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subsidiary of The Upjohn Company

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PETITION FOR DETERMINATION OF REGULATORY STATUS

Gentlemen:

Enclosed is a copy of a petition for determination on the regulatory status of *Cucurbita pepo* L. cultivar YC77E ZW-20 which has been modified to be resistant to watermelon mosaic virus-2 (WMV-2) and zucchini yellow mosaic virus (ZYMV), which is currently deemed a "regulated article". Based on the data and information contained in the enclosed petition, we believe that there is no longer "reason to believe" that the modified squash plant should be deemed to be a regulated article. The modified squash plant does not present a plant pest risk and is not otherwise deleterious to the environment. The enclosed petition does not contain confidential business information.

The undersigned certifies that, to the best of our knowledge and belief, this petition includes all data, information, and views relevant to the matter, whether favorable or unfavorable to the position of the undersigned, which is the subject of the petition.

Sincerely,

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enclosure

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I. Rationale for the Development for Transgenic Squash

Commercial production fields are plagued by a number of virus-related diseases. The four most important of these diseases are caused by cucumber mosaic virus (CMV), watermelon virus 2 (WMV-2), zucchini yellow mosaic virus (ZYMV), and papaya ringspot virus (PRV). All four viruses are transmitted by aphids in a non-persistent manner (Provvidenti 1986); an aphid can efficiently acquire a virus and inoculate a plant during feeding (probing) intervals of as little as two minutes (Gibbs and Harrison 1976). Since fruits that develop on infected plants become highly distorted or discolored, plants infected with these viruses have significantly lower yields of marketable fruit than non-infected plants. Commercially acceptable squash cultivars that exhibit resistance to even a single one of these four viruses are not presently available. Consequently, in an attempt to prevent squash plants from becoming infected with virus, plants are sprayed repeatedly with insecticides or stylet oils in an attempt to control aphids, which are responsible for virus spread in the fields. However, it is impossible to eradicate aphids completely from a field regardless of the amount of insecticide that one applies. Furthermore, stylet oils are not completely effective and must be applied repeatedly. As aphid populations increase during the growing season, these methods of protection against virus infection become increasingly less effective.

Genetic engineering offers a means of developing squash hybrids which are protected from virus infection without altering the plants' commercially or horticulturally desirable characteristics. For example, the expression of the cucumber mosaic virus coat protein gene in transgenic tobacco plants confers resistance against subsequent infection by CMV, one of the important cucurbit viruses. Virus-inoculated transgenic plants exhibit milder or delayed symptoms when compared to inoculated control plants (Cuozzo *et al.*, 1988). Other investigators have demonstrated the same phenomenon by expressing the coat protein genes from other viruses in various species of transgenic plants (Powell-Abel *et al.*, 1986, Tumer, *et al.*, 1990, Nelson *et al.*, 1988, Hoekema *et al.*, 1989). We have extended these observations by utilizing *Agrobacterium tumefaciens*-mediated transformation to introduce plant-expressible CMV, WMV-2, ZYMV, or PRV coat protein gene(s) into the genome of selected Asgrow inbred squash lines.

During field trials conducted in 1990 and 1991, we identified a transgenic squash line that exhibited significant resistance against WMV-2 and ZYMV. The original regenerated plant was transformed with a Ti plasmid vector containing ZYMV and WMV-2 coat protein genes as well as the selectable marker gene for neomycin phosphotransferase (NPT II). Five T-DNA insertions occurred in the original plant. However, not all of the insertions were complete, and in all but one of them, the NPT II gene was absent. Furthermore, not all of the T-DNA insertions were linked, and in subsequent generations, we have been able to select lines which contain and express the viral coat protein gene(s), but not the NPT II gene. We intend to use one

of these virus-resistant, NPT-II-lacking transgenic lines in place of the virus susceptible parental line used in the production of a yellow crookneck squash hybrid now sold by Asgrow. This hybrid should produce fruits of superior quality and quantity in regions where infection by WMV-2 and ZYMV is prevalent.

II. The Family *Cucurbitaceae*

A. Taxonomy of Cucurbita

Plants of the genus *Cucurbita* are members of the family *Cucurbitaceae*. This family has a tropical or subtropical distribution, and its members do not tolerate temperatures below freezing (Whitaker and Robinson 1986). The genus *Cucurbita* includes five domesticated species and 22 wild species, all of which possess 20 pairs of chromosomes (Decker 1988). The genus is indigenous to the Americas with most of the species coming from North America. The two exceptions are the South American species C. *maxima* and C. *andreana*. The center of distribution of the genus is believed to be central or southern Mexico. (Whitaker and Davis 1962).

Although the majority of *Cucubita* species are mesophytic, a number of perennial xerophytic species have evolved, and can be found in Northern Mexico and in the southwestern United States (Whitaker and Davis, 1962). Some of the mesophytic types have adapted to more humid conditions, and one species in particular (C. *okeechobeensis*) can be found as far north as Florida (Whitaker, *et al.*, 1962). Five additional species of *Cucurbita* can be found growing in warmer areas of the United States (Kartesz and Kartesz, 1980), and one species, C. *foetidissima*, is occasionally found in the upper Midwest (Gleason 1952).

B. Squash as a Crop

The four cultivated species of *Cucurbita* which produce fruit for human consumption are C. *pepo*, C. *maxima*, C. *mixta* and C. *moschata*. These species are believed to have originated from the mesophytic complex of gourds that are also indigenous to southern Mexico and northern Central America.

For best growth and development, C. *pepo* and C. *maxima* are adapted to the long days of summer for vegetative growth and the cooler, shorter days of fall for fruit development. In contrast, cultivars of C. *moschata* perform best under the warm conditions of the tropical lowlands, and cultivars of C. *mixta* are adapted mainly to tropical and subtropical conditions (Whitaker and Robinson, 1986).

Edible fruit harvested from cultivated *Cucurbita* species are called squash or pumpkin. The term "squash" is used to refer to all baking cultivars of C. *maxima* Duch., C. *moschata* (Duch). Duch. ex Poir (i.e. Butternut), and the cushaw-type cultivars of C. *mixta* Pang. For all these types, the mature fruits are consumed. The

term "squash" is also used to refer to non-baking cultivars of C. *pepo* in which immature fruits are consumed, as well as certain cultivars of C. *pepo* (i.e. Acorn) in which mature fruits are baked and consumed. The term "pumpkin" is normally applied to the edible fruits of any species of *Cucurbita* used when ripe as forage, as a table vegetable, or in pies (Whitaker and Robinson, 1986, Culter and Whitaker 1961). The squash line under consideration in this application belongs to C. *pepo*. The yellow crookneck squash it produces is mainly consumed as a nonbaked table vegetable, or processed for the frozen food market.

C. Pollination of Cucurbita

C. *pepo* plants are vining or bushy, with five-sided, rough, bristly to prickly stems. The flowers are large and showy with united yellow to orange-yellow petals. The flowers are unisexual. The staminate flowers--possessing three stamens--are located near the center of the plant, and are borne on long, slender pedicels. Pistillate flowers are borne on short pedicels, distal to the staminate flowers, and possess an inferior ovary divided into three loculi. Since all species of *Cucurbita* are monecious and produce heavy sticky pollen grains, pollination requires some agent other than wind to transmit the pollen from the staminate to the pistillate flowers. Under agricultural conditions, this transfer is normally accomplished by domestic honey bees, or by species of wild gourd bees or squash bees (Whitaker and Robinson, 1986).

D. Interspecific Hybridization

Interspecific hybridization has been extensively investigated and is well understood for the four annual cultivated species (C. *pepo*. C. *mixta*, C. *moschata* and C. *maxima*). F_1 hybrids can be obtained in breeding programs, but only with difficulty and such hybrids are usually sterile. There is no evidence for spontaneous hybridization among these four species despite the fact that they have been grown side by side under cultivation for many generations (Whitaker and Robinson, 1986). A century ago, Pammel (1892) interplanted various cultivated species of *Cucurbita* in an attempt to achieve interspecific crosses by natural means, but was unsuccessful. He concluded that "under natural conditions the different species of *Cucurbita* will not produce hybrids as a rule". The crossing relationships between the cultivated species reflect what is known for the genus as a whole. Most species have barriers to interspecific crossing (see Figure 1).

E. Weediness of *Cucurbita*

Various species of the genus *Cucurbita* have been reported as weeds outside the continental United States. C. *andreana*, a medium sized gourd-producing plant, has been reported as a weed which colonizes disturbed areas. C. *pepo* has been reported as a weed in Jamaica and West Polynesia (Holm *et al.*,1979).

Within the continental United States, *C. digitata* and *C. foetdissima* have been reported as a weed. Within the states of Florida and Georgia where yellow crookneck squash are commercially grown, one can find a few specimens of domesticated *Cucurbita* growing without cultivation. The Flora of Georgia (Duncan and Kartesz 1981) include C. *moschata* (Duchn) Duch. ex Poir and C. *pepo* L. var ovifera (L) Alef as part of the flora of the coastal plain of Georgia. C. *pepo* and C. *moschata* have also been occasionally found in disturbed areas of the southern peninsula of Florida (Long and Lakela, 1976), but have not been identified in the Florida panhandle (Clewell,1985).

F. Wild Relatives of Cultivated *Cucurbita*

The majority of wild species of *Cucurbita* are found in areas south of Mexico City to as far south as the Mexico-Guatemala border (Whitaker and Robinson, 1986, Whitaker and Bemis, 1964). These wild species are generally annual mesophytic plants. However, eight perennial xerophytic species are found from Baja California to the Gulf of Mexico, and extend northward to the southwestern United States, including New Mexico, Arizona and Southern California.

The xerophytic perennial C. *foetidissima* has been reported in Utah, Nevada, Kansas, Colorado, Missouri, Texas, Southern California, Arizona and even as far north as Nebraska. Xerophytic perennial species have been shown to be incompatible with mesophytic species (Whitaker and Bemis 1964). Pollinations with cultivated species result in failure of fruit set and embryo development; even after repeated attempts. It has been demonstrated that C. *moschata* will produce F_1 plants when crossed to some xerophytic types, particularly C. *foetidissima*. However, these plants are completely sterile (Whitaker and Bemis, 1964).

In addition to fertility barriers to crossing, temporal barriers exist as well. Many wild species require a short photoperiod to flower while cultivated species flower under long photoperiods. Therefore, even if interspecific pollinations between cultivated and wild species were to occur and viable seeds produced, these would only occur late in the season, and seeds would probably fail to mature before the plants are killed by frost (Whitaker and Davis, 1962).

The only wild species growing within the United States which can cross with *C. pepo* without loss of fertility is *C. texana* (Scheele) (Whitaker and Davis, 1962). It is morphologically very similar to the ornamental gourds (*C. pepo L. var. overfera Alef.*) which has caused some authors to regard them as conspecific (Decker and Wilson, 1986). There is also documentation of spontaneous populations of *texana*-like plants in Alabama, Arkansas, Illinois, and Missouri, mainly around watercourses such as the Arkansas and Red River (Harrison, *et al.*, 1977). There is general uncertainty as to the relatedness of populations of *C. texana* to *C. texana*-like plants found Northeast of the State of Texas. Spontaneous populations occurring within Texas are referred

to as *C. texana* while those occurring outside Texas are usually classified as *C. pepo* var *ovifera* (Steyermark, 1963). This reflects the general assumption that *C. texana* is endemic only to Texas and spontaneous populations occurring elsewhere are escapes from cultivation (Decker and Wilson, 1986).

G. Modes of Escape in Squash

Genetic material of *C. pepo* may escape from areas under cultivation by seed, or pollen.

The escape of genetic material by seed is unlikely, since yellow crookneck squash requires 45 days after pollination to develop mature seeds. When grown under commercial farming practices, the fruits (and seeds) are harvested in an immature state. Therefore the seeds produced in these immature fruits would be nonviable, making the likelihood of escape of genetic material by seed unlikely.

There is, however, the limited likelihood of movement of genetic material by pollen. Within the U.S., movement of genetic material by pollen to wild species is only possible between C. *texana* and C. *pepo*. However, C. *texana*'s geographical distribution is restricted and does not include Georgia or Florida, where the majority of yellow crookneck squash are produced. Therefore, geographical overlap between these two species is limited. In the areas of overlap, transmission of genetic material to *C. texana* should be further limited by the fact that squash pollen only travels short distances. For example, a distance of 400 meters serves to isolate genetically engineered squash varieties for seed production (Figure 2). The limited range of pollen travel appears to be characteristic of cucurbits, for Handel's studies of pollination and bee foraging behavior in cantaloupe and cucumber (Händel, 1982 and 1983) also demonstrate that the pollen in these species also travels only a short distance. The effectiveness of this distance is corroborated by data we have obtained. During our 1990 and 1991 field trials, border rows were sampled in order to monitor the flow of transgenic pollen. Our data show that the frequency of transgenic pollen declines rapidly as one moves away from the transgenic source plants (Figure 2).

H. Uses of the Transgenic Line.

The transgenic line under consideration in this document (ZW-20) was derived by transforming an inbred squash line which is used by Asgrow in the production of the yellow crookneck hybrid, Pavo. Pavo is grown in the southeastern United States particularly in Georgia and Florida. We intend to replace the nontransgenic parent with the transgenic line to produce this hybrid. In addition, new proprietary lines are being developed through backcrossing to other cultivated varieties of *C. pepo*, including zucchini, yellow straightneck, acorn, butternut, and pumpkin types.

III. DESCRIPTION OF THE VECTOR SYSTEM AND THE REGULATORY SEQUENCES TO BE EXEMPTED

Disarmed *Agrobacterium tumefaciens* strain A208.35

The *Agrobacterium tumefaciens* strain used to transfer the engineered coat protein genes of watermelon mosaic virus 2 (WMV2), and zucchini yellow mosaic virus (ZYMV) into the ZW-20 squash line is a non-pathogenic (avirulent) derivative of the A208 vector (Sciaky *et al.*, 1978). A208.35 was constructed by the replacement (via homologous recombination) of the wild-type Ti plasmid of strain A208 with the disarmed T-DNA region of the plasmid pGA482 (An, 1986; see below). During the replacement, one of the borders of the T-DNA region was inactivated, resulting in the inability of the T-DNA in the helper plasmid to transfer into plant cells.

A208.35 possesses the genes necessary for the transfer and integration of a T-DNA region from *Agrobacterium* into the genome of a host plant. These genes (referred to collectively as the virulence genes) are located outside the T-DNA region and encompass approximately 35 kb of DNA (Das *et al.*, 1986). The proteins encoded by these genes serve to transfer DNA fragments--delimited by specific 25 base-pair sequences--into plant genomes (Zambryski 1988; Slightom *et al.*, 1985).

B. Promoter, Transcription Termination, and Polyadenylation Signal Sequences

A key step in the design of a plant expression vector is the identification and characterization of strong promoters (Tempe and Goldmann, 1982; Fraley *et al.*, 1983; Sanders *et al.*, 1987). Promoters are DNA sequences to which RNA polymerase binds in order to initiate transcription. Promoter DNA sequences are located 5' to functional gene sequences, and are not transcribed into mRNA. A strong promoter results in high levels of mRNA synthesis, which may or may not result in high levels of protein production.

Promoters derived from plant pests can be divided into two major classes: those derived from cauliflower mosaic virus (CaMV) or those derived from the Ti plasmid. Ti plasmid derivatives include promoters from the mannopine synthase (Willmitzer *et al.*, 1983), octopine synthase (Koncz *et al.*, 1983), and nopaline synthase (Herrera-Estrella *et al.*, 1983) genes. These promoters have two desirable characteristics: 1) they are among the most highly transcribed Ti plasmid genes (Willmitzer *et al.*, 1983; Velten *et al.*, 1984), and 2) they are constitutively expressed (Bevan *et al.*, 1983). However, stronger promoters possessing these same characteristics have been derived from CaMV. Two major RNA transcripts, designated 35S and 19S (based on their sedimentation coefficients), are produced during CaMV replication (Hull and Covey, 1983). The 35S CaMV promoter sequences have been characterized by Nagy *et al.*, (1985), Odell *et al.*, (1985), Nagata *et al.*, (1987); the 19S promoter has been characterized by Balazs *et al.*, (1985). In the experiments described in this document,

the 35S CaMV promoter was used to drive the expression of the WMV-2 and ZYMV coat protein genes.

Another step in the design of a plant expression vector is identification of transcription termination and polyadenylation signal sequences. Most eukaryotic mRNAs possess a number of polymerized adenosine monophosphate residues (poly A) at their 3' -termini. The number of residues may vary from 20 to 200 bp. The poly A sequences (i.e., series of adenosine residues) are not transcribed, but rather are added posttranscriptionally (Mainwaring *et al.*, 1982). In our vectors, the signal sequences for transcription termination and polyadenylation were derived from the 3'-termini of the 35S CaMV genes.

Although the promoters, transcription termination, and polyadenylation sequences are derived from known plant pests, they cannot cause any disease by themselves nor in conjunction with any sequences located on the Ti plasmid (Rogers *et al.*, 1985; Nagy *et al.*, 1985). Thus, all genes inserted and expressed in plants by the Ti plasmid will contain the following (in 5' to 3' order): a specific plant-expressible promoter, the gene to be expressed, and a polyadenylation/termination signal sequence.

C. Binary Plasmid Vectors

The DNA which was transferred into the plant genomes was contained in binary plasmids (Bevan, 1983). The parent binary plasmid was pGA482, constructed by An (1986). This vector contains the T-DNA border sequences from pTiT37, the selectable marker gene Nos-NPT II (which contains the plant-expressible nopaline gene promoter fused to the bacterial NPT II gene obtained from Tn5), a multiple cloning region, and the cohesive ends of phage lambda (see Figure 3 and An *et al.*, 1985; An, 1986). The following steps were undertaken to construct the vector ZYMV72/ WMBN22, which was used in the transformation of squash.

1. Construction of the plasmid pPRBoriGN

pPRBoriGN was derived from the plasmid pGA482 by the following steps:

1. A bacterial selectable marker, gentamycin resistance, (Allmansberger, *et al.*, 1985) was inserted adjacent to the right border (B_R), but outside the T-DNA region.
2. The Nos-NPTII gene was excised and the multiple cloning site (MCS) was regenerated adjacent to B_R, just inside the T-DNA region.
3. A plant-expressible ß-glucunoridase (GUS) gene cassette (Jefferson, *et al.*, 1987) was inserted within the T-DNA region adjacent to the pBR322 origin of replication.

4. A plant-expressible NPTII cassette produced by insertion of the NPTII coding region into the expression cassette of the *E. coli* plasmid pDH51 (Kay and McPherson, 1987)(see below) was inserted inside the T-DNA region adjacent to the left border (B_L). This NPTII gene is driven by the cauliflower mosaic virus (CaMV) 35S promoter and terminated by the CaMV polyadenylation signal.

A map of this plasmid is shown in Figure 3.

2. Construction of pPRBN from pPRBoriGN

The plasmid pPRBN was derived from pPRBoriGN by the following steps:

1. The region of pPRBoriGN from the beginning of the GUS coding sequence to B_L was deleted. Therefore, the GUS gene and 35S/NPTII cassette were removed as a unit.

2. This region was replaced by a fragment consisting of the 35S/NPTII cassette only.

   The net result of these steps was the removal of the GUS gene plus a short region of pBR322 homology, leaving the plant expressible NPTII gene adjacent to B_L. A map of pPRBN is shown in Figure 4.

3. Construction of ZYMV72/WMBN22 from pPRBN

The vector ZYMV72/WMBN22 was constructed by inserting the plant-expressible genes for WMV2 and ZYMV coat proteins into pPRBN (see section IV. C.).

D. Transfer of Genes into Plant Genomes

The vector ZYMV72/WMBN22 was transformed into *Agrobacterium* strain A208.35 by standard methods (An 1987). Transformed A208.35 bacteria were plated on TY agar plates containing kanamycin and gentamicin, and those containing the binary plasmid were selected because of their ability to grow more rapidly on this medium. The presence of the binary plasmid was confirmed by restriction enzyme digestion and Southern blot analysis. The A208.35 bacteria containing the desired plasmids were then used to infect squash tissues using modifications of the procedure described by Horsch *et al.*, (1985).

E. Gene Insertion Is a One-way Process

The scientific literature supports the view that only the T-region is transferred and integrated into the plant genome (Fraley *et al.*, 1986). The sequence that is

integrated includes the genes which are contained between certain short, well-characterized segments of the Ti plasmid essential for incorporation into the plant genome. Also, border sequences (the 25 base pairs required for transfer) are lost during the process of insertion of T-DNA into the plant cell genome. This means that the inserted DNA is no longer a functional T-DNA capable of being transferred by the same mechanism that originally inserted the T-DNA into the plant genome (Zambryski *et al.*, 1982). The plasmid vector by itself is not viable and can only replicate inside a bacterial cell. Thus, all evidence available since the identification of T-DNA in 1978, and the accumulated knowledge of the epidemiology of crown gall disease, indicates that T-DNA transfer into plant cells by *Agrobacterium* is irreversible.

IV. DESCRIPTION OF DONORS GENES TO BE EXEMPTED

A. Watermelon Mosaic Virus 2

Watermelon mosaic virus 2 (WMV2) is a member of the potyvirus group. It occurs worldwide, and along with zucchini yellow mosaic virus (ZYMV) is one of the major viral pathogens of cucurbit crops in the United States (Alderz *et al.*, 1983; Provvidenti and Gonsalves, 1984; Nemeth et al., 1985; Chala *et al.*, 1987; Davis and Mizuki, 1987). WMV2 is serologically related to ZYMV (Davis, 1986; Purcifull *et al.*, 1984) as well as to Johnson grass mosaic virus (Shukla *et al.*, 1988). Furthermore, the amino acid sequence of WMV2 is extremely similar to that of the N strain of soybean mosaic virus, suggesting that these two viruses are strains of the same virus type (Yu *et al.*, 1989). As with other potyviruses, the genome of this virus is composed of a single RNA molecule, approximately 10,000 nucleotides in length. Proteins are translated from the RNA as a single polyprotein precursor, which is subsequently processed by a viral-encoded protease (Yeh and Gonsalves, 1985), thereby producing the mature protein.

The cloning and characterization of the WMV2 coat protein gene used in our experiments is described in Quemada *et al.*, 1990. From that clone, a plant-expressible WMV2 coat protein gene was constructed (see Figure 5). First, specific oligonucleotide primers and the polymerase chain reaction (PCR) were used to amplify the WMV2 coat protein gene while simultaneously adding the flanking restriction sites AatII and BglII. This gene was ligated to an AatII/BglII-digested derivative of the plasmid pUC19 (Yanisch-Perron *et al., 1985*) modified to possess a BglII restriction enzyme site in its multiple cloning region. A fragment encoding the CaMV 35S promoter, cucumber mosaic (CMV 5' untranslated region, and 48 nucleotides from the 5' terminus of the CMV coat protein gene were then added from the plasmid pUC1813/CP19, containing a plant-expressible CMV coat protein gene cassette (Slightom, 1991). The CaMV 35S polyadenylation signal was also obtained from pUC1813/CP19 coat protein gene construct. The resulting construct should produce a protein which is a fusion between WMV2 coat protein and 16 amino acids

of the NH_3-terminal portion of the CMV coat protein gene. The cassette was designated CPW (Figure 5).

B. Zucchini Yellow Mosaic Virus

Zucchini yellow mosaic virus (ZYMV) is a recently recognized member of the potyvirus group (Lecoq *et al.*, 1983). Despite its relatively recent detection as a viral pathogen, it has become an important disease problem in cucurbit crops (see Provvidenti *et al.*, 1984; Davis & Mizuki, 1987). It has not been characterized extensively at the molecular level, but the existing molecular information suggests that its genome structure is similar to those of other potyviruses (Quemada *et al.*, 1990). It is serologically related to WMV2 (see above).

The cloning and characterization of the ZYMV coat protein gene used in our experiments is described in Quemada, *et al.*, 1990. The strategy employed in the construction of a plant-expressible ZYMV coat protein gene involved the construction of a plant expression cassette designed to provide the necessary elements for expression of potyviral coat protein genes (5' promoter, 5' untranslated leader sequence and ATG translational initiation codon, and 3' polyadenylation signal), and subsequent modification of the ZYMV coat protein gene for insertion into this cassette. The polymerase chain reaction (PCR) technique--with appropriate oligonucleotide primers and pUC1813CP19 (Slightom, 1991) as a template--was used to produce the expression cassette possessing an internal NcoI site and flanking HindIII sites (Slightom, 1991). The NcoI site provided an ATG translational initiation codon, and enabled the insertion of coat protein genes into the cassette. The HindIII site allowed excision of the coat protein gene cassette for transfer to other plasmids. The procedure is briefly diagrammed in Figure 6. This expression cassette is designated pUC18cpexp (Slightom, 1991).

In order to clone the ZYMV coat protein into the expression vector pUC18cpexp, the polymerase chain reaction, using the appropriate oligonucleotide primers, was used to amplify the ZYMV coat protein coding region and to add flanking NcoI sites. This fragment was ligated to pUC18cpexp at the plasmid's NcoI site, producing pUC18cpZYMV. This process is outlined in Figure 7. Because of the site chosen for hybridization of the oligonucleotide primers during the polymerase chain reaction, the protein which will be produced by the pUC18cpZYMV cassette will have two amino acids attached to the NH_3-terminus of the natural coat protein sequence: Met (resulting from the ATG initiation codon) and Gln (the COOH terminus of the adjacent nuclear inclusion protein).

C. ZYMV72/WMBN22

ZYMV72/WMBN22 is the binary plasmid pPRBN (see section III), into which expression cassettes for ZYMV and WMV2 coat proteins have been inserted. To accomplish this, a BamHI site was introduced 5' to the 35S promoter, and a BglII site was introduced 3' to the poly A addition sequence of both the CPW and pUC18cpZYMV expression cassettes. These sites were introduced by the use of appropriate oligonucleotide primers during PCR amplification of the cassettes. The PCR products were then digested with BamHI and BglII to produce the appropriate ends. The CPW cassette carrying BamHI/BglII ends was ligated into the unique BglII site of pPRBN. A construct was selected in which the BamHI end of the CPW cassette was ligated to the BglII site of pPRBN. This construct was again digested with BglII, and the cassette from pUC18cpZYMV fitted with BamHI/BglII termini was then added to yield ZYMV72/WMBN22 (Figure 8).

D. The Transformation Selectable Marker Gene - Neomycin Phosphotransferace

Aside from the viral coat protein genes, a gene encoding the enzyme neomycin phosphotransferase was incorporated into the chromosomal DNA of squash during the transformation process. This gene encodes a protein which confers resistance to the aminoglycoside antibiotic, kanamycin, by phosphorylating the molecule and thereby inactivating it (Fraley *et al.*, 1986). The gene was obtained from *E. coli* transposon Tn5, and functions only as a genetic marker in the cell selection and regeneration process following transformation. The NPT II gene was utilized during the selection of the primary ZW-20 regenerant plant. However, the original regenerant plant was found to contain five inserts of the introduced genes (see below). Four of these inserts had a truncation of the T-DNA in the region of the left border, thus eliminating the NPT II gene (and in one of these cases, the WMV2 coat protein gene as well). The fifth insert consisted of an NPT II gene and WMV2 coat protein gene only. In subsequent generations, we were able to select plants that contained coat protein genes but which lacked the NPT II gene. These plants and their progeny are the subject of this document.

V. Genetic Analysis and Agricultural Performance of Transformed Cultivars

A. Mendelian Inheritance

DNA for the primary (R_0) plant has been examined by Southern blot analysis, and has been determined to contain five inserted T-DNA's (Figure 9). Only one of these inserts contained an NPT II gene (Figure 9a, lane 11). This NPT II gene accounted for R_1 seedling segregation ratios for the NPT II gene activity that were consistent with a single gene insert (Table 1)

TABLE I

Segregation ratios for R_1 seed produced by backcrossing the primary transformants to a non-transgenic plant

	Ratio of NPT II positive plants to NPT II negative plants (assayed by ELISA using anti-NPT II antibodies)		
	Observed	Expected	X^2 (P ~)
Kalamazoo Trial	14:18	16:16	0.14 P~ .70
Tifton Trial	11:14	12.5:12.5	0.08 P ~ .80

When challenged in the field with virus, the inserts unlinked to the NPT II gene were found to confer resistance, even under conditions of heavy virus pressure. These NPT II negative plants were selfed and selected for virus resistance according to standard breeding practice, in order to fix the breeding line ZW-20. Southern blot analysis of the resulting line revealed that two inserts marked by the junction fragments labelled α in Figure 9 had indeed become fixed (homozygous) in this line, but that the other two inserts (marked by the junction fragments labeled β in Figure 9) were still segregating. The α inserts consist of one ZYMV coat protein gene, and one ZYMV/WMV2 coat protein insert. The two β inserts (both consisting of ZYMV/WMV2 coat protein gene pairs) appear to be on the same chromosome since they co-segregate (see Figure 9). Of 20 plants examined in this generation, 13 contained the β set, 7 did not. This ratio is close to the 3:1 expected for Mendelian segregation of this set. Subsequent offspring will therefore contain the α set of genes, but may or may not possess the β set depending upon the selected line.

B. Disease and Pest Characteristics of the Transformed Cultivars

A number of transgenic plant lines including the ZW-20 were field tested in the United States in 1990, 1991, and again in 1992. At the end of the 1992 growing season, these plants will have been grown at a total of 12 different test sites throughout the United States, with some sites having multiple trials within a single year and some sites having been used for trials over three years. During these trials, we did not observe any pleiotropic effects in any of the transgenic lines. Based on visual observation, except for susceptibility to viral infection, the transformed cultivar's susceptibility or resistance to other pathogens was unchanged when compared to non-transformed control or non-coat protein containing sibs. For example, based on physical observation, both transgenic and nontransgenic plants exhibited identical susceptibility to powdery mildew and silverleaf. They were also

equally susceptible to infestation by whiteflies, and cucumber beetles. Other observations have been made on this line by a number of Asgrow workers and no alteration from the original type has been noted (see section V, E below).

C. Nutritional Composition

Studies on the nutritional composition of cultivated cucurbits have revealed that squash are all low in food value, (21 calories/100 gms of edible fruit) and are especially low in protein (~1.4 g protein/100 gms of edible fruit) (Whitaker and Davis, 1962). Based on this information, it is clear that squash does not contribute a significant portion of the consumer's nutrient intake. Therefore, we believe that analysis directed at verifying the nutritional content of the transgenic fruits of these lines need not be addressed.

D. Pollination Characteristics of the Transformed Cultivars

Since 1990, we have collected samples of seeds from plants in the border rows of our field trials. We are in the process of analyzing the movement of pollen from transgenic squash plants transformed with vectors containing the NPT II marker gene. Analysis has been completed for a 1990 Kalamazoo, Michigan and a 1991 Tifton, Georgia field trial site. Our data (Figure 2) indicate that pollen dispersal in transgenic squash plants is inherently no different from that documented for non-transgenic *cucurbits* (Handel 1982 and 1983). The frequency of transgenic pollen declines rapidly with distance from the source. At the 1990 Kalamazoo site, a slight increase at the outer edge of the border was detected, a phenomenon also observed in other nontransgenic cucurbit studies (Handel, 1983).

E. Characteristics of Virus Resistant Squash

The effectiveness of transgenic squash line ZW-20 was first demonstrated in field trials conducted in 1991. This line was tested for WMV-2 resistance in Kalamazoo, Michigan and was tested for WMV2 and ZYMV resistance in Tifton, Georgia. R_1 progeny (produced by backcrossing the primary transformant to a non-transgenic plant of the same inbred) were germinated in the greenhouse and mechanically inoculated with WMV-2 or ZYMV prior to transplantation into the field. The trial plots were planted in a paired plot design, in which a row containing the transgenic inbred line was paired with a row containing a non-transgenic line of the same inbred. Some of the non-transgenic controls were mechanically inoculated prior to planting into the field while others were left non-inoculated in order to monitor for virus spread by aphids. The entire plot was surrounded by a border zone of non-transgenic squash plants in order to contain the transgenic pollen. During these trials the ZW-20 line exhibited significant protection against ZYMV and WMV-2 infection even under conditions where virus spread by aphids was severe, thus

exposing plants to repeated aphid inoculation after the initial mechanical inoculation. These results of these trials are shown in table 2a and 2b.

TABLE 2a

WMV-2 Symptom Development on Progeny of Primary Transgenic Squash Line, ZW-20 (1991)

Line	NPT II	% Symptomatic	
		mechanically inoculated	Non-mechanically inoculated
ZW-20	+	0	-
	-*	33	-
Controls	-	100	100

TABLE 2b

ZYMV Symptom Development on Progeny of Primary Transgenic Squash Line, ZW-20

Line	NPT II	% Symptomatic	
		mechanically inoculated	Non-mechanically inoculated
ZW-20	+	0	-
	-*	40	-
Controls		100	100

* includes plants with WMV2 and ZYMV coat protein genes but no functional NPT II genes

These data show that the ZW-20 line has significant and commercially valuable resistance against WMV2 and ZYMV.

Except for resistance to viruses, examination of the ZW-20 line by Asgrow's plant breeders, plant pathologists, product managers and technical service representatives has determined this line to be horticulturally identical to its non-transgenic counterpart. For example, the non-transgenic inbred has been developed to be highly female flowering, have a moderately crooked fruit shape, a fair colored interior flesh

and a thin yellow outer skin. None of these traits have been altered in the transgenic inbred. USDA inspectors have also examined the plants during the trials and, to our knowledge have not recorded any unusual or unexpected characteristics.

F. Expression levels of CP in the plant tissue and Product

Since the coat protein genes used to generate this transgenic plant line are under the control of the constitutive 35S promoter, most plant tissues, including the fruits, could contain low levels of viral coat proteins. Using enzyme linked immuno-absorbent assays (ELISA), we sampled marketable sized fruits to determine coat protein levels. We found levels of Coat Protein expression in transgenic fruit to be .008 ± .008% and .003 ± .004% of total soluble protein for ZYMV and WMV-2 respectively.

VI. Environmental Consequences of the Introduction of the Transformed cultivars

The recent document by the President's Office of Science and Technology Policy outlining the scope of regulation for the products of biotechnology states that the assessment of risk should address the trait of a genetically engineered organism not the process by which it was produced (Federal Register, Vol. 57 (104) 22983-23005, 1992). We will therefore address issues which we believe may arise as a result of the release of virus-resistant squash.

A. Weediness

Despite being cultivated in the United States since antiquity, *Cucurbita pepo* has never been reported as a weed, probably because it possesses few of the traits that are associated with weediness. Characteristics that define a weed are debatable, but a list of the traits possessed in common by many weeds was compiled by Baker (1974). They include: (1) germination requirement fulfilled in many environments; (2) discontinuous germination and great longevity of seed; (3) rapid growth through vegetative phase to flowering; (4) continuous seed production for as long as growing conditions permit; (5) self-compatible but not completely autogamous and apomictic; (6) cross-pollination accomplished by unspecialized visitors or wind; (7) high seed output in favorable environments and some seed production in a wide range of environments; (8) adaptation for short- and long-distance dispersal; (9) if perennial, capable of vegetative reproduction or regeneration from fragments, accompanied by brittleness (thus not easily removed from the ground); and (10) the ability to compete interspecifically by special means (rosette formation and presence of allelochemicals). The *Cucurbita pepo* cultivar that was selected for transformation with the ZW transgenes possesses only a few of the weedy traits listed above, specifically traits 3,

5, and 6. The insertion of the ZW coat protein gene into a species of *Cucurbita* which has never been reported as a weed within the United States is not likely to significantly alter the weediness of the cucurbit variety.

Because the aim of Asgrow is to produce transgenic lines that are horticulturally identical to the non-transgenic lines they will replace, any change in characteristics--including those which would cause a weediness problem--are undesirable, and would cause a transgenic line to be discarded. Thus far, we have not detected any transgenic line which displays undesirable weedy characteristics. In 1991 the ZW-20 line was tested in field trials in California, Georgia and Michigan. Twelve additional field trials within the United States are presently in progress. We have also tested many other transgenic squash lines not under consideration in this document. These transgenic plants have been examined by a number of different company professionals: plant molecular biologists, plant tissue culture specialists, plant breeders, plant pathologists, product managers, production supervisors and technical service personnel. Scientific personnel normally examine the trial on a weekly basis and collect data at four times during the duration of the test. No change in morphological characteristics associated with weediness has been observed in this or other transgenic cultivars when compared with their non-transformed counterpart.

One trait that is associated with weediness is seed longevity. In temperate regions such as the United States, any alternation in seed longevity that might increase the overwintering ability of a species might affect weediness by increasing the number of volunteer plants that appear the next season. If transgenic cultivars produce seeds that overwintered more efficiently than their non-transgenic counterpart, they may have a selective advantage that could increase their weediness. In this regard we have monitored our field trial sites for volunteer seedlings during the subsequent season. In the spring of 1991, no volunteer seedlings (transgenic or non-transgenic) were observed on sites used for the 1990 trials. Volunteer seedlings did appear in the spring of 1992 on our 1991 Kalamazoo, Michigan field trial sites. Sample seedlings were analyzed for the presence of the inserted NPT II genes and no difference in the survivorship of transgenic versus non-transgenic seed was apparent: Non-transgenic offspring were found in the border zone area, as were transgenic offspring in the experimental area.

It is unlikely that the addition of virus resistance alone could increase the weediness of cultivated *Cucurbita pepo* since it would not enhance any of those traits that are considered characteristic of weedy species. Furthermore, we know of no cases where viruses exclude a plant species from a geographic region or habitat. In fact, examples exists in other cucurbit species which show that virus resistance does not generate a weedy variety. For example, cucumber varieties resistant to cucumber mosaic virus (CMV) have been produced by classical plant breeding techniques (Munger 1950). Today, nearly all commercially grown cucumber varieties are CMV resistant, yet there is no evidence that CMV resistance has enabled the establishment of weed

populations of cucumbers, even though virus resistant varieties have been under cultivation for decades.

The development of squash varieties with multiple virus resistance has been the aim of traditional breeding programs with nontransformed *C. pepo* for decades, without concern for their potential weediness. Cultivated species of *Cucurbita* have been identified which have varying levels of viral resistance. Good sources of resistance to CMV have been identified in a few accessions of *C. moschata* and *C. maxima* from South America. Resistance to PRV have been identified in *C. maxima* from Uruguay and *C. moschata* from Nigeria. Resistance to WMV-2 has been found in a Chinese cultivar of C. *maxima*. Finally, resistance to ZYMV has been identified in *C. moschata* from Nigeria (Provvidenti 1986). Because of sterility barriers and other difficulties in breeding these genes have not yet been successfully transferred to commercial varieties. However, it is clear that genes which confer virus resistance already exist in the gene pool of *Cucurbita*, and these genes will eventually be incorporated into commercial squash--albeit much later than through genetic engineering methods. We are not aware of any case where the existence of these genes has caused the above mentioned species to become weeds.

B. Vertical Transfer of the Gene

The inserted coat protein genes may be transferred from the transgenic ZW-20 cultivar to non-transgenic cultivated cucurbits or to the non-cultivated *Cucurbita* species *C. texana*. Below we discuss the environmental consequences of the transfer of these genes to non-transgenic *Cucurbita*.

1. Between ZW-20 and Cultivated *C. pepo*

Outcrossing from transformed varieties of *C. pepo* to non-transformed *C. pepo* can and will occur in the field. However, given the nature of the transgenes as well as the logistics involved in growing hybrid squash, the consequences of the transfer of transgenic pollen to non-transformed cultivars of C *pepo* should be negligible. For example squash growers generally grow hybrid varieties, and therefore do not save seed to replant the next year. Instead, growers purchase new hybrid seed from commercial seed companies each planting season. Therefore transgenic seed produced from pollinations of non-transgenic cultivars with transgenic pollen will be eliminated from the production process.

Although yellow crookneck squash are harvested at an immature stage of development, imperfect fruit or "culls" may be left on the plant and will contain viable seed at the time the crop is plowed under. Viable seed produced during the spring planting may germinate as volunteers later in the season or in the case of a fall squash planting, they may over-winter to germinate the following spring. However, we have found that volunteer squash is not a problem in growers' fields. First,

following good crop rotation practices, growers will normally not plant a vine crop in a field that was used to produce a spring squash crop, but rather will plant fall beans or leafy greens. Likewise a vine crop is not grown in a field where squash was grown the previous fall, thus allowing over-wintering squash seeds to be readily identified and eliminated. Therefore the few volunteers that may germinate are readily identified and are eradicated by standard tillage practices. Second, overwintering volunteers are often killed after germination by spring frosts. Finally, cultivated varieties are several generations removed from wild or weedy ancestors and have been bred for adaptation to agricultural conditions. Therefore, it is unlikely that volunteers will persist without agricultural intervention, even if they are virus-resistant.

2. Between ZW-20 and the *Cucurbit* species.

In the United States, the only non-cultivated cucurbit known to hybridize with C. *pepo* without loss of fertility is C. *texana*. However, the geographic distribution of the species is limited, thus minimizing the probability of hybridization. In the event that a transgenic squash plant hybridizes with C. *texana*, we believe that because virus resistance can possibly be obtained from conventional breeding material, it is unlikely that the coat protein genes will present selective advantage which will allow any issue different from those presented by conventionally bred resistance. However, we have addressed one specific issue, that of possible enhanced vigor of hybrids between *C. texana* and *C. pepo* (due to the transgene). We have made greenhouse crosses between *C. texana* (female) and virus resistant transgenic YC77E (male) and have examined the vigor of the progeny in the greenhouse. No difference in growth habit or vigor was apparent between segregants possessing or lacking the coat protein insert. Whether this hybrid has a selective advantage in the presence of viruses will be determined by field tests now in progress. It is therefore unlikely that a hybrid between *C. texana* and ZW-20 could create a unique weed pest. However, should it be found that the hybrids or subsequent populations do become weeds, remedial actions such as herbicide applications can be used to eradicate the hybrid, since cucurbits are highly sensitive to many post-emergence herbicides. This would be true for any *C. pepo* or *C. pepo* interspecific hybrid which might arise during the commercial cultivation of transgenic squash.

In the event that our resistance genes are transferred to *C. texana* they would not represent an unique addition to the gene pool of wild *Cucurbits*, since a wide variety of wild species of *Cucurbita* already have resistance to the viruses we are targeting (see above). For example *C. ecuadorensis* is highly resistant to papaya ringspot virus (PRV), ZYMV and WMV-2, *C. ficifolia* and *C. foetidissima* are resistant to PRV and WMV-2, and *C. pedatifolia* is highly resistant to WMV-2. Resistance to CMV has been identified in many non-cultivated genotypes. *C. cordatar, C. cylindrata, C. digitate, C. ecuadorensis, C. ficifolia, C. foetidissima, C. gracilior, C. lunlelliana, C. martinezii, C. okeechobeensis, C. palmata, C. palmeri, and C. pedatifolia* (Provvidenti

1986). *C. martinezii* which produces particularly fertile hybrids with cultivated *C. moschata* also has resistance to CMV. As with the cultivated species the wild species have not become noxious weeds, in spite of the fact that they possess virus resistance. We contend that this is because they possess few morphological or physiological traits associated with weediness (Keeler 1989).

In contrast to these species, a wild cucurbit (*Citrullus sp.* "citron") is found in the southeast U.S. and is a weed in our test fields (presumably in agricultural fields as well). This weed is susceptible to the viruses we are targeting, yet grows well in spite of virus infection. Other characteristics independent of virus infection have therefore allowed its persistence and weediness, even in an area where the viruses we are targeting are prevalent.

Direct evidence that the addition of virus resistance alone to cultivated or non-cultivated cucurbit will not have an impact on the weedy characteristic can be found by examining the non-cultivated cucurbit, *C. foetidissma*. This species is a perennial xeophytic squash which is resistant to PRV, CMV and WMV-2 (Provvidenti, *et al.*, 1978). It is found growing in the southwestern United States, through Colorado, Kansas, Missouri and as far north as Nebraska (Whitaker and Bemis 1964). Like most wild cucurbits, it can often be found along roadside or inhabiting other areas which have been disturbed by man or nature (Kohn and Casper 1992). Evidence from Indian archeological sites indicate that it has been growing in the Southwestern United States since 900 AD (Culter and Whitaker 1961). Despite the fact that this cucurbit is a perennial xerophyte--two characteristics associated with weediness (Keeler 1989)--is resistant to CMV, PRV, and WMV-2, and has been growing in the United States for hundreds of years, it is not known to be a significant weed problem. If a wild perennial, xerophytic, multiple, virus resistant cucurbit is not known to be an invasive weed, it is highly improbable that the addition of virus resistance alone will enhance the weediness of an annual, mesophytic squash, such as *C. texana* or cultivated squash varieties.

Transencapsidation

One recent concern with coat protein-expressing plants has been raised by researchers (DeZoeten, 1991). The concern (which relates to the process of producing the resistance rather than the trait of resistance itself) involves the phenomena of transencapsidation and phenotypic mixing. Transencapsidation refers to the encapsidation of the infecting RNA of one virus within a capsid composed of protein subunits from another virus. Phenotypic mixing is the encapsidation of viral RNA by a capsid composed of protein subunits from both co-infecting viruses. Both are known to occur in naturally infected field plants (Creamer and Falk 1990).

This concern is that transencapsidation or phenotypic mixing may occur in coat protein expressing plants, resulting in increased ecological risk (deZoeten, 1991). It

has been postulated that RNA from viruses which are replicating within infected genetically engineered plants may be transencapsidated or undergo phenotypic mixing by coat proteins which are constitutively expressed in the transgenic host plant. However, if one examines the amount of coat protein produced in transgenic plants, one finds the levels to be extremely low relative to that produced in naturally infected plants. Consequently, the probability for transencapsidation or phenotypic mixing is far less than the probability of these events occurring in nature. In the geographic areas where cucurbits are grown commercially, fields are normally subjected to multiple infection by the potyviruses whose coat protein genes were engineered (Adlerz *et al.*, 1983, Davis and Mizuki, 1987). We have measured the levels of coat protein in virus-infected plants and have found that the level of coat protein in these plants can be up to 500 times higher than the level found in coat protein- expressing transgenic plants (Figure 11). Therefore, the probability of transencapsidation or phenotypic mixing would be far greater in multiply-infected plants than for a genetically engineered plant. In our virus-resistant ZW-20 plants viral replication is prevented. Therefore, coat protein-expressing transgenic plants should not increase the probability of transencapsidation or phenotypic mixing over that which already occurs in nature, and may, in fact, reduce the probability of occurrence of this phenomenon by decreasing the titer of viral RNA and coat protein in resistant plants.

Support for the argument that coat protein expressing plants will not increase ecological risk through transencapsidation can also be obtained from years of experience in classical cross protection work. Classical cross protection, which involves the deliberate inoculation of plants with a mild or attenuated strain of a virus, thus protecting that plant from subsequent infection by a more virulent strain, has been practiced for many years. The technique has been applied to tomato, potato, apple, peach, cocoa, papaya, citrus and recently cucurbits (Fulton, 1980, Wang, 1991). Classical cross protection has been used successfully with potyviruses, specifically PRV in papaya (Yeh, *et al.*, 1988) and ZYMV in zucchini squash (Wang *et al.*, 1991). When using this strategy, mild strains of the virus replicate within the cross protected plant, producing levels of RNA and coat protein which should be much higher than the levels found in genetically engineered CP protected plants. However, to our knowledge there are no reports that this practice has caused environmental harm due to an increase in transencapsidation or phenotypic mixing.

Native Floral Communities

The genus *Cucurbita* is a small closed system (Decker *et al.*, 1988). Except C. *texana*, unrelated plant species cannot be successfully fertilized by C. *pepo* pollen. No weedy species capable of intercrossing with C. *pepo* have been observed in the geographic areas of Georgia, Florida, Michigan or California, the areas where the majority of this material will be commercially grown. Therefore, we anticipate no adverse effect on native floral communities.

Native Faunal Communities

No factor unique to the use of this transgenic plant line can be identified that would have an effect on any animal species.

Impact on Existing Agricultural Uses

Current agricultural practices themselves often have adverse ecological effects because large quantities of chemicals are often used for pest control. Aphids are the natural vector of these virus and can be controlled by a number of pesticides. Pesticidal control for aphids includes spraying plant with diazinon (oral LD_{50} 542/kg, highly toxic to birds, fish, and bees), Lannate (oral LD_{50} 17 mg/kg; highly toxic to birds, fish, and bees), Metasystox-R (oral LD_{50} 75 mg/kg; highly toxic to fish and bees). Phosdrin (oral LD_{50} 3-12 mg/kg highly toxic to birds and bees). Thiodan (oral LD^{50} 70 mg/kg; highly toxic to birds and fish). Parathion (oral LD_{50} 4-13/kg; highly toxic to birds, fish, and bees), Malathion (oral LD_{50} 1,375 mg/kg; highly toxic to fish and bees) or Dibrom (oral LD_{50} 430 mg/kg; highly toxic to fish and bees) (Garrison *et al.*, 1991) (Figure 12). Although these insecticides are effective against aphids they do not kill the insect before it has transmitted the virus. Therefore these products have generally fallen out of favor with commercial growers, who now utilize stylet oil sprays and insecticidal soaps which are often combined with insecticides targeted at other non-aphid insect pests. Stylet oil sprays need to be applied frequently and at very high volume and pressures in order to ensure maximum leaf coverage. For example, oils need to be applied every day for the first three days post emergence, to ensure that every germinating seedling is treated. They are then applied at day 5 and subsequently at 3 to 5 days intervals.

The use of plants constitutively expressing viral coat protein genes which interfere with virus multiplication or movement within or between plants offers a more advantageous mechanism to control viral pathogens. The use of crop plants with specific genes that control virus infection will reduce the number of trips a farmer must make into a field to apply oil sprays or insecticidal soaps which will in turn reduce his labor, fuel, maintenance, and supply costs. Any technology that will reduce the amount of insecticidal application and thereby reduce the occupational exposure to potentially hazardous chemicals will be a benefit to the farmer. The availability of resistant varieties will also reduce the amount of overplanting usually practiced by farmers in order to ensure a certain yield of marketable fruit.

Virus resistant varieties will also extend the growing season for squash. At present the virus pressure is so severe that growers no longer attempt to grow susceptible varieties during the late summer and fall. By extending the growing season for squash we increase the farmer's selection of crops he can chose from when implementing crop rotation practices. This longer growing season will increase the availability of this crop to the consumer.

Impact on Human Health

The commercial production of transgenic plant line ZW-20 should not pose a risk to human health. The presence of virus infected produce on supermarket shelves has been previously documented in cucurbits (Provvidenti and Gonsalves, 1984). Furthermore, we have examined zucchini squash, yellow crookneck squash, cantaloupe, and honeydew melons from supermarket shelves, and found WMV2 and ZYMV coat protein levels up to 4% of total protein, which is equivalent to 8 mg/kg of fruit. The levels of these coat proteins are at least one order of magnitude greater than ZW-20 coat protein levels (Figure 11). Human ingestion of viral coat protein has also occurred during the consumption of crops that have been protected from viral infection by classical cross protection (i.e. the intentional inoculation of a crop with a mild strain of a virus in order to protect the crop from subsequent infection by a more virulent strain of that virus). These plants contain replicating virions which reach titers well in excess of the coat protein levels present our transgenic squash lines. Additionally, plant virologists have been handling plant viruses for many decades and have therefore been externally exposed to high levels of coat protein genes and gene product with no evidence of risk to human health and safety.

Clearly there is a long history of human exposure to viral coat protein genes either through contact or through the consumption of virus infected produce without adverse effects on human health.

Statement of Grounds Unfavorable

The long term durability of coat protein mediated protection is unknown and strains of ZYMV or WMV which can overcome the protection might develop. However, this possibility also exists with conventionally bred disease resistance, and in this respect, our transgenic varieties should be no different from conventionally-bred varieties. On the other hand, CMV-resistance in cucumbers, a trait which has been commercially available for decades and in common use today, presents an example of a virus resistance trait which is durable. If this resistance proves not to have long term durability, no novel consequences will result. The grower will lose a source of virus resistance, but they will not encounter any unique challenge, since at present, they are forced to grow virus-susceptible varieties.

Conclusion

We believe that the availability of virus resistant squash will have a positive benefit to growers and consumers of this crop by allowing more reliable production and thus more reliable supply to the consumer. We have presented evidence to support our claim that the modified squash line in this petition does not present a plant pest risk,

nor is it otherwise deleterious to the environment. Removal of this line from its status as a regulated article will allow Asgrow to make this crop available to the public.

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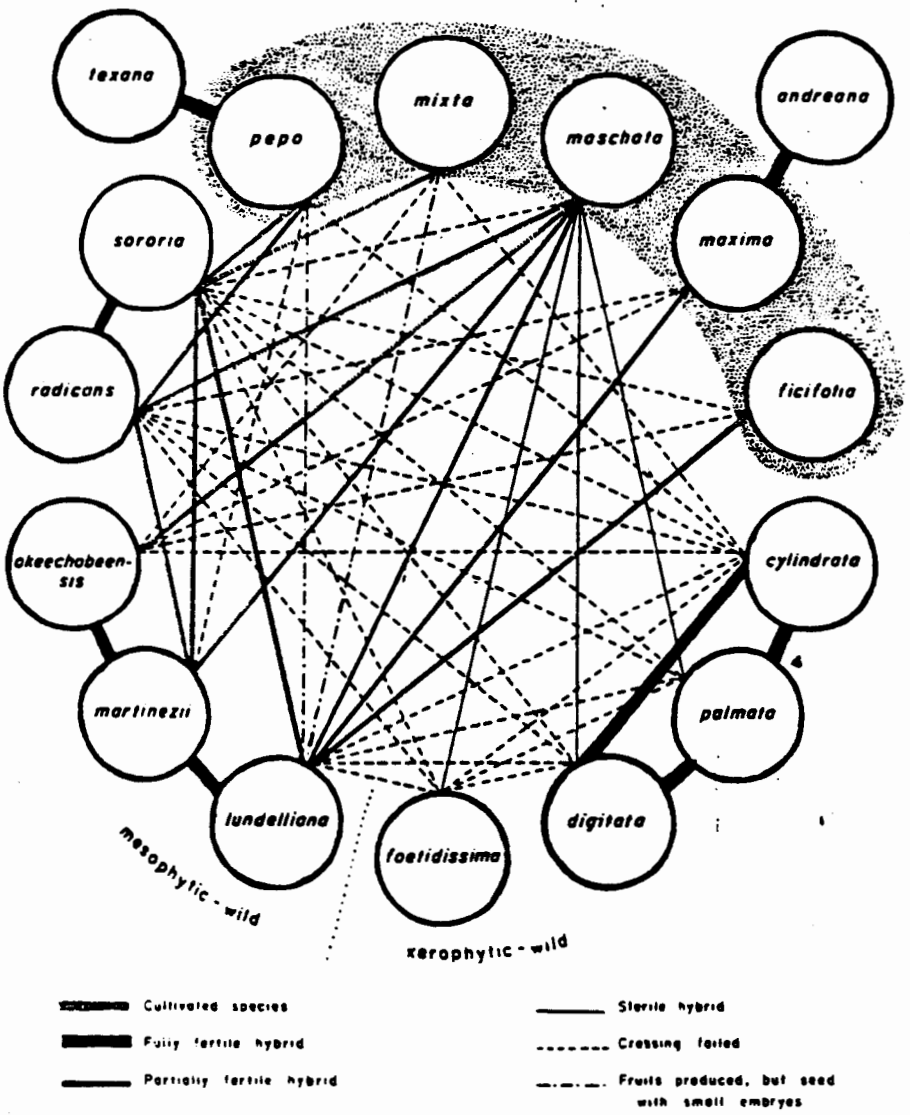


Figure 1. Crossability diagram of different species of *Cucurbita*. The interrelationships among the cultivated species are not shown (from Whitaker, T., and Bemis W. 1964).

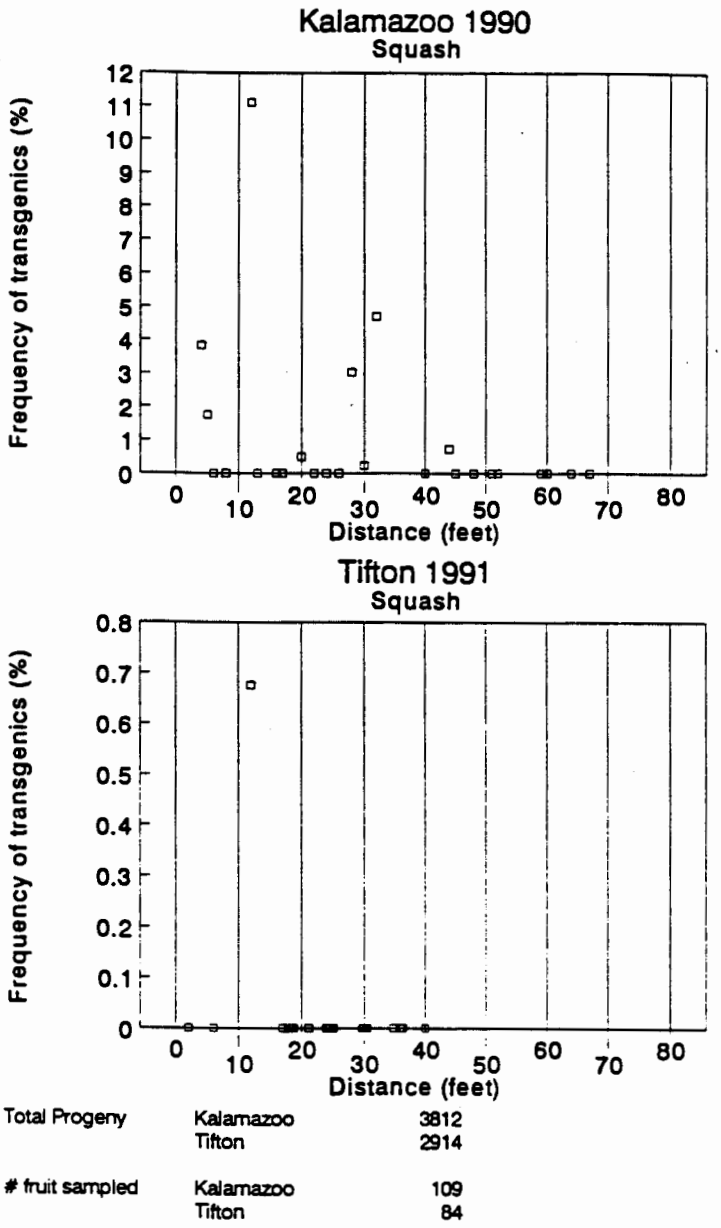


Figure 2. Distribution of transgenic offspring found in sampled fruits from transgenic border plants. The X-axis plots the distance at which fruits were sampled; the Y-axis plots the frequency of transgenic offspring in all the progeny found at that distance. ELISA assays for the NPT II protein were used to determine the frequency of transgenics in germinated seedlings.

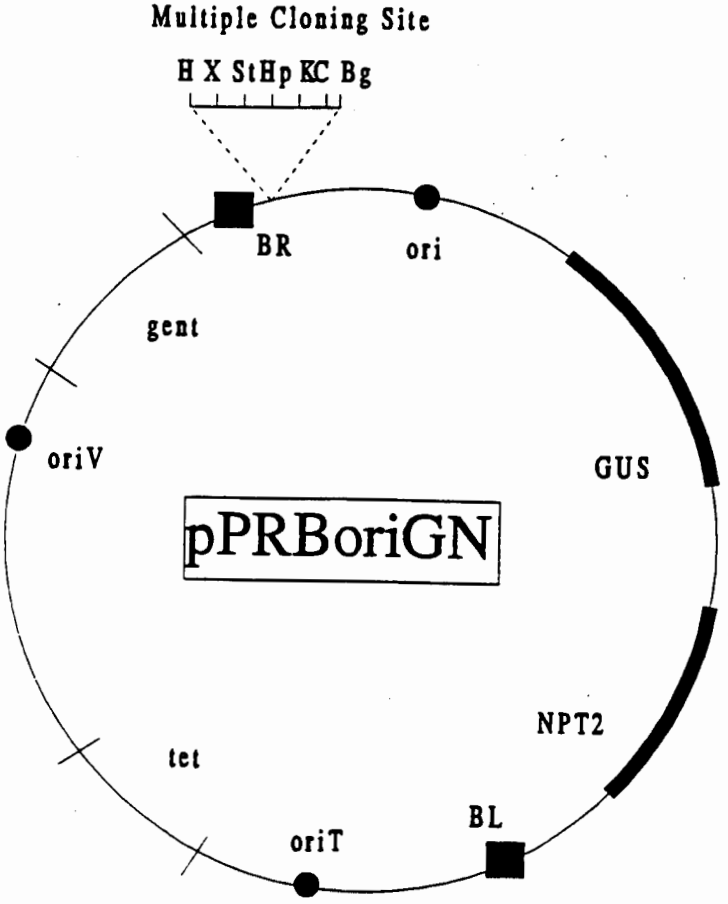


Figure 3. Structure of the binary vector pPRBoriGN. This vector was derived from pGA482 as described in the text.

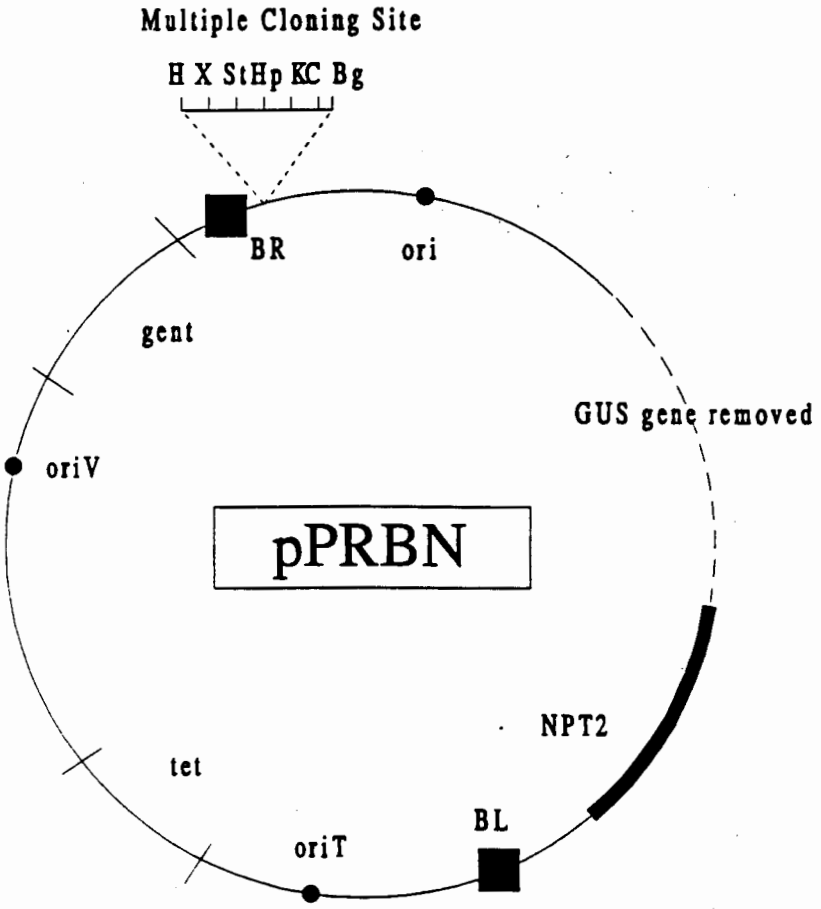


Figure 4. Structure of the binary vector pPRBN. This vector is essentially pPRBoriGN with the GUS gene removed. See text for further explanation.

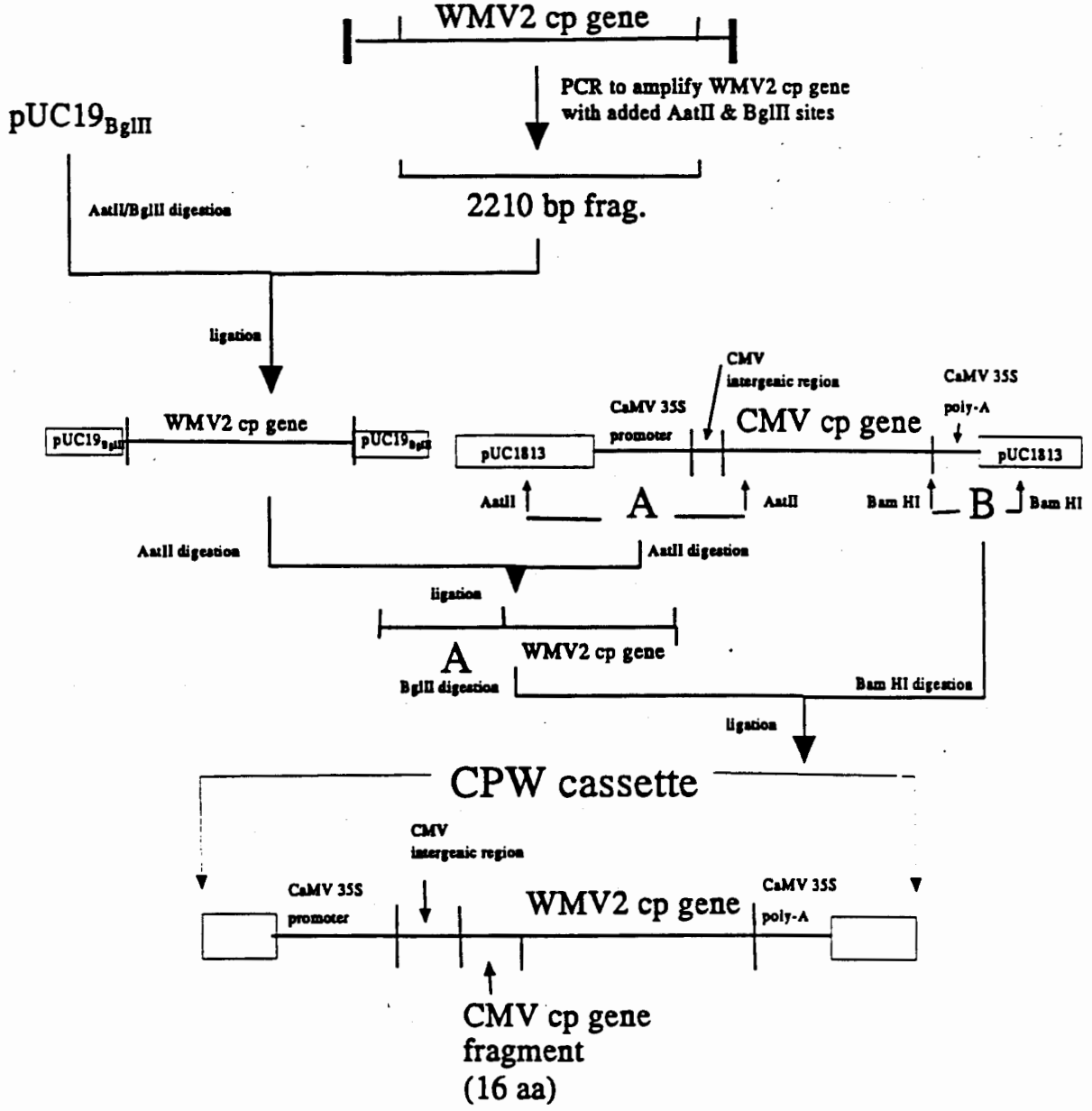


Figure 5. The construction of a plant-expressible WMV2 coat protein gene cassette. The polymerase chain reaction (PCR) was used to produce a WMV2 coat protein fragment with flanking AatII and BglII sites. A CaMV 35S promoter, 5' untranslated region, and 5' portion of a CMV coat protein gene were added, as well as a CAMV 35S poly-a-signal.

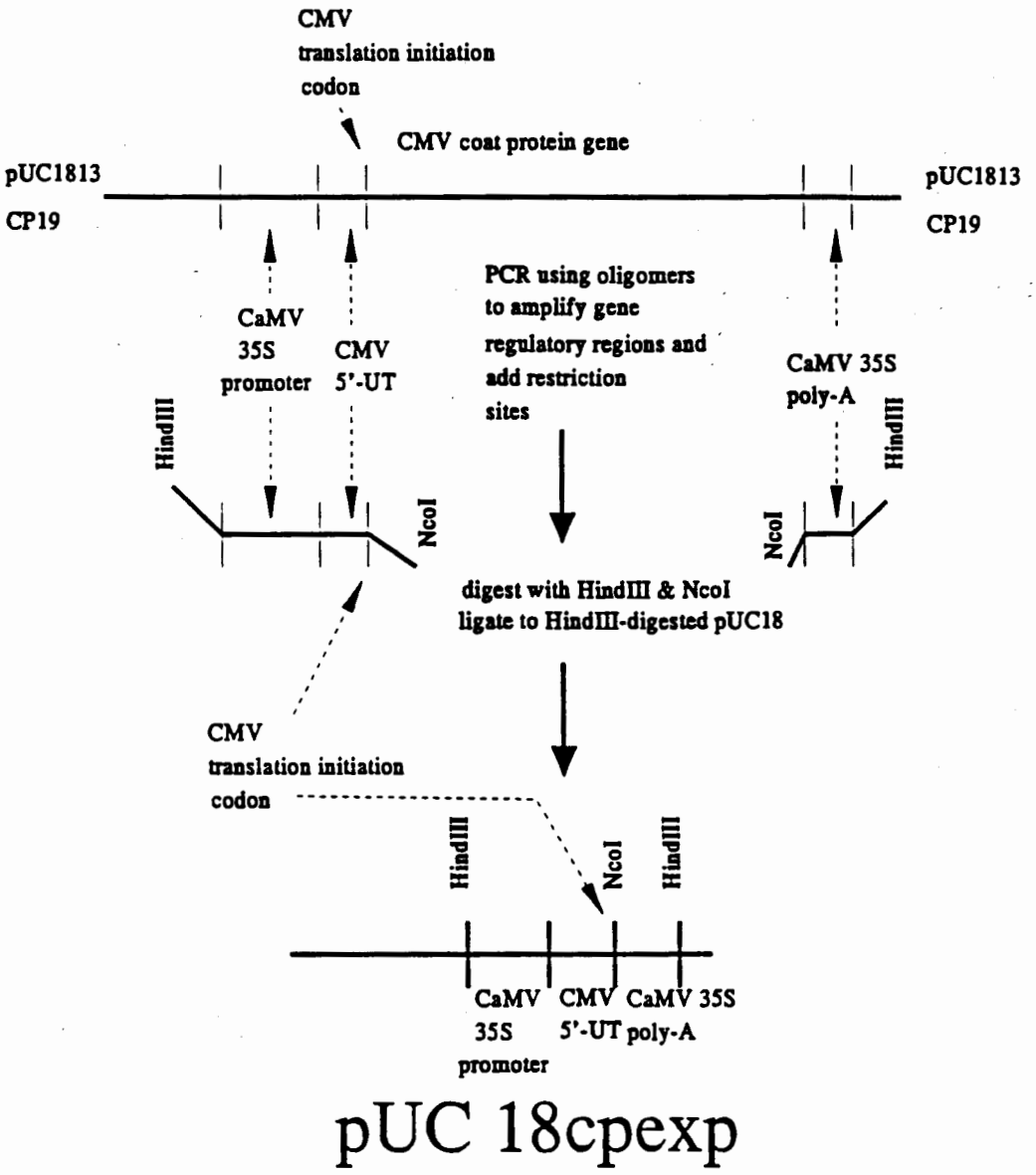


Figure 6. Construction of pUC18cpexp. The polymerase chain reaction technique was employed to construct the expression cassette, which was made to contain an NcoI site for cloning viral coat protein genes. The NcoI site also provides a translation initiation codon.

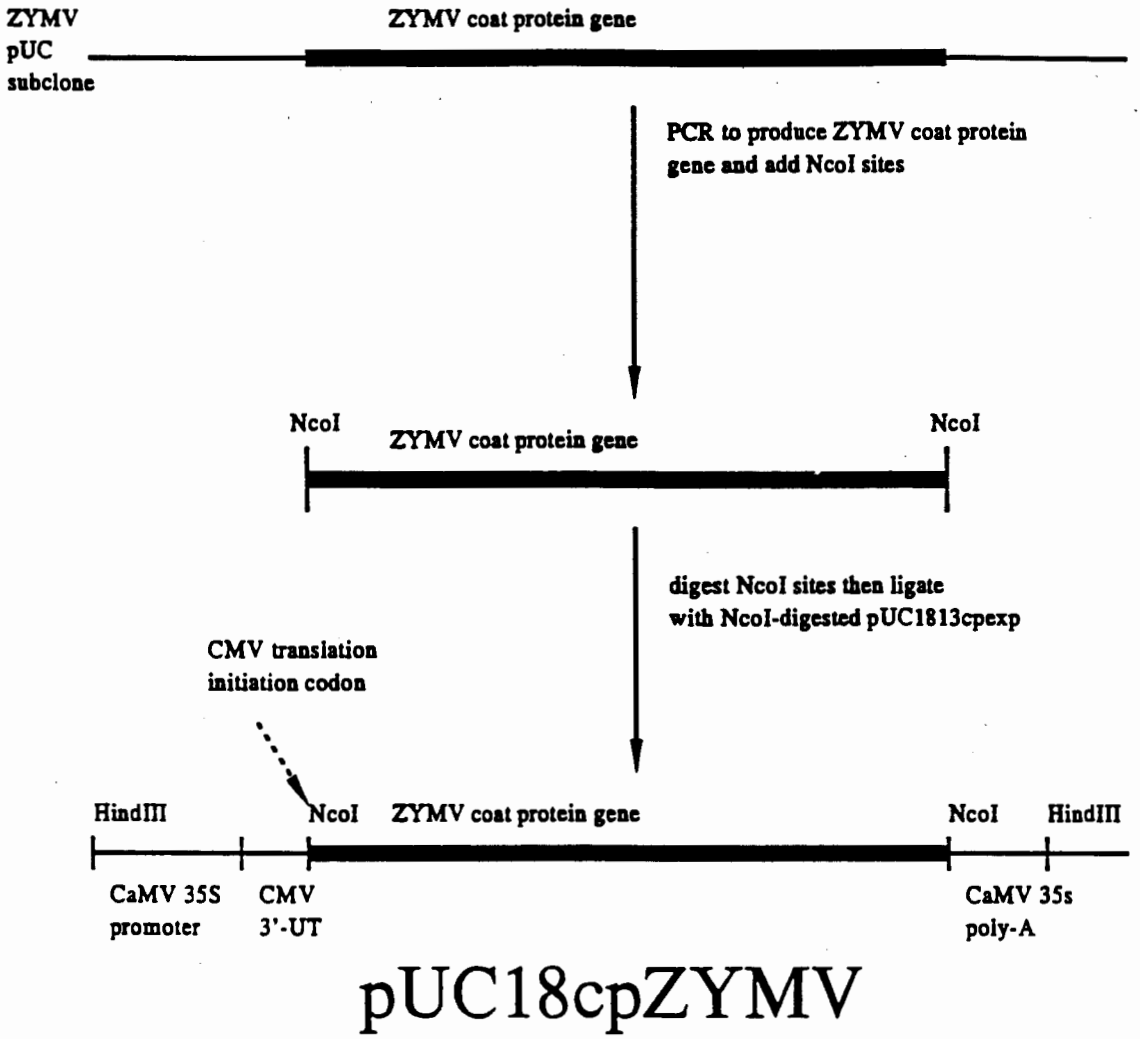


Figure 7. Construction of pUC18cpZYMV. The polymerase chain reaction was used to reproduce the ZYMV coat protein coding region, with flanking NcoI sites. This fragment was inserted into the NcoI site of pUC18cpext.

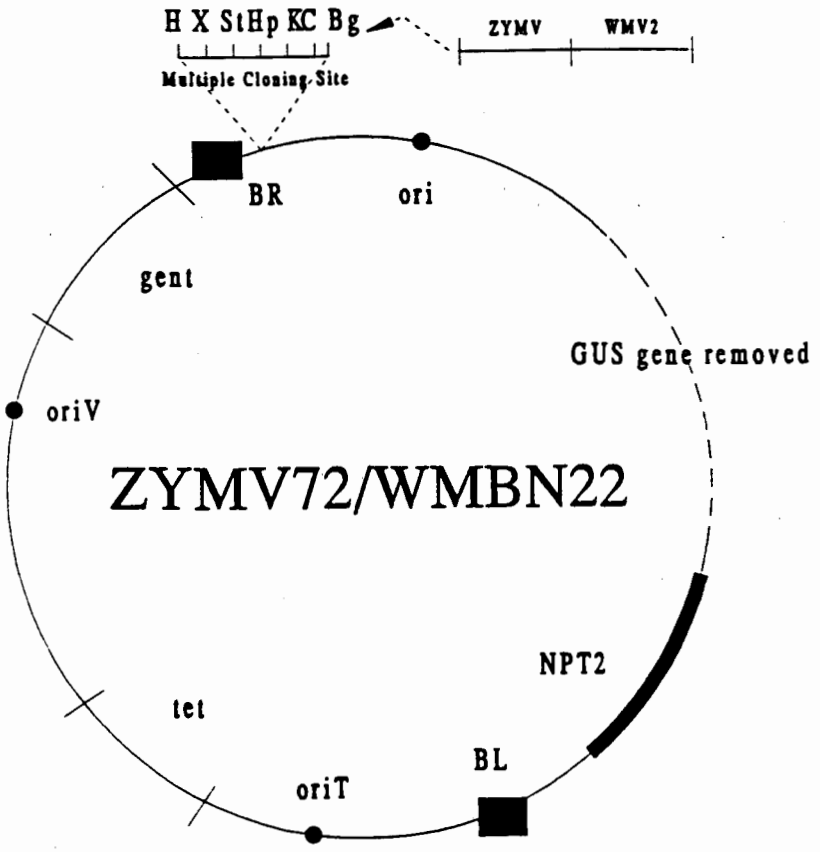


Figure 8. Binary plasmid ZYMV72/WMBN22 includes two viral coat protein expression cassettes: The CPW cassette and cpZYMVexp. Both cassettes were cloned sequentially into the unique BglII site of pPRBN to produce a plasmid containing the genes for the ZYMV and WMV2 coat proteins.

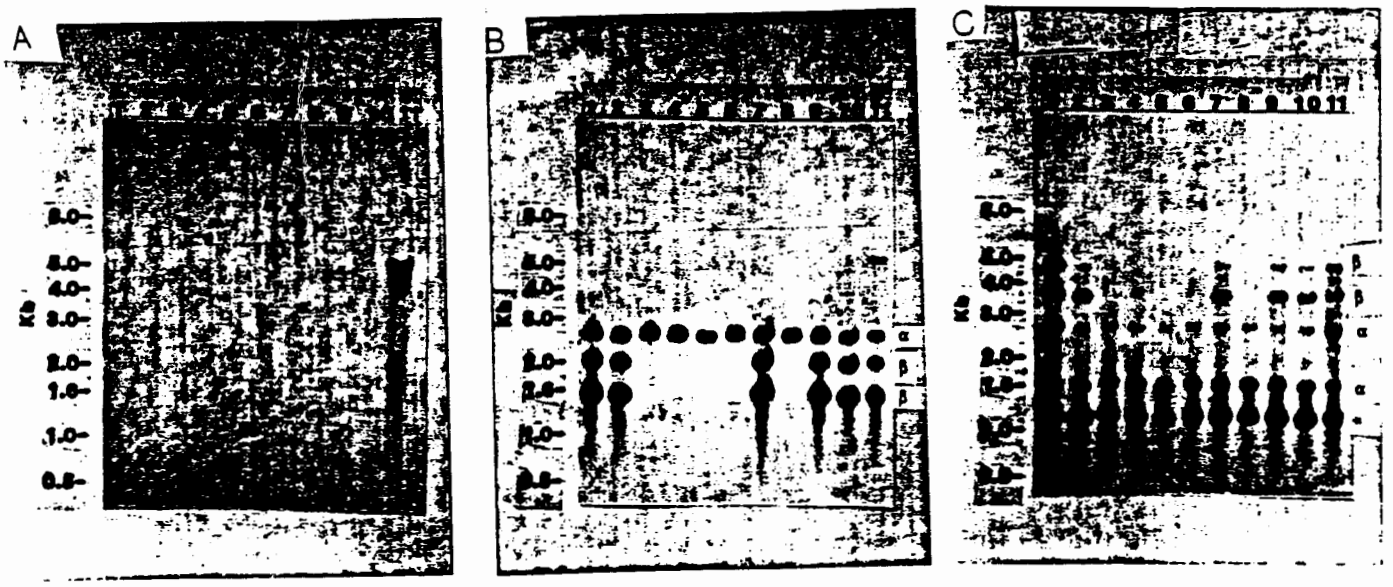


Figure 9. Southern Blot analysis of DNA from the primary regenerant (R_0) plant, and ten plants from the fourth sexual generation of the ZW-20 line, which have been selected for absence of NPT II marker gene activity. Lane 1 in each figure is DNA from the R_0 plants. Lane 2 are fourth generation plants. Digestions were done with NSiI to reveal junctions between inserted DNA and plant genome. A) Probed with NPT II gene to reveal junction fragments from plants containing the NPTII gene at the left T-DNA border. B) Probed with WMV2 coat protein gene to reveal left border fragments containing that gene. C) Probed with the ZYMV coat protein gene to reveal right border junction fragments containing that gene. *= internal T-DNA fragment containing a part of the ZYMV coat protein gene.

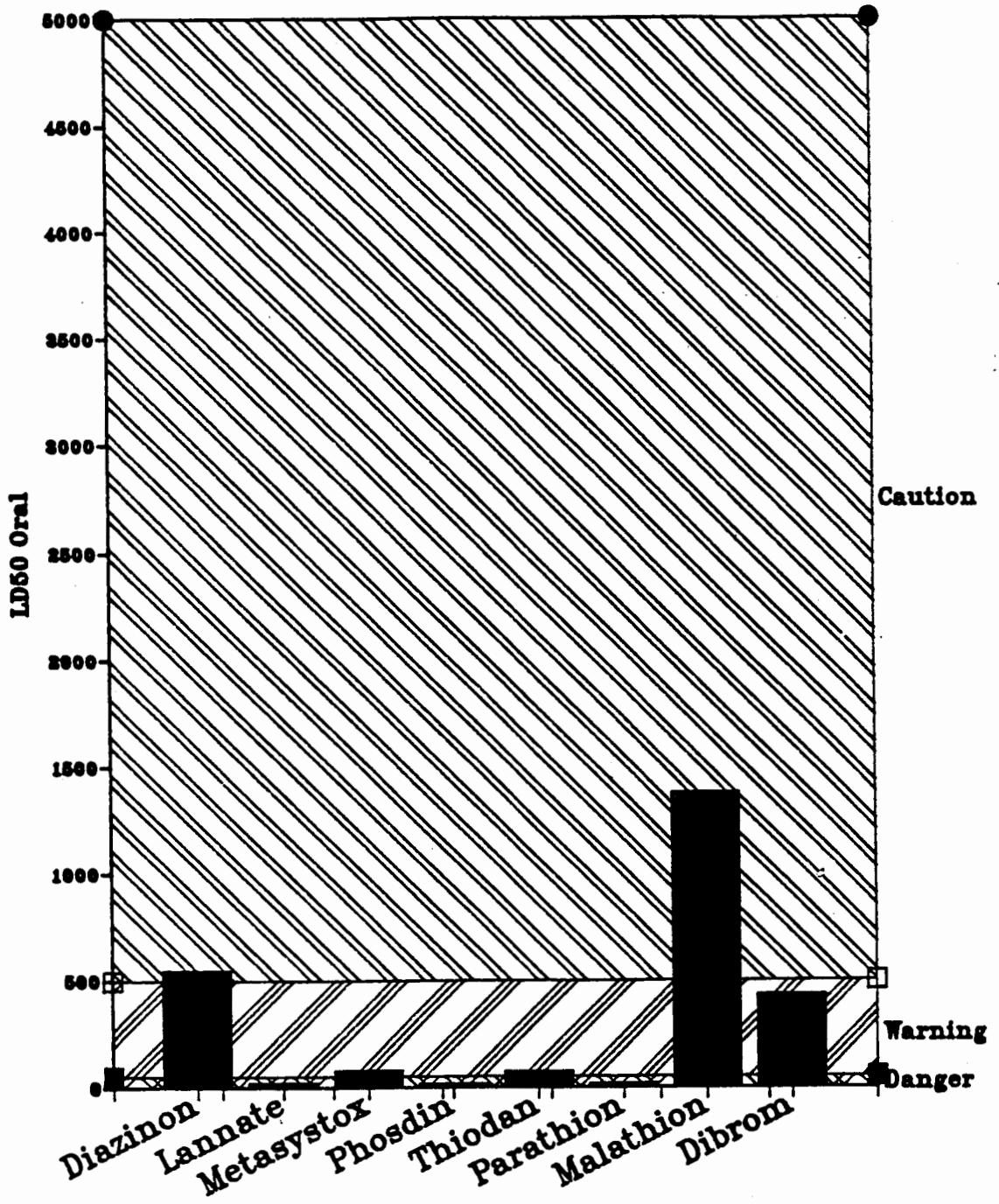


Figure 10. A list of insecticides recommended for aphid control and their oral LD_{50}. Toxicity values are divided into three categories (dashed line) and the accepted EPA warning that must be present on the product label (Toxicity values are expressed as acute oral LD_{50} in mg of substance/kilogram of body weight.

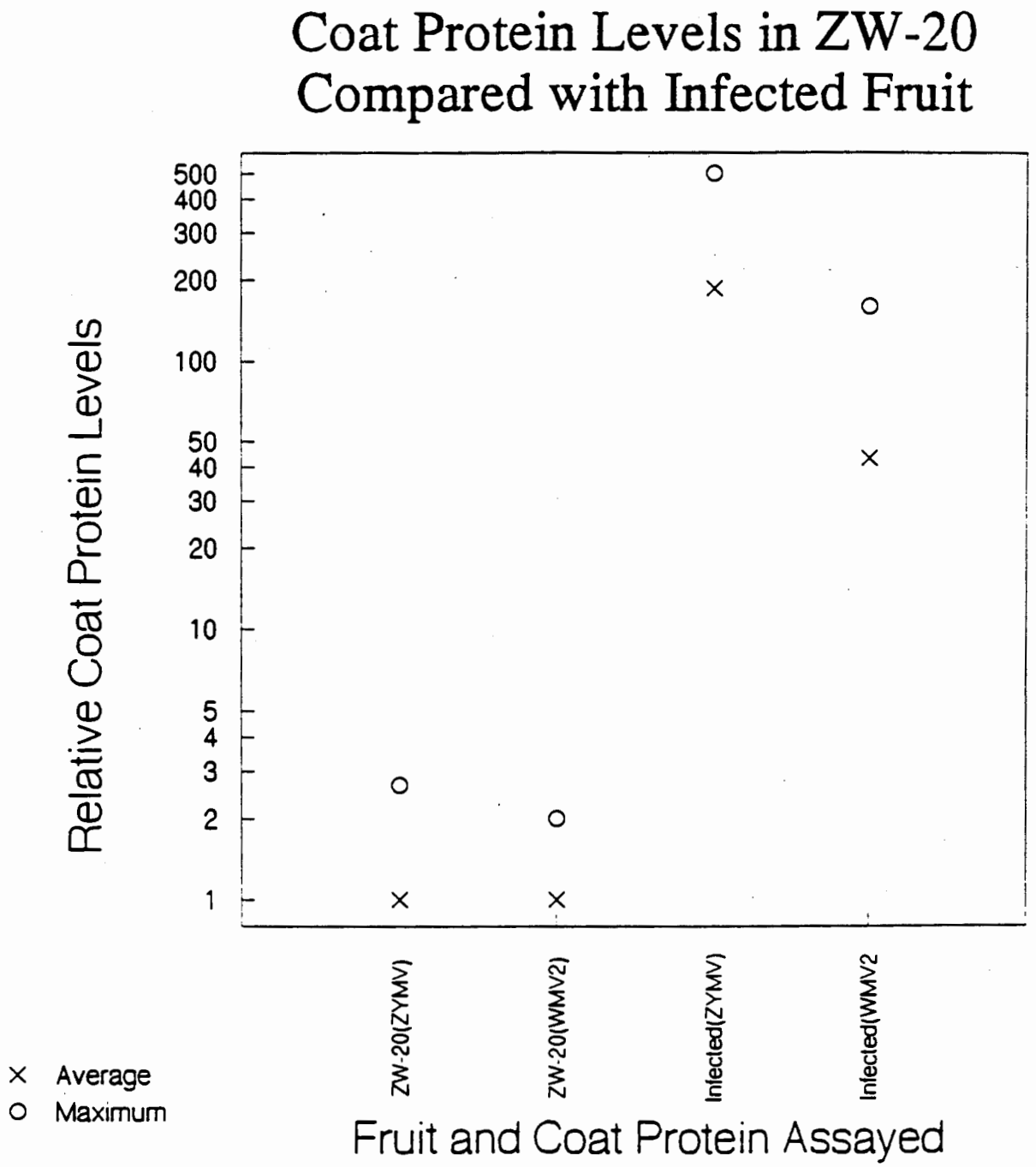
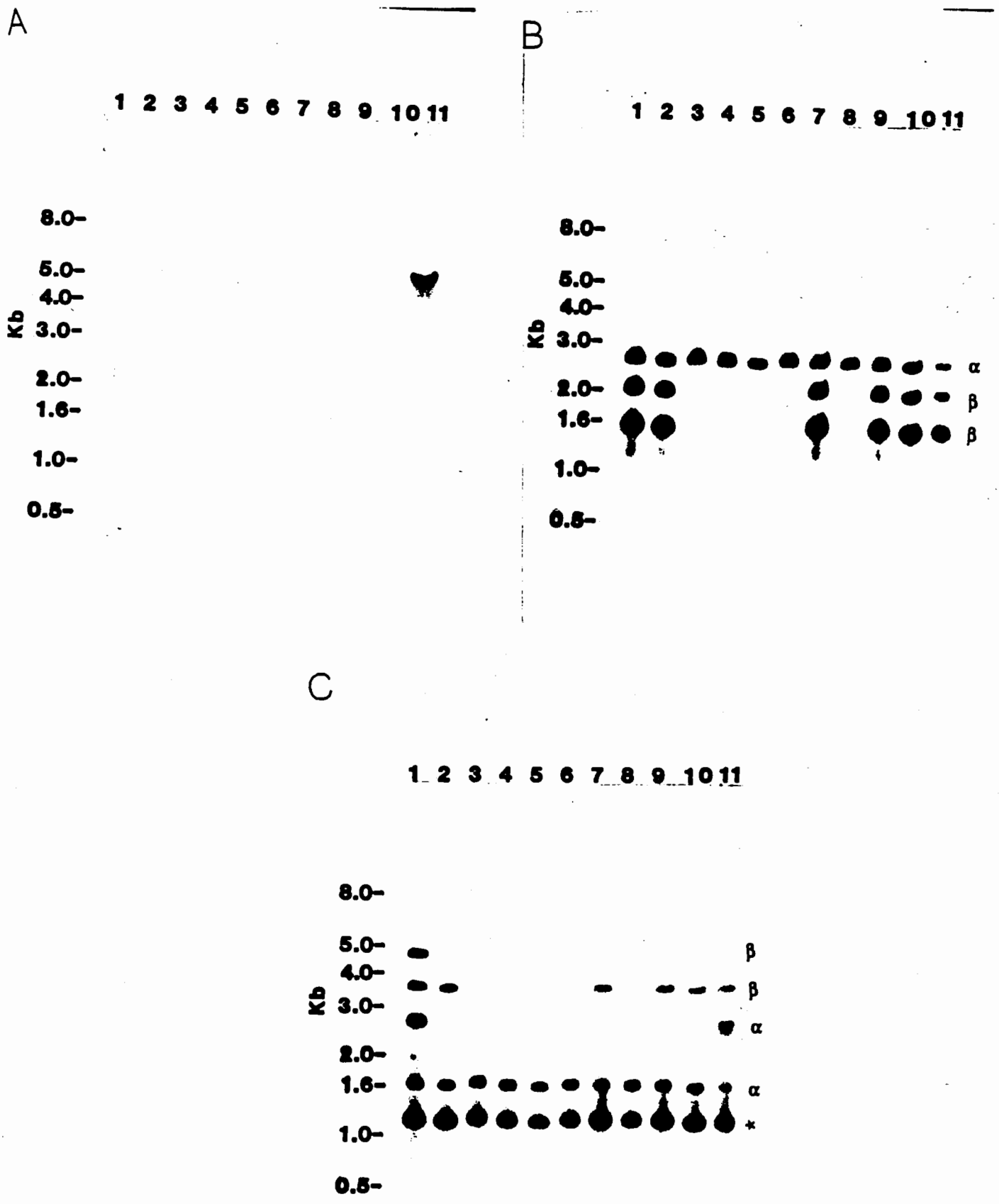


Figure 11. Level of viral coat protein detected in ZW-20 and cucurbit fruits obtained from grocery store (cantaloupe, honeydew melon, yellow crookneck squash, zucchini squash). ELISA assays were conducted on 35 fruits sampled from a local grocery. Thirty one on the fruits showed infection by ZYMV and/or WMV2. The levels of coat protein measured are shown.


A
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Kb
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B
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4.0-
Kb
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1.0-
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α
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8.0-
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92-204-01p

Notices

Federal Register
Vol. 57, No. 173
Friday, September 4, 1992

Animal and Plant Health Inspection Service

[Docket No. 92-127-1]

Notice of Proposed Interpretive Ruling in Connection With The Upjohn Company Petition for Determination of Regulatory Status of ZW-20 Virus Resistant Squash

AGENCY: Animal and Plant Health Inspection Service, USDA.

ACTION: Notice of Proposed Interpretive Ruling.

SUMMARY: We are advising the public that the Animal Health Inspection Service (APHIS) has received a petition from The Upjohn Company seeking a determination regarding the regulatory status of its ZW-20 virus resistant squash. APHIS is requesting comments on its proposal to issue an interpretive ruling that the ZW-20 virus resistant squash does not present a plant pest risk, and therefore, would no longer be considered a regulated article under its regulations.

DATES: Consideration will be given only to written comments that are received on or before October 19, 1992.

ADDRESSES: To help ensure that your written comments are considered, send an original and three copies to Chief, Regulatory Analysis and Development, PPD, APHIS, USDA, room 804, Federal Building, 6505 Belcrest Road, Hyattsville, MD 20782. Please state that your comments refer to Docket No. 92-127-1. A copy of the Upjohn submission and any written comments received may be inspected at USDA, room 1141, South Building, 14th Street and Independence Avenue SW., Washington, DC, between 8 a.m. and 4:30 p.m., Monday through Friday, except holidays. A copy of the Upjohn petition may be obtained by contacting Ms. Kay Peterson at 301-436-7601.

FOR FURTHER INFORMATION CONTACT: Michael A. Lidsky, Deputy Director, Biotechnology Coordination and Technical Assistance, or L. Val Giddings, Chief, International Programs Branch, Biotechnology Coordination and Technical Assistance, BBEP, APHIS, USDA, room 850, Federal Building, 6505 Belcrest Road, Hyattsville, MD 20782, 301-436-7601.

SUPPLEMENTARY INFORMATION: On July 13, 1992, the Animal and Plant Health Inspection Service (APHIS) received a "Petition for Determination of Regulatory Status" from The Upjohn Company (Upjohn), of Kalamazoo, MI. The Upjohn petition seeks a determination from APHIS that its ZW-20 virus resistant squash no longer be considered a "regulated article" under regulations in 7 CFR part 340 (the regulations).

The ZW-20 virus resistant squash (*Cucurbita pepo* L. cultivar YC77E ZW-20) (ZW-20 squash) has been described by Upjohn as a squash line which contains the coat protein genes of watermelon mosaic virus 2 (WMV2) and zucchini yellow mosaic virus (ZYMV), and which exhibits significant field resistance against WMV2 and ZYMV.

The Upjohn petition states that the ZW-20 squash should no longer be regulated by APHIS because it does not present a plant pest risk. The ZW-20 squash is currently considered a regulated article under the regulations because it was developed through the use of vectors, promoters, and terminators from plant pathogenic sources. However, as indicated in the petition, the vectors used in producing the ZW-20 squash were disarmed, and the other plant pathogen derived elements did not present a risk of plant pest introduction or dissemination. The field testing of the ZW-20 squash has demonstrated that it does not present a plant pest risk.

Under the regulations, a genetically engineered plant or other organism is a regulated article, subject to regulatory oversight by APHIS, if it is a plant pest or it is unclassified or the Deputy Administrator has reason to believe it is a plant pest. Based on reviews for a number of field tests of the ZW-20 squash and the information in the petition submitted by Upjohn, APHIS believes that the ZW-20 squash is not a plant pest, and that there is no reason to believe that it may be a plant pest or otherwise presents any plant pest risk. Therefore, APHIS is proposing to issue a ruling that the ZW-20 squash is not a regulated article under its regulations. APHIS is requesting comments on the petition and the proposed ruling.

After reviewing the data submitted by the petitioner, written comments received during the comment period, as well as other relevant literature, and interpreting the application of statutes and regulations to these data and comments, APHIS will issue its interpretive ruling regarding the regulatory status of the ZW-20 squash. A notice of the ruling and its availability will be published in the Federal Register.

Done in Washington, DC, this 1st day of September 1992.

Robert Melland,

Administrator, Animal and Plant Health Inspection Service.

[FR Doc. 92-21379 Filed 9-3-92; 8:45 am]
BILLING CODE 3410-34-M