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Studies on Transformation of Tomato(Solanum lycopersicum L.) and Arabidopsis thaliana using Chimerical constructs of varying Tospoviral Origin

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STUDIES ON TRANSFORMATION OF TOMATO (SOLANUM LYCOPERSICUM L.) AND ARABIDOPSIS THALIANA USING CHIMERICAL CONSTRUCTS OF VARYING TOSPOVIRAL ORIGIN

By

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This dissertation has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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As chair of the candidate's graduate committee, I have read the thesis of Joshua N. Cobb in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

STUDIES ON TRANSFORMATION OF TOMATO (SOLANUM LYCOPERSICUM L.) AND ARABIDOPSIS THALIANA USING CHIMERICAL CONSTRUCTS OF VARYING TOSPOVIRAL ORIGIN

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Master of Science

Pathogen derived resistance (PDR) is a recent breakthrough where plant hosts can be made to be resistant to viral infections through transformation with conserved viral genes. Given the severity of Tospovirus diseases worldwide (particularly in tomato), PDR has the potential to garner large yield returns where pathogen populations have overcome the established resistance. Tomato breeding lines FLA7804, FLA8044, and the

research line MP1 were used in transformation experiments with potions of the *Tomato spotted wilt virus* (TSWV) N-gene, and two other chimerical viral nucleocapsid gene constructs from, *Impatiens necrotic spot virus* (INSV), and *Groundnut ringspot virus* (GRSV). We conducted 19 independent transformations consisting of 300 to 700 14-day old whole cotyledons each for a total number of approximately 9,000 potentially transformed explants. Of those, approximately 6,300 explants failed to produce regenerants, 2,419 explants underwent abnormal development on elongation media, 187 failed to root, and 215 plants to be characterized genetically. Of the 215 plants, 9 were from FLA 7804, 96 from FLA 8044, and 110 from MP1. Both PCR and Southern blot hybridization analysis later confirmed that none of the 215 plants were transgenic.

Opposite to tomato, we were able to transform *Arabidopsis thaliana* ecotype wassilewskija (Ws) via floral dip with the above listed constructs demonstrating that constructs were not deleterious within a plant once fully introgressed. Sixteen independent transformants in the T_0 generation resulted from 19,000 germinated seed from three dipped plants resulting in a total transformation rate of 0.08%. Of the 1,000 T_1 seed germinated on kanamycin media from each of the 16 putative *Arabidopsis* plants transformed with the construct containing elements of the N-gene from all three of the aforementioned tospoviruses, four populations exhibited simple Mendelian inheritance of the transgene. DNA walking analysis yielded amplification of the unknown region outside the *nptII* region of the insert for three of the four remaining transformants, which was subsequently sequenced and mapped to chromosomes 1, 3, and 4. There were 25 T_1 individuals selected from each population and transferred to soil for DNA extraction and zygosity determination. Homozygous T_2 seed was collected for future resistance studies.

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LITERATURE REVIEW

The Tomato

Cultivated tomato (*Solanum lycopersicum* L (2n=2x=24)) is one of several important members of the relatively large taxonomic family *Solanaceae.* Other important members of this family include potato (*Solanum tuberosum* L.), bell pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L. var. *lycopersicum*), tobacco (*Nicotiana tabacum L.*), the garden petunia (*Petunia hybrida L.*), and deadly nightshade (*Atropa belladonna L.*). While the domestication of tomatoes took place by Native Americans in present day Mexico, the original center of diversity for most members of this family has been traced primarily to the Andean regions of western South America (Kalloo 1991, Rick 1982).

The original Linnaean taxonomic classification of tomato was *Solanum lycopersicum,* however Miller (1754) proposed the genus name *Lycopersicon* (Latin-Wolf Peach) and later proposed the designation *Lycopersicon esculentum* for cultivated tomato and *Lycopersicon pimpinellifolium* for wild tomato (Miller 1768). While many other classifications systems have been proposed since then (for a review see Peralta and Spooner 2000), Terrell et al (1983) suggested that the Miller classification become the standard due to its common usage. Despite that suggestion and the common usage of the designation *Lycopersicon esculentum* many people still choose to use the *Solanum* classification (Fosberg 1987, Spooner et al. 1993). This being the case, a true consensus among tomato scientists has yet to emerge (Spooner et al. 1993). Reproductive compatibility studies combined with morphological analysis have demonstrated two species complexes within this genus. The first being the 'esculentum complex' including

S. lycopersicum L.; *S. pimpinellifolium* L.; *S. cheesmaniae* (L. Riley) Fosberg; *S. habrochaites* S. Knapp & D.M Spooner; *S. pennellii* (Corr.); *S. chmielewskii* (C.M. Rick, Kesicki, Fobes & M. Holle) D.M. Spooner, G.J. Anderson & R.K. Jansen; and *S. neorickii* D.M. Spooner, G.J. Anderson & R.K. Jansen. While the second is known as the 'peruvianum complex' consisting of *S. peruvianum* (L.) Mill., and *S. chilense* Dun. While the cultivated species *S. lycopersicum*, remains one of the few economically valuable member of this genus, the other varieties have served as a rich source of genetic resources that have contributed greatly to increased agronomic performance as well as increased disease resistance (Hille et al. 1989, Kalloo 1991, Ricks 1982).

Tomatoes are herbaceous perennials but due to cold frosts and drought-kill tend to behave more like an annual in wild populations (Müller 1940). Cultivated tomatoes are generally self-pollinating species, though controlled crosses can be made by emasculating young flowers before the pollen is fully mature. Many wild species, on the other hand tend to be self-incompatible probably due to the fitness bestowed by increased genetic diversity brought on by cross-pollination. Regardless of the reproductive strategy, an individual plant is capable of producing anywhere from 10,000 to 250,000 seeds in one generation (Peralta and Spooner 2000). All tomato species are initially erect, though due to the weight of the branches many become prostrate later in development. Branches usually have 2-6 sub-opposite sessile or petiolate pairs of leaflets. The basic inflorescence is a cyme with branching morphologies ranging from monochaisal, dichotomous, and polychotomous (Luckwill 1943).

Despite their origin on the American continent, European imperialism ensured that by the year 1800 domesticated tomato varieties were grown in all parts of the world

(Kalloo 1991). Today that trend continues with worldwide tomato production reaching 123,262,380 metric tonnes occupying 4,447,080 hectares with a combined export value of US$ 6,926,510,000 (FAO 2004).

Tospoviruses: A Major Problem of Tomato Production

Since tomato cultivation has been occurring longer than written record, it is not surprising that there are many varied disease organisms that have evolved as parasitic diseases of both wild and domestic tomatoes. To date there are about 200 known diseases of tomato of both parasitic and non-parasitic nature. Among parasitic diseases, fungi form the bulk of the pathogenic organisms as far as species diversity is concerned, but just over 10% of those parasitic diseases are known to be caused by viruses (Barrett et al. 1991). Common viruses known to cause significant decreases in crop yield include, but are not limited to: *Tomato spotted wilt virus* (TSWV)*; Alfalfa mosaic virus* (AMV)*; Tobacco mosaic virus* (TMV)*; Tomato yellow leaf curl virus* (TYLCV)*; Groundnut ringspot virus* (GRSV)*; Cucumber mosaic virus* (CMV)*; Curly top virus* (CTV)*; Potato virus X* (PVX)*;* and *Potato virus Y* (PVY) (Barrett et al. 1991, Blancard 2000, U.S. Department of Agriculture 1972).

From an integrated pest management point of view one important class of virus that commonly infects tomatoes as well as many other hosts are known as *Tospoviruses* (Family: Bunyaviridae). Some of the most prominent members of this genus include TSWV, INSV, and GRSV (de Avila et al. 1993). The earliest description of a tospovirus species occurred in Australia in 1915 (Brittlebank 1919) when it was shown that 'spotted wilt' disease of tomato was of viral origin (Samuel et al. 1930) and thus the pathogen was

named *Tomato spotted wilt virus*. For many years following TSWV remained the only member of this unique group of plant viruses (Matthews 1982). As research continued on the diseases caused by TSWV it soon became clear that TSWV was not alone in this new category of viruses and was re-classified as a member of the genus *Tospovirus*, created to categorize the relatively few plant infecting members of the family *Bunyaviridae*, a diverse and well characterized group of animal viruses (Francki et al. 1991). INSV (formerly TSWV-I) was the second virus classified as a member of the *Tospovirus* genus (Law et al. 1991). There are currently 16 recognized tospovirus species which are described based on less than 90% sequence homology of the nucleocapsid protein, host specificity, and range. Species are further classified based on reactivity with N protein antiserum and thus are separated into serogroups I-IV. Serogroups I and III each contain only one member (TSWV and INSV respectively) while the other serogroups contain multiple members that each cross react with the N protein antisera (de Avila et al. 1993, Elliot et al. 2000, McMichael et al. 2002, Moyer 1999, Persley et al. 2006, Yeh and Chang 1995).

Now tospoviruses are known as a highly ecumenical group of plant viruses with a worldwide distribution. They are notorious for causing a significant amount of damage to economically valuable food and ornamental crop species. Up to 80% crop yield losses have been reported in India groundnut due to GRSV (Ghanekar et al. 1979). Likewise in the US TSWV is ranked as the most threatening pathogen of field crops including tobacco, peanut, and pepper crops. Additionally in Hawaii up to 90% crop losses of lettuce and tomato have been reported in areas where TSWV populations have evolved to overcome the established genetic resistance (Chamberlin et al. 1992, Cho et al. 1987a).

Tospoviruses and most notably the tospovirus type species TSWV, currently infects at least 1090 plant species in 15 families of monocots and 69 families of dicots (Parrella et al. 2003). The ability of tospoviruses to establish infection in such a wide range of hosts (Allen and Matteoni 1988, Best and Gallus 1953, Cho et al. 1986, Cho et al. 1987b, German et al. 1992, and Iwaki et al. 1984) makes them one of the most economically important plant pathogens in the world, and a serious threat to the greenhouse industries of North America and Western Europe (Allen et al. 1986, Allen and Matteoni 1988, Cho et al. 1987a, Cho et al. 1984, German et al. 1992, Goldbach and Peters 1994, Greenough et al. 1985, Hausbeck et al. 1992, Smith 1932, Stobbs et al. 1992).

Tospovirus morphology is unique among plant viruses. It is the only plant virus that is spherical in structure, though this is a common characteristic of the Bunyaviridae virus family (German et al. 1992). The 80-100 nm virion particles consist of four proteins: a 200 kD replicase protein (L protein); Two glycoproteins of 78 and 58 kD (G1 and G2 respectively); and a 29 kD RNA binding protein called the N protein. Both glycoproteins form part of the membrane surrounding the virus particle, and the N proteins are tightly bound to the three molecules making up the single stranded RNA genome (Moyer 1999). These three molecules of RNA are organized as the L (large), M (medium), and S (small) strands. The L RNA is of negative polarity, while the M and the S exhibit an ambisense nature (Tsompana et al. 2005). The terminal ends of each of the M and S RNA are complementary inverted repeats of 65-70 nucleotides that potentially function as a regulatory signal for the recognition of viral polymerase. Furthermore direct cloning analysis has demonstrated that an eight nucleotide sequence ($GAUUGCUCU_{OH}$) is conserved between the terminal end of the each of the L, M and S RNA strands (Moyer

1999). RNA sequencing and cloning of the S fragment have shown it to contain the genetic information for the N protein (necessary for nucleocapsid formation) as well as a non-structural protein designated NSs (Non Structural protein on the S RNA) shown to serve as an RNA silencing suppressor during initial plant infection (Bucher et al. 2003, and Takeda et al. 2002).

Sequencing of the M strand has demonstrated it to contain the genetic information for the two glycoproteins and another non-structural protein designated NSm implicated as a viral movement protein necessary for navigation through the plasmodesmata of the plant cell wall (Mumford et al. 1996 and Soelick et al. 1999). The glycoproteins are suspected to function in viral binding during receptor mediated endocytosis, as well as transport signals to membranes within the interior of the infected cell (Elliot et al. 2000).

As previously mentioned, the L RNA segment encodes an RNA dependent RNA polymerase in the negative sense. Additionally, no subgenomic RNAs are produced from this strand unlike the other two. Evidence suggests that the entire strand is transcribed and translated to produce the polymerase protein, thereby eliminating any non-structural proteins that might have been encoded on this strand (Moyer 1999, and Tsompana et al. 2005).

Tospovirus symptomology is diverse and varied. Largely the symptoms produced are dependent upon the species and isolate of the tospovirus involved, the particular type of plant host, the time of year, and in some cases even the relative temperature at the time of infection. Necrosis on several different plant parts, chlorosis, ring patterns, mottling, silvering, stunting, line patterns, wilting, bronzing, and the production of lesions have all been reported (German et al. 1992, Mumford et al. 1996).

Thrips: The Vectors for Tospoviruses

The only known insect vector of tospoviruses are thrips species. Even as far back as 1935, thrips were recognized as an important vector of plant viral diseases in general (Bailey 1935). Thrips are small insects with an adult body size of no more than 5 mm in length with four slender wings. Over 5000 species of thrips have been identified, though it is suspected that this is only about half of the true number of extant species. The thrips family Thripidae is the most widely characterized of all taxonomic families with over 95% of the member species identified (Jones 2005). All species of thrips known to vector plant disease are members of this family, and even then they are all classified together in the subfamily Thripidae (Lewis 1997, Mound 1997). The genus *Frankliniella* harbors the most pertinent species of plant virus vectors and is so ancient with respect to others in the same family that it is suspected to have originated on the prehistoric super-continent of Gondwanaland (Jones 2005).

Tospovirus transmission by thrips was first observed by Pittman (1927) by demonstrating that *Thrips tabaci* transmitted TSWV. Worldwide 11 species of thrips have been documented to transmit at least one type of tospovirus with *Frankliniella occidentalis* (Western Flower Thrips) being the most damaging species because it is capable of efficiently transmitting at least five different tospovirus species including TSWV (Ullman et al. 2002). The complete life cycle of a thrip can take place in 40 to 60 days beginning with two larval stages when the insect feeds on plant tissue using a mechanism of rasping and freeing and feeding on the cellular fluids of the mesophyll. This is followed by two relatively dormant pupal stages where, depending on the species, feeding does not occur. Infection of thrips by tospoviruses only occurs during the initial

two larval periods due to a temporary connection between mid-gut visceral muscles and the salivary gland present in juvenile individuals (Whitfield et al. 2005). For a detailed review of tospovirus-thrip interactions at the cellular level see Whitfield et al (2005). Once infected, a larval thrip that reaches adulthood remains infectious for the remainder of its lifespan (Jones 2005). Further complicating the problem, Maris et al (2004) found evidence that thrips may preferentially feed and reproduce on tospovirus infected plants.

Control of Tospovirus Infection via Thrip Management

Efforts to manage tospovirus infection via thrip control is a varied approach. Since virus acquisition occurs during the larval stage and because the virus is able to replicate within the thrip itself (Ullman et al. 2002) control of viral infection by thrip management is not as effective as direct control of the virus via plant genetic resistance. Nevertheless, thrip control has resulted in some management of the virus when other safeguards cannot be put in place. The most obvious management technique is to use insecticides to control the thrip population. This has proven effective to reduce thrip populations to levels of relative tolerance, but enough viruliferous thrips still survive to initiate a significant number of novel infection events (Cho et al. 1989, Riley 2004). Furthermore there is some evidence to suggest that the use of pesticides can increase tospovirus infection due to the dispersion of thrips upon application and the fact that the thrips may still transfer the virus before being infected by the insecticide (Reddy and Wightman 1988). Treatment with foliar insecticides have been shown to not significantly affect thrip mortality, but still partially reduce virus infection by altering the feeding behavior of infected thrips (Chaisuekul and Riley 2001, Joost and Riley, and Pappu 2004). Another strategy employed by Cook et al (1996) involves the use of the

anthocorid bug (*Orius armatus*) as a field predator of thrips. While this has proven useful, it cannot be combined with other methods like insecticide application because the biocontrol predator populations are as devastated by the insecticide as are the thrips populations (Cook et al. 1996).

Other cultural strategies for thrips control, including UV reflective mulch, later planting dates, elimination of alternate weed hosts, and rotation with non-susceptible crops, have been shown to be somewhat effective though not sufficiently to control tospovirus infection (Cho et al. 1989, Riley 2004, and Riley and Pappu 2000). These alternative treatments are still not popular or effective enough to be considered mainstream, and so individual growers generally respond to tospovirus infections by applying broad spectrum insecticides (Momol et al. 2004). This remains the case even in the face of research demonstrating that most of the crop loss due to tospoviruses are the result of primary infections, which are not prevented by insecticide treatments (McPherson et al. 1995, McPherson et al. 1997, and Puche et al. 1995). Due to the lack of effectiveness of any of these treatments against thrip populations, to date, no single control measure has been put forth to significantly reduce the incidence of tospovirus infection (Momol et al. 2004).

Tospovirus Resistance through Conventional Breeding

Given the difficulty presented in controlling tospovirus infection by managing vector populations, the next best available option is to breed for tospovirus resistance in the economically important hosts. Additionally genetic resistance is preferable because it reduces the negative effects of pesticides on plant health and presents clear ecological

benefits limiting the risks to growers, consumers, and the environment. If resistance proves durable, then the use of resistant crop varieties is certainly the most cost effective control mechanism (Kang et al. 2005 and Langella et al. 2004).

In tomato the first record of tospovirus resistance was isolated from the wild tomato species *S. pimpinellifolium* (Samuel et al. 1930). Later (Holmes 1948) found that Argentine *S. lycopersicum* cultivars 'Rey de los Tempranos' and 'Manzana' harbored an isolate specific resistance to TSWV when grown in New Jersey (Cho et al. 1996). In Hawaii, TSWV resistant cultivated tomato variety 'Pearl Harbor' was developed using these resistances found in line BC-10 in the genetic background of cultivar 'Bounty' (Kikuta et al. 1945). However, when the 'Rey de los tempranos' and 'Manzana' varieties were grown in Hawaii, and when 'Pearl Harbor' was grown in New Jersey the local TSWV populations overcame each of the resistances (Cho et al. 1996). Finlay (1951) found that all three varieties were susceptible when grown in Australia, but a cross between 'Pearl Harbor' and 'Rey de los Tempranos' demonstrated high field resistance. A few years following, Finlay (1953) demonstrated that all of these resistances are controlled by two dominant genes (*Sw-1*^{a} and *Sw-1*^{b}) and three recessive genes (sw-2, sw-3, and sw-4). Use of these genes has been discontinued in tomato breeding programs due to the isolate specific nature of the resistance, which was quickly overcome by wild virus populations (Roselló et al. 1998).

Since then, some resistance has been identified in *S. hirsutum* and introgressed into *S. lycopersicum*, but again this resistance was isolate specific and a subsequent loss of resistance has been observed (Kumar and Irulappan 1992, Maluf et al. 1991, and Roselló et al. 1998). Accession LA-2931 of *S. chilense* was reported to be symptomless

after tospovirus infection, but the genetic control of this resistance has never been elucidated (Kumar et al. 1993). Many accessions of *S. peruvianum* have demonstrated resistances to a variety of different tospovirus isolates, however the relative expression in *S. lycopersicum* genetic background is generally reduced and depends on the accession used (Kumar et al. 1993, Kumar and Irulappan 1992, Maluf et al. 1991, and Paterson et al. 1989). The most common resistance used in modern breeding programs is that of *Sw-5* derived from *S. peruvianum* cultivar 'Stevens', expressed as a hypersensitive response to infection (Roselló et al. 1998, Stevens et al. 1992 and van Zijl et al. 1986). This resistance is preferred as it confers resistance to a variety of tospovirus species and to several isolates of each of those species (Boiteux and Giordano 1993). Additionally it is inherited in a simple, monogenic, Mendelian dominant pattern which facilitates its introgression into economically valuable lines of tomato (Boiteux and Giordano 1993 and Langella et al. 2004). As useful as *Sw-5* has been as a source of tospovirus resistance, it is important to recognized that *Sw-5* does not confer immunity (Roselló et al. 1998).

The nature of the interaction between pathogens and resistant cultivars in a monoculture cropping system is such that no resistance can stay in place very long before the pathogen population evolves to render the cultivar susceptible. To date, five strains from across the world have been reported as overcoming *Sw-5* resistance: TSWV-6 in Hawaii (Cho et al. 1996); JF in South Africa (Thompson and van Zijl 1996); To_{TAS}-1d and Da_{WA}-1d in Australia (Latham and Jones 1998); GRAU in Australia (Aramburu and Martí 2003); and T992 in Italy (Ciuffo et al. 2005). This being the case, there is a significant need for a new tospovirus resistance to be identified. Furthermore, this new resistance needs to be simply inherited and robust enough to be challenged by multiple

isolates or even species of tospoviruses in order for it to exhibit enough effectiveness to be economically viable.

Pathogen Derived Resistance

Originally defined by Sanford and Johnston (1985) 'parasite derived resistance' (or pathogen derived resistance—PDR as it has come to be known) is a mechanism of engineering plant virus resistance in susceptible varieties by transforming those varieties with genes derived from the viral genome itself. A year following that landmark paper Powell-Abel (1986) became the first to confirm that genetic transformation of plant virus genes into the host is actually a viable method for engineering plant virus resistance when they characterized TMV resistant tobacco plants that were transgenic for the TMV coat protein gene (Powell-Abel et al. 1986). Since then PDR has blossomed into a diverse field of research and a new paradigm for achieving viral disease resistance.

The mechanism of PDR can be varied and is often dependent on how and to what extent the plant host expresses the transgene, as well as the physiological mode of infection presented by an invading virus. The prevailing hypothesis is that this type of engineered resistance comes as a result of having viral gene products present in the host at the wrong time, in the wrong quantity, or in the wrong form as to disrupt the physiological processes unique to the invading pathogen (Sanford and Johnston 1985). Research has since demonstrated that protein mediated resistance against most plant viruses is due to an inhibition of virion disassembly. Much of this evidence comes from experiments with TMV where plants transgenic for the viral coat protein exhibit resistance against functional virus particles but not against RNA inocula, since RNA

inocula does not require disassembly of the virion, and can infect neighboring plant cells without the need to be encapsulated (Clark et al. 1995, Osbourn et al. 1989, and Register and Beachy 1988). In other plant viruses, like AMV, the mechanism for protein mediated pathogen derived resistance is thought to occur at two different stages. It is clear that the first stage (the inhibition of virion disassembly) is at work in this system, but plants transgenic for the coat protein exhibit resistance to both virion and RNA incocula (Taschner et al. 1994). The second stage must therefore come at some point in the virus life cycle dependent on functional coat protein (Baulcombe 1996). It has been suggested that such a resistance mechanism might operate by inhibiting interactions necessary for the viral RNA to properly assemble virions (Taschner et al. 1994), or by interfering with host plant receptors for the coat protein (Reusken et al. 1994). Studies with PVX have shown that the the origin of virion assembly is likely the 5' region of the viral genome, implying that the presence of the coat protein might inhibit the translation of the viral RNA-dependent RNA polymerase (RdRp) which is encoded in the open reading frame (ORF) closest to the 5' end (Sit et al. 1994). However Chapman (1992) also suggest that resistance may also be conferred by inhibiting cell to cell movement of viral particles since the coat protein is often a necessary cofactor in that reaction (Chapman et al. 1992, Schwach et al. 2004).

PDR mechanisms are not limited to proteins encoded by the viral transgene, but also may involve the transgene itself or its RNA transcript. Such nucleic acid mediated resistance may come as a result of competition between the transgene or its transcript with the invading viral genome. In this way the transgene acts as a decoy for proteins of either host or viral origin. In so doing, proteins that would otherwise be necessary for

viral replication are tied up interacting with the decoy-transgene (Baulcombe 1996). This type of resistance interaction has been found to be the case in PDR studies involving geminiviruses, *Cymbidium ringspot virus*, and *Turnip yellow mosaic virus* (Kollár et al. 1993, Stanley et al. 1990, and Zaccomer et al. 1993).

The protein and nucleic acid mediated resistances discussed thus far come as a result of the transgene or a product of the transgene interfering directly with the viral life cycle and physiology. There remains one other proposed mechanism of PDR that involves a more indirect interaction that actually prevents the invading viral genome from ever initiating its life cycle inside an newly infected plant cell. This mechanism operates on the basis of post-transcriptional gene silencing (PTGS). The proposal of this mechanism followed the initially confusing findings that untranslatable constructs were still able to bestow resistance (de Haan et al. 1992, Lindbo and Daugherty 1992, and van der Vlugt et al. 1992) and that transgenic resistance often was associated with post-transcriptional silencing of the transgene itself (Lawson et al. 1990). Later Lindbo et al (1993)'s work with *Tobacco etch virus* (TEV) suggested that these two processes were both caused by the same chemical process. In his model the transcript from the viral transgene is copied into small RNA fragments by host RdRp and these small RNA fragments then bind to homologous copies of RNA of viral or transgenic origin. RNAase molecules of host origin are subsequently recruited by the double stranded RNA and then digest the targeted construct. Since this resistance operates at the RNA level it would have the ability to suppress the expression of any RNA sharing sequence homology with the transgene. However, due to the homology dependent nature of this kind of resistance, the protection conferred by the transgene is highly specific to the strain of virus from

which the transgene was taken (Marano and Baulcombe 1998, Mueler et al. 1995, and van den Boogaart et al. 1998).

To date, PDR has been shown to be an effective means of engineering plant virus resistance to as many as 13 different taxonomic groups of plant viruses affecting dozens of agronomically and horticulturally important crops (Accotto et al. 2005, Anderson et al. 1992, Bau et al. 2002, Bau et al. 2004, Brunetti et al. 1997, de Haan et al. 1992, Fuchs et al. 1998, Fuchs et al. 1996, Gal-On et al. 1998, Golemboski et al. 1990, Gonsalves et al. 1996, Gonsalves 1998, Gonsalves 2006,, Gonsalves 2002,Grumet 1995, Gubba et al. 2002, Herrero et al. 1999, Jan et al. 2000, Jan et al. 1999, Lennefors et al. 2006, Levin et al. 2005, Li et al. 1996, Ling et al. 1991, Lommonossof 1995, Malinowski et al. 2006, Marano and Baulcombe 1998, Nervo et al. 2003, Pang et al. 1996, Pang et al. 2000, Praveen et al. 2005, Schwach et al. 2004, Sherman et al. 1998, Ultzen et al. 1995, and Yepes et al. 1995).

Pathogen derived resistance in tomato against tospoviruses has largely focused on TSWV due to the high economic cost of its pathogenicity. Due to the ease of transformation associated with tobacco and the potential economic benefits, PDR against tospoviruses began against TSWV infection in tobacco plants using the TSWV N gene sequence (Gielen et al. 1991) and marked the first instance of PDR against a virus with negative RNA polarity. Since then it has since been successfully implemented in TSWV hosts such as tomato (Accotto et al. 2005, Fedorowicz et al. 2005, Gonsalves et al. 1996, Hoffman et al. 2001, Kim et al. 1994, Nervo et al. 2003, and Ultzen et al. 1995), tobacco (de Haan et al. 1992, Levin et al. 2005, MacKenzie and Ellis 1992, Prins et al. 1995, and

Vaira et al. 2000), *gerbera* plants (Korbin et al. 2002), peanut (Li et al. 1996), lettuce (Pang et al. 1996), and chrysanthemum (Sherman et al. 1998, Yepes et al. 1995).

For tomato the first limited transgenic resistance was obtained in the R_1 generation of a line containing the TSWV N gene sequence (Kim et al. 1994). Later an inbred line of tomato also containing the TSWV N gene sequence demonstrated high levels of resistance specifically to isolate BR-01 of TSWV (Ultzen et al. 1995). The following year it was reported that TMV resistant tomato line 'Geneva 80' was transformed with the TSWV N gene from the lettuce isolate of TSWV (TSWV-BL). Resulting transformants from the R_1 generation were 100% resistant to TSWV-BL (based on lines resistant/lines inoculated), 84% resistant to the closely related isolate TSWV-91, 52% resistant to the related but taxonomically distinct GRSV-BR strain of GRSV (Gonsalves et al. 1996). In order to overcome the generally specific nature of the resistance obtained from plants transgenic for TSWV genes Gubba et al (2002) designed to combine transgenic and natural resistance to TSWV in the same plant. R_5 plants transgenic for the N gene of the Hawaiian TSWV isolate (TSWV-H) showing high resistance to TSWV-BL and TSWV-H, but susceptible to GRSV-BR were crossed with line S-R containing the *Sw-5* gene which confers resistance to GRSV-BR and TSWV-BL, but not TSWV-H. The resulting progeny showed either a resistant or a tolerant phenotype when challenged with any one of the three viruses in question. Nervo et al (2003) further reported high levels of resistance in two elite fresh market tomato lines transformed with the N gene sequence of an unspecified Italian TSWV isolate. The resistance obtained against this isolate of TSWV was determined to be caused primarily by PTGS, thus explaining the strength of the resistance as well as its isolate specific nature. The

transgenic line created by Nervo et al (2003) was recently evaluated for field performance where it significantly out performed its non-transgenic counterparts (Accotto et al. 2005). Most recently Fedorowicz et al (2005) transformed two tomato breeding lines with the full length sequence of the N gene from the Bulgarian L3 isolate of TSWV. They found that 10 out of 42 primary transformants were highly resistant to infection from both the Bulgarian L3 isolate of TSWV as well as the closely related Polish isolate. The remaining 32 individuals showed a range of intermediate resistant phenotypes. Interestingly half of the highly resistant plants showed no presence of the transgene transcript while the other half did, implying that the mechanism of resistance included both protein mediated resistance and PTGS.

PDR has therefore been proven successful in tomato against tospoviruses, but the resistance obtained remains isolate specific unless combined with other forms of natural resistance (Gubba et al. 2002). The mechanism for tospovirus resistance through PDR is generally PTGS which provides the most probable explanation of the specificity of the resistance. Any kind of broad pathogen derived tospovirus resistance conferring protection against several species of tospoviruses (and not just related isolates) has yet to be achieved in tomato.

It has been previously reported that the rapid adaptability of tospoviruses as well as their propensity towards genomic re-assortment make the establishment of a stable TSWV resistance difficult at best. These mechanisms have been demonstrated to be the primary strategies employed by virus populations to overcome both the established genetic resistance as well as N-gene based pathogen derived resistance (Hoffman et al. 2001, Qiu and Moyer 1999, and Qiu et al. 1998). Despite this apparent persistence of

viral populations, we believe pathogen derived resistance of the nature herein described to be valuable due to the sequence diversity of the fusion construct. Having N-gene sequences from several different species of tospoviruses present in the same genome should make it more complicated for any one isolate of the virus to reassemble its genome sufficiently to fully overcome this type of transgenic resistance. Additionally, this type of pathogen derived resistance can be bred into elite tomato lines in order to reinforce the presently predominant *Sw-5* resistance gene (Gubba et al. 2002). This is particularly important when the fact that *Sw-5* overcoming strains of TSWV may come with a fitness disadvantage in comparison to non-*Sw-5* breaking isolates is taken into consideration (Gordillo et al. 2008).

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RESULTS OF USING CHIMERICAL TOSPOVIRUS CONSTRUCTS TO TRANSFORM TOMATO (*SOLANUM LYCOPERSICUM* L.) FOR PATHOGEN DERIVED RESISTANCE

Abstract

Tomato breeding lines FLA7804 FLA8044, and the research line MP1 were used in transformation experiments with the *Tomato spotted wilt virus* (TSWV) N-gene sequence, and two other chimerical tospovirus nucleocapsid gene constructs from *Impatiens necrotic spot virus* (INSV), and *Groundnut ringspot virus* (GRSV). This was done with the intention of rendering the above listed lines resistant to Tospoviruses via pathogen derived resistance (PDR). MP1 was used as a control to test the effectiveness of the transformation protocol. We conducted 19 independent transformations consisting of 300 to 700 14-day old whole cotyledons each for a total number of approximately 9,000 potentially transformed explants. Of those, approximately 6,300 explants did not produce regenerants, succumbed to fungal contamination, or only produced leafy regenerants that lacked shoot apical meristems. There were 2,419 explants which underwent abnormal development on elongation media such as unusual tissue swelling and premature senescence, or also succumbed to fungal contamination. Of the 402 fully developed plantlets, 187 plants failed to produce roots, produced insufficient root systems, or were lost to fungal contamination and 215 plants survived through rooting to be characterized genetically (9 from FLA 7804, 96 from FLA 8044, and 110 from MP1).

Inconclusive PCR verification of transformation prompted initial questioning of the 215 putatively transformed tomato plants and Southern blot hybridization later confirmed that none were transgenic. The lack of transformed plants was most likely due to a number of different reasons including, but not limited to: fungal contamination, genotype specific subtleties incumbent to the transformation protocol, and possibly a poor interaction with the vector insert.

Introduction

Cultivated tomato (*Solanum lycopersicum* L. (2n=2x=24)) is one of several important members of the relatively large taxonomic family *Solanaceae.* The economic value of this crop cannot be understated considering worldwide tomato production has reached at least 123,262,380 metric tonnes occupying 4,447,080 hectares with a combined export value of $6,926,510,000 US dollars (FAO 2004). Consistent with its high export value it is not surprising that the control of major tomato pathogens is an area of great intellectual interest.

Tospoviruses at present represent one of the most severe disease categories facing tomato cultivation and results in yield losses as high as 90% where virus populations have evolved to overcome the established genetic resistance (Chamberlin et al. 1992, Cho et al. 1987, Goldbach and Peters 1994). Tospoviruses, most notably the tospovirus type species *Tomato spotted wilt virus* (TSWV) is particularly difficult to control considering it can maintain itself in at least 1090 reservoir plant species in 15 families of monocots and 69 families of dicots (Parrella et al. 2003). Pittman (1927) was the first to observe that thrips in the insect family Thripidae are capable of transmiting tospovirus infection. Worldwide 11 species of thrips have been documented to transmit at least one type of tospovirus with *Frankliniella occidentalis* (Western Flower Thrips) being the most damaging species because it is capable of efficiently transmitting at least five different tospoviruses including TSWV (Ullman et al. 2002).

Thrip management is not as effective a control as the implementation of plant genetic resistance since the size of thrips species lends itself to avoiding pesticide sprays and tospovirus acquisition in the vector species occurs during the larval stage but can be

transmitted in the adult stage when the insects feed (Ullman et al. 2002). Some sources of genetic resistance to tospovirus infection have been identified in wild populations with cultivated relatives, but these resistances have been short lived due to the isolate specific nature of the resistance, which was quickly overcome by wild virus populations (Roselló et al. 1998). The most common resistance used in modern breeding programs is that of *Sw-5* derived from *S. peruvianum* and expressed as a hypersensitive response to infection (Roselló et al. 1998, Stevens et al. 1992 and van Zijl et al. 1986). However, to date, five TSWV isolates from across the world have been reported as overcoming *Sw-5*: TSWV-6 in Hawaii (Cho et al. 1996); JF in South Africa (Thompson and van Zijl 1996); To_{TAS}-1d and Da_{WA}-1d in Australia (Latham and Jones 1998); GRAU in Australia (Aramburu and Martí 2003); and T992 in Italy (Ciuffo et al. 2005). Thus, there is a great need for new tospovirus resistance that is simply inherited and robust enough to be challenged by multiple tospovirus isolates in order for it to exhibit enough effectiveness to be economically viable.

Originally defined by Sanford and Johnston (1985), pathogen derived resistance (PDR) has emerged as a potent source of transgenic resistance against viral diseases (Lommonossof 1995). Due to the ease of transformation associated with tobacco, PDR to tospoviruses began with resistance to TSWV in tobacco plants using the TSWV N gene and marked the first instance of PDR to a negative-strand RNA virus (Gielen et al. 1991). Since then it has been successfully implemented in TSWV hosts such as tomato (Accotto et al. 2005, Fedorowicz et al. 2005, Gonsalves et al. 1996, Hoffman et al. 2001, Kim et al. 1994, Nervo et al. 2003, and Ultzen et al. 1995), tobacco (de Haan et al. 1992, Levin et al. 2005, MacKenzie and Ellis 1992, Prins et al. 1995, and Vaira et al. 2000), *gerbera*

plants (Korbin et al. 2002), peanut (Li et al. 1996), lettuce (Pang et al. 1996), and chrysanthemum (Sherman et al. 1998, Yepes et al. 1995). However, PDR to tospoviruses remains isolate specific unless combined with other forms of natural resistance (Gubba et al. 2002).

The mechanism for PDR to tospoviruses has been shown to generally be post-transcriptional gene silencing, thus providing the most probable explanation of the isolate specificity of the resistance (Marano and Baulcombe 1998, Mueler et al. 1995, and van den Boogaart et al. 1998). Jan et al. (2000) demonstrated resistance to multiple virus species using a chimerical transgene consisting of portions of each virus' nucleocapsid gene (N-gene), but found that the resistant phenotype only surfaced when the transgene was also fused to a green fluorescent protein (GFP) sequence. In this study our objective was to transform an elite breeding line of cultivated tomato with a chimerical construct consisting of portions of the N-gene from three tospovirus species; namely TSWV, *groundnut ringspot virus* (GRSV), and *impatiens necrotic spot virus* (INSV).

Materials and Methods

Binary vector construction and bacterial transformation

Five chimerical binary vectors were provided in the form of air dried DNA by Dennis Gonsalves at the Pacific Basin Agricultural Research Center of the United States Department of Agriculture (USDA) in Hilo, Hawaii (Fig. 1). Each vector was independently transformed into ElectroMAX™ LBA4404 *Agrobacteria tumafaciens* cells (Invitrogen Corp., Carlsbad, CA) via electroporation carried out on a BTX® ECM® 600 electroporator using the following conditions: 20.0 kV, 200 Ω, 25 μF. After 56 hrs of incubation, one colony from each construct was grown overnight in 2 ml of yeast-mannitol (YM) broth containing 50 mg/l of kanamycin and 50 mg/l of gentamicin. 400 μl of the overnight culture was mixed with 600 μl of 20% glycerol and stored at -80°C.

Plant material and plant transformation

MP1 (Barg et al. 1997) tomato transformation protocols were obtained from Naim Iraki and Omar DarIssa of the UNESCO Biotechnology center at Bethlehem University. Since we desired the resistance to be conferred into tomatoes better adapted to Hawaii, transformation attempts were made with two cultivated inbred breeding lines (FLA 7804 and FLA 8044 from Jay Scott; University of Florida) along with MP1 tomatoes as a positive control.

All three lines were germinated under sterile conditions for two weeks on germination media (Table 1). At which point explants taken from cotyledon tissue were placed abaxial side up (ten per plate) on regeneration media (Table 1) without antibiotics and placed in the dark. Twenty-four hours later explants were incubated for 2 hrs with 5-7 ml per plate of *Agrobacteria* solution containing one of the five chimerical constructs of interest.

The *Agrobacteria* solution was made by streaking stock cultures on selective YM media (Table 1) and growing at 29°C for 48 hrs. One colony for each construct was then grown in 5 ml of YM broth for 48 hrs (29°C and 200 rpm), diluted to 25 ml and grown for an additional 6 hrs under the same conditions. Following centrifugation (4°C, 1700 g, 20 m) the bacterial pellet was re-suspended in 30 mls of germination broth containing acetosyringone (100 μM final concentration). Following this incubation, the liquid was removed from each Petri dish, the explants were rearranged such that they touched each other and were incubated at 25°C in the dark for 48 hrs exactly. Explants were then placed on regeneration media (Table 1) for approximately 30 days at 25°C with a 16 hr photoperiod. Any regenerants were excised and moved to elongation media (Table 1) for 30 days under the same conditions to promote foliage development. After each plantlet reached a height of 2 cm, they were transferred to rooting media (Table 1) until they developed a sufficiently strong root system and were hardened under a gradually opened plastic bag. Following a 10-day hardening period, the plants were moved to the greenhouse until they grew large enough to excise tissue for DNA extraction.

DNA extraction, PCR, and Southern blot analysis

Genomic DNA was extracted from 100 mg of lyophilized leaf tissue as described by Sambrook et al. (1989) with modifications from Todd and Vodkin (1996). Following extraction, each sample was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Montchanin, DE) and adjusted to 1μg/μl of TE.

PCR amplification of the chimerical inserts and *nptII* region was carried out in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). Each reaction was constituted as follows: PCR buffer buffer (10 mM TRIS-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin; Sigma-Aldrich Corp., St. Lois, MO), 0.2 mM dNTPs, 0.5 mM forward primer, 0.5 mM reverse primer (Tables 2 and 3), 50-100 ng of template DNA or bacterial stock solution, 0.5 U of JumpStart™ Taq DNA polymerase (Sigma-Aldrich Corp., St. Lois, MO), and sterile ddH_2O to volume. The amplification protocol included: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 53°C for 30 sec, 72°C for 3 min; followed by a final 72°C incubation for 10 min. Products were separated in a 0.5X TBE (0.001 M EDTA, 0.045 M Tris, 0.045 M Boric Acid) on 1% agarose gels run at 100 V for 1 hr and visualized using ethidium bromide staining techniques.

Southern blot preparation was carried out by digesting 5 μg of tomato genomic DNA using *Eco*RI as per Sambrook et al. (1989). Radioactive probe was prepared using the Prime-a-Gene® Labeling System (Promega Corp., Fitchburg, WI) using 25 ng of *nptII*.

Results and Discussion

We conducted 19 independent transformations consisting of 300 to 700 14-day old whole cotyledons each for a total number of approximately 9,000 potentially transformed explants. Of those, approximately 6,300 explants never produced regenerants, succumbed to fungal contamination, or only produced leafy regenerants that lacked shoot apical meristems. Of the viable regenerants, 2,419 underwent abnormal development on elongation media such as unusual tissue swelling and premature senescence, or also succumbed to fungal contamination. Of the 402 fully developed plantlets, 187 plants failed to produce roots, produced insufficient root systems, or perished due to fungal contamination and 215 plants survived through rooting to be characterized genetically and grown in the greenhouse. Concerning the plants in the greenhouse, 9 were FLA 7804, 96 were of line FLA 8044, and 110 were variety MP1 (Table 4). Inconclusive PCR verification of transformation (Fig. 2 and 3) prompted initial questioning of the putatively transformed plants and Southern blot hybridization later confirmed that none of the tomato plants were transgenic (Fig. 4).

PCR amplification of both the *nptII* gene as well as of the chimerical inserts was carried out on the *Agrobacteria* stocks resulting in amplification products of the expected sizes (Fig. 5). Due to the high success rate of modern tomato transformation protocols most studies involving tomato transformation do not comment on variations of success rate and only mention that most protocols vary by genotype. This being the case, it is possible that the unknown genetic heritage of the two uncharacterized breeding lines (FLA 7804 and FLA 8044) we used may account for some of the reduced transformation

success in those lines. Gal-on et al. (1998) and Nervo et al. (2003) both used uncharacterized breeding lines and experienced high transformation rates (92% for Gal-on et al. 1998). However, genotype specificity in this case cannot fully account for the overall low transformation rate considering our protocol is designed specifically for MP1 variety tomatoes and there is not a significant difference between the transformation rate of MP1 and the other two advanced breeding lines (0% for all lines). However, it has been shown that minute variations in reagent concentration or alternative reagent choices can have significant effects on the transformation rate of a given protocol (Frary and Earle 1996).

One example is rooted in the fact that our protocol calls for cotyledon explants to be oriented abaxial side up on regeneration media as suggested by McCormick (1991) who found that placing cotyledon explants (cv Moneymaker) abaxial side up yielded a higher transformation rate (91% vs. 77%) than the inverse orientation. However, Frary and Earle (1996) yielded a two-fold increase in transformation rate in a similar cultivar (VF36) by placing the cotyledon abaxial side down. They suggest this might be due to the inevitable curling of the tissue, and with the abaxial side down the cotyledon will turn into the media rather than away from it. While this probably does not fully explain our 0% transformation rate, it may have contributed to it.

Additionally, Frary and Earle (1996) point out that transgenic cells from a given explant will regenerate more slowly than their non-transgenic counterparts. As such they demonstrated that the transformation rate among regenerants first pulled from the explants (39%) is notably lower than the transformation rate after 101-120 days (77%). We found that after explants had been maintained on fresh media for more than 60 days

endogenous fungal contamination became uncontrollable and regeneration yields were reduced to zero. This may have pre-empted the time needed for transformed cells to fully regenerate.

Frary and Earle (1996) additionally support that micropore tape contributes to a higher transformation rate of regenerating plantlets when used to seal the Petri dishes than does parafilm (11.7% vs. 7.6%). Since all of our plates were wrapped with parafilm, this likely contributed to a lack of gas exchange and may have formed a small part of our reduced transformation efficiency.

It is noteworthy to mention at this point that the slight differences in protocol discussed thus far generally represent non-significant variables individually, but when all the best treatments are applied together significant gains can be made. Frary and Earle (1996) for example were able to increase the overall transformation rate of their protocol from 0.9% to 10.6% by manipulating only a few of the variables discussed here. This indicates that the most important consideration when trying to trouble-shoot or improve a protocol cannot be found in any one of its elements, but rather in the interaction between the major variables involved, underscoring the overall importance of accuracy and precision in measurement and timing.

While the transformation protocol itself may have not have been fully optimized, it is unlikely that this alone can account for complete failure given our sample size. Despite the fact that rooting in selective media is a good indicator of transformation (Frary and Earle 1996) molecular characterization of putative transformants is essential. T-DNA rearrangements, mutations, and improper vector construction may have also contributed to our extreme results. While the pGA482G binary vector is published (Chee

et al. 1989), the nature of the construction of the chimerical insert is not well understood and may be in question (Savarni Tripathi, USDA Hilo, HI personal communication).

Two more explanations worthy of note without being heavily implicated as causal agents are the relative pathogenicity of our N-gene protein products as well as the possible effects the transgene might have on the ability of the tomato to regenerate. Hou et al. (2000) reported that when they attempted tomato transformation with geminivirus movement proteins the specific sequence of the transgene insert itself could have an effect on the transformation rate (2% - 14% between the four cassettes they used). Additionally they found that 40-44% of their primary transformants lacked an insert while maintaining *nptII* activity and 67% of the plants with an insert showed non-expression of the transgene indicating host suppression. They explain that this variation of transformation rate due to differences in transgene sequence is probably due to the viral pathogenicity of the resulting protein. Given the pathological importance of viral movement proteins to infection, it may be that this is not an issue when N-gene sequences are used as transgenes as in the case of this study, but the question remains unanswered and thus a potential contributor.

Another unlikely contributor worthy of mention is the potential for the transgene to adversely affect the ability of transformed cells to properly regenerate. Fedorowicz et al. (2005) reported such problems when attempting tomato transformations with a chimerical TSWV N-gene/UTR plum pox virus construct. Only 12 primary transformants had a normal seed set, and of those only eight showed proper transgene integration. The remaining plants showed integration of only fragments of the transgene or suppressed transgene expression all together. Considering the chimerical cassettes used in this study

are based on N-gene sequences from three virus species, this could be part of the explanation as to why any transformant that might have been generated did not survive. However, the fact that constructs EPJ and EPN in our study did not show any improved results over the other three rebuts this argument, considering they transfer only the T-DNA vector elements and no virus sequence at all. This information taken with the technicality of transformation protocols highlights that even subtle differences in protocol and experimental design all taken together can have significant negative (or positive) effects on the overall transformation efficiency.

To clear up the apparent discrepancies of protocol new tomato germplasm proven to transform (cv. Moneymaker and cv. Geneva 80) should be transformed along side transgenic lines of the same cultivars to serve as a direct comparison. These should be independently transformed with a GUS reporter gene, the chimerical construct used in this study, and empty vector sequence to test for pathogenicity and lethality of the transgene. This would allow for the elimination or implication of variables associated with protocol and the insert and narrow down the possible points at which the MP1 protocol might be deficient with respect to the FLA 8044 germplasm.

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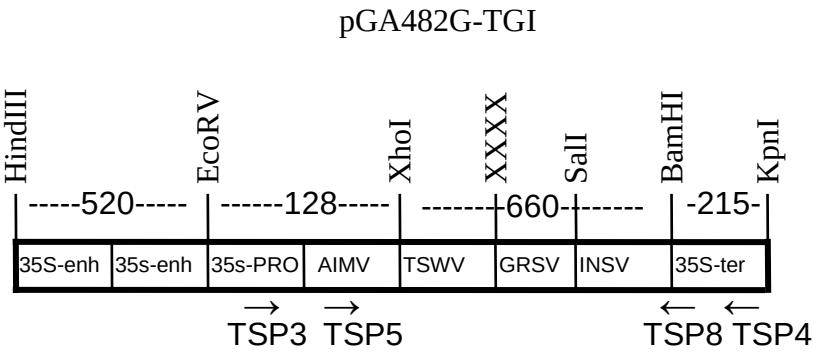
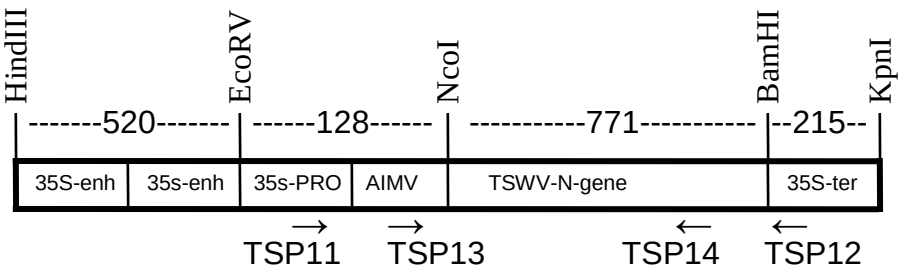
Figures and Tables

Fig. 1 Diagramatic representations of the region of the T-DNA containing viral sequences of interest for pathogen derived resistance against tospoviruses. (a) TGI was constructed as follows: 35S double enhancer and promoter region from *Cauliflower mosaic virus* (CaMV) followed by an *Alfalfa mosaic virus* (AlMV) promoter and the respective regions of the nucleocapsid genes from each of the following viruses: third quarter of the *Tomato spotted wilt virus* (TSWV) N gene sequence**,** first quarter of the *Groundnut ringspot virus* (GRSV) N gene sequence, second quarter of the *Impatiens necrocitc spot virus* (INSV) N gene sequence, and a 35S CaMV terminator ; (b) TSW contains the 35S double enhancer and promoter region from CaMV followed by an AlMV promoter and the full length of the TSWV N gene sequence with the 35S terminator; (c) TGN contains the 35S double enhancer and promoter region from CaMV followed by an AlMV promoter and the same regions of the respective viral N gene sequences as TGI followed by the m/2 N universal gene silencer sequence and a 35S terminator; (d) EPN was similarly constructed with the 35S double enhancer and promoter region from CaMV followed by an AlMV promoter and only the m/2 N universal gene silencer with a 35S terminator; (e) EPJ contains the 35S double enhancer and promoter region from CaMV followed by an AlMV promoter and a 35S terminator region and as such does not make use of any viral N gene sequence.

(a)

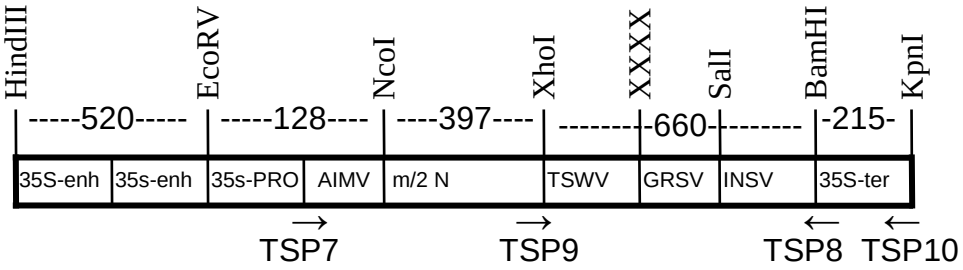


(b)



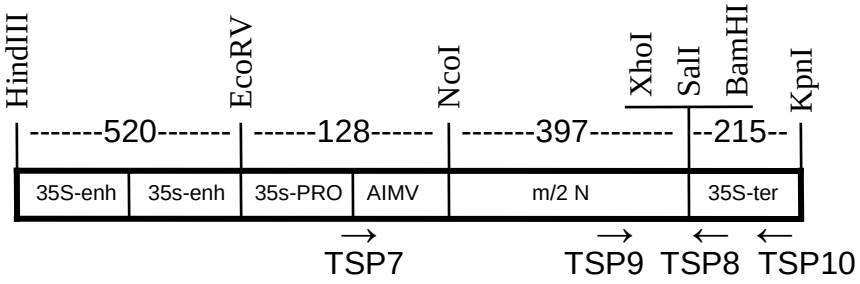
(c)

pGA482G-TGN



(d)

pGA482G-EPN



(e)

pGA482G-EPJ

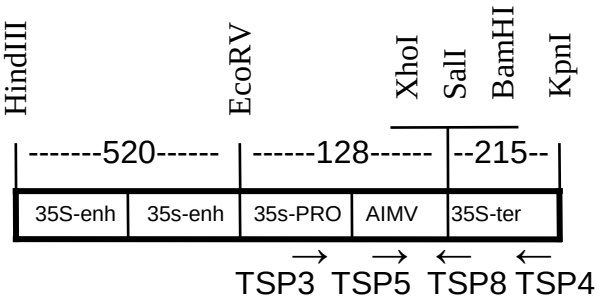


Fig. 2 *nptII* PCR amplification results from selected FLA 8044 putative tomato transformants showing the construct used for each sample (Fig. 1) and the expected product sizes.

Fig. 3 Viral T-DNA insert PCR amplification results from selected FLA 8044 putative tomato transformants showing the construct used for each sample (Fig. 1) and the expected product sizes.

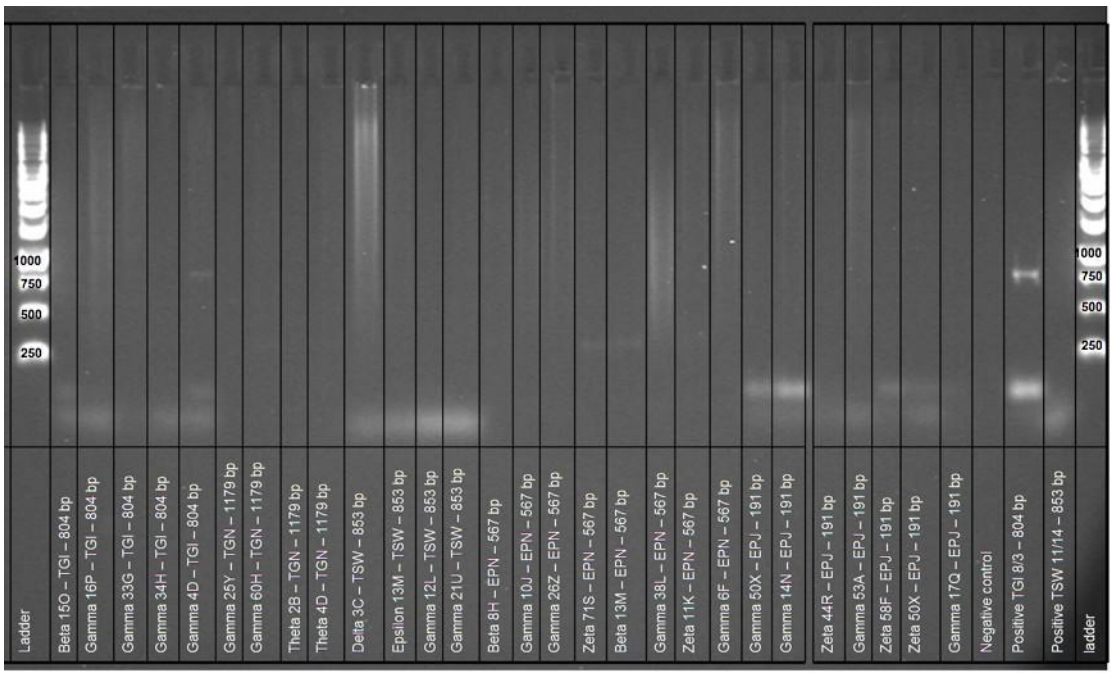


Fig. 4 Southern Blot hybridization with a P^{32} labeled *nptII* probe results from positive control lanes using normalized vector DNA for each of the five constructs of interest. All putative tomato samples showed no banding (data not shown).

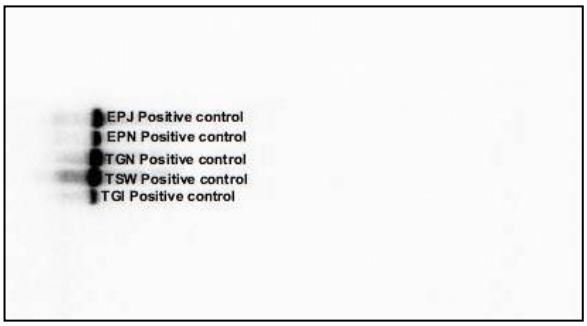


Fig. 5 PCR results from *Agrobacteria* for both the *nptII* gene and the viral inserts (a) EPN—JYS100/JYS101 (b) TGN—JYS100/JYS101 (c) TSW—JYS100/JYS101 (d) EPJ—JYS100/JYS101 (e) TGI—JYS100/JYS101 (f) EPN—TSP7/TSP10 (g) TGN—TSP7/TSP10 (h) TSW—TSP11/TSP12 (i) EPJ—TSP3/TSP4 (j) TGI—TSP3/TSP4 (k) Negative Control—JYS100/JYS101 (l) Negative Control—TSP11/TSP12 (m) Negative Control—TSP7/TSP10 (n) Negative Control—TSP3/TSP4 (o) Positive Control EPN DNA—TSP7/TSP10 (p) Positive Control TGN DNA—TSP7/TSP10 (q) Positive Control TSW DNA—TSP11/TSP12 (r) Positive Control EPJ DNA—TSP3/TSP4 (s) Positive Control TGI DNA—TSP3/TSP4 (t) Positive Control EPN DNA— JYS100/JYS101.

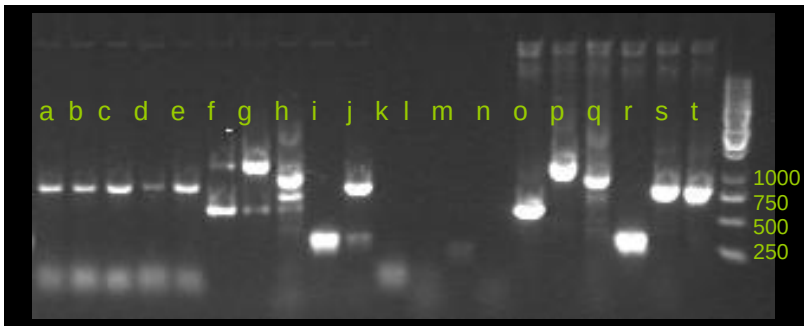


Table 1 Media recipes used for the regeneration of MP1 variety tomatoes as well as for breeding lines FLA 8044 and FLA 7804 after putative *Agrobacterium* mediated transformation of cotyledon explants with one of five constructs implicated for Pathogen Derived Resistance to Tospoviruses.

	Germination Media	Regeneration Media	Elongation Media	Rooting Media
MS Medium plus Vitamins[a]	4.3 g/l	4.3 g/l	4.3 g/l	4.3 g/l
Glycine[b]	2 mg/l	2 mg/l	2 mg/l	2 mg/l
Sucrose[c]	15 g/l	-	-	15 g/l
Glucose[d]	-	30 g/l	30 g/l	-
Agar[e]	8 g/l	-	-	-
Phytagel[f]	-	2.5 g/l	2.5 g/l	2.5 g/l
Kanamycin[a]	-	50 mg/l	50 mg/l	-
Gentamicin[g]	-	50 mg/l	50 mg/l	-
Cefotaxime[a]	-	500 mg/l	500 mg/l	500 mg/l
Carbenicillin[a]	-	500 mg/l	500 mg/l	500 mg/l
Indole-3-butyric acid[f]	-	-	-	2 mg/l
Indole-3-acetic acid[g]	-	0.1 mg/l	0.04 mg/l	-
Zeatin[g]	-	1 mg/l	0.1 mg/l	-
pH	5.8	5.8	5.8	5.8

[a]Bio-World Corp., Dublin, OH

[b]USB Corp., Cleveland, OH (16405)

[c]ThermoFisher Scientific Inc. Waltham, MA

[d]EMD Biosciences Inc., San Diego, CA

[e]Spectrum Laboratory Products Inc., Gardena, CA

[f]Sigma-Aldrich Corp., St. Lois, MO

[g]Research Products International Corp., Mt. Prospect, IL

Table 2 Predicted product sizes for each primer combination with each of the five constructs (Fig. 1) implicated for Pathogen Derived Resistance against Tospoviruses, as well as for the neomycin phosphotransferase gene (*nptII*) used as a selectable marker with kanamycin.

	TGI	TSW	TGN	EPJ	EPN	*nptII*
TSP3/TSP4	850 bp	-	-	237 bp	-	-
TSP3/TSP8	804 bp	-	-	191 bp	-	-
TSP5/TSP4	809 bp	-	-	151 bp	-	-
TSP5/TSP8	763 bp	-	-	197 bp	-	-
TSP7/TSP8	-	-	1179 bp	-	567 bp	-
TSP7/TSP10	-	-	1187 bp	-	575 bp	-
TSP9/TSP8	-	-	740 bp	-	128 bp	-
TSP9/TSP10	-	-	748 bp	-	136 bp	-
TSP11/TSP12	-	973 bp	-	-	-	-
TSP11/TSP14	-	853 bp	-	-	-	-
TSP13/TSP12	-	890 bp	-	-	-	-
TSP13/TSP14	-	770 bp	-	-	-	-
JYS100/JYS101	-	-	-	-	-	822 bp

Table 3 Primer sequences of all primers used to amplify selected regions of the five T-DNA inserts used to engineer Pathogen Derived Resistance against Tospoviruses in tomato, as well as the neomycin phosphotransferase gene (*nptII*) used as a selectable marker with kanamycin.

	Oligo sequence 5'-3'	Length (bp)
TSP3	GCAAGACCCTTCCTCTATATAGGGAAGT	28
TSP4	TAAGAACCCTAATTCCCTTATCTGG	25
TSP5	GAGAGGACACGTTTTTATTTTT	22
TSP7	GGGAAGTTCATTTCATTTGGAGAG	24
TSP8	AGAGAGAGTAGATTTGTAGAGAGAGA	26
TSP9	AAAAGAAAACTAGGTAACTAACCATGGTC	29
TSP10	ATTATTATAGAGAGAGATAGATTTGTAGAG	30
TSP11	CAGACCTTCCTCTATATAGGGAAGTTC	27
TSP12	ACTCTTTCCCTTCTCACCTGATCT	24
TSP13	ATGGTTAAGCTCACTAAGGAAAGC	24
TSP14	TTAAGCAAGTTCTGTGAGTTTTGCC	25
JYS100	TCAGAAGAACTCGTCAAGAAGGCG	24
JYS101	ATGGCAATTACCTTATCCGCAACTTC	26

Table 4 Total number of successful tomato cotyledon explants/regenerates/seedlings from each of the three tomato lines used at varying points in the *Agrobacterium* mediated tomato transformation protocol reflecting not only the sample size used in this study but also the rate at which each line individually regenerated and transformed.

	Total # explants	Regenerated shoots	Fully elongated plantlets	Viably rooted plants	Overall Regeneration rate[a]	Overall Transformation Rate[b]
FLA 8044	~3500	1209	164	96	7.9%	0%
FLA 7804	~1500	336	56	9	2.6%	0%
MP1	~3500	1276	182	110	8.6%	0%

[a]Total number of regenerated shoots/number of viably rooted plants
[b]Total number of regenerated shoots/number of transgenic individuals

RESULTS OF USING CHIMERICAL TOSPOVIRUS CONSTRUCTS TO TRANSFORM *ARABIDOPSIS THALIANA* FOR PATHOGEN DERIVED RESISTANCE

Abstract

Arabidopsis thaliana ecotype wassilewskija (Ws) has been successfully transformed via floral dip with a chimerical construct consisting of regions of the nucleocapsid gene from *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), and *Groundnut ringspot virus* (GRSV) with the intention of rendering transformants resistant to tospoviruses via pathogen derived resistance (PDR). Sixteen independent transformants in the T_0 generation resulted from 19,000 germinated seeds from three dipped plants resulting in a total transformation rate of 0.08%. One-thousand T_1 generation seeds from each individual were germinated on kanamycin media where four populations (3, 9, 10, and 16) exhibited a wild-type phenotype with simple Mendelian inheritance patterns indicating a single transgene introgression or multiple tightly linked introgressions. DNA walking analysis mapped three of the four transformants to chromosomes 1, 3, and 4. Twenty-five T_1 individuals were selected from each population were transferred to soil for DNA extraction and zygosity determination. T_2 seed from homozygous plants was collected for future replicated tospovirus resistance studies.

Introduction

Tospoviruses, at present, represent one of the most severe disease categories facing vegetable cultivation and often results in yield losses as high as 90% where virus populations have evolved to overcome the established genetic resistance (Chamberlin et al. 1992, Cho et al. 1987, Goldbach and Peters 1994). Tospoviruses, most notably the tospovirus type species *Tomato spotted wilt virus* (TSWV) is particularly difficult to control considering it can maintain itself in at least 1090 reservoir plant species in 15 families of monocots and 69 families of dicots (Parrella et al. 2003). Pittman (1927) was the first to observe that thrips in the insect family Thripidae are capable of transmiting tospovirus infection. Worldwide 11 species of thrips have been documented to transmit at least one type of tospovirus with *Frankliniella occidentalis* (Western Flower Thrips) being the most damaging species because it is capable of efficiently transmitting at least five different tospoviruses including TSWV (Ullman et al. 2002).

Thrip management is not as effective a control as the implementation of plant genetic resistance since the size of thrips species lends itself to avoiding pesticide sprays and tospovirus acquisition in the vector species occurs during the larval stage but can be transmitted in the adult stage when the insects feed (Ullman et al. 2002). Some sources of genetic resistance to tospovirus infection have been identified in wild populations with cultivated relatives, but these resistances have been short lived due to the isolate specific nature of the resistance, which was quickly overcome by wild virus populations (Roselló et al. 1998). In the *Solanaceae* genus for example, the most common resistance used in modern breeding programs is that of *Sw-5* derived from *S. peruvianum* cultivar 'Stevens',

expressed as a hypersensitive response to infection (Roselló et al. 1998, Stevens et al. 1992 and van Zijl et al. 1986). However, to date, five TSWV isolates from across the world have been reported as overcoming *Sw-5*: TSWV-6 in Hawaii (Cho et al. 1996); JF in South Africa (Thompson and van Zijl 1996); To_{TAS}-1d and Da_{WA}-1d in Australia (Latham and Jones 1998); GRAU in Australia (Aramburu and Martí 2003); and T992 in Italy (Ciuffo et al. 2005). Thus, there is a significant necessity for new tospovirus resistance that must be simply inherited and robust enough to be challenged by multiple tospovirus isolates in order for it to exhibit enough effectiveness to be economically viable.

Originally defined by Sanford and Johnston (1985), pathogen derived resistance (PDR) has emerged as a potent source of transgenic resistance against viral diseases (Lomonossoff 1995). Due to the ease of transformation associated with tobacco, PDR to tospoviruses began with resistance to TSWV in tobacco plants using the TSWV N gene and marked the first instance of PDR to a negative-strand RNA virus (Gielen et al. 1991). Since then it has been successfully implemented in TSWV hosts such as tomato (Accotto et al. 2005, Fedorowicz et al. 2005, Gonsalves et al. 1996, Hoffman et al. 2001, Kim et al. 1994, Nervo et al. 2003, and Ultzen et al. 1995), tobacco (de Haan et al. 1992, Levin et al. 2005, MacKenzie and Ellis 1992, Prins et al. 1995, and Vaira et al. 2000), *gerbera* plants (Korbin et al. 2002), peanut (Li et al. 1996), lettuce (Pang et al. 1996), and chrysanthemum (Sherman et al. 1998, Yepes et al. 1995). However, PDR to tospoviruses remains isolate specific unless combined with other forms of natural resistance (Gubba et al. 2002).

PDR to tospoviruses has been shown to generally be post-transcriptional gene silencing, thus providing the most probable explanation of the isolate specificity of the resistance (Marano and Baulcombe 1998, Mueler et al. 1995, and van den Boogaart et al. 1998). Jan et al (2000) demonstrated resistance to multiple virus species using a chimerical transgene consisting of portions of each virus' nucleocapsid gene (N-gene), but found that the resistant phenotype only surfaced when the transgene was also fused to a green fluorescent protein (GFP) sequence.

In this study we report on the transformation of the model plant species *Arabidopsis thaliana* (ecotype Wassilewskija) with a chimerical construct consisting of portions of the N-gene from three tospovirus species; namely TSWV, *groundnut ringspot virus* (GRSV), and *impatiens necrotic spot virus* (INSV). Our previous studies in tomato with this vector yielded no transformants and raised questions about the efficacy of the chimerical construct, as such our objectives were to asses the transformation efficiency in an alternate target organism and develop homozygous populations to be assessed for possible resistance phenotype(s).

Materials and Methods

Binary vector construction and preparation of engineered *Agrobacteria*

Five chimerical binary vectors were provided in the form of air dried DNA by Dennis Gonsalves at the Pacific Basin Agricultural Research Center of the United States Department of Agriculture (USDA) in Hilo, Hawaii (Fig. 1). Each vector was independently transformed into ElectroMAX™ LBA4404 *Agrobacteria tumafaciens* cells (Invitrogen Corp., Carlsbad, CA) via electroporation carried out on a BTX® ECM® 600 electroporator using the following conditions: 20.0 kV, 200 Ω, 25 μF. After 56 hrs of incubation, one colony from each construct was grown overnight in 2 ml of yeast-mannitol (YM) broth containing 50 mg/l of kanamycin and 50 mg/l of gentamicin. 400 μl of the overnight culture was mixed with 600 μl of 20% glycerol and stored at -80°C.

Plant material and plant transformation

Floral dip of *Arabidopsis* was carried out as per Bent (2006). All five constructs were transformed independently using *Arabidopsis* ecotype Wassilewskija (Ws). Control plants were transformed at the same time using the pCambia 1305.1 GUSplus™ vector containing *nptII* and a GUS reporter gene. Putatively transgenic seeds were sterilized by bathing 20 mg of seeds in isopropanol for 45-60 sec followed by a 5 min wash in 50% bleach/50% water/0.05% Tween-20 solution. After three to four rinses in sterile water,

the seeds were suspended in 0.1% agarose and spread evenly over 0.5x MS media with 50 mg/l of kanamycin. The Petri dishes were sealed with porous tape, vernalized at 4°C for 48 hrs, and left under fluorescent lights for 14 days before resistant plants were transplanted to soil.

DNA extraction and PCR amplification

Genomic DNA was extracted from 100 mg of lyophilized leaf tissue as described by Sambrook et al. (1989) with modifications from Todd and Vodkin (1996). Following extraction, each sample was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Montchanin, DE) and adjusted to 100 ng/µl of TE.

PCR amplification of the chimerical inserts and *nptII* region was carried out in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). Each reaction was constituted as follows: PCR buffer (10 mM TRIS-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin; Sigma-Aldrich Corp., St. Lois, MO), 0.2 mM dNTPs, 0.5 mM forward primer, 0.5 mM reverse primer (Tables 2 and 3), 50-100 ng of template DNA or bacterial stock solution, 0.5 U of JumpStart™ Taq DNA polymerase (Sigma-Aldrich Corp., St. Lois, MO), and sterile ddH_2O to volume. The amplification protocol included: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 53°C for 30 sec, 72°C for 3 min; and a final 72°C incubation for 10 min. Products were separated in a 0.5X TBE (0.001 M EDTA, 0.045 M Tris, 0.045 M Boric Acid) on 1% agarose gel and visualized with ethidium bromide staining techniques.

Transgene mapping analysis

Mapping of the transgene introgression sites was accomplished by amplifying regions of unknown genomic DNA with three transgene specific primers (DW_TSP1, DW_TSP2, DW_TSP3 [see table 2 for primer sequences]) pointed away from the insert on the 5' end of the *nptII* gene using the DNA walking SpeedUp™ Premix Kit II (Seegene Inc., Seol, South Korea). PCR reactions were run on an a GeneAmp® PCR System 9700 thermocylcer (Applied Biosystems, Foster City, CA） and products were analyzed in a 0.5X TBE solution (0.001 M EDTA, 0.045 M Tris, 0.045 M Boric Acid) on a 2% Agarose gel. Samples showing clear amplification were chosen for sequencing and purified DNA fragments were submitted to the DNA sequencing center at Brigham Young University (Provo, UT). BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) was used for cycle sequencing reactions analyzed on an ABI 3730xl DNA analyzer. Sequence from each band was scored against the *Arabidopsis thaliana* genomic database on the National Center for Biotechnology Information (NCBI, Bethesda, MD) website using the BLASTN algorithm.

Results and Discussion

Two independent transformations yielded approximately 19,000 seeds from 3 plants and produced 16 independent TGI transformants in the T_0 generation. This resulted in a total transformation rate of 0.08%, but due to a fungal infection, only 8 of 16 plants produced sufficient seeds for continued analysis and enough biomass for DNA extraction. Duplication of the TGI transformation experiment with independent aliquots of the construct DNA yielded a transformation rate of 0.06% [4 transgenic plants from 6,000 seeds (Table 3)]. Due to the low transformation rate of both experiments (compared to 2.6% for the pCambia 1305.1 GUSplus™ positive control), PCR verifications of both the chimerical inserts and the *nptII* gene were conducted on the *Agrobacteria* stock solutions with results consistent with expected amplification product sizes (Fig. 2).

While such a low transformation rate is not common, it is also not surprising considering that transformation success rates often vary from laboratory to laboratory and the causal variables are not always identified (Bent 2006). Furthermore, it has come to our attention that the nature of the construction of the T-DNA inserts is not well documented and may be in question as to its accuracy (Savarni Tripathi; USDA Hilo, HI personal communication). These two factors whether individually or combined are likely the primary contributors to our reduced transformation rate.

An additional explanation worthy of note is the potential pathogenicity of the transgene or transgene product itself. While this is not as likely a culprit as variation between labs or unknown vector construction, it does merit some discussion. Hou et al. (2000) reported that when they transformed tomato with geminivirus movement proteins the specific sequence of the transgene insert itself had an effect on the transformation rate

(2% - 14% between the four cassettes they used). They explain that this variation of transformation rate due to differences in transgene sequence is probably due to the viral pathogenicity of the resulting protein. It is important to consider that the chimerical cassettes used in this study are based on N-gene sequences and not viral movement proteins, so while this explanation is both interesting and intriguing, the data is not sufficient to draw the conclusion that transgene pathogenicity affected our transformation rate. However, this argument is, to an extent, rebutted considering the fact that constructs EPJ and EPN in our study did not show any improved results over the other three constructs and that these two both transfer only the T-DNA vector elements (35S enhancers, promoters, terminator, and/or m/2 N universal gene silencer) and no viral sequence at all.

Despite the low transformation rate, 1,000 T_1 generation seeds from each of the eight primary transformants were germinated on kanamycin media. Of those two populations exhibited a non-standard phenotype in the progeny; one segregated in a 15:1 ratio for kanamycin resistance consistent with two transgene insertions; another segregated in a 1:1 ratio indicating that most likely only the heterozygotes survived; and four populations exhibited a wild-type phenotype with simple Mendelian inheritance patterns indicating a single transgene introgression or multiple tightly linked introgressions (Table 4). DNA walking analysis mapped the transgene for three of the four remaining transformants to chromosomes 1, 3, and 4. (Table 5).

Twenty-five T_1 individuals were randomly selected from each population and transferred to soil for DNA extraction and zygosity determination. Further research endeavors should include a replicated resistance study with TSWV isolates that can

overcome *Sw-5* resistance using the homozygous T_2 seed collected in this study. If resistance is to be found, northern blot analysis should be considered to determine if post-transcriptional gene silencing is responsible for the resistant phenotype.

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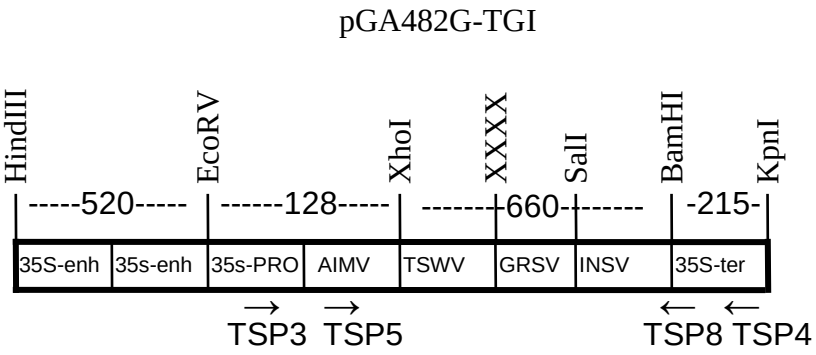
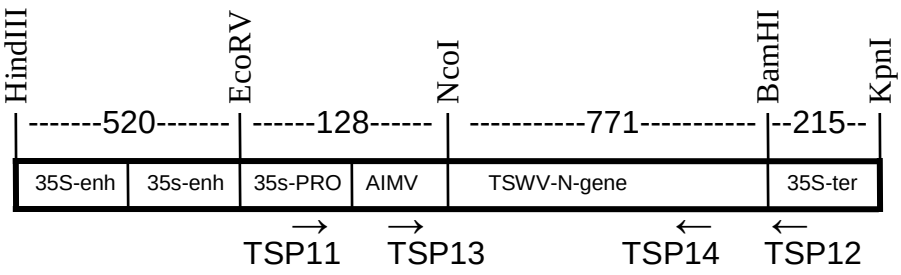
Figures and Tables

Fig. 1 Diagramatic representations of the region of the T-DNA containing viral sequences of interest for pathogen derived resistance against tospoviruses. (a) TGI was constructed as follows: 35S double enhancer and promoter region from *Cauliflower mosaic virus* (CaMV) followed by an *Alfalfa mosaic virus* (AlMV) promoter and the respective regions of the nucleocapsid genes from each of the following viruses: third quarter of the *Tomato spotted wilt virus* (TSWV) N gene sequence**,** first quarter of the *Groundnut ringspot virus* (GRSV) N gene sequence, second quarter of the *Impatiens necrocitc spot virus* (INSV) N gene sequence, and a 35S CaMV terminator ; (b) TSW contains the 35S double enhancer and promoter region from CaMV followed by an AlMV promoter and the full length of the TSWV N gene sequence with the 35S terminator; (c) TGN contains the 35S double enhancer and promoter region from CaMV followed by an AlMV promoter and the same regions of the respective viral N gene sequences as TGI followed by the m/2 N universal gene silencer sequence and a 35S terminator; (d) EPN was similarly constructed with the 35S double enhancer and promoter region from CaMV followed by an AlMV promoter and only the m/2 N universal gene silencer with a 35S terminator; (e) EPJ contains the 35S double enhancer and promoter region from CaMV followed by an AlMV promoter and a 35S terminator region and as such does not make use of any viral N gene sequence.

(a)

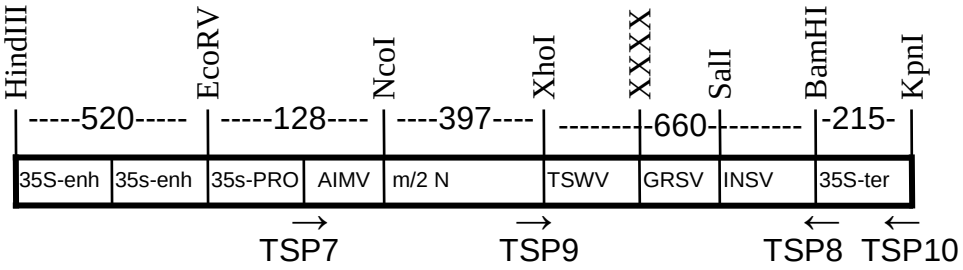


(b)



(c)

pGA482G-TGN



(d)

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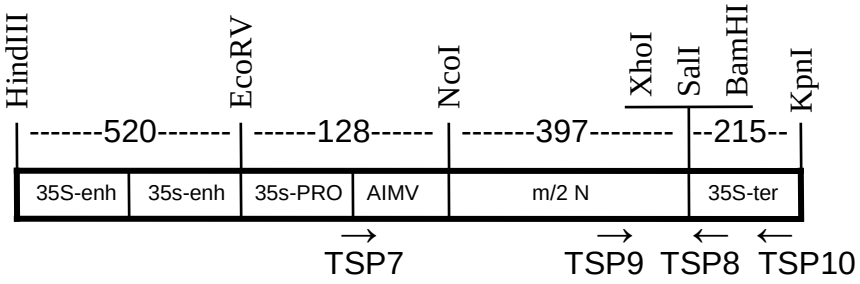

(e)

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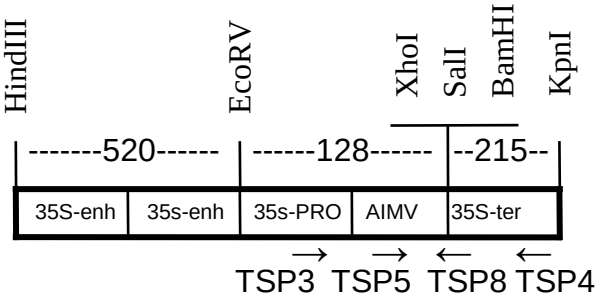

Fig. 2 PCR results from *Agrobacteria* for both the *nptII* gene and the viral inserts. (a) EPN—JYS100/JYS101. (b) TGN—JYS100/JYS101. (c) TSW—JYS100/JYS101. (d) EPJ—JYS100/JYS101. (e) TGI—JYS100/JYS101. (f) EPN—TSP7/TSP10. (g) TGN—TSP7/TSP10. (h) TSW—TSP11/TSP12. (i) EPJ—TSP3/TSP4. (j) TGI—TSP3/TSP4. (k) Negative Control—JYS100/JYS101. (l) Negative Control—TSP11/TSP12. (m) Negative Control—TSP7/TSP10. (n) Negative Control—TSP3/TSP4. (o) Positive Control EPN DNA—TSP7/TSP10. (p) Positive Control TGN DNA—TSP7/TSP10. (q) Positive Control TSW DNA—TSP11/TSP12. (r) Positive Control EPJ DNA—TSP3/TSP4. (s) Positive Control TGI DNA—TSP3/TSP4. (t) Positive Control EPN DNA—JYS100/JYS101.

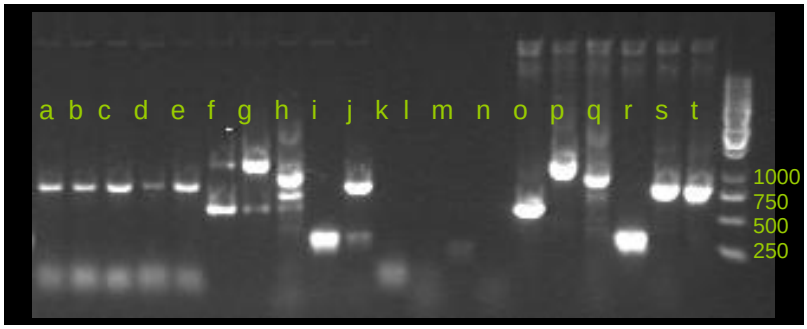

Table 1 Predicted product sizes for each primer combination with each of the five constructs (Fig. 1) implicated for Pathogen Derived Resistance against Tospoviruses, as well as for the neomycin phosphotransferase gene (*nptII*) used as a selectable marker with kanamycin.

	TGI	TSW	TGN	EPJ	EPN	*nptII*
TSP3/TSP4	850 bp	-	-	237 bp	-	-
TSP3/TSP8	804 bp	-	-	191 bp	-	-
TSP5/TSP4	809 bp	-	-	151 bp	-	-
TSP5/TSP8	763 bp	-	-	197 bp	-	-
TSP7/TSP8	-	-	1179 bp	-	567 bp	-
TSP7/TSP10	-	-	1187 bp	-	575 bp	-
TSP9/TSP8	-	-	740 bp	-	128 bp	-
TSP9/TSP10	-	-	748 bp	-	136 bp	-
TSP11/TSP12	-	973 bp	-	-	-	-
TSP11/TSP14	-	853 bp	-	-	-	-
TSP13/TSP12	-	890 bp	-	-	-	-
TSP13/TSP14	-	770 bp	-	-	-	-
JYS100/JYS101	-	-	-	-	-	822 bp

Table 2 Primer sequences of all primers used to amplify selected regions of the five T-DNA inserts used to engineer Pathogen Derived Resistance against Tospoviruses, as well as the neomycin phosphotransferase gene (*nptII*) used as a selectable marker with kanamycin, and the three target specific primers used to map the transgene in the DNA walking analysis.

	Oligo sequence 5'-3'	Length (bp)
TSP3	GCAAGACCCTTCCTCTATATAGGGAAGT	28
TSP4	TAAGAACCCTAATTCCCTTATCTGG	25
TSP5	GAGAGGACACGTTTTTATTTTT	22
TSP7	GGGAAGTTCATTTCATTTGGAGAG	24
TSP8	AGAGAGAGTAGATTTGTAGAGAGAGA	26
TSP9	AAAAGAAAACTAGGTAACTAACCATGGTC	29
TSP10	ATTATTATAGAGAGAGATAGATTTGTAGAG	30
TSP11	CAGACCTTCCTCTATATAGGGAAGTTC	27
TSP12	ACTCTTTCCCTTCTCACCTGATCT	24
TSP13	ATGGTTAAGCTCACTAAGGAAAGC	24
TSP14	TTAAGCAAGTTCTGTGAGTTTTGCC	25
JYS100	TCAGAAGAACTCGTCAAGAAGGCG	24
JYS101	ATGGCAATTACCTTATCCGCAACTTC	26
DW_TSP1	GACATCATTCTGTGGCGGGTA	21
DW_TSP2	GCAGGAGATGCTGGCTGAAC	20
DW_TSP3	CGATTTACCGCTGGGTTCAG	20

Table 3 Individual transformation rates for each of the five constructs (Fig. 1) implicated for Pathogen Derived Resistance against Tospoviruses in *Arabidopsis thaliana* ecotype Wassilewskija. Parenthesis indicate the number of initial transformants/the number of putatively transformed seeds germinated.

	Original DNA	**New DNA**
TGI	0.08% (16/19000)	0.06% (4/6000)
TSW	0.04% (3/8000)	-
TGN	0.02% (3/13000)	-
EPJ	0.03% (4/8000)	-
EPN	0.04% (3/13000)	-
Control	2.6% (78/3000)	-

Table 4 Segregation values for each of the six *Arabidopsis thaliana* transformants containing the TGI (Fig. 1) chimerical insert for Tospovirus N gene sequences.

	Total Pop #	# Survived selection	X^2 at p=0.05
Transformant #1[a]	-	-	-
Transformant #3	1,063	748	3.0
Transformant #4	468	409	9.5
Transformant #9	696	505	0.55
Transformant #10	988	751	0.13
Transformant #12	494	225	56.8
Transformant #16	888	594	7.7
Transformant # 17[a]	-	-	-

[a]data not collected due to non-standard phenotype

Table 5 Chromosome numbers, base pair positions, and flanking molecular markers of the TGI (Fig. 1) transgene insert from each of the three mapped *Arabidopsis thaliana* transformants.

	Chromosome Number	bp position	Flanking Markers	
			North	South
Transformant #9	4	12855276	SGCSNP215	SM120_126,2
Transformant #10	1	16545493	SGCSNP163	SM218_156,8
Transformant #16	3	18059993	ALS	CDC2A