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Dr. John Payne
Associate Director
BBEP/APHIS/USDA
4700 River Road, Unit 147
Riverdale, MD 20737-1237

Subject: Petition for Determination of Nonregulated Status: Squash Containing the Coat Protein Genes from Cucumber Mosaic Virus (CMV), Watermelon Mosaic Virus 2 (WMV 2), and Zucchini Yellow Mosaic Virus (ZYMV).

Dear Dr. Payne;

Asgrow Seed Company is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) regarding an inbred squash line containing the coat protein genes from cucumber mosaic virus, watermelon mosaic virus 2 and zucchini yellow mosaic virus. This petition request a determination form APHIS that the virus resistant line CZW-3 no longer be considered a regulated article under regulations in CRF part 340.

If you have any questions regarding this submission, please do not hesitate to contact Dr. Quemada or myself.

Sincerely,

David M. Tricoli
Regulatory Specialist

Petition for Determination of Nonregulatory Status

SQUASH LINE CZW-3 Containing the Coat Protein Genes from Cucumber Mosaic Virus (CMV), Watermelon Mosaic Virus 2 (WMV 2) and Zucchini Yellow Mosaic Virus (ZYMV).

Submitted by

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Asgrow Seed Company

CERTIFICATION

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.

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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 mosaic 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. Depending on the season of the year and the geographic location, losses can range from 20-80%.

Cultural practices have been used to help lessen the impact of virus epidemics by removing reservoirs of infection and reducing vector populations. Growers have sprayed plants 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 throughout the season as new, unprotected foliage develops. As aphid populations increase during the growing season, these methods of protection against virus infection become increasingly less effective.

It has long been recognized by plant breeders that the best method for dealing with disease problems is the development of disease resistant cultivars. Consequently, the incorporation of virus resistance genes into cucurbits has for decades been the goal of breeding programs. Resistance genes for CMV, WMV-2 and ZYMV have been reported in *Cucurbita pepo* or related species, and breeders in the public and private sectors are developing virus resistant cultivars of *C. pepo* using traditional breeding techniques to backcross these genes into commercially acceptable cultivars. However, because these sources of resistance are derived from wild relatives, several generations of backcrossing are required before these genes can be introgressed into horticulturally acceptable genotypes.

Genetic engineering allows breeders to rapidly develop virus resistant varieties by introducing engineered resistance genes directly into inbred parents of commercially proven hybrids. For example, Asgrow has developed a transgenic squash line, ZW-20 that is resistant to infection by WMV-2 and ZYMV. This line was produced by introducing the coat protein genes of these two viruses into the parent of a hybrid which was already commercially acceptable. In December of 1994, APHIS determined that this line was no longer a regulated article, and sale of the commercial hybrid, named Freedom II, began in July, 1995. The ability to introduce the viral coat protein genes into a line which had already been determined to be commercially

acceptable was a significant factor in the timely introduction of this new virus-resistant variety.

In 1993, a transgenic squash line containing the CMV, WMV-2 and ZYMV coat protein genes was identified as being resistant to all three viruses. This line has been designated CZW-3. Line CZW-3 is similar to ZW-20 in that both lines contain the coat protein genes from ZYMV FL strain and WMV 2 NY strain. The only difference between lines ZW-20 and CZW-3 is that CZW-3 also contains the coat protein gene from the C strain of CMV, and the selectable marker gene, NPT II.

As we expected with Freedom II, we also expect that the introduction of hybrid squash varieties with resistance to all three viruses will have a positive impact on both growers and consumers. The virus resistant hybrid will produce more consistent yields on fewer production acres. Since fewer production acres require fewer inputs, the use of resistant hybrids should result in decreased pesticide, fungicide, fertilizer and energy inputs. The new variety will allow production over a longer time period, increasing the time consumers will be able to buy fresh squash. Virus resistant squash will also lead to a more consistent supply of produce, helping to moderate severe price fluctuations common in the market.

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 *Cucurbita* 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 and Davis, 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).

The common ancestor of all cucurbits are probably an annual gourd-producing plant which was first used in New World agriculture approximately 10,000 years ago. *C.*

pepo spp. includes pumpkins, acorn squashes, zucchini, and ornamental gourds. The *C. pepo* lineage appears to be composed of two subsets, formally identified as subspecies *ovifera* and subspecies *pepo*. Subspecies *pepo* includes domesticated types (including pumpkins and marrow cultivars) and some ornamental gourds, whereas the subspecies *ovifera* includes the remainder of the ornamental gourds as well as acorn, crookneck, straightneck, scallop and yellow squash (Wilson 1993).

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*. The transgenic squash line, CZW-3 was derived by transforming an inbred squash line which belongs to *C. pepo ssp ovifera var. ovifera* and is used by Asgrow in the production of hybrid yellow crookneck squash cultivars. The hybrids are grown in the southeastern United States particularly in Georgia and Florida. The yellow crookneck squash fruits these plants produce are grown for domestic consumption as a fresh table vegetable.

We intend to replace the nontransgenic parent with the transgenic line to produce these hybrids. 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.

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 flower, 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 flower. Under agricultural conditions, this transfer is normally accomplished by domestic honey bees or by species of wild gourd 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).

E. Weediness of Cultivated Squash

Yellow crookneck squash is not listed as a weed in the Federal Noxious Weed Act and is not reported by the Weed Society of America to be a common or troublesome weed anywhere in the United States (Bridges and Baumman, 1992). Although squash volunteers may appear adjacent to production fields they do not readily establish feral or free-living populations. Volunteer plants can be readily identified and eliminated by cultivation or herbicide application.

We have trialled CZW-3 and hybrids made from CZW-3 since 1993, and have not observed any change in seed germination, seed set viability, susceptibility or resistance to pathogens (other than CMV, WMV-2 or ZYMV), or insects. We have observed no differences in overwintering survivability between CZW-3 and its corresponding nontransgenic line nor between hybrids produced using this line and other yellow crookneck hybrids. These observations are based on trials conducted in Michigan, Texas, Georgia, Pennsylvania, New Jersey, Maryland, North Carolina, South Carolina, Florida and Oregon.

Squash cultivars with genes conferring resistance to CMV have been produced using conventional breeding techniques and have been on the market for several years and are available from Thompson and Morgan, Inc. of Jackson, New Jersey. Furthermore, cultivars traditionally bred with resistance genes to ZYMV and WMV-2 are being sold during the 1995 growing season. These products of traditional breeding which provide the same virus resistance traits as CZW-3 are not regulated articles. Therefore, since the virus resistance traits in CZW-3 are equivalent to the virus resistance traits of conventionally bred varieties, the transgenic virus resistance should also not be viewed as conferring any plant pest characteristics on transgenic varieties.

Asgrow further wishes to point out that ZW-20, a yellow crookneck squash line with two of the three resistances that CZW-3 possesses, has been ruled a non-regulated article by APHIS. We see no issues presented by CZW-3 which are not addressed by APHIS decision regarding ZW-20, and which were answered favorably by APHIS in its ruling.

F. Potential for Outcrossing

1. Hybridization with free-living gourds

   There exists in the United States two free-living subspecies (Free-living *Cucurbita pepo*, or FLCP) of *C. pepo* which can cross with cultivated varieties of *C. pepo* without loss of fertility. These include free living gourds in Texas, designated *C. pepo ssp. ovifera var. texana* and free-living gourds in Illinois, Missouri, Arkansas, Oklahoma and Louisiana designated *C. pepo ssp ovifera var*

ozarkana. These subspecies freely interbreed with cultivated squash and therefore genes bred into cultivated squash have the potential to be transferred via pollen to free-living gourds. A detailed analysis of free-living C. pepo has been written (Wilson, 1993)

2. Hybridization with related species

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 results 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).

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 fruit (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. It is possible that nonharvested material may be left on the plants long enough for viable seed to develop, but germination seedlings can be readily identified and removed by cultivation or applications of herbicide.

There is the potential for movement of genetic material by pollen. Within the U.S., movement of genetic material by pollen is possible between free-living gourds and *C. pepo*. However foreign genes are most likely to be retained in a population if they confer a reproductive advantage to the plant containing the foreign gene over their competitors. However surveys conducted on natural stands of FLCP at 14 locations in the United States have failed to detect the presence of CMV, ZYMV, or WMV 2 in these populations(see APHIS/USDA ZW-20 Determination Appendix I Table 6, 1994). Therefore, virus infection did not appear to be limiting the size of the populations surveyed. If, these populations are representative of other FLCP populations, then it is unlikely that virus resistant genes will confer a competitive advantage in natural FLCP stands, and should not be favored by natural selection in the wild.

III. TAXONOMY OF THE VIRUSES

CUCUMBER MOSAIC VIRUS

Cucumber mosaic virus (CMV) is endemic to all temperate regions of the world. The virus belongs to the cucumovirus group. It has an extremely wide host range which includes cereals, forages, woody and herbaceous ornamental, vegetable and fruit crops. CMV is transmitted non-persistently by a number of aphid species and is readily transmitted by inoculation of sap. The virus consists of four different RNA components, designated RNAs 1 to 4. Only RNAs 1 to 3 are required for infectivity (Peden and Symons, 1973) because the coat protein, which is encoded by RNA 4, is also encoded by RNA 3. Two subclasses of CMV have been identified; the Wt subclass, with at least 17 representatives, and the S subclass with at least three members (Piazzolla *et al.*, 1979). The coat protein used to confer CMV resistance in line CZW-3 came from the CMV-C strain which belongs to the WT class (Quemada *et al.*, 1989). Synergistic effects on symptom severity have been reported in plants with mixed infection of CMV and ZYMV (TABLE Ia).

WATERMELON MOSAIC VIRUS 2

Watermelon mosaic virus 2 (WMV 2) a member of the potyvirus group (Purcifull and Hiebert, 1984). It occurs in many areas of the world including many cucurbit growing areas in the United States (Alderz *et al.*, 1983; Purcifull and Hiebert, 1984; Provvidenti *et al.*,1984; Nameth *et al.*,1985; Chala *et al.*, 1987; Davis and Mizuki, 1987). The virus consists of a flexuous filamentous particle. As with other potiviruses, the genome is composed of a single RNA molecule, approximately 10,000 nucleotides in length. Proteins are translated from this RNA as a single polyprotein precursor, which is subsequently processed by a viral-encode protease (Yeh and Gonsalves, 1985) into five or six functional proteins (Dougherty and Carrington 1988). The virus is readily mechanically transmitted and is

transmissible by many species of aphids in a non-persistent manner (Purcifull and Hiebert, 1984). The strain of WMV 2 used for the isolation of the coat protein gene was the Florida strain. Sequence data for the coat protein gene has been published by Quemada *et al* 1990.(TABLE Ib).

ZUCCHINI YELLOW MOSAIC VIRUS

Zucchini yellow mosaic virus (ZYMV) is serologically related to WMV 2. Like WMV 2, it is an important pathogen of cucurbits, and occurs in many cucurbit growing areas in the United States, Central Europe, and the Mediterranean (Lisa *et al.*, 1984). It also belongs to the potyvirus group. The virus consists of a flexuous filamentous particle the genome consisting of a single-stranded positive-sense RNA, which is processed as described above for WMV 2. Several different strains of the virus have been described (Lecoq *et al.*,1981; Risser *et al.*, 1981; Pitrat and Lecoq, 1984). The coat protein used to confer resistance in line CZW-3 was isolated from the Florida strain of ZYMV. The pathogen is transmitted in a non-persistent manner by numerous species of aphids. Synergistic effects have been reported in mixed infections of CMV and ZYMV (Poolpol and Inouye, 1986) (TABLE Ic).

TABLE 1a
Taxonomy of CMV

VIRUS	Cucumber Mosaic Virus (CMV)
GROUP	Cucumovirus
SUBGROUP	I
STRAIN	C Strain
SYNONYMS	Cucumber virus I (Rev. appl. Mycol. 6:501) *Cucumis* virus 1 (Rev. appl. Mycol. 17:52) *Marmor cucumeris* (Rev. appl. Mycol. 28:514) Spinach blight virus (F. agri. Res. 14:1) Tomato fern leaf virus (Rev. appl. Mycol. 9:41)
NUCLEIC ACID	Single stranded RNA components; RNAs 1-3 required for infectivity (Peden and Symons, 1973).
TISSUE RELATION	Particles found in cytoplasm, nuclei, and vacuoles (Gerola, Bassi and Belli, 1965; Lyons and Allen, 1969; Honda and Matsui, 1968, 1974).
SATELLITES	Present in some isolates (Kaper, Tousignant and Lot, 1976; Mossop and Francki, 1978; Gould *et al.*, 1978.)

TABLE 1a Taxonomy of CMV	
HOST RANGE	Extremely wide; 191 species in over 40 families (Price, 1940).
TRANSMISSION	Transmitted by more than 60 species of aphids in a non-persistent manner (Kennedy, Day and Eastop, 1962). Readily transmissible in inoculum sap.
SYNERGY	Synergistic effects have been reported in mixed infections with ZYMV. (Poolpol and Inouyne, 1986.)

TABLE 1b Taxonomy of WMV 2	
VIRUS	Watermelon Mosaic Virus (WMV 2)
GROUP	Potyvirus group
STRAIN	Florida Strain
SYNONYMS	Watermelon mosaic virus (Rev. appl. Mycol. 46, 818).
NUCLEIC ACID	Single RNA particle approximately 10,000 nucleotides in length (Purcifull and Hiebert, 1984; Yeh and Gonsalves, 1985.)
TISSUE RELATIONS	Cytoplasic inclusions and nuclear inclusions (Christie and Edwardson, 1977).
SATELLITES/ HELPER VIRUSES	None
HOST RANGE	Over 160 dicotyledonous species in 23 families (Molnar and Schmelzer, 1964; Edwardson, 1974).
TRANSMISSION	Readily transmitted in a non-persistent manner by at least 38 species of aphids Karl and Schmelzer, 1971; Alderz, 1974; Greber, 1978; Yamamoto and Ishii, 1980; Yamamoto *et al.*, 1982; Perring *et al.*, 1992.)
SYNERGY	To our knowledge, none reported.

TABLE 1c Taxonomy of ZYMV	
VIRUS	Zucchini Yellow Mosaic Virus (ZYMV)
GROUP	Potyvirus group
STRAIN	Florida strain
SYNONYMS	Muskmelon yellow stunt virus (Rev. Pl. Path. 61, 3749.
NUCLEIC ACID	Single stranded RNA (Lisa *et al.*, 1981).
TISSUE RELATIONS	Virus induces cytoplasmic pinwheel inclusions (Lisa *et al.*, 1981), accumulation of endoplasmic reticulum and vesicles containing fibrillar material (Lesemann *et al.*, 1983).
SATELLITES/ HELPER VIRUS	none
HOST RANGE	members of the *Aizoaceae, Amaranthaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Labiatae, Leguminosae, Ranunculaceae, Scrophulariaceae, Solanaceae, and Umbelliferae* (Lecoq, Pitrat, and Clement, 1981; Lisa *et al.*, 1981 and Perring *et al.*, 1992.)
TRANSMISSION	Transmitted in a non-persistent manner by at least nine species of aphids (Perring *et al.*, 1992). Transmissible in inoculum spa (Perring *et al.*, 1992).
SYNERGY	Synergistic effects have been reported in mixed infections with CMV. (Poolpol and Inouyne, 1986.)

IV. THE TRANSFORMATION SYSTEM

A. Construction of the Binary Plasmid

The DNA that was transferred into the plant genome was contained in a binary plasmid (Bevin, 1984). The parent plasmid was pGA482 constructed by An (1986). This binary vector contains the T-DNA border sequence from pTiT37, the selectable marker gene Nos-NPT II a multiple cloning region, and the cohesive ends of phage lambda (An 1985, 1986).

B. *Agrobacterium tumefaciens* Mediated Transformation

The *Agrobacterium tumefaciens* strain used to transfer the engineered coat protein genes of cucumber mosaic virus (CMV), watermelon mosaic virus 2 (WMV2), and zucchini yellow mosaic virus (ZYMV) into the CZW-3 squash line is a non-pathogenic (avirulent) derivative of the C58 vector (Sciaky *et al.*, 1978). A non-pathogenic strain of C58 was constructed by Hepburn *et al.*, (1985) by using marker exchange techniques to replace the C58 pTi T-DNA (region with the neomycin phosphotransferase I (NPT-I gene of Tn903).

The C58Z707 bacteria containing the desired plasmids were then used to infect squash tissues using modifications of the procedure described by Horsch *et al.*, 1985.

Following the use of *Agrobacterium* for plant transformation, the *Agrobacterium* were killed by incorporating the antibiotics carbenicillin and cefotaxime into the medium.

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 crow gall disease, indicates that T-DNA transfer into plant cells by *Agrobacterium* is irreversible.

V. DESCRIPTION OF DONOR GENES TO BE EXEMPTED

A. Viral Coat Protein Genes

The cloning and characterization of the WMV-2 (isolate FL-1656, provided by Dr. D. E Purciful, University of Florida) coat protein used is described by Quemada *et*

al., (1990). A plant-expressible WMV-2 gene was constructed by using specific oligonucleotide primer to generate a fragment consisting of the WMV-2 coat protein coding region and flanking AatII (5') and BglII (3') restriction enzyme sites. This fragment was ligated to AatII/BglII-digested pUC19B, which is the plasmid pUC19 modified by the addition of the cauliflower mosaic virus 35S promoter and polyadenylation signal obtained from pUC1813/CP19, in order to produce a plant expressible coat protein cassette. The protein produced by the expression of the gene is a fusion between the WMV-2 coat protein and the NH3 terminal portion of the CMV coat protein gene. This cassette, CPW was then excised by BamH1 digestion and ligated to the BglII site of pPRBoriGN to produce the binary plasmid designated pPRCPW.

The cloning and characterization of the ZYMV coat protein gene (ZYMV-FL strain) is described by Quemada *et al.*, 1990 and Namba et al. 1992. The strategy employed in the construction of a plant expressible ZYMV coat protein gene has been previously described (Namba *et al.*, 1992).

The C strain of CMV was used as the source of the coat protein gene. The cloning and characterization of the CMV coat protein gene has also been previously described (Quemada *et al.*, 1991).

B ZYMV22/WMBN22

ZYMV22/WMBN22 is the binary plasmid pPRBN, into which the expression cassettes for ZYMV and WMV 2 were inserted. The expression cassettes were inserted sequentially into a unique Bgl II restriction site of pPRBN t yield pPRBN-ZYMV72/WMBN22. To accomplish this, a BamH I site was introduced 5" to the CaMV 35 S promoter, and a Bgl II site was introduced 3" to the polyadenylation signal sequence of the WMV 2 and the ZYMV coat protein expression cassettes. BamH I and Bgl II sites were introduced by the use of appropriate oligonucleotide primers during PCR amplification of the cassettes. PCR products were digested with BamH I and Bgl II, and inserted into the unique site of pPRBN to yield ZYMV72/WMBN22.

C. CMV73/ZYMV72/WMBN22

The vector CMV73/ZYMV72/WNBN22 was used to introduce the three coat protein genes. This vector was derived from ZYMV72/WMBN22 the vector used to produce line ZW-20) by inserting the CMV coat protein expression cassette into a unique HindII site of ZYMV72/WMBN22 (Tricoli et. al 1995). The elements of CMV73/ ZYMV72/WMBN22 are summarized in Table II.

TABLE II

Summary of Sequences of CMV73/ZYMV72/WMBN22

GENETIC ELEMENT	FUNCTION
LB	The left border region of the T-DNA from *Agrobacterium tumefaciens* (Barker *et al.*, 1983; van den Elzan *et al.*, 1985.
p35S	The 35S gene promoter from cauliflower mosaic virus (CaMV) (Odell *et al.*, 1985). Controls expression of the NPT II gene.
NPT II	The neomycin phosphotransferase II gene from Tn5 (Beck *et al.*, 1982). Encodes a protein which renders transformed cells resistant to kanamycin.
t35S	The 35S terminator from CaMV.
p35S	The 35S promoter from the cauliflower mosaic. Controls expression of the WMV-2 coat protein gene.
WMBN22	The coat protein gene from WMV-2 FL strain fused to the CMV 5' untranslated region and 48 nucleotides from the 5' terminus of the CMV coat protein gene (Quemada *et al.*, 1990).
t35s	The 35S terminator from CaMV.
p35S	The 35S promoter from CaMV.
ZYMB72	The coat protein gene from ZYMV-FL strain fused to the 5' untranslated region and the CMV translation initiation codon from the CMV coat protein gene (Quemada *et al.*, 1990, Namba et al. 1992.).
t35S	The 35S terminator from CaMV.
p35S	The 35S promoter from CaMV.
CMV73	The coat protein gene from CMV C strain and 64 nucleotides of the 5' untranslated region (Quemada et al. 1991)
t35S	The 35S terminator from CaMV.
RB	The right border region of the T-DNA from *Agrobacterium tumefaciens* (Barker *et al.*, 1983, van den Elzen *et al.*, 1985).

C. The Transformation Selectable Marker Gene - Neomycin Phosphotransferase

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 CZW-3 regenerant plant.

VI. Genetic Analysis and Agricultural Performance of Transformed Cultivars

A. Mendelian Inheritance

DNA for the primary (R_O) plant has been examined by Southern blot analysis, and has been determined to contain a single intact insert (Figure 1). The original transgenic R_O plant was backcrossed to the non-transgenic version of the same inbred, producing the R_1 generation. Segregants containing coat protein genes were identified by assaying for the linked NPT II gene using ELISA. Positive segregants were then self-pollinated to produce the R_2 inbred generation or were crossed to the appropriate non-transgenic inbred line to create the segregating progeny of the hybrids Dixie or Pavo. The R_2 and hybrid progeny were tested for resistance to virus infection in multiple field trials conducted in various locations throughout the United States during 1993, 1994 and 1995, including major testing sites in Tifton, GA and Kalamazoo, MI. During trials conducted in Kalamazoo MI, segregation ratios were determined for inbred and hybrid lines by assaying for the presence of the linked NPT II selectable marker gene using ELISA. The results of those assays are given in table III and IV below. The segregation ratios are consistent with those expected for a single gene insert.

TABLE III

Segregation ratios for R_1 seed produced by self-pollinating a heterozygous R_1 transformant

Ratio of NPT II positive plants to NPT II negative plants (assayed by ELISA using anti-NPT II antibodies)				
	Observed	Expected	X^{2}*	P
CZW-3	40:15	41.25:13.75	$X^2 = 0.05$	0.80-0.70

*Chi-square goodness-of-fit for hypothesis for 3:1 segregation.
$X^2 = 3.84$, for a 3 to 1 ratio at p = 0.05; df = 1.

TABLE IV

Segregation ratios for hybrid seed produced by crossing a heterozygous transformant inbred parent to a non-transformed inbred parent.

Ratio of NPT II positive plants to NPT II negative plants (assayed by ELISA using anti-NPT II antibodies)				
	Observed	Expected	X^{2}*	P
HYBRID A-CZW-3	27:30	28.5:28:5	$X^2 = 0.06$	.90 - .70
HYBRID B-CZW-3	27:33	30:30	$X^2 = 0.4$	.70 - .50

*Chi-square goodness-of-fit test for hypothesis of 1:1 segregation.
$X^2 = 3.84$, for a 1 to 1 ratio at p - 0.05; df = 1

B. Disease and Pest Characteristics of the Transformed Cultivars

Line CZW-3 has been included in field trials in over 13 test sites during 1993 and 1994. Trials have been conducted in Michigan, Georgia, North Carolina, Florida, Oregon, and Texas. During these trials, we did not observe any pleiotropic effects in this or any of the other transgenic lines tested. Based on visual observation of the Kalamazoo trials, 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 are susceptible to powdery mildew whiteflies, and cucumber beetles.

C. Nutritional Composition

Studies on the nutritional composition of cultivated cucurbits have shown that squash are all low in calories, (21 calories/100 gms of edible fruit) and protein (~ 1.4 g protein/100 gms of edible fruit) (Whitaker and Davis, 1962). Fruits harvested from CZW-3 and its non-transgenic counterpart have been analyzed for total protein, fat and carbohydrates and have found no significant difference between the transgenic and non-transgenic inbred line. Likewise levels of vitamin C, beta carotene, vitamin A, calcium, iron sodium, ash, dietary fiber,moisture and sugar profiles (fructose, glucose,galactose,sucrose,maltose, and lactose) were also examined and no significant changes between the transgenic and non-transgenic version of this inbred were seen (Table V).

D. Cucurbitacin Levels

Cucurbits are known to produce alkaloids knows as cucurbitacins. Cucurbiticin E is the primary cucubitacin found in squash. Cucurbitacin B and E are readily detectable by taste at levels as low as 1 to 10 ppb (Rymal *et al.*, 1984). The standard test in breeding programs for identify cucurbitacins in breeding lines is to evaluate by taste. Both CZW-3 and the non-transgenic inbred from which CZW-3 was derived are non-bitter.

E. Pollination Characteristics of the Transformed Cultivars

Since 1990, samples of seeds have been harvested from plants in the border rows of our field trials. The movement of pollen from transgenic squash plants from various squash lines was assayed for the expression of the NPT II marker gene. Analyses are shown for a 1990 Kalamazoo, Michigan and a 1991 Tifton, Georgia field trial site. The data indicate that pollen dispersal in transgenic squash plants is 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). It is unlikely that the pollination characteristics of CZW-3 differ from these already observed with other transgenic lines.

TABLE V

A Nutritional Analysis of Transgenic Squash Line CZW-3 and Non-Transgenic inbred control.

ASSAY	Analysis		Unit
	CZW-3*	Control**	
Protein (N x 6.25)	.7	.7	G/100 G
Moisture, 70° vac. oven	95.0	94.9	G/100 G
Total Fat	.1	.2	G/100 G
Ash	.5	.4	G/100 G
Dietary Fiber	21.0	21.0	G/100 G
Total Carbohydrate	3.7	3.8	G/100 G
Calories	18.5	19.8	Calories/100 G
Sugar Profile			
-Fructose by HPLC	1.1	1.1	G/100 G
- Glucose by HPLC	1.1	1.0	G/100 G
- Glucose by HPLC	2.1	2.1	G/100 G
-Sucrose by HPLC	2.1	2.1	G/100 G
-Maltose by HPLC	2.1	2.1	G/100 G
-Lactose by HPLC	2.1	2.1	G/100 G
Vitamin C, Total	19.5	21.0	mg/100 G
Beta Carotene by HPLC	0.3	20.3	mg/100 G
Vitamin A from Carotene	50	25.0	IU/100 G
Calcium	16.0	16.5	mg/100 G
Iron	.266	.307	mg/100 G
Sodium	22.50	5.39	mg/100 G

*composite sample consisting of 20 fruits
**composite sample consisting of 11 fruits

F. Horticultural Performance of the Transformed Cultivar

1. Field Performance

The effectiveness of transgenic squash line CZW-3 was first demonstrated in field trials conducted in 1993. This line was tested for CMV, ZYMV and WMV-2 resistance in Kalamazoo, Michigan and in Tifton, Georgia. R_1 or R_2 progeny or hybrid lines generated using a transgenic lines as one of the parents were germinated in the greenhouse. Carborundum dusted cotyledons on six-day-old seedlings, were mechanically inoculated by rubbing the leaves with a 1/10 wt/vol (fresh weight of infected plant tissue/mls of virus buffer) of CMV strain C, ZYMV strain FL, or WMV-2 strain NY, which were propagated in *Cucumis sativus, c.v. amcogreen, Cucurbita pepo, c.v. corsair,* and *Phaseolus vulgaris, c.v.* black turtle 2, respectively. Plants were re-inoculated 5 days later. Approximately 7-10 days post-inoculation, plants were transplanted into the field. The trial plots were planted in a paired plot design, in which each row containing a transgenic line was paired with a row containing its non-transgenic counterpart as a control. Each row consisted of 15 plants, two feet apart, with five feet between rows. In general, two replications of each transgenic line were incorporated into each trial. This design allowed for a side by side comparison between the transgenic line and its nontransgenic counterpart for horticultural as well as virus resistance traits. The use of segregating R_1 transgenic progeny provided nontransgenic segregant which allowed us to distinguish between traits resulting from the introduction of the transgene, from traits that may have been introduced by somaclonal variation. The entire plot was surrounded by a border zone of non-transgenic squash plants in order to contain the transgenic pollen.

The results of four different trials are presented in Table VI. In order to limit cross-contamination between the plots by natural aphid spread, plantings were carried out separated from each other by space and time. The CMV trial was planted first, the ZYMV trial was planted about one month later and at a location about 3.5 km away, and the WMV 2 trial was planted last on the same farm as the CMV trial but about 1 km away and after the CMV trial was terminated.

The results presented in Table VI are based on data accumulated at our Kalamazoo location. However these results reflect observations obtained at other locations as well. Once the efficacy of line CZW-3 was identified it was extensively trialled at numerous locations throughout the United States including North Carolina, Texas, Georgia, New York and Oregon and similar results were obtained. In all these trials, CZW-3 exhibited significant protection against CMV, ZYMV and WMV-2 infection. In some

trials, very mild symptoms were observed on some transgenic plants, however overall CZW-3 consistently provided a high level of resistance to CMV, ZYMV and WMV 2. This line was also evaluated in trials that demonstrated the efficacy of the line even under conditions where virus spread by aphids was severe, thus exposing plant to repeated aphid inoculation after the initial mechanical inoculation. Line CZW-3 has not only been shown to be resistant to infection when inoculated with each virus individually, but has also been shown to be highly resistant when simultaneously inoculated with all three viruses (Table VI).

Breeder trials in Tifton GA. have verified the results presented in table V and have also demonstrated that except for virus resistance, CZW-3 is horticulturally identical to its non-transgenic counterpart.

Field performance of hybrids made using CZW-3 was also demonstrated by Arce-Ochoa *et al.*, (1995). Under production conditions typical for Texas, a significant increase in marketable fruit was observed on the transgenic hybrid as compared to its nontransgenic counterpart.

TABLE VI

Field Trial Results

Symptom development on transgenic inbred CZW-3 or hybrid squash 60 to 71 days post inoculation using a 1/10 wt/vol (wt of tissue per mls of buffer) dilution of single virus preparation of CMV-C strain (CMV Trial), ZYMV-FL strain (ZYMV Trial) or WMV-2 NY strain (WMV-2 Trial) 55 days post inoculation with a multiple virus preparation of CMV-C + ZYMV-FL + WMV-2-NY (CZW Trial).

		CMV Trial				WMV-2 Trial				ZYMV Trial				CZW Trial			
Line	NPT II	Symptomatic fraction	%	Disease Rating foliage	fruit	Symptomatic fraction	%	Disease Rating foliage	fruit	Symptomatic fraction	%	Disease Rating foliage	fruit	Symptomatic fraction	%	Disease Rating foliage	fruit
Hybrid A	+	0/20	0	0.0	NT	0/5	0	0.0	0.0	0/14	0	0.0	0.0	0/27	0	0.0	0.0
CZW-3	-	6/6	100	9.0	NT	9/9	100	7.2	7.2	4/4	100	8.0	7.0	30/30	100	8.4	7.0
Hybrid A nontransformed	+	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	-	9/9	100	9.0	NT	15/15	100	7.0	6.1	11/11	100	7.4	8.8	14/14	100	8.7	7.0
Hybrid B	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0/27	0	0.0	0.0
CZW-3	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	33/33	100	7.0	7.0
Hybrid B nontransformed	+	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	-	35/35	100	8.1	NT	30/30	100	7.0	7.0	28/28	100	7.2	8.7	24/24	100	8.9	7.0
CZW-3	+	1/13	0/7	0.4	NT	NT	NT	NT	NT	NT	NT	NT	NT	0/40	0	0.0	0.0
	-	6/6	100	8.5	NT	NT	NT	NT	NT	NT	NT	NT	NT	15/15	100	7.0	7.0
control	+	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	-	15/15	100	8.1	NT	15/15	100	9.0	6.9	43/43	100	7.4	8.4	20/20	100	9.0	7.0

2. Greenhouse Performance

Greenhouse challenges were conducted on heterozygous or homozygous lines of CZW-3 for resistance to CMV, ZYMV, WMV 2 and PRV. Carborundum dusted cotyledons on six day old seedlings were mechanically inoculated by applying inoculum sap prepared by grinding virus infected tissue in phosphate buffer at a rate of 10mls of buffer per gram of fresh weigh of infected leaf tissue. Inbred line CZW-3 was resistant to CMV, ZYMV, and WMV 2 regardless of whether the line was homozygous or heterozygous. As expected the material was susceptible to PRV (Table VII).

TABLE VII Greenhouse Screens

Symptom development on transgenic inbred CZW-3 after greenhouse inoculations using a 1/10 wt/vol dilution of CMV-C strain, ZYMV-FL strain, WMV-2 CA strain or PRV-FL strain.

		CMV-C Symptomatic		WMV-2-CA Symptomatic		ZYMV-FL Symptomatic		PRV-FL Symptomatic	
Line	NPT II	fraction	%	fraction	%	fraction	%	fraction	%
CZW-3	+	0/15	0	0/13	0	0/7	0	--	--
Heterozygous	-	--		2/3	67	5/5	100	--	--
CZW-3-2	+	0/10	0	0/10	0	0/10	0	10/10	100
Homozygous	-	--	--	--	--	--	--	--	--

*Homozygous line

These data show that line CZW-3 has significant and commercially valuable resistance against CMV, WMV2 and ZYMV.

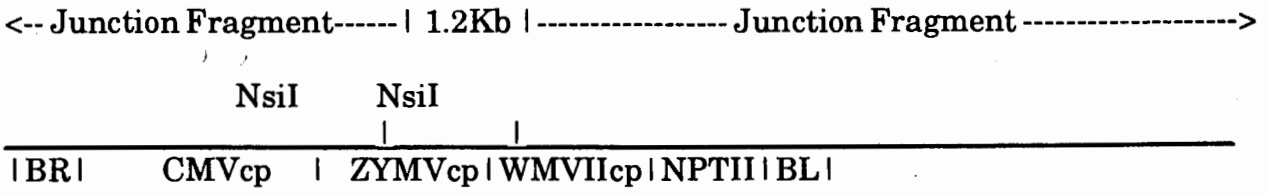
Except for resistance to viruses, examination of the CZW-3 line by Asgrow's plant breeders, plant pathologists, product manager and technical service representatives, as well as cooperating researchers outside the company, has determined this line to be horticulturally identical to its non-transgenic 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. When the transgenic line is used in place of the non-transgenic inbred for hybrid seed product,

the resulting hybrids posses the transgenic inbred for hybrid seed production, the resulting hybrids posses the identical horticultural characteristics as the counterparts.

G. DNA Analysis of CZW-3

1. T-DNA Analysis of Virus Resistant CZW-3 Squash

Southern hybridization was used to verify the integrity and copy number of T-DNAs transferred into transgenic squash line CZW-3. The T-DNA structure consists of four gene cassettes; cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV), watermelon virus 2 (WMV 2) coat protein genes and selectable marker neomycin phosphtransferase (NPT II), flanked by right (BR) and left border (BL) sequences (see map below). This T-DNA map illustrates the gene cassettes and border regions and identifies two NsiI restriction sites. The restriction endonuclease NsiI was used to cut the T-DNA into diagnostic fragments that could be mapped by hybridizing specific DNA fragments (probes) to identify the 1.2Kb internal fragment, and BR and BL junction fragments. The probes consist of coding regions specific for CMV, ZYMV, WMV 2 and NPT II. The digested T-DNA fragments were identified with the following probes: internal 1.2Kb fragment with the ZYMV probe, right border junction fragment with the CMV and ZYMV probe, and the left border junction fragment with the WMVII and NPT II probe.



T-DNA Map Not Drawn to Scale

Total squash-leaf DNAs (~8µg) from five CZW-3-R_3 progeny were digested with NsiI restriction enzyme, electrophoresed, transferred to nylon membrane, and hybridized with ^{32}P-labelled DNA probe (CMV, ZYMV, WMVII or NPT II coding sequences). Four replica blots were made as described and each blot hybridized with one of four different ^{32}P-labelled coding regions. Following hybridization the blots were subjected to autoradiography to visualize the digested T-DNA fragments by hybridization signals on x-ray film. The hybridization results are described below:

The NPT II probe hybridized to one Left border junction fragment approximately 6.0Kb for each of the five CZW-3 siblings tested (see Figure 1a). The WMVII probe also hybridized with this junction fragment, see map above and WMVII hybridization data. This data is consistent with one intact junction fragment.

The CMV probe hybridized to one Right border junction fragment approximately 4.5Kb for each of the five CZW-3 siblings tested (see Figure 1b). The ZYMV probe also hybridized to this junction fragment, see map above and ZYMV hybridization data. This data is consistent with one intact right border junction fragment.

The ZYMV probe hybridized to one Right border junction fragment approximately 4.5Kb and one 1.2Kb internal fragment of expected size for each of the five CZW-3 siblings tested (see Figure 1c). The CMV probe also hybridized with this junction fragment, see map above and CMV hybridization data. This data is consistent with one intact right border junction fragment and the expected internal T-DNA fragment of 1.2Kb. The ZYMV probe hybridizes to the same right border junction fragment as CMV because the ZYMV coding region used for probe spans both sides of the first NsiI site.

The WMVII probe hybridized to one Left border junction fragment approximately 6.0Kb for each of the five CZW-3 siblings tested (see Figure 1d). The NPT II probe also hybridized with this junction fragment, see map above and NPT II hybridization data. This data is consistent with one intact left border junction fragment.

In summary these hybridization data indicate a single T-DNA structure for CZW-3. The results support that CZW-3 squash harbors a single-complete-integrated T-DNA consisting of CMV, ZYMV, WMVII, and NPT II gene cassettes.

2. Binary Plasmid Sequences Minus The T-DNA Region

We verified CZW-3 squash harbors no binary plasmid sequences outside the T-DNA borders by southern hybridization. One of the replica blots, from the previous experiment was stripped to remove hybridization probe. The blot was rehybridized with ^{32}P-labelled binary plasmid sequences minus the T-DNA (BN-).

The BN-probe hybridized to the binary plasmid positive control (see Figure 1e) only. No hybridization signal was detected for the negative control or five CZW-3 siblings tested. This data indicates that CZW-3 squash does not harbor any binary plasmid DNA sequences outside the T-DNA border region.

H. 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. We determined the level of viral coat protein in squash fruit and leaf tissue by double antibody sandwich ELISA. Polyclonal antibodies were raised against each respective virus, and used to determine the levels of coat protein in the fruit and leaves. Levels of viral coat protein were quantitated by comparison to standard curves produced by serial dilution of known amounts of purified viral coat protein.

Fruits were collected from four progeny of greenhouse grown CZW-3 plants in order to determine the average level of coat proteins in the edible portion of the plant. The average level of coat protein expression of the CMV, WMV 2, and ZYMV coat protein in transgenic fruit were determines to be 8.28, 7.25 and 20.25 µg of protein per kg of fruit respectively (Table VIIIa). These level when compare to the level of viral coat protein found in fruit samples collect from a local grocery store. The amount of coat protein in samples taken from fruit purchased at a local supermarket and these were compared to the levels of coat protein produced in line CZW-3. Samples of equivalent mass from several different plants, were pooled to produce each extract. Bradford assays were performed on each sample and samples were standardized to ensure that similar amounts of protein were being compared among the samples. The levels of viral coat proteins in the transgenic fruits were found to be significantly lower than viral coat protein levels found in virus infected cucurbit fruits collected from grocery store shelves (TABLE VIIIb).

TABLE VIIIa

NPT II AND VIRAL COAT PROTEIN LEVELS IN TRANSGENIC FRUIT

		µg NPT II or Viral Coat Protein per kg Fruit			
Line	Plant	NPT II	CMV	WMVII	ZYMV
CZW-3	1	33.0	8.3	14.0	32.0
	2	32.9	5.9	4.0	7.0
	3	52.0	10.8	5.0	16.0
	4	59.0	8.1	6.0	26.0
	Ave.	44.0	8.28	7.25	20.25

TABLE VIIIb

C= cantaloupe; CJ= cantaloupe (second source); H= honeydew melon; Y= yellow crookneck squash; Z= zucchini squash; ND= assay conducted but no virus detected.

FRUIT	µg Viral Coat Protein per kg Fruit			
	CMV	PRV	WMV2	ZYMV
C-251	355,200	18,000	14,400	10,320
C-252	130,464	5,472	10,944	115,488
C-253	ND	252,000	28,800	720
C-255	ND	ND	864	ND
CJ-1	>2,400,000	1,200	8,400	ND
CJ-2	>3,216,000	ND	14,000	ND
CJ-3	>3,216,000	ND	12,864	ND
H-1	ND	7,200	9,480	ND
H-2	ND	6,840	1,800	ND
H-3	ND	ND	2,200	ND
H-4	359	4,752	3,888	173
H-5	269	3,168	3,168	260
H-6	238	ND	2,592	ND
U-7	ND	5,928	1,824	137
H-8	664	13,272	1,896	190
H-9	82	960	24	24
H-10	ND	ND	250	ND
H-11	ND	ND	1,560	ND
H-12	ND	ND	480	ND
H-13	ND	ND	2,200	ND
H-14	ND	3,120	720	ND

TABLE VIIIb

C= cantaloupe; CJ= cantaloupe (second source); H= honeydew melon; Y= yellow crookneck squash; Z= zucchini squash; ND= assay conducted but no virus detected.

FRUIT	µg Viral Coat Protein per kg Fruit			
	CMV	PRV	WMV2	ZYMV
H-15	ND	10,080	1,700	ND
H-16	ND	ND	3,100	ND
Y-1	ND	ND	11,424	ND
Y-2	ND	ND	ND	ND
Y-3	ND	ND	1,152	ND
Y-4	ND	ND	13,056	ND
Z-1	ND	ND	140	ND
Z-2	ND	ND	ND	ND
Z-3	ND	ND	454	ND
Z-4	ND	ND	ND	ND
Z-5	ND	ND	ND	ND
Z-6	ND	ND	576	ND
Z-7	43	ND	2,592	ND
Z-8	14	ND	2,900	ND
				ND

These data demonstrates that overall the levels of viral coat proteins can be significantly higher in locally grown were significantly higher than those seen in the transgenic line CZW-3.

CMV, WMV 2, and ZYMV coat protein levels were also measured in leaves taken from transgenic progeny of CZW-3. Leaf samples were harvested from four non-inoculated greenhouse grown transgenic plants. The averaged coat protein levels were found to be 372.9, 19.3 and 318.2 ug per kg of leaf tissue for CMV, WMV 2, and ZYMV respectively (Table VIIIc).

TABLE VIIIc
Level of Viral Coat Protein in noninoculated transgenic CZW-3 squash leaves

Line	µg of viral coat protein per kg of leaf tissue			
	Plant	CMV-Cp	WMV-2-CP	ZYMV-cp
CZW-3	1	0.0130	0.0012	0.0106
	2	0.0165	0.0004	0.0093
	3	0.0096	0.0006	0.0103
	4	0.0073	--	0.0095
	ave	0.0116	0.0006	0.0099
	µg/kg	372.9	19.3	318.2

I. Expression levels of NPT II

The level of expression of endogenous NPT II was calculated using antibody sandwich ELISA using a kit commercially available from 5 Prime → 3 Prime, Inc. Assays were performed according to the company's protocol. Endogenous levels were quantified by comparison to a standard curve produced from dilutions of purified NPT II protein provided by the company. The level of NPT II in transgenic fruit samples was determined to average 44.0 µg/kg fruit (Table VIIIa). This data indicates that there is very low levels of NPT II protein in fruit from transgenic line CZW-3.

VII. Environmental Consequences of the Introduction of the Transformed cultivars

A. Weediness

Despite being cultivated in the United States since antiquity, cultivated *Cucurbita pepo* has is not listed in any of the standard texts and lists or weed (Holms et al 1979; Muenscher,1980, Weed Society of America, 1992). Yellow crookneck squash has been bred for agricultural use and has few traits that are associated with weediness.

Thus far, we have not detected any transgenic lines which display undesirable weedy characteristics. 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, as well as cooperating researchers outside the company. Scientific personnel normally examine the trial on a weekly basis and collect data on performance and horticultural type throughout the duration of the test. No change in horticultural characteristics have been observed in this or other transgenic cultivars when compared with their non-transformed counterpart during trials conducted by Asgrow personnel or outside collaborators. Therefore, no change in horticultural characteristics leading to increased weediness have been observed.

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. In fact, examples exist 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 through virus resistant varieties have been under cultivation for decades. Furthermore, for the past few years, cucumber varieties have been marketed which are resistant to CMV, ZYMV, WMV 2 and PRV. There has been no report of increased weediness of these varieties.

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 has 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, 1993).

Traditionally bred squash with resistance to CMV has been on the market for a number of years. Commercial squash hybrids with resistance to ZYMV and WMV-2 produced by traditional plant breeding were released for sale in 1995. Therefore the release of virus resistant squash plants produced by recombinant DNA techniques does not present a different risk of weediness than virus resistant squash developed via traditional breeding methods and already released commercially.

B. Vertical Transfer of the Gene

The inserted coat protein genes may be transferred from the transgenic CZW-3 cultivar to non-transgenic cultivated cucurbits or to the non-cultivated FLCP. Below we discuss the environmental consequences of the transfer of these genes to non-transgenic *Cucurbita*.

1. Between CZW-3 and Cultivated *C. pepo*

Yellow crookneck squash is not listed in the Federal Noxious Weed Act (7 U.S.C. 2801-2813) and is not reported by the Weed Society of America to be a common or troublesome weed anywhere in the United States. 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 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.

Commercial growers harvest yellow crookneck squash at an immature stage of development and at this stage the seeds are non-viable. However, imperfect fruit or "culls" may be left on the plant and will contain viable seed when 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, volunteer squash is generally not a problem in or around growers' fields since they do not produce feral populations. Following good crop rotation practices, a vine crop is generally not grown in a field where squash was grown the previous fall. Therefore, over-wintering squash seeds can be readily identified and eliminated. The few volunteers that may germinate are readily identified and are eliminated by standard tillage practices. Additionally, overwintering volunteers are often killed after germination by spring frosts. Finally, cultivated varieties are several generations removed from wild or weedy ancestors and are adapted to agricultural conditions. Therefore, it is unlikely that volunteers will persist without agricultural intervention, even if they are virus-resistant.

2. Between CZW-3 and Free-living *Cucurbita pepo*

In the United States there are free living Cucurbit pepo (FLCP) which are

known to hybridize with cultivated *C. pepo* without loss of fertility. However, it is unlikely that progeny from a cross between FLCP and CZW-3 would pose a weed problem. Hybrid squash varieties have been bred for growing under cultivation and is less adapted than FLCP to grow without cultivation. Therefore, any cross between CZW-3 and FLCP will not only transfer viral coat protein genes but also numerous traits that are characteristic of cultivated squash. This includes such traits as low levels of cucurbitacin, thin skinned fruits, bush type growth habit; traits that would make the hybrid progeny less suitable from survival in the absence of cultivation. In fact, cultivated squash is routinely grown in close proximity with FLCP yet there is no evidence that improvements bred into cultivated varieties have resulted in the emergence of weedy hybrid populations.

It is also unlikely that resistance to CMV, ZYMV and WMV 2 infection will confer a selective advantage or be maintained in FLCP. Surveys conducted in 1993 on FLCP populations in Louisiana, Arkansas and Mississippi for the presence of CMV, ZYMV, or WMV-2, suggested that resistance to CMV, ZYMV or WMV 2 would have little selective advantage to these populations, since these viruses do not appear to be prevalent in these wild populations. This information suggests that FLCP are not exposed to significant levels of viral infection and that the selective pressure to maintain the virus resistance genes in natural populations should be minimal.

Should it be found that FLCP populations increase, 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 FLCP hybrid which might arise during the commercial cultivation of transgenic squash.

C. Transencapsidation and Recombination

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).

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.

The amount of coat protein produced may be an important factor in transencapsidation since the greater the concentration of coat protein the greater the probability that the endogenous coat proteins will encapsidate an infecting viral RNA. However, when 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 occurring in transgenic plants is far less than the probability of these events occurring in nature where mixed virus infections are common. In the geographic areas where cucurbits are grown commercially, fields are normally subjected to multiple infection by the potyviruses under consideration in this document (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 3). Therefore, the probability of transencapsidation or phenotypic mixing could be far greater in multiply-infected plants than for a genetically engineered plant. In our virus-resistant CZW-3 plants CMV, ZYMV and WMV 2 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.

Even if a masked virus was generated, it could only be maintained in the population as long as it replicated in the transgenic plants since one transferred outside of the transgenic plant since once transferred outside of the transgenic crop it would recreate the wildtype virus particle.

In the event that a resistance breaking isolate of CMV infects CZW-3, transencapsidation between CMV and potyviruses has not been reported and would not be expected due to the architectural differences of the two types of viral particle. CMV is icosahedral whereas potyviruses are flexuous rods. The likelihood of transencapsidation would be greater given infection with PRSV or a resistance breaking strain of ZYMV or WMV 2. However transencapsidation between potyviruses would not enhance viral transmission since the three viruses share common insect vectors.

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.

Recombination is defined as the formation of new genetic combinations by exchange of genes. For recombination to occur in CZW-3 at least two different viruses must be replicating in the same plant. The fact that CZW-3 inhibits replication of three of the major viruses of squash (see Figure 4) should decrease the probability of recombination.

The level of the coat protein RNA transcript in CZW-3 is significantly lower than the level of transcript in single or multiply-infected squash plants (Figure 2). Therefore, if the probability of recombination is correlated to the level of RNA transcript, the probability of recombination occurring between a transgenic plant containing a low transcript level and an infecting virus particle would be lower than the probability of recombination between two replicating particles in a multiply infected plant. The probability of recombination would be even further reduced since CZW-3 is resistant to infection by three of the four major viruses that infect cucurbits.

Recombination between the CMV coat protein and an infecting potyvirus or between the potyvirus coat protein gene and an infecting CMV is unlikely since most evidence suggests that recombination occurs at higher frequency between viruses or virus strains that share a significant nucleotide homology (Lai, 1992) which is not the case between CMV and potyviruses.

Recombination between ZYMV and WMV 2 coat protein genes and an infecting PRSV would be more likely than between the CMV coat protein and an infecting PRSV since potyviruses share sequence homology. However recombination has not been demonstrated within the potyvirus group (Lai,1992).

Synergistic interactions have been known to occur between infecting viruses in plants with multiple virus infections. In order to demonstrate that no synergy exists between the endogenous coat protein transcripts and viruses known to commonly infect squash, line CZW-3 was inoculated with a number of plant viruses either singly or in combination. Progeny of CZW-3 were germinated in the greenhouse and mechanically inoculated using various combinations of virus preparations. Four plants were used per inoculation. Test

inoculations included; CMV, ZYMV, WMV 2, PRV, CMV+ZYMV+WMV 2 or CMV+ZYMV+WMV 2+PRV. Using ELISA or RNA dot blots (White and Bancroft 1992), the level of coat protein and coat protein RNA transcript in inoculated CZW-3 plants were compared to levels in uninoculated CZW-3 plant as well as inoculated and uninoculated nontransgenic controls. No evidence of synergy was observed for any of the combinations of viruses tested. For example, when CZW-3 was inoculated with ZYMV, no increase was observed in RNA transcript levels or coat protein concentration of the WMV 2 transgene. (Table IX and figure 2 a-d).

These results were expected since coat protein genes have not been shown to be involved in synergistic effects. Recently, the work of Vance et.al (1995) demonstrated that the expression of the coat protein gene of TVMV in transgenic plants does not support any aspects of PVX/potyviral synergism.

TABLE IX

Coat protein levels (µg/kg leaf tissue) in CZW-3 plants after inoculations with CMV-C, ZYMV-FL, WMV-2-NY, PRV-FL, CMV-C+ZYMV-FL+WMV-2 NY (C+Z+W), or CMV-C+ZYMV-FL+WMV-2-NY+PRV-FL (C+Z+W+P)

	Inoculating Virus													
Coat protein levels ug/kg leaf tissue	Non-inoculated		CMV-C		ZYMV-FL		WMV-2-NY		PRV-FL		C+Z+W		C+Z+W+P	
	CZW-3	control *	CZW-3	control	CZW-3	control	CZW-3	control	CZW-3	control	CZW-3	control	CZW-3	control
CMV	400	--	196	48,652	282		290		--	--	270	71,148	161	29,799
ZYMV	150	--	82		102	31,725	111		--	--	40	26,063	47	16,771
WMV2	300	--	65		128		111	335	--	--	27	18,392	38	579
PRV	--	--	--		--		--		4,337	4,725	--	--	2,627	3,194

* non-transgenic controls

D. Native Floral Communities

The genus *Cucurbita* is a small closed system (Decker *et al.*, 1988). Therefore it is unlikely that CZW-3 will have an impact on native floral communities. Since the evidence suggests that FLCP are not under significant selective pressure from virus infection, the probability of maintaining virus resistance genes in natural populations should be minimal.

E. 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. CZW-3 contains no known pathogenic properties that would impact native fauna. High levels of CMV, ZYMV and WMV 2 coat proteins are already present in fruits of naturally infected squash plants. There has been no detectable alteration of cucurbitacin levels in the transgenic line.

F. Neomycin Phosphotransferase

The NPT II protein is ubiquitous in the environment (Flavell 1992). The safety of NPT II was detailed in a recent publication by Fuchs *et al.* 1993a and 1993b, Flavell *et al.* 1992 and Nap *et al.* 1992. Data in support of the safety of NPT II has been reviewed by the Environmental Protection Agency resulting in the establishment of an exemption from the requirement of a tolerance for residues of NPT II and the genetic material necessary for its production when used as a plant pesticide inert ingredient (Federal Register notice Vol 59 No. 187).

G. 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. One of the most commonly used pesticides for controlling aphid is Thiodan. Thiodan has an oral LD_{50} 70 mg/kg, and is highly toxic to birds and fish. 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 insecticide targeted at other non-aphid insect pests. Stylet oil sprays need to be applied frequently and at a 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 virus 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 over planting usually practiced by farmers in order to ensure a certain yield of marketable fruit.

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

Virus resistant cultivars will allow the production squash using less agricultural land. Depending on the geographic location and the season a grower can loose 20 to 80% a crop to virus disease. By using virus resistant hybrids growers can produce greater yields on less land. Cultivating less acreage results in less inputs of fertilizers, fungicides, insecticides and energy, all of which will be a benefit to the grower, consumer, and the environment.

H. Impact on Human Health

The commercial production of transgenic plant line CZW-3 should not pose a risk to human health. The presence of virus infected produce on supermarket shelves have been previously documented in cucurbits (Provvidenti *et al.* 1984). Furthermore, we have examined zucchini squash, yellow crookneck squash, cantaloupe, and honeydew melons from supermarket shelves, and found CMV, WMV2 and ZYMV coat protein levels up to 4% of total plant protein. The level of these coat proteins are at least one order of magnitude greater than CZW-3 coat proteins levels (TABLE VIIIa and b). 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 in 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.

Taste testing has determined that cucurbitacin levels were not significantly altered in CZW-3 as compared to the non-transformed inbred.

VIII. Statement of Grounds Unfavorable

The long term durability of coat protein mediated protection is unknown and strains of CMV, 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.

IX. Conclusion

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. Evidence has been presented to support our claim that the modified squash line in this petition does not present a plant pest risk. Removal of this line from its status as a regulated article will allow Asgrow to make this crop available to the public.

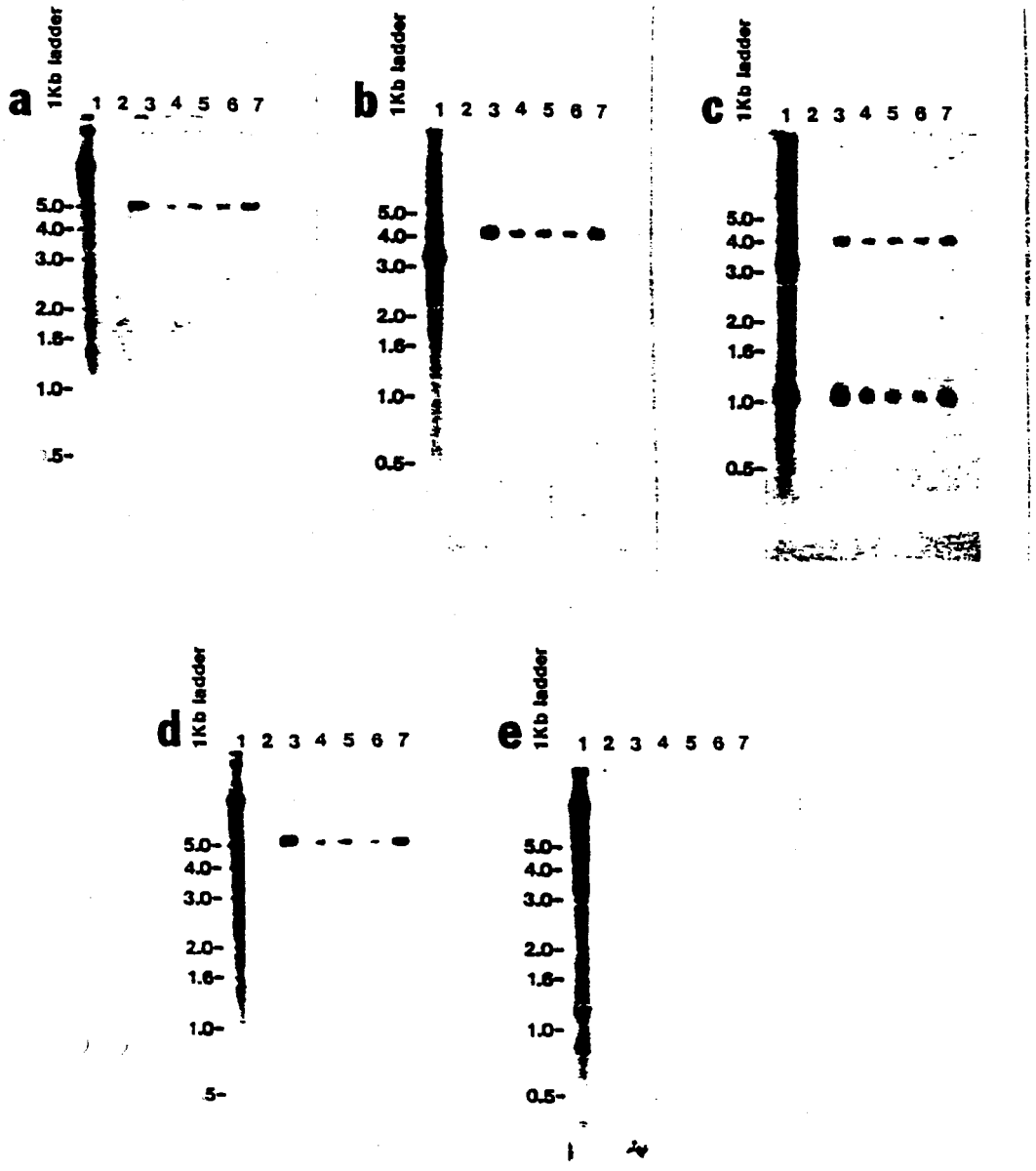


Figure 1.a-e Genomic DNA from progeny of CZW-3 (lanes 3-7) were digested with NsiI and hybridized to 32P-labelled: Npt II (a), CMV coat protein (b), ZYMV coat protein (c), WMV 2 coat protein (d), or binary plasmid (e) probe. Lane 2 contains DNA from the nontransgenic counterpart of CZW-3.

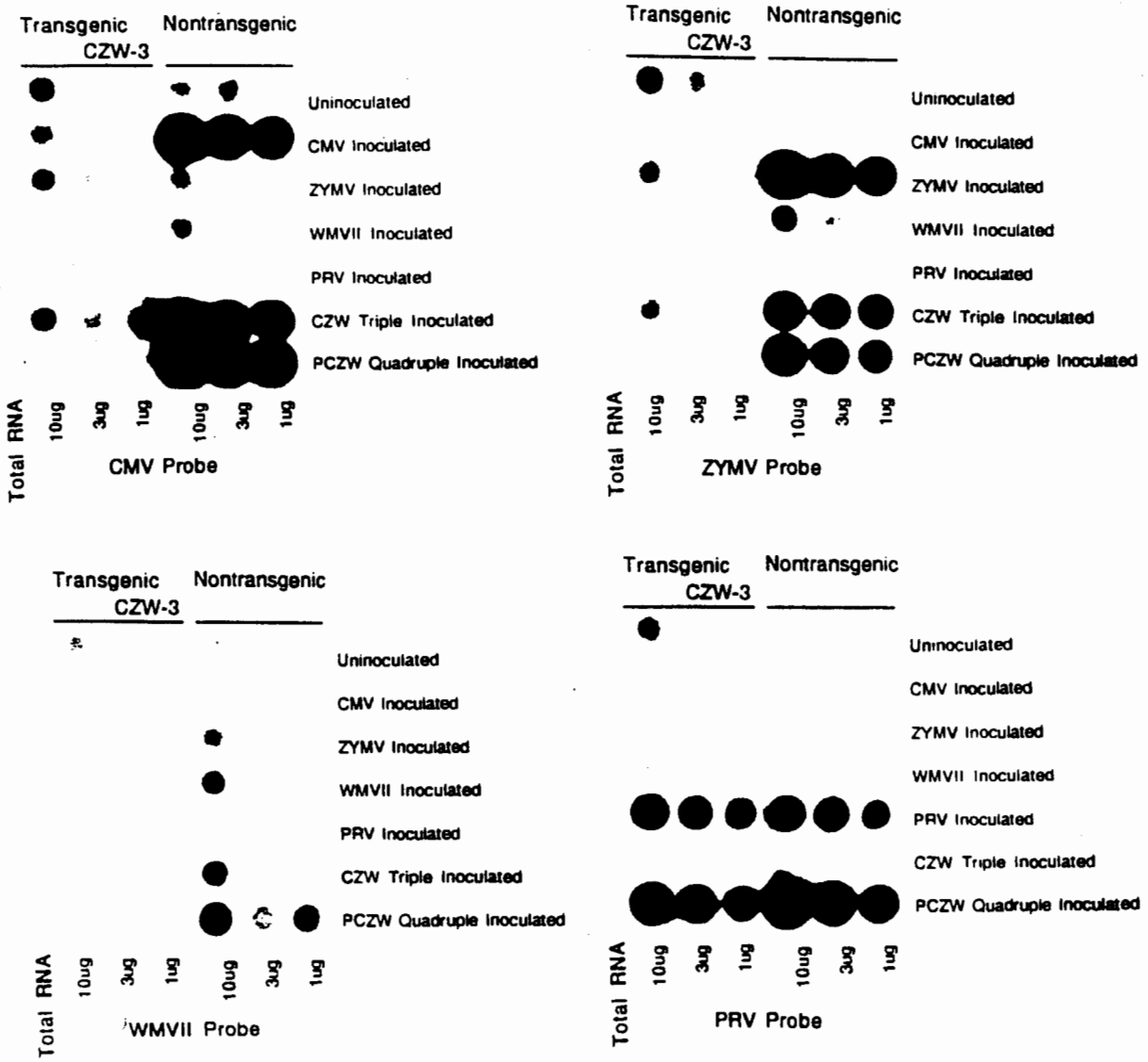


Figure 2 RNA dot blots from leaf tissue to determine transcript levels in CZW-3 and non-transgenic control plants after inoculation with CMV, ZYMV, WMV-2, PRV, CMV+ZYMV+WMV2 CMV+ZYMV+WMV-2+PRV. Blots were probed with CMV coat protein transcript (2a), ZYMV coat protein transcript (2b), WMV2 coat protein transcript (2c), and PRV coat protein transcript (2d). RNA was isolated from leaves of plants as indicated and probed with P-32 labelled cDNA clones for CMV, ZYMV, WMV 2, PRV. The CMV probe was 654 bp in length, or 89% of the coat protein coding region; the PRV probe was 891 bp long, or 98% of the coat protein coding region; the WMV 2 probe was 879 bp ling, or 96% of the coat protein coding region; the ZYMV probe was 881 bp long, or 96% of the coding region. Total RNA loaded in each well are indicated in the figure.

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