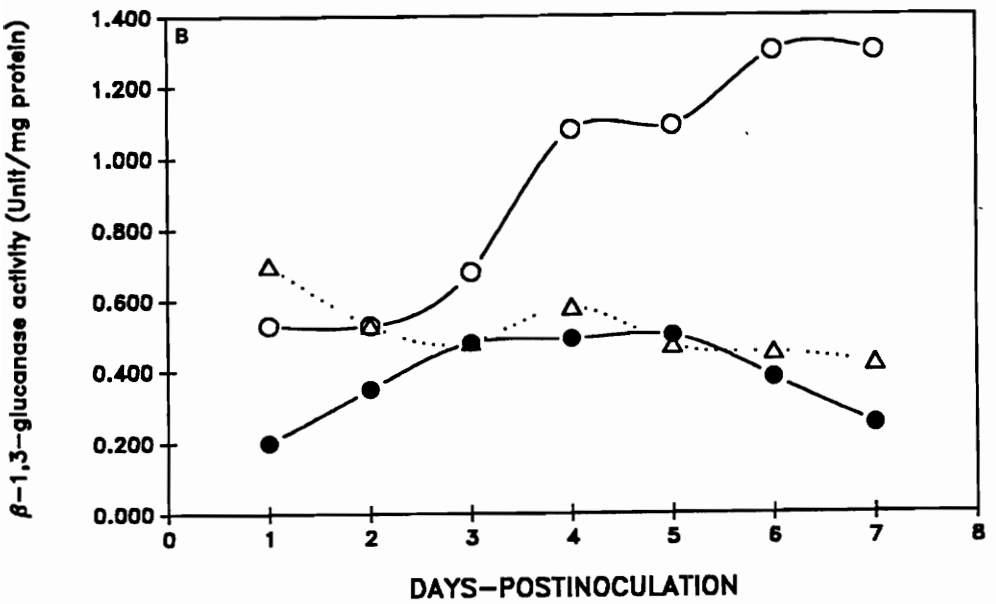
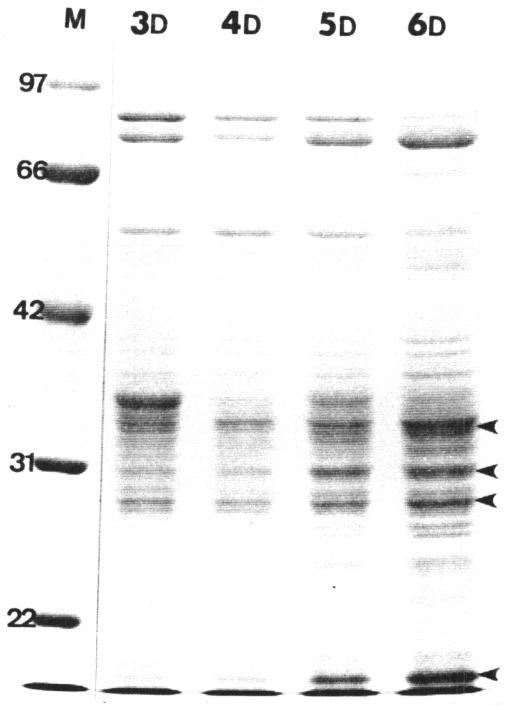
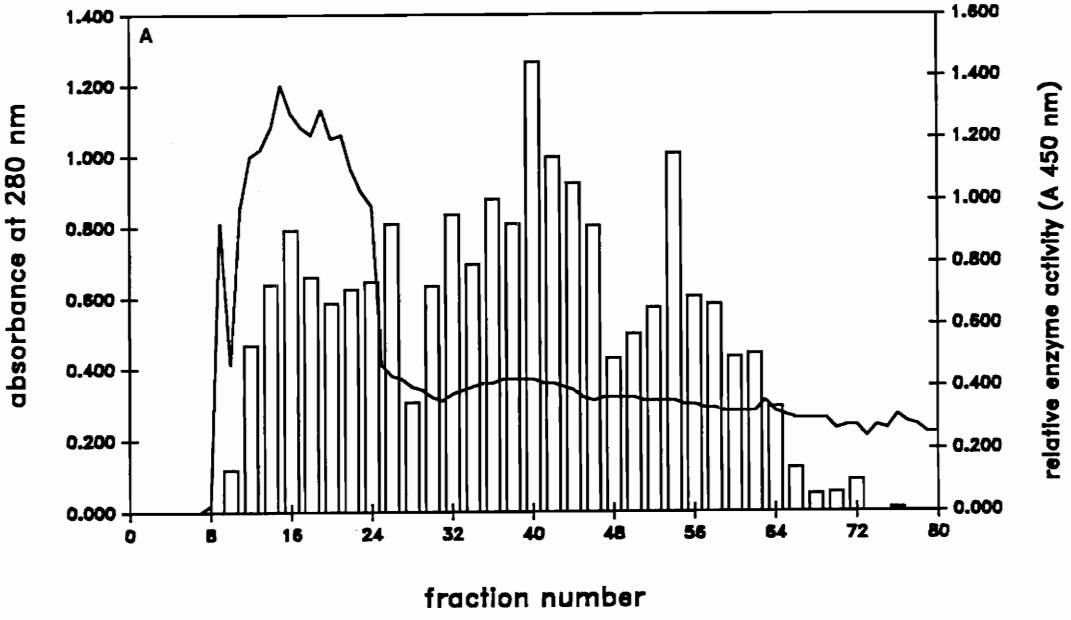

B
β–1,3–glucanase activity (Unit/mg protein)
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
1
2
3
4
5
6
7
8
DAYS–POSTINOCULATION

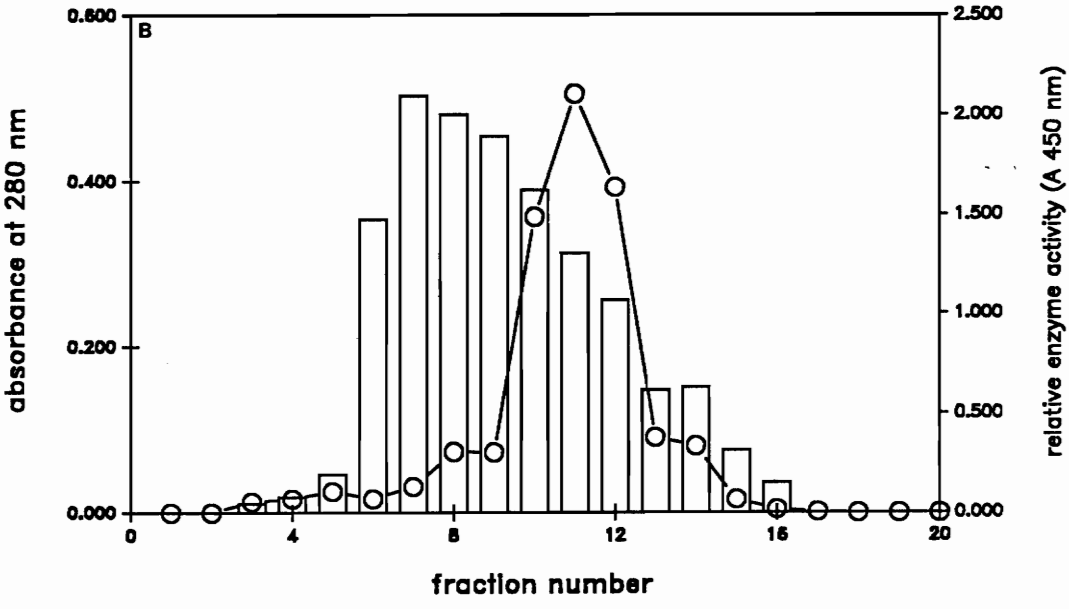
**Fig. 5.** Electrophoretic patterns of pH 5.0 extracts from N response showing the accumulation of the 15 kD (SPR1), 32 kD (SPR2) and 29 and 30 kD (SPR3) during the progress of SMV-G4 infection on York. Three to six days after inoculation (D) SPRs accumulating the most are indicated by arrows. The SPR proteins were separated on 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Molecular weight markers (M) are in kD.




10 % SDS – PAGE

**Fig. 6.** Chromatography of soybean $\beta$-1,3-glucanase on hydroxyapatite column (**A**) and Sephadex G-100 column (**B**). The fractions of both columns were monitored for protein by determining absorbance at 280 nm (line) and glucanase activity (absorbance at 450 nm in the neocuproine assay method; Bar) using laminarin as substrate.


A
absorbance at 280 nm
relative enzyme activity (A 450 nm)
fraction number
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
8
16
24
32
40
48
56
64
72
80


B
absorbance at 280 nm
relative enzyme activity (A 450 nm)
fraction number
0.600
0.400
0.200
0.000
2.500
2.000
1.500
1.000
0.500
0.000
0
4
8
12
16
20

staining of tobacco PR proteins revealed the presence of two minor and one major acidic $\beta$-1,3-glucanases, which correspond to PR-2, -N, and -O position visualized by subsequent staining with Coomassie blue and silver nitrate.

The SPR proteins in the N response with this method also revealed the presence of two major bands. One was designated GLN3 correponded to SPR2c, and the others were combined basic isoforms GLN(1+2) corresponded to SPR2(a+b) (Fig. 8A). The estimated molecular weights of $\beta$-1,3-glucanase polypeptides were SPR2a (33.5 kD), -2b (34 kD), and -2c (32 kD) (Fig. 3A). Three isoforms with enhanced activity of $\beta$-1,3-glucanases in SPR proteins were detected from the N response. However, basal level or less amount of three isoforms were present in R- or S response as well as mock-inoculated control (Fig. 8A and Fig. 3A,B).

**Gel assay for chitinase activity and correlation with enzyme isoforms.** There are certain levels of constitutively expressed chitinase activity in two major bands (CHN1 and CHN2) which migrated at the position of SPR3a and -3b in extracts of the mock-inoculated leaves, R- and S response (Table 2). In contrast, four major bands (CHN1, CHN2, CHN3 and CHN5) and one minor band (CHN4) were detected in the extracts of N response (Fig. 9A). Results thus indicated that three additional isoforms (CHN3, CHN4, and CHN5) were induced after SMV-G4 infection of cv. York leading to localized necrosis, and that these migrated at the position of

**Fig. 7.** Protein profiles on 10% SDS-PAGE gels during various steps in purification of basic $\beta$-1,3-glucanases. Lane 1, mol wt standards (kD); lane 2, 20 ug of protein after ammmonium sulfate precipitation; lanes 3, 5 ug of protein eluted from hydroxyapatite column; lane 4-5, 2 ug and 1 ug of proteins eluted from Sephadex G-100 column.

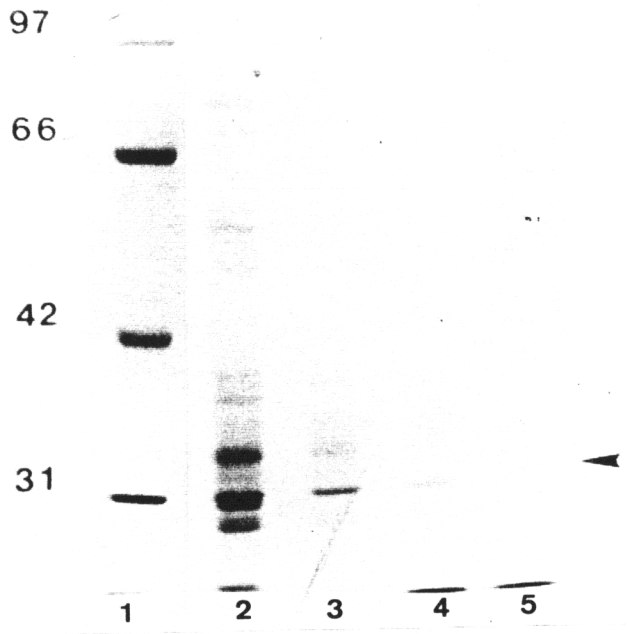
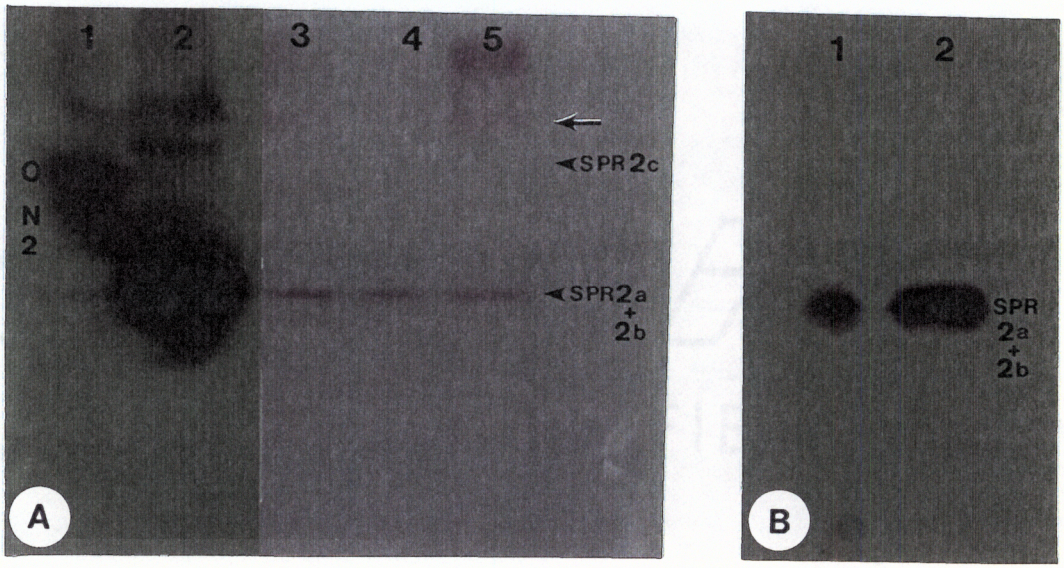

97
66
42
31
1
2
3
4
5

**Fig. 8.** Gel assay for detection of $\beta$-1,3-glucanase activities in SPR proteins after electrophoresis in a 15% PAGE. **A**, crude acid-soluble extracts (100 ug total protein) were loaded onto the gels; tobacco PR proteins (lane 1), N response (lane 2), Mock-inoculated (lane 3), R response (lane 4) and S response (lane 5). Arrow indicates false band. **B**, Purified $\beta$-1,3-glucanase from N response from soybean York inoculated with SMV-G4 (10 ug and 20 ug), appeared basic form of single activity band corresponding to SPR2(a+b).

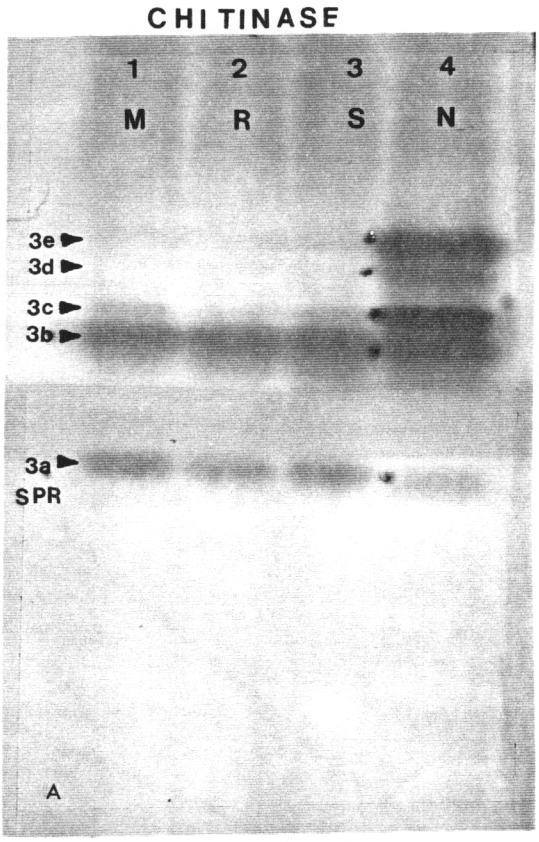

1
2
3
4
5
ON2
SPR 2c
SPR 2a + 2b
A
1
2
SPR 2a + 2b
B

the SPR3c, -3d and -3e.

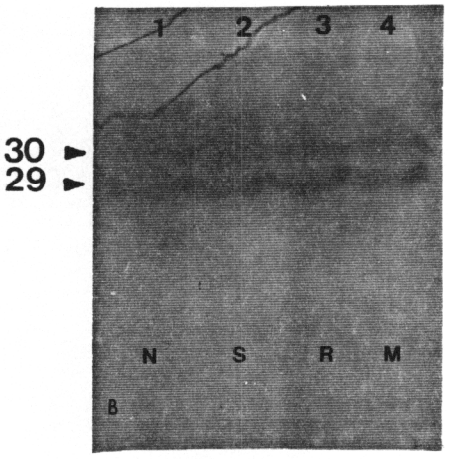
The SPR proteins were also analysed in denaturing gels to estimate the molecular weights of chitinases. The same leaf extracts as those used in Figure 9A, when subjected to SDS-PAGE and activity staining (Trudel and Asselin, 1989), indicated that two bands near 30 and 29 kD were present in the extracts of all responses after renaturation of enzyme activity (Fig. 9B). In order to obtain a better resolution of the chitinases, the methods for detecting chitinase activity as previously described (Trudel et al., 1989) was used to identify individual proteins with chitinase activity in the 7.5-15% SDS-linear gradient 2D-gel containing glycol chitin as substrates (Fig. 10). Results in Figure 10 indicated that acid-soluble PR proteins from the N response had six chitinase isoforms in contrast to the five forms in native one-dimensional gels. It appears that SPR3a and -3a', detected as a single band in native gels, are represented as dimeric forms with chitinase activities in 2D-gels (Fig. 10 A,B). The intensity of individual activity was also detected after Calcofluor white, Coomassie blue, and following silver nitrate staining method in 2D-gel system (Trudel et al., 1989). The system revealed negative staining of lysis zones visualized as dark (nonfluorescent) bands against a UV fluorescent background by calcofluor staining, turned into white lysis zone against a silver stained background. Therefore, the lysis zone within which no protein remained could

be detected by silver staining (Fig. 10B). Comparisons among individual chitinases displayed differences in the relative intensity of chitinases.

**Fig. 9. A,** Gel assay for detection of chitinase activity after electrophoresis in 15% polyacrylamide gel at pH 8.9. Acid-soluble extracts from Mock-inoculated (M) and R-, S- and N response inoculated with SMV strains were subjected to PAGE. **B,** Gel assay for detection of chitinase activity after electrophoresis in a 15% SDS-PAGE gel containing 0.01% (w/v) glycol chitin as substrate. The same extracts were subjected to SDS-PAGE from N-, S-, and R response, and Mock-inoculated leaves (M). Arrows indicate chitinases bands (29 and 30 kD).

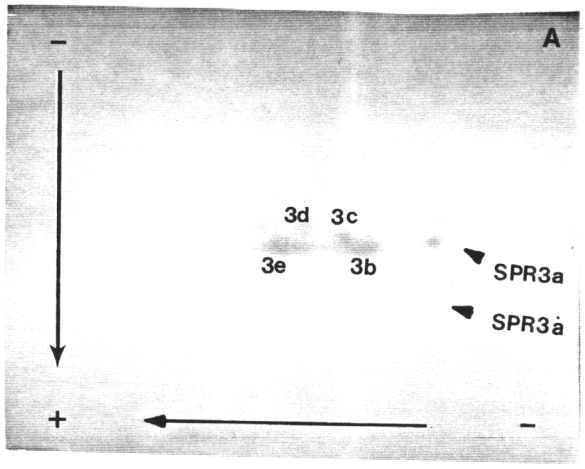



15 % PAGE

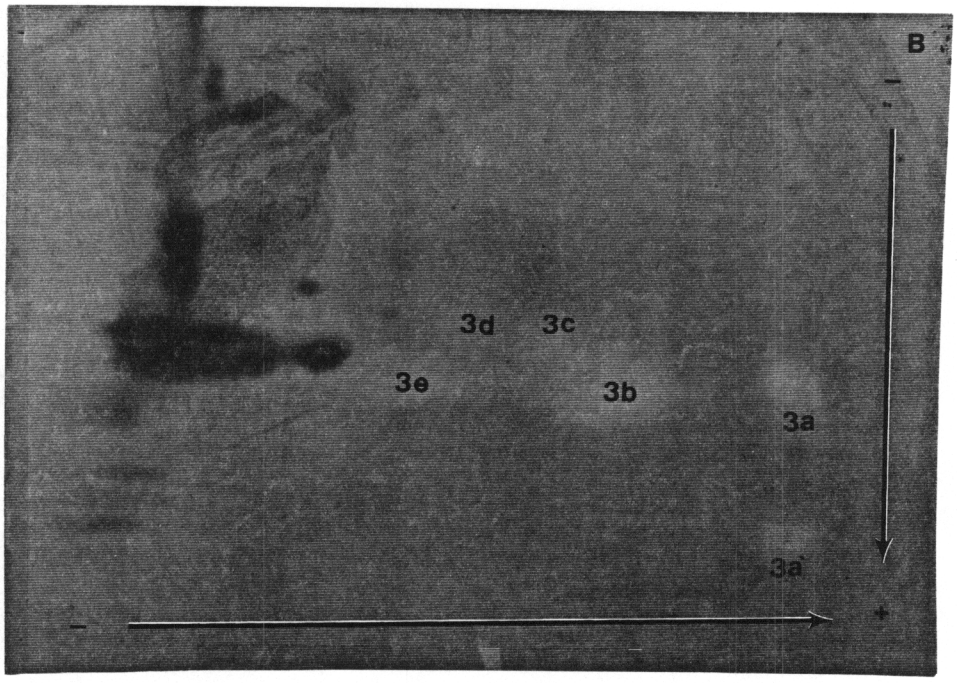



15% SDS - PAGE

**Fig. 10.** Gel assay after separation of proteins from N response in two-dimensional gel system. The SPRs were subjected to non-denaturing PAGE in the first dimension (horizontal arrows). After PAGE, gel slice was incubated in SDS buffer and subjected to 7.5-15% gradient of SDS-PAGE (top to bottom of panels). After SDS-PAGE, the gels were allowed to renaturate for 18 hr and stained with Calcofluor White M2R only (**A**), and following Coomassie Brilliant Blue R 250 and aqueous silver nitrate (**B**). Lytic activity against glycol chitin embedded in the SDS gel appears as dark spots in panel **A**, and as light zones in panel **B**.




7.5—15% 2D



## DISCUSSION

There are certain correlations between the concentrations of SMV in inoculated plants and the reaction of plant genotypes to the SMV strains. Based on ELISA values and symptoms (Table 1), the results on York corresponded to Cho and Goodman's (1979) classification system in which G1 represents the least virulent strain; G4 represents an intermediate virulent strain; and G5 represents a highly virulent strain. Thus, multiplication of the avirulent strain G1 may be inhibited by the host resistance gene, while strain G4 and G5 contain a virulence factor allowing multiplication to overcome the inhibitory effects of $Rsv^y$. The N response induced by G4 strain resulted in negative values by ELISA, suggesting that G4 strain had not multiplied sufficiently to give a positive reaction. These results provide additional support for the conclusion that necrotic responses should be classed as resistant and confirms results of Buss et al. (1989). However, the value obtained at 1:50 dilution for the upper leaves of York inoculated with G4 strain was weakly positive, suggesting that virus spread partially in plants expressing the N response.

It must be taken into consideration, however, that the two types of reaction differ in some respects. The R response progresses to a level giving complete protection, whereas in the N response protection is not complete since there is some virus movement. The high virus concentration

obtained in the S responses in both inoculated leaves and uninoculated upper leaves indicated that the virus multiplied and systemically spread from cell to cell and through the plant. Resistance mechanisms conditioned by the resistance gene may be more related to inhibition of virus replication than blocking of virus spread.

On the basis of major similarities in molecular weight characteristics and enzyme-substrate specificities, it is proposed that SPR proteins are classed into four groups: SPR1: 1a, 1b, 1c and 1d; SPR2: 2a (GLN1), 2b (GLN2) and 2c (GLN3); SPR3: 3a (CHN1), 3a', 3b (CHN2), 3c (CHN3), 3d (CHN4), and 3e (CHN5); SPR4 (Table 2). The acidic SPR1 group consisted of 1a, 1b, 1c and 1d, showing a single homogeneous band with low molecular weight of 15.5 kD (Fig. 1B) and four isoforms with apparent molecular weights in the range of about 14.5-17.5 kD. Relative to tobacco PR-1 group, the SPR1 group had similar molecular weights, and was predominantly accumulated in the N response. The function of SPR1 has not been determined. It will be interesting to test structural relationships between the tobacco PR-1 group and SPR1 group. Immunologically crossreacting homologues of tobacco PR-1 proteins have been found in tomato, potato, cowpea, maize and barley (Nassuth and Sänger, 1986; White et al., 1987), while a structurally unrelated PR-1 class has been found in parsley based on nucleotide sequence homology (Somssich et al., 1988).

Because several PR proteins in tobacco (Legrand et al., 1987; Kauffmann et al., 1987), potato (Kombrink et al., 1988), and maize (Nasser et al., 1988) were chitinases and/or β-1,3-glucanases, we tested enzymatic activities for the SPR proteins. The SPR2 group, consisting of SPR2a, -2b and -2c, was identified as β-1,3-glucanases, since they possess specificities to substrate and molecular weights similar to known soybean β-1,3-glucanases induced by fungal elicitors (Keen and Yoshikawa, 1983). Among them, basic isoforms, SPR2a and -2b, appeared as a single band in the native gel following activity staining, however they were resolved into distinct dimeric forms in the 2-D gel system. In the N response, isoform SPR2a was markedly enhanced in activity when combined with SPR2b, compared to constitutive expression of those in other responses. Three isoforms, SPR2a, -2b and -2C, are induced markedly in the N response. The purified basic β-1,3-glucanases corresponded to SPR2a and -2b, which appeared as combined form as a single band with enhanced activities. However, SPR2c appeared to have been lost by DEAE-cellulose adsortion. The absence of this isoform in the activity fractions after gel filtration chromatography resulted from separation of acidic and basic isoforms by anion exchange adsorption. It is possible that SPR2c is an acidic protein and localized distinct from SPR2a and -2b. Therefore, we can classify three isoforms into two subgroups of β-1,3-glucanase which may be encoded by differ-

ent structural genes and localized in different cellular compartments.

The SPR3 group, consisting of six isoforms, was identified as having chitinase activity, based on substrate specificities and molecular weights similar to known chitinases. Based on the analysis of differential induction, three distinct subgroups of chitinases can be classified. The first class, SPR3a and -3b, appeared to be constitutively expressed. Expression of the second class, SPR3c, -3d and -3e, appears to be induced only in the N response. The unusual isoform SPR3a′ possesses a different molecular weight and represents a third structural molecular form, possibly because of post-translational modification of SPR3a.

It has been previously demonstrated that a highly hydrophobic signal peptide with polysaccharide-binding domain is present in the N-terminus of basic chitinases, but is absent in acidic chitinases (Linthorst et al., 1989). In the $\beta$-1,3-glucanase, its basic form is synthesized as preproenzyme and is processed to the mature form with the processing of N-terminal hydrophobic signal peptide and C-terminal extension (Shinshi et al., 1988; Payne et al., 1990). Chitinases and $\beta$-1,3-glucanases exist in multiple isoforms which exhibit either acidic or basic isoelectric points, and are encoded by different genes. The enzymes are thought to be localized in different compartments of the plant (Hooft

van Huijsduijnen et al., 1987; Shinshi et al., 1987, 1988, 1990; Boller, 1988; van den Bulcke et al., 1989; Payne et al., 1990). It is questionable whether the different molecular forms of chitinase and $\beta$-1,3-glucanase of SPR's are products of different genes or different processing products of the same gene.

The question can be asked as to whether any N-specific isoforms of both glucanase and chitinase were elicited by the recognition signals of the G4 strain. Alternatively, they may represent forms of signal molecules that are specifically induced in plant cells as a secondary response to general stress. Both enzyme activities are induced with similar kinetics in ethylene-treated bean leaves, by various fungal elicitors and infection, and in hormonal regulation (Joosten and De Wit, 1989; Kombrink et al., 1988; Vögeli et al., 1988; Mauch et al., 1988; Boller, 1985; Boller et al., 1983). However, the induction of $\beta$-1,3-glucanase and chitinase activities with necrosis is an indirect argument for their role in defense. Neither of the enzyme activities increased with wounding (mock-inoculated), suggesting that they were infection-specific. The kinetics of induction of both enzymes was similar to that reported for tobacco chitinase and $\beta$-1,3-glucanase upon infection with TMV (Kauffmann et al., 1987; Legrand et al., 1987) or cucumber chitinase upon infection with tobacco necrosis virus (Metraux and Boller, 1986). The increases in $\beta$-1,3-glucanase and chitinase

activity in the N response include the induction of different molecular forms of $\beta$-1,3-glucanase and chitinase. Since SPR2b, -2c, -3c, -3d, and -3e are specific forms induced only in the N response, we can speculate that the induction of these may be related to a mechanism for necrotic symptom expression.

The basic assumptions may be questioned; Are SPR proteins involved in *Rsv* resistance? If they are involved in *Rsv* resistance, the degree of resistance between R- and N response would show temporal and quantitative relationships to SPR protein accumulation. If SPR proteins are not involved in resistance, what is their possible role? To address those questions, we assessed the accumulation of SPR proteins in differential responses of soybean inoculated with SMV strains. The accumulation of SPR proteins, chitinases, and $\beta$-1,3-glucanases in R response was much less than in the N response. It seems unlikely that these proteins directly protect soybean plants against SMV, and there was no significant relationship in R response between accumulation of SPR proteins and lack of infection with SMV. Therefore, our results indicate that there is no proportional or direct relationship between the accumulation of SPR proteins and the degree of resistance.

However, SPR proteins are associated with expression of the N response and may be involved in an active defense mechanism against SMV. The SPR proteins in the N response

from different cultivar-strain interactions were quite similar in protein profiles, and in the S response were similar as the mock-inoculated (data not shown). This observation provides additional evidence that SPR proteins are more likely involved in the necrogenesis rather than directly involved in the *Rsv* resistance or antiviral activity. However it seems evident that the essential requirement for resistance is for localization of virus from the primary infection. Even though SPR proteins are involved in virus-induced necrosis, their role in localization of the virus is still an open question.

In the case of induced tobacco PR proteins, studies with transgenic plants constitutively expressing PR-1 or PR-S genes have failed to show any direct involvement of PR proteins in the resistance to viruses or insects and to express a necrotic symptom (Linthorst et al., 1989; Cutt et al., 1989). Another recent observation has shown other PR proteins, such as $\beta$-1,3-glucanase and chitinase, accumulated in tobacco leaves not undergoing necrosis as well as in leaves undergoing necrosis, suggesting that these proteins were not sufficient to induce the formation of necrotic lesions associated with hypersensitivity (Godiard et al., 1990).

In preliminary tests for acquired systemic resistance, we observed a delayed development of fewer and much smaller lesions in response to subsequent challenging inoculation with SMV-G4 than developed in comparably inoculated healthy

plants (data not shown). In general, SPR proteins were induced in the inoculated lower leaves, when the interaction caused a necrotic reaction. Following systemic necrotic infection, the SPR proteins were also found even in the upper leaves when they had necrosis (data not shown). Like the HR response, accumulation of SPR proteins in the N response may be related a localization mechanism to block virus movement, but it is ineffective and results in systemic necrosis following veinal virus spread and limited virus replication. Based on these observations, we may assume that synthesis of the SPR proteins associated with tissues undergoing necrosis may be a series of molecular events or complex factors involved in development of necrosis. To our knowledge, this is first report to demonstrate induction of SPR proteins as well as enzyme activities in soybean infected with SMV.

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## Chapter V.

# SMV-Soybean Interaction III: Necrosis-triggered Defense Gene Expression of a Soybean Cultivar Inoculated with a Resistance Breaking Strain of Soybean Mosaic Virus

Chang W. Choi[1], Carole L. Cramer[1], Glenn R. Buss[2] and Sue A. Tolin[1]

[1]Department of Plant Pathology, Physiology and Weed Science;
[2]Department of Crop and Soil Environmental Sciences,
Virginia Polytechnic Institute & State University,
Blacksburg, VA 24061, USA

**ABSTRACT**

In order to understand viral resistance mechanism, we investigated soybean mosaic virus (SMV) accumulation and induction of well known defense genes, enzymes, and products in differential responses. Strain-specific resistance conditioned by a single dominant gene in soybean cv. York inoculated with SMV-G1, revealed no phenotypic symptoms and no detectable viral replication (R). Unlike the hypersensitive response (HR), the R response did not result in localized virus and induction of a cascade defense mechanism, but in inhibition of virus replication at the virus entry level. However, this resistance was overcome by resistance-breaking strain, SMV-G4, which induced lethal necrosis (N). Like HR, the N response induced a cascade defense mechanism. Unlike HR, G4 strain was not restricted but spread in a restricted pattern along the vein, stem and into upper uninoculated leaves where it induced necrosis. In this N response, there are marked increases, in a bimodal pattern, of phenylalanine ammonia-lyase (PAL) and peroxidase (POX) activities, bimodal pattern of PAL mRNA and chalcone synthase (CHS) mRNA induction, a single pattern of chitinase (CHT) mRNA induction, and glyceollin and lignin accumulation. These marked increases occurred at or just before the time of initial appearance of necrotic lesions, or following necrosis development for localization of virus.

## INTRODUCTION

The phenotypic symptom changes evident in a particular soybean cultivar (cv.) following inoculation depend upon the genotype of the cv. and the strain of virus. The soybean mosaic virus (SMV) resistance breaking strain, SMV-G4, overcomes resistance of soybean (*Glycine max* (L.) Merr) cv. York and often induces local and systemic necrosis (N), whereas SMV-G5 produces systemic mosaic symptoms (S). This form of strain-specific resistance (R) is conditioned by a single dominant gene in cv. York, which when inoculated with SMV-G1, produces no phenotypic symptoms and no detectable viral replication (Buss et al., 1989; Chen et al., 1991). This genetic basis for response suggests that soybean cultivars differ in their ability to recognize SMV strains and raises questions concerning the mechanism of the differential responses. A recognition event between a host resistance gene or its products and an interactive virus avirulence gene has been postulated to induce the resistance mechanism (Fraser, 1990). SMV-soybean interactions serve as an appropriate model system to study the molecular basis of the strain-specific resistance mechanism.

The incompatible plant-pathogen interaction characterized by a hypersensitive response (HR), is known to induce phytoalexin accumulation and lignification, and is among the best analyzed post-infection defense mechanisms. A good correlation has been demonstrated between expression of resis-

tance and onset of isoflavonoid phytoalexin accumulation concomitant with the rapid and coordinated, but transient, increases in the rates of synthesis of key regulatory phenylpropanoid-isoflavonoid pathway enzymes and corresponding mRNA, phenylalanine ammonia-lyase (PAL) or chalcone synthase (CHS) upon inoculation with fungal spores and hyphae, or treatment of different types of elicitors (Keen et al., 1983; Dixon et al., 1983; Lawton et al., 1983 a,b; Ryder et al., 1984; Bell et al., 1984; Cramer et al., 1985a,b; Hahn et al., 1985; Bonhoff et al., 1986a,b; Bhattacharyya and Ward, 1986,1987; Kochs et al., 1987; Latunde-Data et al., 1987; Tepper et al., 1989).

An activation of enzymes of the phenylpropanoid pathway has also been correlated with the induction of lignification (Moerschbacher et al., 1988). In contrast to phytoalexin accumulation, induced lignification of cell walls has been shown to occur in response to pathogens without also being elicited by a range of abiotic stress (Pearce and Ride, 1980). Thus lignification has been proposed as a structural defense expression of disease resistance (Vance et al., 1980; Moerschbacher et al., 1988). Peroxidases (POX) were shown to catalyze lignin polymerization *in vitro* (Mäder et al., 1980; Mäder and Füssl, 1982; Lagrimini and Rothstein, 1987). Chitinase (CHT) also has been shown to stimulate the biosynthesis of precursors of lignin (Pearce and Ride, 1982; Kurosaki et al., 1986). Although tobacco plants infected

with tobacco mosaic virus (TMV) were shown a number of years ago to have a marked increase of PAL activity in the hypersensitive response (Legrand et al., 1976; Massala et al., 1980), the induction of further steps in this pathway in response to viral infection has not been described in detail.

Viruses also trigger multiple changes involved in the defense system of host cells. Similar elicitation signals or early molecular events of metabolic pathways involved in virus resistance mechanisms are still in question. We describe the pathways involved and the expression of defense-related genes and products, and relate these results with SMV accumulation in differential responses of the cv. York inoculated with three SMV strains. The research was specifically aimed at determining the involvement of the necrotizing strain G4 in inducing key enzymes of the phenylpropanoid-isoflavonoid pathway and lignification. We investigated the induction of PAL and POX activities, as well as expression of PAL, CHS, and CHT mRNA in the differentially responding tissues.

## MATERIALS AND METHODS

**Virus.** Soybean mosaic virus strains G1 (Hunst and Tolin, 1982), G4 and G5 were maintained in the greenhouse by mechanical inoculation on soybean seedlings (Cho and Goodman, 1979). Soybean cv. Lee was used for G1 and G4, and cv. York was used for G5. On cv. York, G1, G4 and G5 gave R, N, and S responses, respectively (Choi et al., in preparation).

**Inoculation of soybean with SMV.** Inoculum was prepared by homogenizing infected soybean leaves in a chilled mortar and pestle in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% celite and rubbed with a pestle onto leaves with 1:10 sap dilution (Hunst and Tolin, 1982). Plants for virus maintenance were inoculated at the unifoliolate stage, but all other test plants were at the second or third trifoliolate stage. Buffer with celite alone were used for mock inoculations.

**Dot-Immunobinding assay (DIBA).** The DIBA was carried out following the procedure of Hammond and Jordan (1990), with minor modifications, to determine SMV presence in differential responses. SMV-G1, -G4 and -G5 strains were purified from systemically infected leaf tissues of cv. Lee or York using the methods of Hunst and Tolin (1982). Crude extracts of the viruses from cv. York at 4 and 12 days after inoculation (DAI) with G1, G4 and G5, were prepared by grinding leaf tissues at 1:10 (w/v) in TBS buffer (0.02 M Tris, 0.15 M NaCl, pH 7.5) containing 50 mM DIECA (diethyldithiocarba-

mate). After filtering through cheesecloth, the homogenates were clarified with chloroform, followed by low speed centrifugation (10,000 g for 10 min). One hundred ul of diluted samples (diluted with TBS + 100 mM DIECA) were applied to Nytran membrane (Schleicher & Schuell, 0.45 um pore diameter) using the MilliBlot-D system (Millipore). After blotting, the membrane was blocked by soaking in 5% nonfat dry milk (Carnation) in TBS for 10 hr at room temperature with shaking, and then incubated 2 hr in rabbit IgG against intact SMV-G1 diluted 1:1000 with TBS. After three rinses of 10 min each with TBS, the membrane was incubated in alkaline phosphatase (Sigma) conjugated rabbit IgG against SMV-G1 diluted 1:1000 in TBS, containing 5 mM $MgCl_2$, for 2 hr with gentle shaking. After four rinses with TBS, the membrane was incubated in 5 mg of nitroblue tetrazolium (NBT) in 15 ml of 0.1 M Tris-HCl buffer, pH 9.5, with 0.1 M NaCl and 5 mM $MgCl_2$, plus 2.5 mg of 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) in 50 ul of N,N-dimethyl-formamide.

**PAL enzyme extraction and assay.** All procedures were carried out at 0-4$^{o}$C. After the appropriate period after inoculation, 4 g of inoculated leaves were homogenized in 20 ml of 0.1 M Tris-HCl (pH 7.5), containing 5 mM 2-mercaptoethanol with 0.4 g of insoluble polyvinylpyrrolidone, in a chilled mortar. Homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000 g for 20 min. The supernatant solution was stirred for 20 min with 10% of

Dowex 1X2 ($Cl^-$form, equilibrated with Tris-HCl buffer), and filtered through glass wool. The precipitate was removed by centrifugation and the supernatant was used as enzyme extract. Phenylalanine ammonia-lyase activity was determined by a spectrophotometric assay (Latunde-Data et al., 1987). The reaction mixture (1.2 ml) consisted of 0.4 ml of 30 mM L-phenylalanine, 0.4 ml of 50 mM Tris-HCl buffer (pH 8.8), and 0.4 ml of enzyme extract. D-phenylalanine was substituted as the substrate in control tubes. The assay mixtures were incubated at 40°C for 2hr and the conversion of cinnamic acid monitored at 290 nm. The specific activity of PAL was expressed as ukat/mg protein. One unit of enzyme activity (1 uKat) is defined as the amount of enzyme required for the synthesis of one umol of product in one second under the assay conditions specified.

**Extraction and determination of glyceollin content.** One gram of tissue was extracted by boiling 2 min in 95% ethanol, which was then passed through Whatman No.1 paper. The residual tissue was transferred to a preweighed aluminum foil boat and dried overnight in an oven at 80°C. The filtrates were concentrated to dryness under reduced pressure at 40°C, and the dried residues were extracted three times with 2 ml of ethyl acetate. The combined ethyl acetate fractions were reduced to dryness, redissolved in 100 ul of 95% ethanol, and applied to a TLC (Whatman LK5DF silica gel) plate using a benzene:methanol (95:8) solvent system. The

glyceollin isomers were detected as a single band by fluorescence quenching under UV light, and identified by comparison with published reports (Bhattacharyya and Ward, 1987). The corresponding silica gel was scraped from the plate and eluted with 95% ethanol. The glyceollin concentration was calculated from the maximum absorbance at 285 nm and the extinction coefficient (=10,300) as previously described (Ayers et al., 1976; Lazarovits and Ward, 1982).

**POX extraction, assay and electrophoretic analysis.** Tissue was homogenized in cold (-20°C) 70% acetone (15 ml/g fresh wt tissue) for 2 min at top speed with a polytron homogenizer (Tekmar). The homogenate was filtered through Whatman No.1 paper on a pre-chilled Buchner funnel. The homogenate was rinsed three times with 100% acetone. After air drying the residues, acetone powders were dissolved in 1 ml of 0.1 M sodium phosphate buffer (pH 6.0) and 1% sodium bisulfite as a reducing agent. The slurry was centrifuged at 14,000 g for 10 min and the supernatant was used in enzyme assays. Total POX activity was determined by the increase in absorbance at 470 nm in 0.28% guaiacol, 0.05 M sodium phosphate buffer (pH 6.0), and 0.3% $H_2O_2$ (Lagrimini and Rothstein, 1987). Activity was expressed as the increase in absorbance at 470 nm $min^{-1}$ $mg^{-1}$ protein. Polyacrylamide gel electrophoresis (PAGE) under native conditions was performed at pH 8.3 according to Davis (1964) using 15% (w/v) polyacrylamide resolving gels (1.5 mm thickness) and 4.5% (w/v)

stacking gel. The gels were stained for 15 min in a solution containing 50 mg of 3-amino-9-ethylcarbazole, 10 ml of N,N,dimethylformamide, 63 ul of 30% $H_2O_2$, and 190 ml of 0.05 M sodium acetate buffer (pH 5.5) and rinsed in 10% methanol, 5% acetic acid (Kerby and Somerville, 1989)

**Protein determination.** Protein concentration was determined by the Bio-Rad protein microassay procedure according to Bradford (1976), with bovine serum albumin as a standard.

**Lignin extraction and determination.** Lignin was assayed quantitatively by derivatization with thioglycolic acid (Bruce and West, 1989) from alcohol-insoluble residues (AIR) of SMV-G4 and mock inoculated leaf tissues. The 4 g of leaf tissues were homogenized in 80 ml of absolute MeOH, filtered over Whatman GF/A, rinsed with MeOH, and dried for 24 hr at 60$^{o}$C in an oven. The resulting AIR was used for lignin determination. To 50 mg of AIR in glass screw-cap tubes was added 5 ml of 2N HCl and 0.5 ml of thioglycolic acid. The sealed tubes were placed in an oven for 4 hr at 95$^{o}$C. After cooling, the contents were centrifuged at 30,000 g for 10 min and the supernatant discarded. The resulting pellet was resuspended in 5 ml of 0.5 N NaOH, sealed with parafilm, and agitated gently at 25$^{o}$C for 18 hr to extract the lignin thioglycolate (LTGA). The samples were centrifuged at 30,000 g for 10 min and the supernatant solutions transferred to glass conical centrifuge tubes. One ml of concentrated HCl was added to each tube and the LTGA allowed to precipitate

at 4°C for 4 hr. Following centrifugation in a clinical centrifuge at top speed for 10 min, the orange-brown pellet was dissolved in 10 ml of 0.5 N NaOH, and the A at 280 nm was measured.

**RNA extraction.** Three grams (fresh weight) of the leaf tissues at 0, 3, 6, 9 and 12 hr after inoculation at the second trifoliate stages with SMV-G4 or SMV-G5 were frozen and stored at -70°C until used for the short term time course. For the long term time course, leaves from plants 1 through 4 DAI with SMV-G1 and SMV-G5, or 1 through 5 DAI with SMV-G4 were used. The frozen tissues were ground to a powder with liquid nitrogen and homogenized in a mixture of phenol and 0.1 M Tris-HCl (pH 9.0) as described previously (Haffner et al., 1978).

**Preparation of cDNA probes.** Bean cDNA probes for PAL, CHS and CHT were provided by Dr. C. J. Lamb (Salk Institute, La Jolla, CA). Plasmid DNA was purified by equilibrium centrifugation in cesium chloride and ethidium bromide. Prior to nick translation, pPAL5 was digested with *PstI* yielding a 1.8 kb insert (Edwards et al., 1985), pCHS1 was digested with *EcoRI* yielding a 1.4 kb insert (Ryder et al., 1984) and pCHT12.3 was digested with *KpnI* yielding 0.65 kb (Hedrick et al., 1988). All fragments representing only the bean cDNA inserts were isolated via electrophoresis, staining with ethidium bromide, and eluting gel-purified fragment using "geneclean" (Bio 101, Inc.).

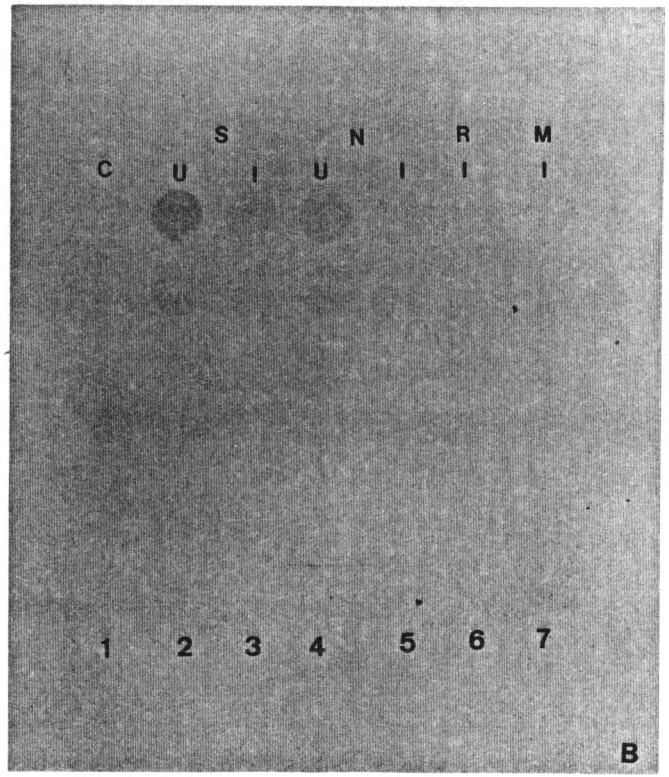
**Northern hybridizations.** Total RNA was denatured with glyoxal, and separated by electrophoresis on a 1.2% agarose gel in 10 mM sodium phosphate buffer (pH 7.0), and then blotted onto Nytran membranes (Schleicher & Schuell). Hybridizations under moderate (or low) stringency conditions was conducted at 42°C (37°C) in a buffer containing 40% (or 30 %) formamide, 5x (or 6x) SSC, 5x Denhardt's solution, 5mM EDTA, 0.1% SDS, and 100 ug denatured salmon sperm DNA. Filters were prehybridized for 24 hr in the same buffer without probe, followed by hybridization for 48 hr in the presence of $P^{32}$-labelled probe prepared by random-primer labeling (Multi-prime Labeling System, Amersham, UK). Filters were washed twice in 2x SSC plus 0.1% SDS for 15 min at room temperature and rinsed for 15 min in 0.1x SSC plus 0.1% SDS at room temperature. Autoradiography was performed using Kodak X-Omat AR film with intensifying screen at -70 C.

## RESULTS

**Determination of SMV concentration in differential responses by DIBA.** In addition to specifically recognizing its corresponding antigen, the anti SMV-G1 antiserum was found to cross-react with the purified virus of SMV strains (Fig. 1A). As little as 20 ng of the purified SMV was detected by the antiserum. Dilution of crude sap (1:100) from S- and N response yielded positive reaction with a decreasing dot color intensity. In both responses more virus particles accumulated in upper uninoculated leaves than in lower inoculated ones at 4 and 12 DAI (Fig. 1A,B). The highest virus concentration could be detected in upper leaves of plants exhibiting the S response at 12 DAI. No virus was detected in leaves from plants showing the R response and mock-inoculated leaves at either 4 or 12 DAI. No cross-reactivity was observed with TBS buffer (Fig. 1A lane 12, 1B lane 1).

**SMV-induced changes in PAL enzyme activity and glyceollin accumulation.** Significant differences in PAL activity among extracts from York plants inoculated with the three SMV strains interactions and mock inoculated controls could be detected over the time intervals studied (Fig. 2A). The PAL activity increased markedly in both the R- and N responses, showing bimodal activities over time. In the R response the transient first and slower second peaks in activity were found at 24 and 48 hr after inoculation, and

Figure 1. Identification of soybean mosaic virus (SMV) in crude sap of differential responses by dot-blot immunobinding assays. (A). Inoculated lower leaves (I) and uninoculated upper leaves (U) with SMV-G1 (R, lane 1, 2); -G4 (N, lane 3, 4); -G5 (S, lane 5,6); mock-inoculated control (M, lane 7, 8) were extracted in 1:10 (w/v) in TBS + 50 mM DIECA 4 days after inoculation. Samples were diluted in TBS + 100 mM DIECA and applied in the wells at final dilutions of 1:50, 1:100, 1:500, 1:1,000, and 1:10,000. Purified SMV-G1 (lane 9), -G4 (lane 10), and -G5 (lane 11) was diluted in TBS + 100 mM DIECA and applied in 100 ul at final dilution of 2 ug, 400 ng, 20 ng, 2 ng, and 0.4 ng (top to bottom). (B). Crude saps were extracted from leaf tissues 12 days after inoculation in S- (lane 2, 3), N- (lane 4, 5), R response (lane 6), and mock-inoculated control (lane 7). After blocking the non-specific binding sites, the membranes were sequentially incubated with polyclonal antibody (anti SMV-G1 IgG), anti SMV-G1 IgG-conjugated to alkaline phosphatase, and NBT-BCIP as described in Materials and Methods.


S N R M
C U I U I I I
1 2 3 4 5 6 7
B

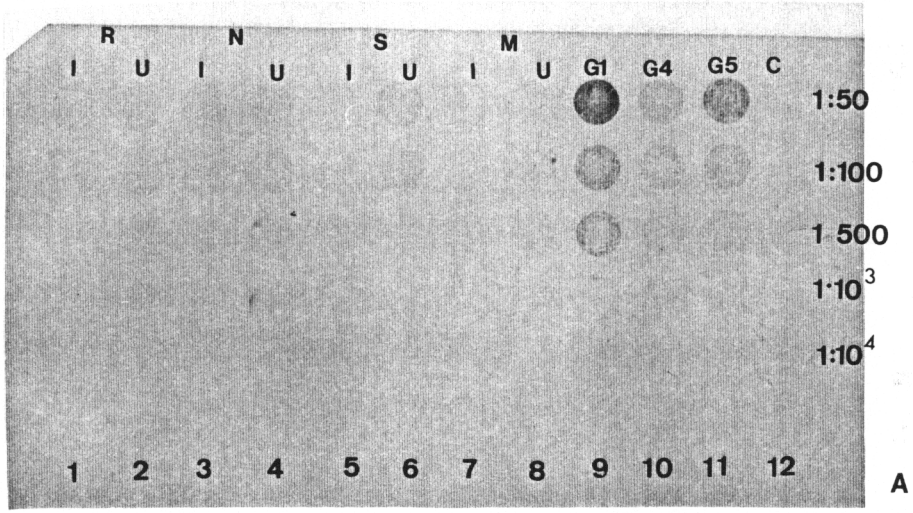
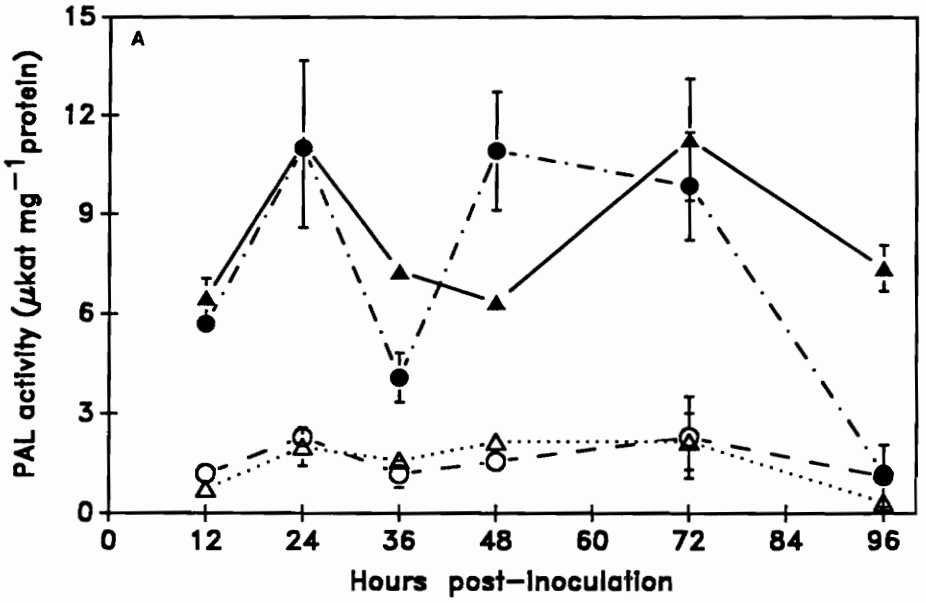
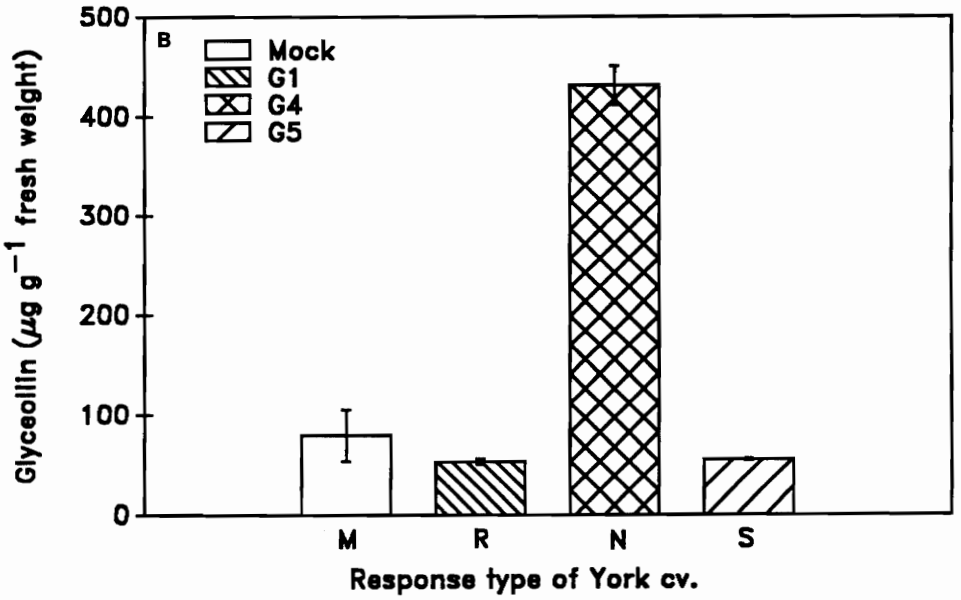

R N S M
I U I U I U I U G1 G4 G5 C
1:50
1:100
1 500
1·10³
1:10⁴
1 2 3 4 5 6 7 8 9 10 11 12
A

Figure 2. (A). SMV-induced changes in phenylalanine ammonia-lyase (PAL) with respect to time after inoculation with SMV-G1 (dashed-dot-dashed line), -G4 (solid line), -G5 (dotted line), mock-inoculated leaves (dashed line). Conversion from L-phenylalanine into cinnamic acid was measured as absorbance at 290 nm at 0, 12, 24, 36, 48, 72, and 96 hr after inoculation with SMV strains. Each plotted value represents the mean of triplicate experiments with standard error (I bar). (B). Glyceollin accumulation in SMV-G1 (R), -G4 (N), and -G5 (S) inoculated leaf tissues 6 days after inoculation. The concentration of glyceollin was determined by thin layer chromatography and spectrophotometric assays described in Materials and Methods. Each bar represents the mean values of triplicate experiments with standard error (I bar).


A
PAL activity (μkat mg$^{-1}$protein)
15
12
9
6
3
0
0
12
24
36
48
60
72
84
96
Hours post-Inoculation


B
Mock
G1
G4
G5
Glyceollin (μg g$^{-1}$ fresh weight)
500
400
300
200
100
0
M
R
N
S
Response type of York cv.

then declined slowly until 72 hr after inoculation. In the N response the first and second peaks were found at 24 and 72 hr after inoculation, and then declined just before appearance of the initial necrotic lesion. Maximum PAL activity was about 5 times higher with both R- and N response than in those of either S response or mock-inoculated control.

The concentrations of glyceollins were determined on leaves of cv. York inoculated with three strains of SMV, as well as mock inoculated control, at 6 DAI. The level of glyceollins produced by the N response was sharply higher (approximately 8-9 fold) than in other responses (Fig. 2B). Even though high PAL enzyme activity was found in leaf tissues of R response, no increase above the basal level of glyceollins was observed.

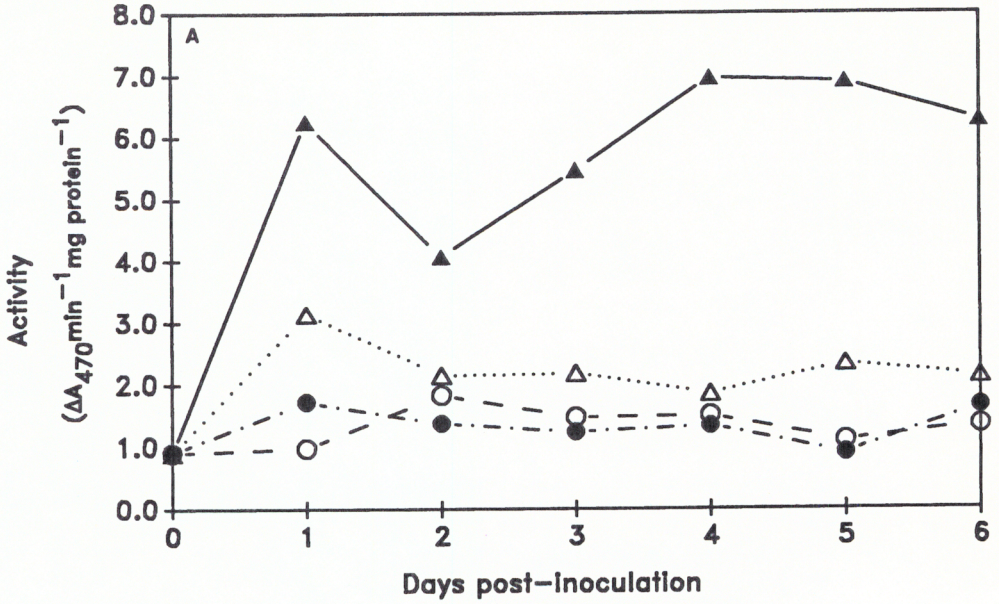
**SMV-induced changes in POX activity and isoforms.** As shown in Fig. 3A, POX activities of R response as well as mock-inoculated control remained constant throughout the time course at 0-6 DAI. No increase due to surface wounding damage by abrasives used in inoculation was observed. In the N response, we could observe two peaks in POX activity similar to those of PAL activity. An early and transient peak appeared rapidly at 1 DAI, but declined at 2 DAI. POX increased gradually to a second peak at 4 DAI. At this time, the N response was 7 times higher than those of other responses and continued up to 5 DAI. Rapid but weak induction of POX activity was found in S response at 1 DAI. PAGE

gel with enzyme staining revealed that elevated peroxidase activity in the N response was the result of increased synthesis of a specific subset of the anionic isoforms, as shown in Fig. 3B. There are 8 isoforms of POX in N response, while 6 isoforms are present in other responses as well as mock-inoculated control. Isoforms 5 and 6 were detected as additional bands and isoforms 3, 4, 7 and 8 appeared to have enhanced activities in N response (Fig. 3B).

**Time course of lignin biosynthesis in necrotic response.** The UV spectrum of a solution containing 100 ug/ml of the LTGA preparation in 0.5 N NaOH from 6 days after inoculation with SMV-G4 is presented in Fig. 4A. It is very similar to previously reported spectra for LTGA (Bruce and West 1989). Lignin contents were measured from AIR of necrotic response for 1-6 DAI. The time course of lignin accumulation is very similar in profile to the pattern of time course of POX activity. The first detectable increase in lignin occurred at 5 DAI in N response. Lignin levels continue to increase following necrosis development (Fig. 4B).

**mRNA induction of PAL, CHS, and CHT by Northern analysis.** The effects of SMV strains on accumulation of PAL and CHS mRNA in the York cv. were compared in a short-term time course. To determine if the increase in PAL activity was correlated with increased level of mRNA encoding these enzymes induced by SMV, total RNA was isolated at the time of inoculation (0 hr) or 3-12 hr after inoculation for short

Figure 3. The appearance of enhanced peroxidase activity (POX) and isoforms. (A). Time-course of SMV-induced POX activities. Dashed-dot-dashed lines represent leaves in which R response was induced by inoculation with SMV-G1; solid lines represent leaves in which N response was induced by inoculation with SMV-G4; dotted lines represent leaves in which S response was induced by inoculation with SMV-G5; dashed line represents mock-inoculated leaves. POX activity is expressed as changes in absorbance (470 nm) $min^{-1}mg^{-1}$ protein using guaiacol as the hydrogen donor. Values represent the mean of two experiments. (B). Induction of peroxidase isoforms in 15% native polyacrylamide gel electrophoresis. Enzymes extracted 6 days after inoculation from mock-inoculated (lane 1), R- (lane 2), S- (lane 3), N response (lane 4) and tobacco Xanthi inoculated with TMV (lane 5). Gels were stained for POX activity using 3-amino, 9-ethylcarbazole as described in the Materials and Methods. Numbers represent individual POX isoforms and are numbered from the bottom to top. Arrowheads indicate the distinctive tobacco anionic isozymes.


A
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Activity
($\Delta A_{470} min^{-1} mg\ protein^{-1}$)
0
1
2
3
4
5
6
Days post-Inoculation

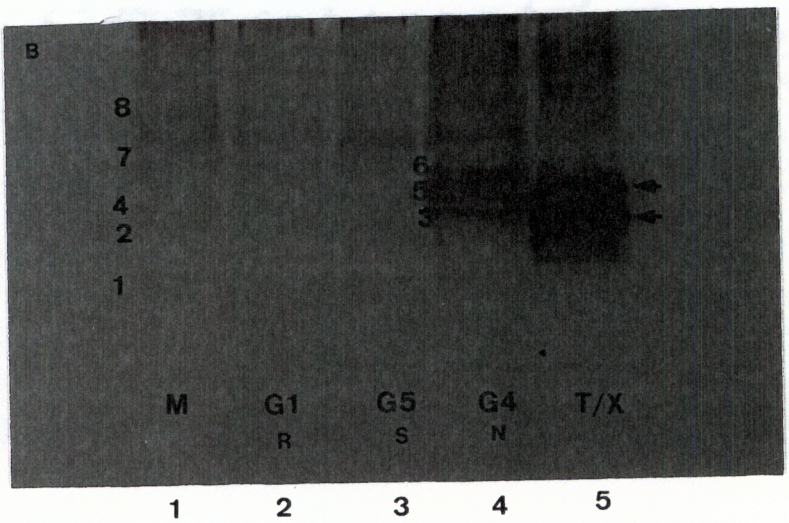
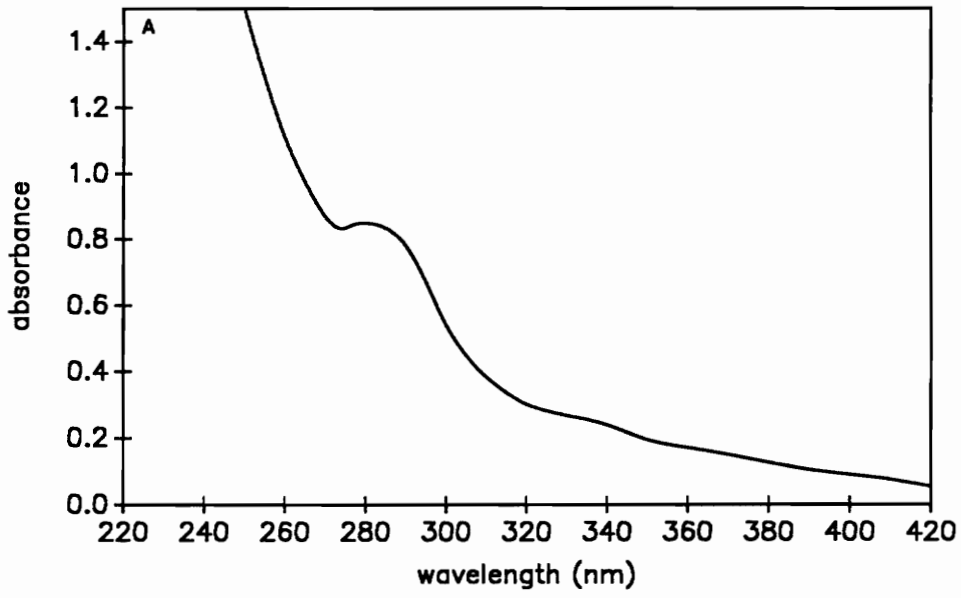
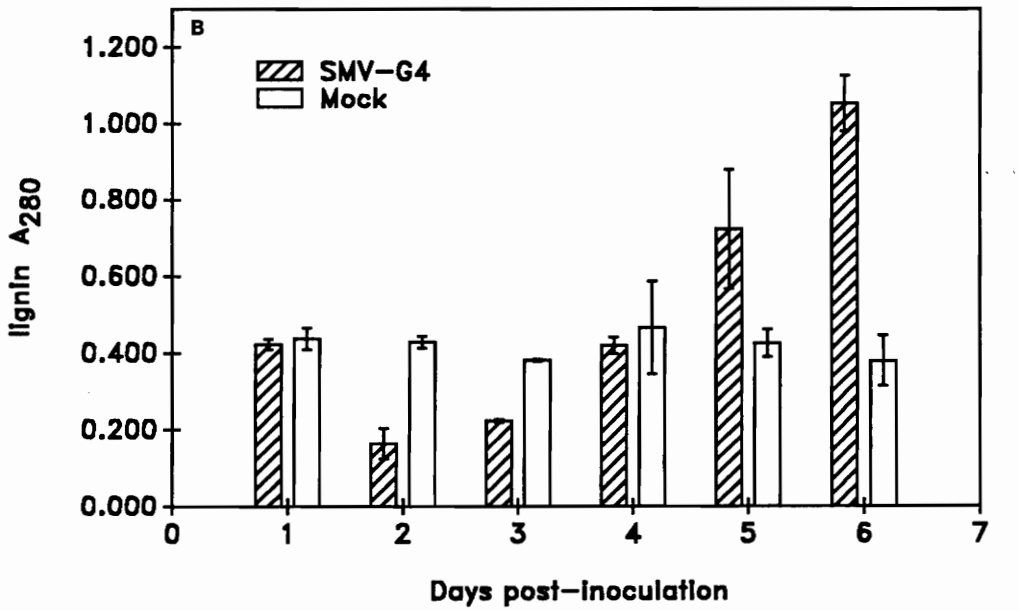

B
8
7
6
5
4
3
2
1
M
G1
R
G5
S
G4
N
T/X
1
2
3
4
5

Figure 4. (A). UV spectrum of 100 ug/ml LTGA in 0.5N NaOH. $A_{280nm}$: 0.849 (B). Time-course of lignin accumulation in N response (filled bar) and mock-inoculated leaves (blank bar) measured from 50 mg alcohol insoluble residues. Values represent means with standard error (I bar) for three replicate plants in the same experiment.


A
absorbance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
220 240 260 280 300 320 340 360 380 400 420
wavelength (nm)

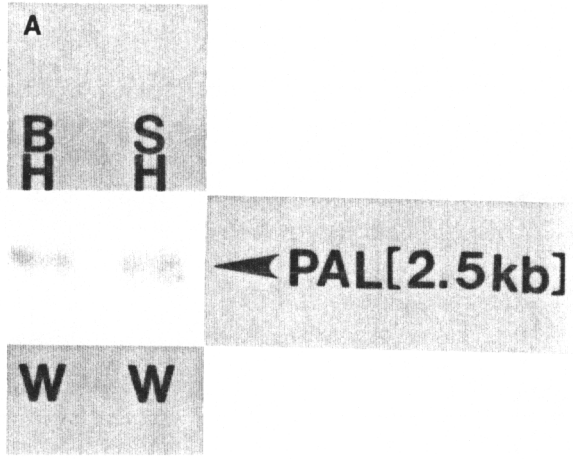

B
SMV-G4
Mock
lignin $A_{280}$
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0 1 2 3 4 5 6 7
Days post-inoculation

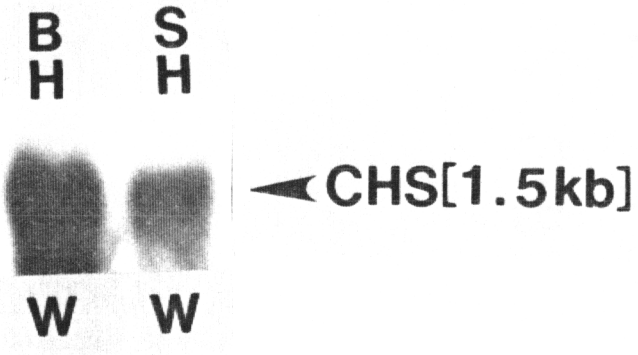
term time course with a 3 hr interval and subjected to RNA blot analysis using heterologous bean PAL and CHS cDNA as probes. Northern blots showing mRNA induction of soybean hypocotyls 8 hr after wounding treatment and hybridized with heterologous bean cDNA probe (Fig. 5A,B). RNA blots were hybridized with a $P^{32}$-labelled cDNA fragment of bean pPAL5, revealing that the sizes of mRNAs were 2.5 kb (Fig. 6) and 1.5 kb (Fig. 7 A,B), respectively. These sizes are very similar to the sizes of corresponding PAL and CHS mRNAs in bean (Ryder et al., 1984; Edwards et al., 1985; Tepper et al., 1989). These soybean PAL and CHS mRNAs were rapidly and coordinately induced in N response caused by SMV-G4, showing biphasic pattern of induction compared to mock-inoculated control and the S response.

The early induction of PAL mRNA at 6 hr after inoculation, relatively low levels at 9 hr after inoculation, and then second induction at 12 hr after inoculation in the N response (Fig. 6), exhibited a similar pattern to that of PAL enzyme activity (Fig. 2A). The mock-inoculated control induced PAL mRNA at 6 hr after inoculation due to surface wounding by celite and rapidly decayed (Fig. 6). Constitutive levels of PAL mRNA were detected in the S response 3-6 hr after inoculation.

For the analysis of CHS mRNA induction for the short and long term time courses, $P^{32}$-labeled bean CHS cDNA fragment was hybridized in the same way as in the analysis of PAL

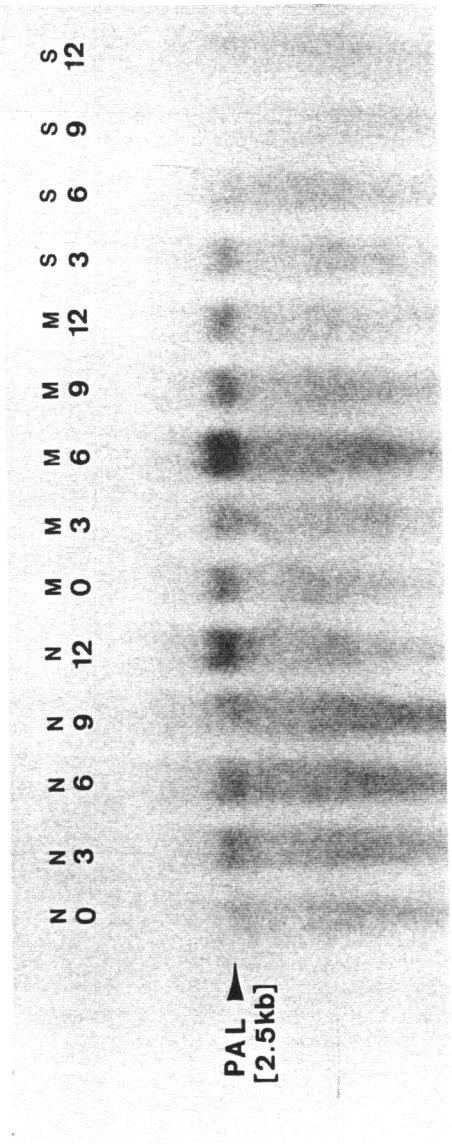
Figure 5. Northern blots showing messenger RNA induction 8 hr after wounding treatments of bean hypocotyls (BH) and hybridized with homologous bean cDNA probes, and 8 hr after wounding treatment soybean (SH) hypocotyls and hybridized with a heterologous bean PAL cDNA under moderate stringency condition. (A). PAL (phenylalanine ammonia-lyase) (B). CHS (chalcone synthase) (C). CHT (chitinase).


A
B
H
S
H
PAL[2.5kb]
W
W


B
B
H
S
H
CHS[1.5kb]
W
W

C
B
H
S
H
CHT
[1.2kb]
W
W

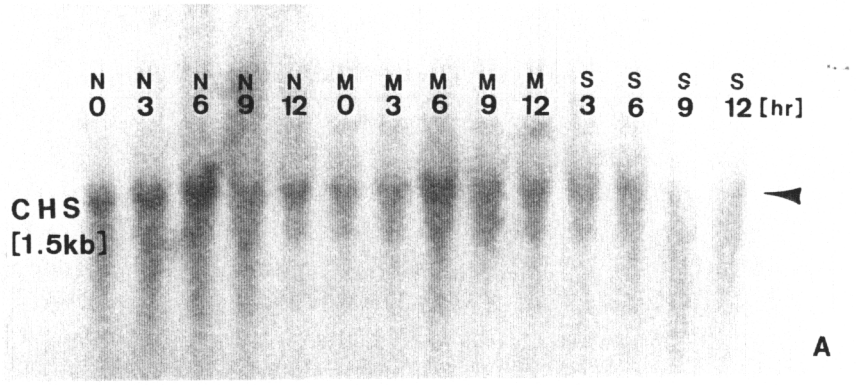
Figure 6. Induction of PAL mRNA during short term time course (0-12 hr) after inoculation with SMV strains. Total RNA (20 ug/lane) isolated from leaves from N response (G4) (lane 1-5), mock-inoculated leaves (lane 6-10), and S response (G5) (lane 11-14) and hybridized with the $P^{32}$-labeled CHS cDNA probe under moderate stringency and washed at low stringency (2x SSC, 0.1% SDS).


N 0
N 3
N 6
N 9
N 12
M 0
M 3
M 6
M 9
M 12
S 3
S 6
S 9
S 12
PAL
[2.5kb]

mRNA. CHS mRNA increased rapidly at 6 hr after inoculation in the N response, but in the S response induction declined to below that of the control in short-term time course and remained suppressed until 4 DAI (Fig. 7 A,B). Similar to that of PAL mRNA induction, mock-inoculated control induced one peak at 6 hr after inoculation. In CHS mRNA, induction and decay of earliest peak was very similar to that of PAL mRNA, however a second peak slowly increased up to 5 DAI following necrosis development (Fig. 7 A,B). There was a close correlation between kinetics of induction of CHS mRNA and accumulation of isoflavonoid phytoalexins (glyceollins) (Fig. 2B). Inoculation with SMV-G1 produced R response, but did not result in a detectable induction of CHS mRNA (Fig. 7B) even though PAL enzyme activity was induced to high levels (Fig. 2B). This is consistent and expected because the accumulation of flavonoid phytoalexins (glyceollins) have never been detected in the R response.

To determine whether another putative defense response was induced in leaves inoculated with SMV strains, a similar RNA blot analysis was conducted using bean cDNAs of CHT as a probe. The bean chitinase cDNA did not hybridize to a soybean mRNA under moderate stringency condition (Fig. 5C), but some cross-hybridization could be observed under low stringency condition (Fig. 8A,B). The size of detected CHT mRNA, 1.2 kb, was similar to the size of bean CHT mRNA (Hedrick et al. 1988). Similar to those of PAL- and CHS mRNA induction,

Figure 7. (A). Autoradiogram of a Northern blot showing the induction of chalcone synthase message during the short term time-course (0-12 hr after inoculation). (B). Long term time course of CHS-mRNA induction. Total RNA (15 ug/lane) is isolated from leaves inoculated with SMV-G1 (R, lane 1-4); -G4 (N, lane 5-9); -G5 (S, lane 10-13); mock-inoculated control (M, lane 14-17), and size fractionated in a 1.2% agarose gel. After blotting, the membranes were hybridized with the $P^{32}$-labelled CHS cDNA probe under moderate stringency and were washed at low stringency (2x SSC, 0.1% SDS).


N 0
N 3
N 6
N 9
N 12
M 0
M 3
M 6
M 9
M 12
S 3
S 6
S 9
S 12 [hr]
CHS
[1.5kb]
A

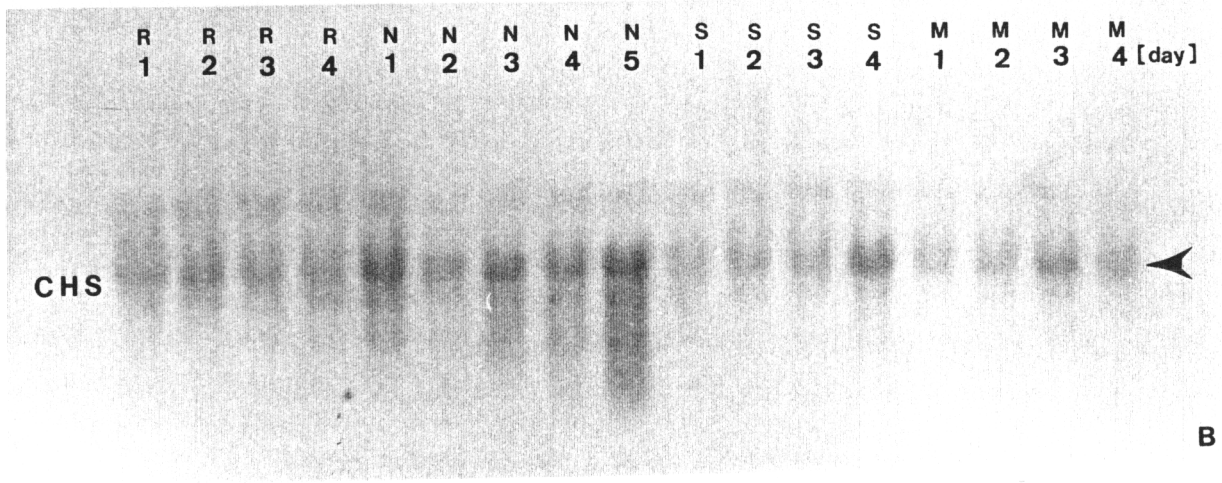
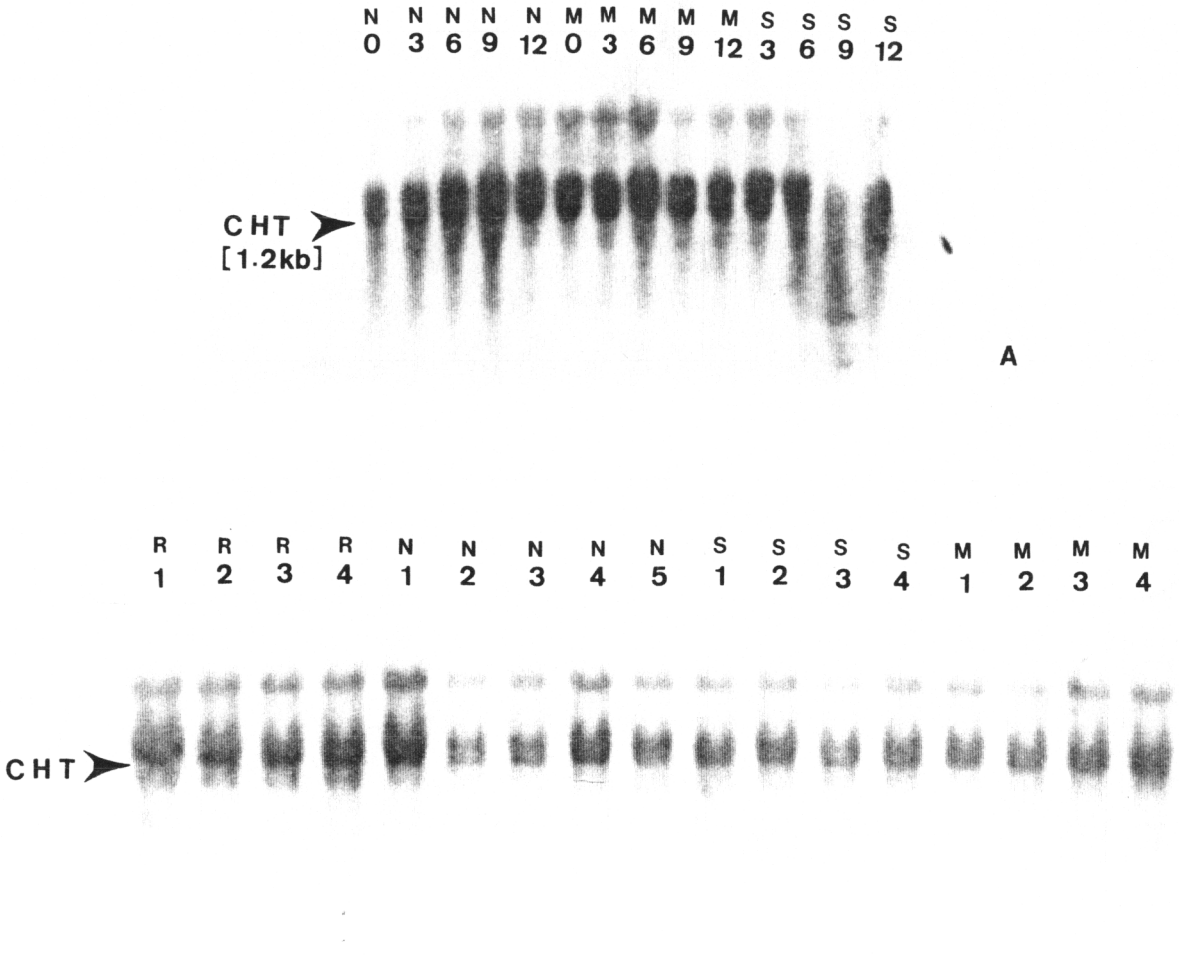

R 1
R 2
R 3
R 4
N 1
N 2
N 3
N 4
N 5
S 1
S 2
S 3
S 4
M 1
M 2
M 3
M 4 [day]
CHS
B

Figure 8. (A). Autoradiogram of a Northern blot showing the induction of CHT mRNA during the short term time-course. After blotting, the membranes were hybridized with the $P^{32}$-labeled CHT cDNA probe under low stringency and were washed at high stringency (6x SSC, 0.1% SDS; 2x SSC, 0.1% SDS). (B). Long term time-course of CHT mRNA induction in R-(lane 1-4), N-(lane 5-9), S response (lane 10-13) and mock-inoculated leaves (lane 10-13).


N 0
N 3
N 6
N 9
N 12
M 0
M 3
M 6
M 9
M 12
S 3
S 6
S 9
S 12
CHT
[1.2kb]
A
R 1
R 2
R 3
R 4
N 1
N 2
N 3
N 4
N 5
S 1
S 2
S 3
S 4
M 1
M 2
M 3
M 4
CHT
B

CHT mRNA in N response also shows bimodal induction. The transient early peak increased up to 1 DAI and a slower second peak could be detected at 4 DAI, corresponding to appearance of initial necrotic lesions (Fig 8 A,B). Mock-inoculated leaf tissues induced one peak up to 12 hr after inoculation and declined to the constitutive level. In the S response, we could observe CHT mRNA induction at 3 hr after inoculation, which then declined rapidly (Fig 8 A). These differences may be caused by incomplete transfer from gel to membranes during blotting. Even though we could not conduct CHT mRNA induction with R response in short term time course, it was not detectable in the long term time course. In leaf tissues inoculated with SMV strains, there are clear differential patterns of accumulation of hybridizable CHT mRNA in the N response compared to the R- or S responses.

## DISCUSSION

DIBA confirmed the presence of viral antigen in the leaf tissues of both N- and S response of cv. York. From the data presented in Fig. 1A and 1B, SMV-G4 apparently moves up to the uninoculated leaves, which develop necrosis in both stems and leaf blades. The different types of responses, caused by G1 and G4 strains, may be the result of two different mechanisms based on distinct symptomatology (Choi et al., 1991) and virus accumulation. The lack of detectable virus in the R response suggests that G1 did not follow the normal events allowing viral infection. This form of resistance may be operating at the level of blocking virus entry or suppression of virus replication. In this case, resistance would be constitutive, and may fit Fraser's "positive" type of mechanism hypothesis (Fraser, 1990). Since there is a certain accumulation of G4 strain in upper uninoculated leaves, it can be characterized as an ineffective resistance mechanism. Results suggest that the G4 strain is replicated, and the type of resistance to the G4 strain may be a suppression of cell-to-cell movement within the plant. Therefore, this type of resistance mechanism may be associated with activation of host defense responses.

Induction kinetics of PAL activity in the N response was very similar to that observed for POX. Our results demonstrate that the resistant and susceptible disease interactions with SMV strains could be distinguished on the basis

of changes in PAL activities but not on the basis of changes in POX activities. Both R- and N responses displayed two peak periods of PAL enzyme activities, while only the N response showed two peaks of POX activities. In addition, early peaks of PAL and POX activities are similar at 24 hr after inoculation, but the second peak of POX activity reached maximum levels 4 DAI, was maintained at that level for a few days following necrosis development, and decreased more slowly than those of PAL. The second peaks at 48 hr in the R response and at 72 hr in the N response declined at 4 DAI. Accumulation of mRNAs corresponding to the enzymes PAL and CHS was coordinate. This is evident in the N response, showing increase of PAL enzyme activity, and induced accumulation of PAL mRNA during the short term time course and of CHS mRNA during the short and long term time courses. Hence, it is expected that increased amounts of these enzymes are involved in isoflavonoid phytoalexin synthesis in the N response. However, the pattern of PAL and CHS mRNA accumulation in inoculated leaves was complex. The N response displayed bimodal induction of CHS mRNA, while in the S response induction was delayed until 4 DAI. Interestingly, CHS mRNA was induced in the S response at 4 DAI, but only a low level of glyceollin accumulated at this time.

Tissue- and cell-type-specific PAL expression during development and in response to wounding has been demonstrated (Bevan et al., 1989). Likewise, high induction of

PAL and CHS mRNA at 6 hr after inoculation in mock-inoculated control could be observed, probably because of surface wounding damage in the inoculated leaves by Celite. However, we could not observe a detectable level of PAL activity and glyceollin accumulation.

The accumulation of lignin-like compounds in infected plant tissue is well documented (Vance et al., 1980). The time course of lignin accumulation in the N response was not as rapid as induced by other plant-pathogen interactions (Bruce and West, 1989). Coincident with lignification is a striking pattern of appearance in the enhanced activity form or additional new isoform profiles of POX, which is not present at readily detectable levels in either the R or S responses or in the mock-inoculated control.

The appearance of new isoforms of POX in infected tissue has also been reported (Moerschbacher et al., 1986; Bruce and West, 1989). In cucumber seedlings, the systemic accumulation of an anionic isozyme of peroxidase has been associated with induced systemic protection against pathogen attack (Hammerschmidt et al., 1982), however a specific function for plant isoperoxidases has not yet been clearly demonstrated. The molecular analysis of POX began with cloning and characterization of the anionic POX isozyme from tobacco (Lagrimini et al., 1987). Recently, transformed tobacco plants with a chimeric gene containing tobacco anionic peroxidase cDNA introduced, revealed overproduction

of two anionic POX and production of chronic severe wilting through loss of tugor in leaves (Lagrimini et al., 1990).

Our results also confirm that an activation of PAL, and in general the phenylpropanoid pathway, could be correlated with the induced glyceollin accumulation and lignification. Unlike the S response, the N response appears to be characterized by rapid and transient increases of PAL and corresponding enzyme activity, followed by the accumulation of glyceollin and lignin. In some reports, the rapid appearance of glyceollin (Keen and Yoshikawa, 1983; Darvill and Albersheim, 1984; Hahn et al., 1985; Ebel, 1986; Bhattacharyya and Ward, 1986) and lignin or lignin-like polymers (Hammerschmidt, 1984) has been associated with the expression of disease resistance in race- and cultivar-specific interactions. Although a certain relationship between resistance and glyceollin accumulation has been demonstrated, the association between glyceollin and necrosis remains consistent and supports previous conclusions that glyceollin production is associated more closely with necrosis than with resistance (Lazarovits et al., 1981; Ward et al., 1981; Lazarovits and Ward, 1982).

A particular striking feature of the present data is the bimodal pattern of enzyme activities and mRNA induction in the N response. This is distinct from that observed in other host-pathogen interactions. Two possible explanations can be proposed; one, the early peak may be triggered by the inocu-

lating virus entry enhanced by wounding, while the later peak is solely triggered by virus replication or spread. Another explanation may be the induction of different forms of multigene families which may be induced, with the two peaks being under differential regulation. This is consistent with reports that PAL is encoded by a family of three to four genes in bean (Cramer et al., 1989), parsley (Lois et al., 1989), and rice (Minami et al., 1989), and the transcripts of individual PAL genes show highly different patterns of accumulation (Liang et al., 1989; Lois et al., 1989). Complex patterns of expression regulated by multiple factors is also a distinctive character of CHS genes with extensive polymorphism. Petunia has eight to ten (Koes et al., 1989), bean has six to eight (Ryder et al., 1987), and soybean has six encoded CHS gene families (Wingender et al., 1989).

Inhibition of virus replication and movement occurs in the R response resulting from inoculation with G1, but induction of known defense-related pathways was not observed except for PAL enzyme activity. However, partially effective supression of virus replication and movement, induction of PAL-, CHS- and CHT mRNA, induction of PAL and POX activities, and glyceollin and lignin accumulation were closely correlated to N response resulting from inoculation with G4 strain. These results are consistant with the findings of soybean PR protein studies (Choi et al., in preparation).

Whether the resistance mechanism is constitutive or induced, dominant alleles associated with the R response tend to be constitutive, whereas incomplete dominant alleles (gene-dosage dependent) operating in the N response appear to be functioning through an induced mechanism. Based on our observations thus far, we assume two possibilities for resistance mechanisms. One is constitutive expression of a resistance gene with a dual role as a signal molecule to induce defense-related pathways. In the R response, a resistance gene that plays a role in the recognition event was proposed to be the inhibitor (Fraser, 1990). For example, *Tm-1* gene in tomato inhibits TMV replication (Watanabe et al., 1987) and *Tm-2* gene prevents cell-to-cell spread (Meshi et al., 1988; 1989). The level of virus accumulation in differential responses in SMV-soybean interaction suggests that the R response operates to inhibit virus replication similar to *Tm-1* resistance. However in the N response, the resistance gene does not act as an inhibitor, rather it may have signal perception molecule activity in transduction chains to induce a massive change and the activation of multiple defense gene activation signals and expression to block virus movement. This hypothesis also parallels the induction of several other defense responses including glyceollin accumulation and lignin deposition (Chappell and Hahlbrock, 1984; Cramer et al., 1985b; Lawton and Lamb, 1987). This is a very similar mechanism to that

operating in tomato *Tm-2* resistance. Another possibility is that there may be two distinct genes but tightly linked, that have dominant effects, and interact with viral products to produce differential responses analogous with *Tm-1* and *Tm-2* resistance genes. Genetic studies to date, however, support the single gene, multiple allele model (Chen et al., 1991).

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## Chapter VI

# Soybean mosaic virus-Soybean Interaction IV. *Rsv* Resistance-triggered Expression of Soybean 3-Hydroxy-3-methylglutaryl A Reductase in Differential Responses Induced by Soybean Mosaic Virus

Chang W. Choi, Heesung Park, Xueshu Yu, Sue A. Tolin, and Carole L. Cramer.

Department of Plant Pathology, Physiology, and Weed Science,
Virginia Polytechnic Institute & State University,
Blacksburg, VA 24061, USA

## Short Communications

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR; EC 1.1.1.34) catalyzes conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonic acid, the major precursor of isoprenoid compounds (Durr and Rudney, 1960). The isoprenoid pathway in plants is associated with synthesis of terpenoid phytoalexin related to pathogen defense (Kuc and Rush, 1985; Stermer and Bostock, 1987), pigments such as cartenoids, growth regulators such as gibberellins and abscisic acid, and electron transport components such as plastoquinone and ubiquinone (Threlfall, 1980). Recently, HMGR genes have been isolated from *Arabidopsis thaliana* (Caelles et al., 1989; Learned and Fink, 1989) and tomato (Narita and Gruissem, 1989; Park, 1990). Park (1990) cloned and sequenced a tomato HMGR gene that shows defense-related expression. In studies to correlate this gene with resistance to tuber soft rot in potato, the HMGR gene-specific probe was used to demonstrate that one isogene of the HMGR family is pathogen activated, and is distinct from wound-activated isogenes (Yang et al., 1991). In soybean, the phenylpropanoid-isoflavonoid phytoalexin glyceollin is well characterized through the decades, however nothing is known of the terpenoid pathway. We demonstrate here that expression of HMGR mRNA in soybean is correlated with *Rsv* resistance to SMV-G1 (Chen et al., 1991).

The previous results reported for defense-related genes and proteins are associated with hypersensitive or necrosis responses (Choi et al., 1991a,b), In contrast, soybean HMGR mRNA expression is strongly activated with symptomless resistance (R response), has an intermediate level of activation with necrosis (N response), and shows only constitutive levels of expression with systemic mosaic symptoms (S response) and mock-inoculated control.

**Methods:** Total RNA was isolated from frozen soybean leaves at 12 hr after inoculation with SMV-G4 (1 g) by the method of Haffner et al. (1978) and used for first strand cDNA synthesis in a 20 ul mixture containing cDNA synthesis buffer (BRL), RNA template (20 ug), 0.5 mM each of dNTP, 100 *p*moles of oligo(dT) primers (BRL), and 200 units of Moloney Murine Leukemia Virus reverse transcriptase (BRL). The reaction was incubated for 1 hr at 37°C and 1000 units of $T_1$ RNase was added for 1 hr additional incubation at 37°C. For amplication using polymerase chain reaction (PCR), the oligonucleotide primer sets 5'-GATAAGCTTGGGTACGTGCAGA-TACCAG-3' (primer A) and 5'-AACAAGCTTCCGATGACATCCATGTCGG-3' (primer B) were used (Park, 1990). To 20 ul of cDNA reaction, 80 ul containing 10 ul of 10X GeneAmp buffer (Perkin Elmer Cetus; Norwalk, CT), 50 *p*moles each of primer A and B, 2 ul each of dNTP (2.5 mM), and 1 unit of *Taq* polymerase (Promega, Madison WI) were added. Temperature cycling was established by programming a TwinBlock thermo-

cycler (model TCX15A, Ericomp, Inc., San Diego, CA). Cycle 1 was 95°C (5 min), 72°C (5 min), 58°C (2 min), and 72°C (3 min). Cycle 2, 95°C (1 min), 58°C (3 min), and 72°C (3 min), was repeated 34 times. Cycle 3 was 72°C for 15 min. After completion, top-layered mineral oil used during the reaction was extracted with TE (10 mM Tris-HCl, pH 8.0, and 1mM EDTA)-saturated chloroform. PCR product was processed by phenol extraction, ethanol precipitation, *HindIII* digestion, electrophoresis in 1% agarose gel, and excision of DNA band of interest from gel (Fig. 1). Amplified cDNAs were cloned into the *SmaI/HindIII* site of the Bluescript vector (pKS$^-$, Stratagene), designated pSOY HMGR, and the ligate transformed (pKS$^-$/PCR product) into *E. coli* DH5$\alpha$ (Maniatis et al., 1982).

Transformed colonies were screened on a LB (Luria-Bertani) media plate containing ampicillin (100 ug/ml of LB media) by spreading 40 ul of a stock solution of X-gal (25 mg/ml in N,N-dimethylformamide) and 100 ul of a solution of isopropylthio-$\beta$-D-galactoside (IPTG) (23.8 mg/ml in distilled water). The overnight culture in LB media containing ampicillin was centrifuged for 1 min, and the pellets resuspended by vortexing in 200 ul of cold lysis-buffer (25 mM Tris, pH 8.0, 10 mM EDTA, 50 mM glucose). pSOY HMGR was isolated by phenol/chloroform extraction, followed by ethanol precipitation, digested with *SmaI*, and loaded onto 1% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, and

Fig. 1. Arrow indicate PCR-generated cDNA using primer sets (primer A and B in the text) from total RNA: soybean cv. Kanggyo treated with herbicides (lane 1), Soybean cv. York at 12 hr after inoculation with SMV-G4 (lane 2). Lane 3 and 4 represent DNA ladder marker and tomato HMGR, respectively.

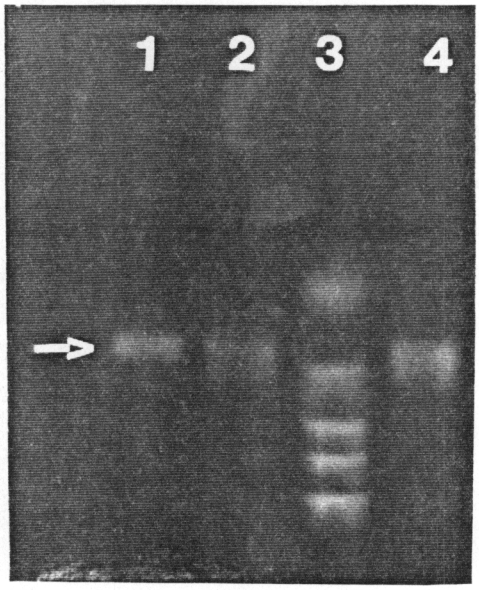
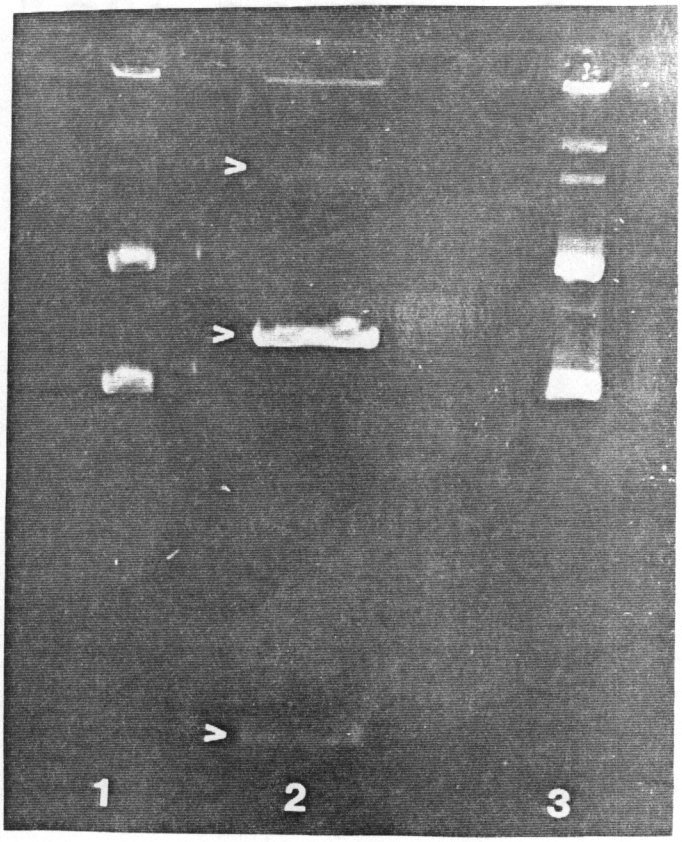

1
2
3
4

Fig. 2. pSOY HMGR (from soybean cv. York at 12 hr after inoculation with SMV-G4) cloned into the Bluescript vector (pKS$^-$, Stratagene), is isolated by digestion with *SmaI* and loading onto 1% agarose gel in TBE buffer (lane 2). Arrowheads indicate undigested pKS$^-$/PCR product (top), digested pKS$^-$ vector itself (middle), and 500 bp of pSOY HMGR (bottom). Lane 1 and 3 represent DNA markers.


1
2
3

0.5 M EDTA). A band of 500 bp DNA fragment (Fig. 2) was cut, extracted with TE-saturated phenol, and used as a probe for Northern analysis.

To determine expression of HMGR mRNA in differential responses induced by SMV inoculation, total RNA was isolated from York leaves 1-4 days after inoculation (DAI) with SMV-G1 and -G5, and 1-5 DAI with SMV-G4. For Northern analysis, total RNA (15 ug/lane) was denatured by treatment with glyoxal and separated on 1.2% agarose gels, and transferred to Nytran membranes (Schleicher and Schuell, Keene, NH) as described (Maniatis et al., 1982). Hybridization under moderate stringency conditions was conducted as described by Choi et al. (1991b).

**Result:** We can detect strong induction of HMGR mRNA at 1 DAI which decreased levels at 3 DAI for the R response. The N response was characterized by intermediate induction at 1 DAI which rapidly decreased at 2DAI. However, both the S response and mock-inoculated leaves displayed a constitutive level of expression throughout the time course (Fig. 3). Interestingly, a tomato cDNA clone used as a heterologous probe, designated pCD1 (Park, 1990), did not cross-hybridize with soybean HMGR mRNA (data not shown). These results indicate that soybean HMGR may not be highly homologous to tomato HMGR, but may be associated with expression of race- and cultivar-specific resistance. This is the first report of induction of the terpenoid pathway in soybean. Future

studies still remain to perform sequence analysis of this fragment and to compare it to the sequence of known HMGR genes.

Fig. 3. Autoradiogram of a Northern blot showing the induction of HMGR-mRNA during the long-term time course. Total RNA (15 ug) isolated from leaves inoculated with SMV-G1 (R, lane 1-4), -G4 (N, lane 5-9), -G5 (S, lane 10-13), and mock-inoculated (M, lane 14-17), was blotted onto Nytran membrane, and hybridized with the $P^{32}$-labelled cDNA fragments of pSOY HMGR under high stringency.

R 1
R 2
R 3
R 4
N 1
N 2
N 3
N 4
N 5
S 1
S 2
S 3
S 4
M 1
M 2
M 3
M 4
HMGR

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## Chapter VII

# Soybean mosaic virus-Soybean Interaction V. Immunological Properties with Peptide Cleavage of Soybean Mosaic Virus Coat Protein.

Chang W. Choi and Sue A. Tolin

Department of Plant Pathology, Physiology, and Weed Science,
Virginia Polytechnic Institute & State University,
Blacksburg, VA 24061, USA

**Abstract**

Soybean mosaic virus coat protein degraded *in vitro* by proteolysis during purification. The coat protein of SMV-G1 and -G5 purified from infected leaves had an apparent size of 34 kD in 10% sodium dodecyl sulfate-polyacrylamide gels with minor peptides of 32 and 31 kD proteins. The proportion of minor proteins increased during the storage at 4°C and treatment of trypsin and reacted with antiserum to intact virions. This heterogeneity of protein profiles was not removed by alkaline phosphatase treatment for varying time intervals, was not related with phosphorylation and dephosphorylation and virus maturation, and revealed another evidence of N- and C-terminal loss exposed on the particles' surface. For the discriminating capacity and sensitivity of Western blot analysis, SMV-G1 coat protein is trapped by intact rabbit-IgG against G1 strain and detected by i) an enzyme-conjugated virus-specific IgG (same animal source), ii) an enzyme-conjugated IgG whole molecule (different animal source), and iii) an enzyme-conjugated $F(ab')_2$ IgG fragments. All three types of IgG-enzyme conjugates could detect antigen with certain differences in sensitivity.

## Introduction

There have been numerous reports on the heterogeneity in coat protein (CP) size of potyviruses using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Values ranged from 26 to 34 kD molecular weight (MW) depending in part on gel concentration used in the estimation (Moghal and Francki, 1976; Gough and Shukla, 1981; Hiebert et al., 1984; Brakke et al., 1990). The variable size of the CP of tobacco etch virus (TEV) during storage (Hiebert et al., 1984) and degrading multiple bands of CP of wheat streak mosaic virus (WSMV) during leaf senesence (Brakke et al., 1990) have been reported. The recent findings suggest that the N- and C-terminal regions are located on the surface of the virus particles and are the most highly variable between viruses and among strains of the same potyvirus (Allison et al., 1985; Dougherty et al., 1985; Shukla et al., 1988 a,b,c,d). In addition, mild trypsin digestion of several potyviruses removed the variable N- and C-terminal regions of the CP without destroying the rod-shaped structure of the virus or its infectivity (Shukla et al., 1988b).

Viral proteins have also been shown to give a different apparent molecular weights for other reasons. Proteins isolated from core particles of hepatitis B virus (HBV), contained a kinase activity which is capable of phosphorylating the core protein (Roossinck and Siddiqui, 1987; Gerlich et al., 1982). These proteins displayed heterogeneity in SDS-

PAGE, however incubation of native cores with alkaline phosphatase removed this heterogeneity (Pugh et al., 1989). It has been suggested that phosphorylation of external amino acids was responsible and dephosphorylation plays a role in virus maturation (Pugh et al., 1989).

Plant viruses are generally classified within groups on the basis of host range, symptomatology, and serological properties, but these approaches would appear inconsistent and unsatisfactory to classify members of the potyvirus group. Recently, some potyviruses and their strains have been reclassified as strains of different viruses (Frenkel et al., 1989; McKern et al., 1990; Shukla and Ward, 1989; Yu et al., 1989). However, structural properties of CP with immunological analysis can be used to differentiate distinctively between individual potyviruses and their strains (Shukla et al., 1986; Shukla et al., 1987; Shukla et al., 1988a,b,c,d; Shukla et al., 1989a,b).

Soybean mosaic virus (SMV), a member of the potyvirus group, has been classified into seven strain groups based on differential reaction on soybean cultivars (Cho and Goodman, 1979). Earlier work done in our lab has characterized and sequenced a cDNA clone containing an insert of 1443 nucleotides representing the 3'terminus of the viral RNA genome SMV-VA/G1 (Gunyuzlu et al., 1987). Comparative studies of CP amino acid sequences among potyvirus, SMV-G1 strain shows the differences in amino acid sequences to another SMV

strain (Shukla and Ward, 1988). Twelve monoclonal antibodies were tested to differentiate 11 isolates of SMV by immunoblot analysis of one-dimensional trypsin peptide maps of the CP (Hill et al., 1989).

Through the years many variations have been developed for double-antibody sandwich (DAS) forms of immunoassay, and have suggested that conjugation of the enzyme to the second antibody can induce conformational modification that may alter the affinity of the antibody molecule for the virus and modify strain specificity (Koenig, 1978). This problem may overcome using primary capture antibody and second detecting antibody prepared in a different animal source (van Regenmortel, 1982).

In this paper, we examine the CP heterogeneity among SMV strains and the proteolytic nature of CP heterogeneity in SDS-PAGE by comparing immunological properties, peptide maps by trypsin cleavage, and dephosphorylation of CP of SMV by alkaline phosphatase treatment. The sensitivity and discriminating capacity of DAS and indirect assay for Western blots are reported.

## Materials and Methods

**Virus.** Inoculum was prepared by homogenizing infected leaves of soybean cultivar Lee for G1 and G4, and York for G5, in 0.01 M sodium phosphate buffer (pH 7.0) containing 1 % celite (Hunst and Tolin, 1982). A 1:10 sap dilution was rubbed with a pestle onto leaves at the unifoliolate stage.

**Virus purification.** SMV strains were purified by clarification of sap with n-butanol, differential centrifugation, centrifugation on sucrose density gradients, and concentration by ultra centrifugation (Hunst and Tolin, 1982). Southern bean mosaic virus (SBMV) and cowpea mosaic virus (CPMV) were used from long-time storage preparations kept at 4°C in our laboratory. All virus preparations were adjusted to 1.5 mg/ml concentration and used immediately or stored at 4°C for several months.

**Antisera.** Polyclonal antisera produced in rabbits against intact SMV-G1 was the same as described previously (Hunst and Tolin, 1982). Alkaline phosphatase conjugated goat anti-rabbit IgG wholemolecule (Sigma No. A-8025, 0.28 mg/ml) and $F(ab')_2$ fragments (Sigma No. A-7778, 0.47 mg/ml) were available commercially.

**Trypsin cleavage for peptide analysis.** Purified preparations, of SMV-G1 and -G5 were used immediately after purification for tryptic peptide mapping. The preparations, in 0.05 M sodium borate buffer (pH 8.0), were incubated with trypsin (Sigma, type XIII, treated with L-1-tosylamide-

2-phenylethyl chloromethyl ketone) at the ratio of 2 ug of enzyme per mg of virus at 37°C for varying time intervals (Shukla et al., 1988b). Proteolysis was terminated by heating at 100°C for 5 min.

**Alkaline phosphatase treatment.** Purified preparation of SMV-G1 and -G5 were incubated at 37°C with alkaline phosphatase (4U/ml) (type VII-N, Sigma) in 0.05 M Tris hydrochloride (pH 7.5) containing 0.005 M dithiothreitol, 0.01 M $MgCl_2$ and 0.1 M $Na_2SO_4$ (Pugh et al., 1989). One ul of 0.1 M phenylmethylsulfonyl fluoride in 2-propanol was added as a protease inhibitor. Reactions were stopped by addition of an equal volume of Laemmli (1970) sample buffer and heating for 5 min in boiling water.

**SDS-PAGE.** For denaturing conditions, SDS-PAGE was performed according to Laemmli (1970), using a 10% separation gel and 4.5% (w/v) stacking gel with Bio-Rad SDS-PAGE Low Molecular Weight Standards. Protein subunits were obtained by heating the virus preparation for 5 min at 100°C in 0.125 M Tris-HCl, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.001% (w/v) bromophenol blue. Electrophoresis was carried out at constant current of 30 mA at room temperature. The gels were stained with Coomassie Brilliant Blue R-250.

**Western blots.** After electrophoresis, polypeptides were electro-blotted to Nytran membrane (0.22 um pore size, Schleicher & Schuell) in 0.025 M Tris-HCl, 0.192 M glycine,

pH 8.3, containing 20% methanol by the method of Towbin et al (1979) in a Transphor-unit cell (Hoefer Scientific Instruments, San Francisco, CA) at 80 V for 6 hr. After transfer, the membrane was blocked by soaking in 5% nonfat dry milk (Carnation) in TBS buffer (0.02 M Tris, 0.15 M NaCl, pH 7.5) and incubated for 2 hr with rabbit IgG against intact SMV-G1 diluted 1:1000 in TBS. After three-10 min rinses with TBS, the membrane was then incubated in alkaline phosphatase conjugated virus-specific rabbit IgG (1:1,000 dilution), alkaline phosphatase conjugated goat anti-rabbit IgG wholemolecule (1:500 dilution, Sigma), or alkaline phosphatase conjugated goat anti-rabbit IgG $F(ab')_2$ (1:500 dilution). After washing four times (10 min each) with TBS, alkaline phosphotase activity was detected by the nitro blue tetrazolium method (Hammond and Jordan, 1990).

## Results and Discussion

**Coat protein profiles.** A major polypeptide species of about 34 kD with minor amounts of 32 kD and 31 kD were detected from native SMV-G1 strain (Fig. 1 lane 6). Similarly, a major polypeptide of 34 kD with minor 31 kD were detected from native G5 strain (Fig. 1 lane 4). However only a single 31 kD band could be detected from long-term storage preparations of SMV-G4 strain (Fig. 1 lane 5). This occurrence of variable size and multiple bands may be caused by a partial degradation of the polypeptide by proteolytic enzymes of host or viral origin (Moghal and Francki, 1976). Long-term storage SBMV displayed 29 kD of CP, which represents loss of 2 kD (Fig. 1 lane 3) compared with published data (Wu et al., 1987). Interestingly, CPMV, the type member of comovirus, displayed two stable coat proteins with MW of 37 and 22 kD during long-time storage (Fig. 1 lane 2), very similar to weights of CP coded on M-RNA (Franssen et al., 1982).

There are possible correlations between degradation effects and structure of virus particles. SMV is characterized by long, flexuous rod-shaped particles (Shukla and Ward, 1989), SBMV is characterized by isometric particles (Hull, 1988), and CPMV is characterized by isometric particles and multipartite genome structure (Goldbach and Van Kammen, 1985). Isometric particle structures may be more resistant to the proteolytic degradation than rod-shape

structures.

**Limited proteolysis of SMV.** Purified SMV-G1 stain was treated with trypsin for limited proteolysis and compared to long-time storage preparations at 4°C. Trypsin treatment of the native particles trimmed the size of the CP up to 30 kD during the time course of treatment (Fig. 2). In the storage preparation the molecular weights of CP subunits reduced from 34 to 31.5 kD, which was a 2.5-3 kD loss during storage (Fig. 2, lane 3). About 8.8% (3 kD) of CP subunit of G4 and G1 strains (Fig. 1 lane 5; Fig. 2 lane 3) was readily susceptible to proteolysis. This loss is very similar to digestion effect of trypsin 5 min after treatment (Fig. 2, lane 4).

Similar results have been reported for storage at 4°C of a purified preparation of tobacco etch virus (TEV), which was found to change the molecular weights of the CP ranging from 32 kD to 26 kD (Hiebert et al., 1984). Recently, it has been demonstrated that mild trypsin digestion of several potyviruses removed the variable N-terminal region (29-67 amino acids depending on the virus) and 18-20 amino acids from the C-terminal region of the CP (Shukla et al., 1988b). The increasing accumulation of minor and smaller proteins in storage preparation suggests that processing forms arise by proteolysis. Degrading wheat streak mosaic virus CP in vivo by proteolysis following leaf senesence has been reported and suggested that proteolysis took place in vivo rather

Fig. 1. SDS-PAGE (10%, w/v) of dissociated coat proteins of native and long-term storage virus preparations (Lane 2-6). Protein molecular weight standards are in kilodalton (Lane 1). Lane 2, long-term storage cowpea mosaic virus (CPMV) showing two coat proteins (37 kD and 22 kD) indicated by arrows. Lane 3, long-term storage southern bean mosaic virus (CPMV) showing degraded 29 kD coat protein indicated by arrow. Lane 4, native soybean mosaic virus G5 strain (SMV-G5) showing partially degraded coat protein (34 kD) and minor peptides (31 kD) indicated by arrow. Lane 5, long-term storage SMV-G4 showing degraded coat protein (31 kD) indicated by arrow. Lane 6, native SMV-G1 showing partially degraded coat protein (34 kD) and minor peptides (32 kD and 31 kD) indicated by arrow.

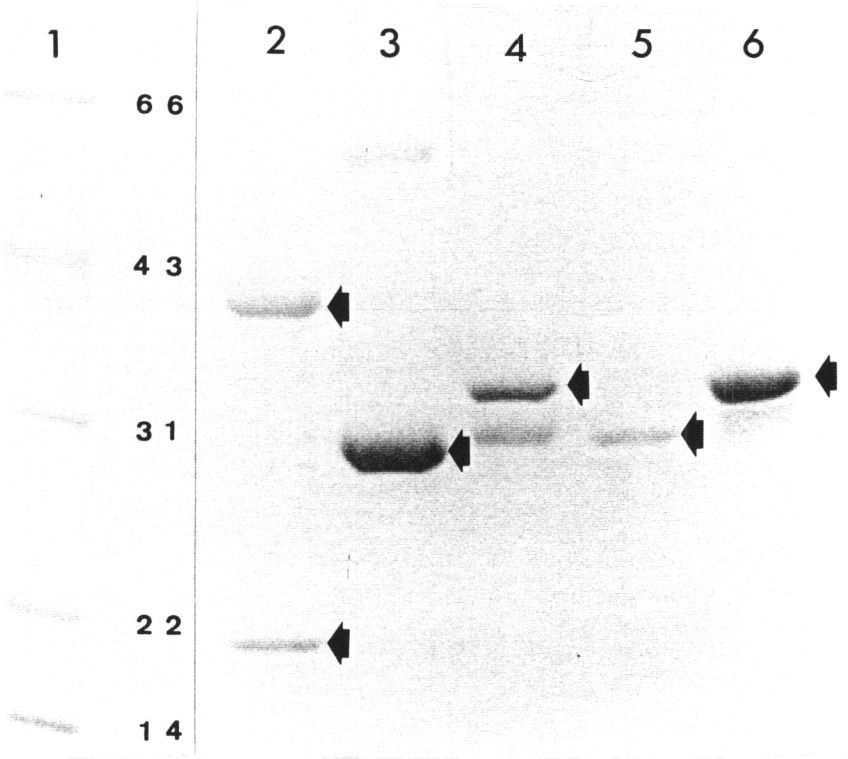
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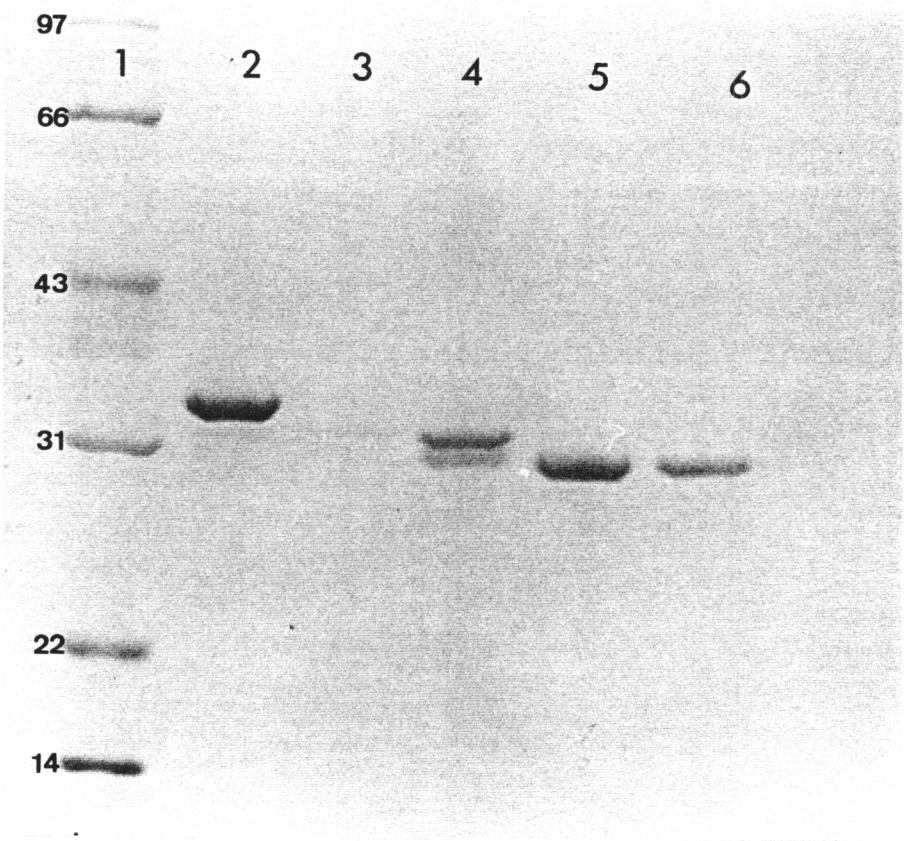
Fig. 2. Trypsin treatment of native soybean mosaic virus G1 strain (SMV-G1). Native SMV-G1 was incubated with trypsin at 37°C; samples were removed after 5 min (lane 4), 30 min (lane 5) and 60 min (lane 6) and added directly to SDS-PAGE sample buffer at 100°C for 5 min, and compared to native SMV-G1 (lane 2) and long-term storage SMV-G1 (lane 3). All samples were resolved on a 10% (w/v) SDS-PAGE and analyzed by Coomassie blue staining. The molecular size markers are shown on the lane 1 in kilodalton (kD).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than during purification (Brakke et al., 1990).

**Alkaline phosphatase treatments of CP.** We investigated the possibility that phosphorylation might be responsible for the observed heterogeneity due to maturation stages of virus particles. The heterogeneity of duck hepatitis B virus core particle protein in SDS-PAGE was removed by incubation with alkaline phosphatase (Pugh et al., 1989). This result indicates that phosphorylation of external amino acids was responsible and that dephosphorylation plays a role in virus maturation (Pugh et al., 1989).

Native SMV-G1 and -G5 were incubated with alkaline phosphatase for varying intervals. CP was resolved on a 10% (w/v) SDS-PAGE and detected either by Coomassie blue staining (Fig. 3A) or by Western blot (Fig. 3B) with a enzyme conjugated-rabbit IgG against intact SMV-G1. The results in Fig. 3A indicate that heterogeneity of CP was not removed by phosphatase treatment and even prolonged incubation of CP with alkaline phosphatase resulted in no effects (Fig 3B). Therefore, the heterogeneity of CP is likely due to loss of N- and C terminal regions as previously described (Shukla et al., 1988b).

**Sensitivity of Western blots using three types of alkaline phosphatase-conjugated IgG.** In an indirect enzyme-linked immunosorbent assay (ELISA), coating IgG $F(ab')_2$ fragments trapped virus and detected it by intact IgG (from same or different antiserum) and $F_C$ portion of IgG (Barbara

and Clark, 1982). Recently, IgM as capture and IgG as detecting antibodies were used and allowed sufficient discrimination of the antigens in ELISA (Jones et al., 1988).

We tested the discriminating capacity and sensitivity of various enzyme-conjugated second antibodies with Western blots. The three types of IgG conjugates compared for their suitability each gave detection of the CP of long-term stored SMV-G1, and displayed 32 kD and 31 kD polypeptides on the membrane (Fig. 4A,B,C). However, the IgG-based (i.e. goat anti-rabbit IgG wholemolecule and goat anti-rabbit IgG $F(ab')_2$ fragments) conjugates could not detect small amount of peptide (31 kD), which was detected with virus-specific IgG conjugate (Fig. 4A). In terms of sensitivity of the antigen detection, the virus-specific IgG conjugate was the most sensitive for detecting small amount of antigen, but resulted in higher background than those of two commercial IgG conjugates. Both commercial IgG conjugates were equally effective in detection, but gave a lower sensitivity to antigen. It may be necessary to use the both commercial enzyme-conjugated IgG from different animal sources at higher concentrations than the 1:500 dilution we used.

Even though it has been reported that indirect assays are more sensitive (Bar-Joseph and Malkinson, 1980) and able to detect a wider range of related virus strains (van Regenmortel and Burckard, 1980) than the DAS with ELISA, we have observed that DAS is more sensitive in the Western blots.

Fig. 3. Alkaline phosphatase treatment of native soybean mosaic virus G1 strain (SMV-G1) and G5 strain (SMV-G5). (A). SMV-G1 was incubated with alkaline phosphatase at 37°C and removed after 0 (lane 1), 0.5 (lane 2), 1 (lane 3), and 3 (lane 4) hrs. SMV-G5 was incubated and removed after 0 (lane 5), 0.5 (lane 6), 1 (lane 7), 3 (lane 8) hrs indicated by arrow. All samples were added directly to SDS-PAGE sample buffer at 100°C for 5 min, resolved on a 10% (w/v) SDS-PAGE and analyzed by Coomassie blue staining. The portions of molecular size markers are shown on the left in kilodalton (kD). (B). After alkaline phosphatase treatment for 18 hr of native SMV-G5 (lane 1) and SMV-G1 (lane 2), samples were resolved 10% SDS-PAGE, analyzed by Western blot procedure, and displayed polypeptides as indicated by arrow.

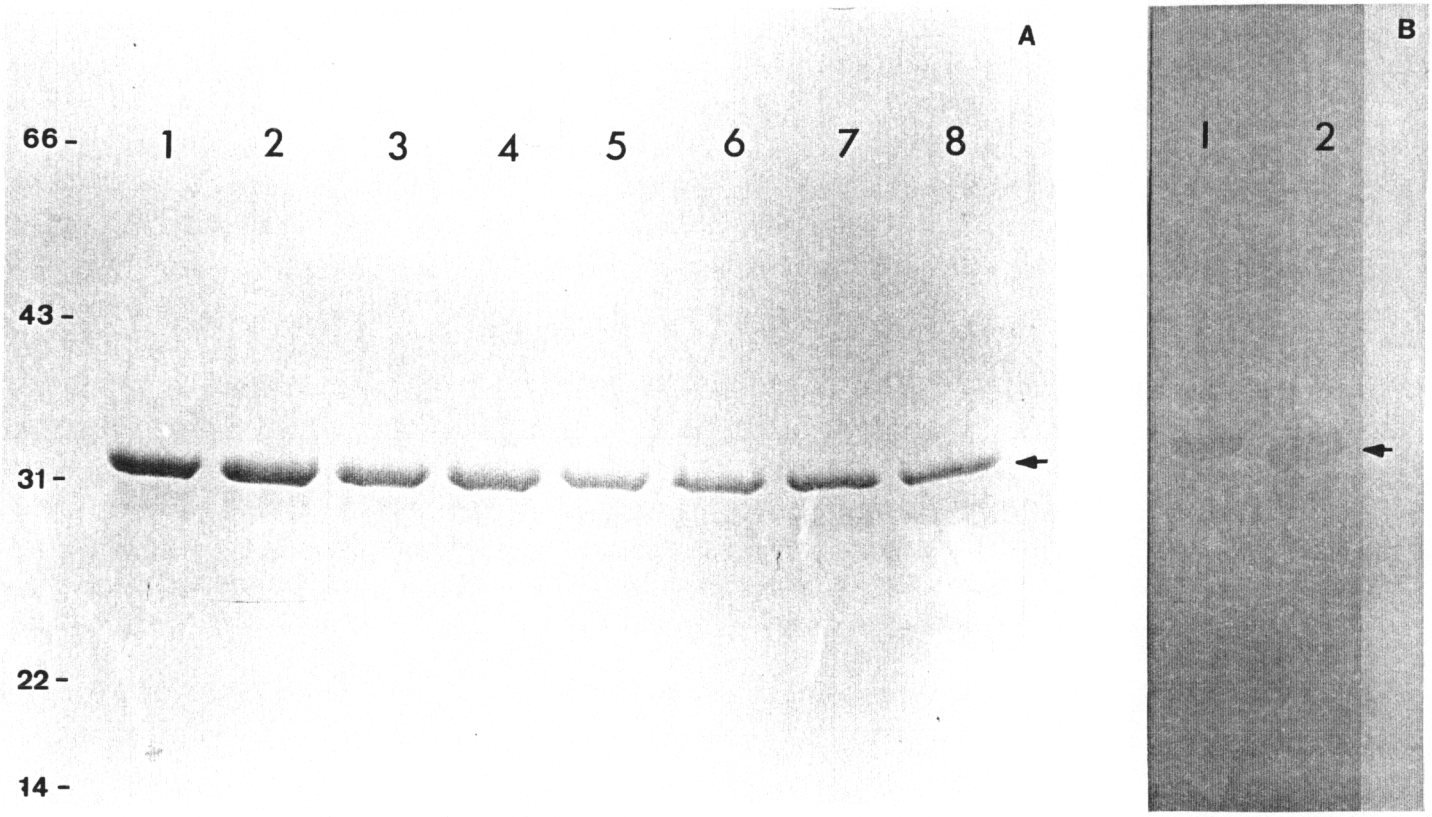
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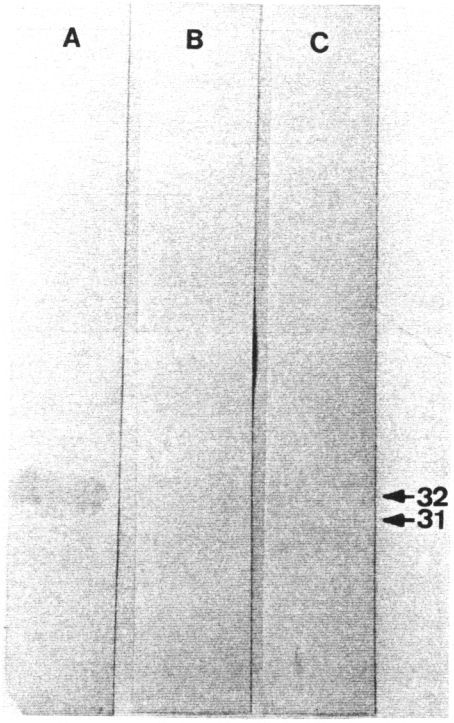
Fig. 4. Different sensitivity of Western blots using three types of alkaline phosphatase-conjugated IgG. Coat proteins from long-term storage SMV-G1 were electrophoresed on 10% (w/v) SDS-PAGE, blotted to Nytran membrane, trapped with polyclonal antiserum against intact SMV-G1, and detected by enzyme-conjugated rabbit IgG against SMV-G1 (lane A, 1:1000 dilution of 1 mg/ml); enzyme-conjugated goat anti-rabbit IgG wholemolecule (lane B, 1:500 dilution of 0.28 mg/ml); enzyme-conjugated goat anti-rabbit IgG $F(ab')_2$ fragments (lane C, 1:500 dilution of 0.47 mg/ml). The numbers indicated polypeptides of SMV-G1 are in kilodalton (kD).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**Western blots and immunological detection of trypsin-digested peptides.** Antiserum to intact virions, containing predominantly 34 kD protein, reacted with 34, 32 and 31 kD proteins of SMV-G5 and -G1, but not CP of southern bean mosaic virus (SBMV) on Western blots (Fig. 5 A,B). We find that minor proteins are unlikely to reflect virus mixtures since both G1 and G5 strains contained similar minor proteins. Additionally, based on cross-reactivity with antiviral antiserum, the minor proteins are independent of the host contaminants. Antiserum against PMV, one of the potyviruses, did not cross-react with SMV CP, while it reacted slightly with the CP of southern bean mosaic virus (SBMV). This finding confirmed a lack of immunological relationship between SMV and PMV by agar immunodiffusion tests containing SDS (Tolin and Ford, 1983).

Based on the previous reports to obtain maximum differentiation of polypeptides (Hill et al., 1989), coat proteins of both strains were digested with trypsin for 15 hr and analyzed on SDS-PAGE followed by Western blots. No reactions were observed between trypsin and antibodies or polypeptides (data not shown). In the immunoreactive pattern produced by using polyclonal antibody, major 6 fragments (34 kD, 32 kD, 31 kD, 29 kD, 21.5 kD, 19.5 kD, and 17.5 kD) could be detected. However small fragments less than 15 kD ranges could not be detected due to limit of amount of small peptides (Fig. 6). Even though we failed to detect small

fragments, it is quite similar pattern shown with monoclonal antibody S3 (IgG1) with SMV-G5 and -G1 (Hill et al., 1989). Both strains showed unique cleavage pattern by trypsin that reflect insufficient discrimination of the epitopic specificity of the viral CP by polyclonal antibody. Even though both strains showed unique patterns that reflected epitopic specificity of the CP, the approaches enhance the potential for identification of strain differences. Since the N-terminal region contains the virus-specific epitopes, its gradual loss from the surface of particles would render virus particles containing only nonspecific epitopes (Shukla et al., 1988b). Interestingly, 34 kD major protein in the native peptides decreased to minor one, while minor 32 and 31 kD proteins increased as major peptides (Fig. 6). These results provide strong evidence that the heterogeniety of SMV-CP is induced by proteolytic processing.

Fig. 5. Western blot analysis of coat proteins of soybean mosaic virus strain G1 (SMV-G1) (lane 1), SMV-G5 (lane 2), and southern bean mosaic virus (SBMV) (lane 3). Coat proteins of viruses were electrophoresed on 10% SDS-PAGE, blotted onto Nytran membranes, probed with polyclonal antiserum against SMV-G1 (panel A) and antiserum against peanut mottle virus (PMV) (panel B), and polypeptides are indicated by arrows.

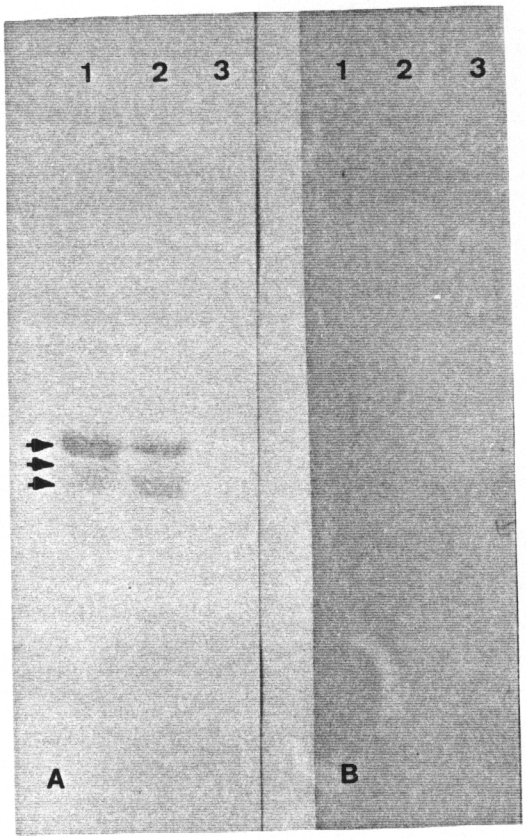
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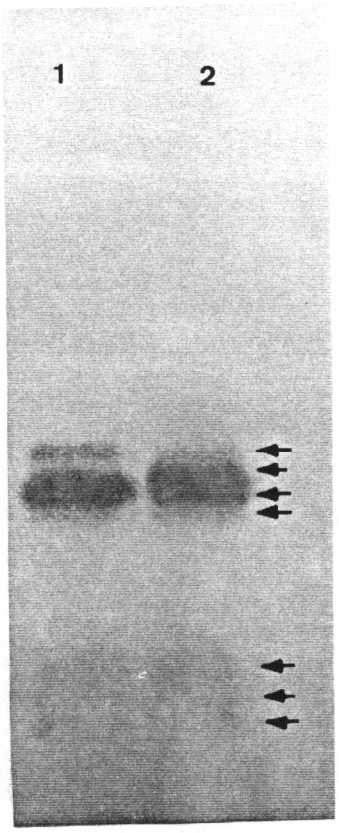
Fig. 6. Pattern produced by polyclonal antiserum against intact SMV-G1 from Western blot analysis of one-dimensional trypsin peptide mapping of coat proteins of SMV-G5 (lane 1) and SMV-G1 (lane 2). Polypeptides from tryptic digests of coat proteins indicated by arrows.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## CHAPTER VIII.

### Summary and Discussion

In various early chapters I have reported present knowledge of SMV-soybean interaction concerning immunological properties of virus coat protein, immunological assays of virus replication and spread, and symptomatology, physiological, biochemical, and molecular analysis of resistance mechanisms associated with differential responses based on genetic background. At present, little is known about what viral and host factors are triggered by *Rsv* resistance to SMV, however we discuss and elucidate the possible theoretical mechanism.

A single dominant gene, $Rsv^y$, provides operational resistance in soybean cv. York to SMV-G1, is race-specific and permits no visible symptom or virus replication (R response). This R response is very similar to that of $R_y$ resistance showing immunity, that is operative in potato to potato virus Y (PVY) (Barker et al., 1984) and a more resistant response than the hypersensitive response (HR) type of well-known tobacco *N* and *N'* resistance to TMV or tomato *Tm* resistance. Unlike the HR response, the R response did not localize virus and induce a series of cascade defense mechanisms, but inhibited virus replication at the recognition level. However, this resistance was overcome by a resistance-breaking strain, SMV-G4, which induced lethal

systemic necrosis. This necrosis-inducing strain is replicated on a limited basis, spreads through restricted portions of vein, stem, and even uninoculated upper leaves, and produces severe systemic necrosis (N response). Based on genetic studies, this N response has been classed as resistance (Buss et al., 1989a,b; Chen et al., 1991). Like the HR response, the N response induced a series of cascade defense mechanisms involving secondary responses and induced well known defense genes, proteins, lytic or toxic enzymes, and other compounds. Unlike the HR response, this response can not localize virus at the infection site, and is represented as less resistant than HR.

Gene-dosage dependent resistance is rather strongly associated with a phenomenon that gives partial localization of the virus commonly restricted to the inoculated leaf (Dufour et al., 1988) but rarely allowing the virus to spread throughout the host, but with inhibited multiplication (Fraser, 1987; Fraser, 1990; Matthews, 1991). The latter mechanism, similar to the N response of soybean, was suggested as fitting the quantitative interaction between a constitutive inhibitor and a viral function (Fraser, 1987).

The *Tm-1* gene in tomato appears to inhibit virus replication (Watanabe et al., 1987). Virulence against this gene is located at the two base changes in the putative replicase gene (Meshi et al., 1988). The *Tm-2* gene in tomato prevents cell-to-cell spread, which requires TMV 30

kD protein (Meshi et al., 1987). Thus two systems operate in tomato, and are thought to be the mode of action in the resistance mechanism (Fraser, 1990).

Operationally, the resistance mechanism of soybean appears very similar to that of the tomato two gene system, *Tm-1* and *Tm-2* genes. However the R and N responses in York operate as single system at same locus as the gene $Rsv^y$ gene, but different alleles. An alternative possibility is that there may be two distinct, but tightly linked, genes that have dominant effects. Current genetic studies to date, however, support the single gene with multiple allele model (Chen et al., 1991). At this moment, we may propose a resistance mechanism based on the results obtained by the series of SMV-soybean interaction studies with genetic backgrounds thus far, in which the $Rsv^y$ gene that plays a role in the strain-specific recognition event may be an inhibitor in the R response. However, this gene may not inhibit resistance-breaking or necrosis-inducing strains containing different virulence factors, rather it may be a signal perception molecule to induce defense-related pathways.

Resistance mechanism in the N response can be considered under two categories: i) a signal transduction pathway that induces physiological and biochemical changes in metabolism; ii) a localization mechanism that induces lesion formation to block the virus movement, but that is ineffective

and results in systemic necrosis following veinal virus spread and limited virus replication.

A major problem in all previous investigations through the decades has been necrogenesis that causes secondary changes in metabolism which are not directly involved in the primary localization of viruses. Based on our observations, we can discuss the primary or secondary factors involved in necrogenesis or resistance mechanisms and classify primary and secondary responses. Summarized results are as follows:

**PAL, CHS, and glyceollin:** A biphasic pattern of PAL activity was observed. A transient and early peak at 1 DAI was observed in both the R and N responses. A secondary peak 2 DAI in the R response and 3 DAI in the N response, was followed by a rapid decrease just before initial appearance of necrotic lesions (primary, R- and N response-associated). There was also a biphasic pattern of PAL mRNA induction, i.e., transient, early, and weak peak induction of PAL mRNA at 6 hr after inoculation and a secondary stronger peak at 12 hr after inoculation (primary, N response-associated). Induction of CHS mRNA, also followed a biphasic pattern, with a transient, early, and strong peak at 6 hr after inoculation and secondary weaker peak 5 DAI, following necrosis development (primary and secondary, N response-associated). Glyceollin accumulated at 6 DAI appearing as N response-associated and a secondary compound.

**POX and lignin:** The pattern of POX activity was also biphasic, with a transient and early peak at 1 DAI, and a secondary peak 4 DAI corresponding to initial appearance of necrotic lesions, followed by a slow decrease (primary, N response-associated). Lignin accumulation followed necrosis development (secondary, N response-associated).

**SPR proteins, CHN, and GLN:** There was a single pattern of SPR protein accumulation, CHN activity and an increase in isoforms following necrosis development. A single pattern of GLN activity and increase in isoforms were observed following necrosis development (secondary, N response-associated). CHN mRNA induction increased in the N response from 9 hr to 24 hr (primary, N response-associated).

**HMGR:** Soybean HMGR mRNA expression is strongly activated with race-specific resistance.

**SMV coat protein:** The heterogeneity and degradation of CP is related with N- and C-terminal loss exposed on the particles' surface.

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**SOYBEAN MOSAIC VIRUS-SOYBEAN INTERACTION: MOLECULAR, BIOCHEMICAL, PHYSIOLOGICAL, & IMMUNOLOGICAL ANALYSIS OF RESISTANCE OF SOYBEAN TO SOYBEAN MOSAIC VIRUS**

by

Chang W. Choi

(ABSTRACT)

Strain-specific resistance conditioned by a single dominant gene in soybean cv. York inoculated with SMV-G1, revealed no symptoms and no detectable viral replication (R). Unlike the hypersensitive response (HR), the R response did not result in localized virus and induction of a series of defense responses, but in inhibition of virus replication. However, this resistance was overcome by the resistance-breaking strain, SMV-G4, which induced lethal necrosis (N). Unlike HR, G4 strain was not restricted but spread in a restricted pattern along the vein, stem and into upper uninoculated leaves where it induced necrosis.

Like HR, PR proteins were found to accumulate in the N response and were named SPR (soybean pathogenesis-related) proteins. On the basis of major similarities in molecular weight characteristics and enzyme-substrate specificities, SPRs are proposed to be classed into four groups: SPR1; 1a, 1b, 1c, 1d; SPR2: 2a, 2b, 2c; SPR3: 3a, 3a′, 3b, 3c, 3d, 3e; SPR4. The functions of SPR1 and SPR4 groups have not yet been determined. The SPR2 group was identified as $\beta$-1,3-glucanases (GLN) and classed two subgroups. The SPR3

group was identified as chitinases (CHN) and classed three distinct subgroups.

Like HR, the N response induced a series of defense responses with marked temporal increases, in a bimodal pattern, of phenylalanine ammonia-lyase (PAL) and peroxidase (POX) activities, bimodal pattern of PAL mRNA and chalcone synthase (CHS) mRNA induction, a single pattern of chitinase mRNA induction, and glyceollin and lignin accumulation. These marked increases occurred at or just before the time of initial appearance of necrotic lesions, or following necrosis development for localization of virus.

Total RNA isolated from soybean inoculated with SMV-G4 were used for synthesis of cDNA by reverse transcription, and amplified using 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR)-specific primer sets by polymerase chain reaction. Amplified cDNAs were cloned into Bluescript vector ($pKS^-$), transformed into *E. coli*, isolated pSOY HMGR and used as a probe for Northern analysis. We demonstrate that expression of HMGR mRNA is correlated with strain-specific resistance (R).

SMV coat protein (CP) degraded *in vitro* by proteolysis during purification. The CP of SMV purified from infected leaves had an major size of 34 kD in SDS-PAGE with minor peptides of 32 and 31 kD. The minor peptides increased during the storage at $4^oC$ or with trypsin treatment, and reacted with antiserum to intact virions. This heterogeniety

of protein was not removed by alkaline phosphatase treatment for varying time intervals, and was not related with phosphorylation and dephosphorylation, or with virus maturation. These studies provided additional evidence of N- or C-terminal exposure on the particle's surface.

## VITA

Chang Won Choi was born January 27, 1959 in Seoul, Korea. He received his elementary and secondary education in Seoul. Mr. Choi received his B.S. in Agronomy from Korea University (Seoul, Korea) in 1982 and continued graduate studies for a year in Plant Protection major at same department. He transferred and received his M.S. in Plant Pathology from South Dakota State University (Brookings, SD) in 1986. Prior to attending Virginia Polytechnic Institute and State University, he completed military service as a second lieutenant in Korea. In April of 1987, he enrolled in VPI & SU to pursue the Ph.D. degree in the Department of Plant Pathology, Physiology, & Weed Science, specializing in the area of plant pathology and plant virology. While at VPI & SU, the author has been employed as a graduate research and teaching assistant in the Dept. PPWS. He completed the requirements for the Ph.D. degree in June of 1991.