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Viral stress activation of retrotransposons in sweetpotato [Ipomoea batatas (L.) Lam.]

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VIRAL STRESS ACTIVATION OF RETROTRANSPOSONS IN SWEETPOTATO [*IPOMOEA BATATAS* (L.) LAM.]

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DEDICATION

This work is dedicated to my beloved grandmother Maria Porpaxia, who passed away in May of 1999.

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ABSTRACT

Mutations in sweetpotato [*Ipomoea batatas* (L.) Lam.], which are poorly understood, are widely implicated as a component in cultivar decline. In sweetpotato, mutations are also likely to result from the activity of plant retrotransposons, which are known to be present in the sweetpotato genome. The majority of the transcriptional activity of plant retrotransposons that has been characterized to date, is stress-induced, involving both biotic and abiotic stresses. Viral infection, a form of biotic stress, is also one that commonly occurs in sweetpotato. The objective of this study is to test whether viral stress, in the form of single and combined viral infections, transcriptionally activates *Ty1-copia* elements, a family of LTR retrotransposons, at the whole-plant level. To assess activation, transcripts from the *RT* domain of *Ty1-copia* elements were relatively quantified using Real-Time Quantitative PCR. Relative amounts of transcripts from five viral inoculation treatments were then compared to those from the virus-free plants of the control treatment. Results showed that the relative amount of *Ty1–copia* transcripts from the viral combination treatment of BWFT-3 (SPCSV isolate) + SPFMV, which produced the most severe symptoms on both varieties tested, was significantly higher than the other viral treatments and the control. No significant difference was observed among the other treatments. No significant difference was also observed in transcript amounts between the two cultivars inoculated with the same virus. This represents the first study examining the effect of stress on relative transposon transcriptional activation.

CHAPTER 1

INTRODUCTION

Sweetpotato (*Ipomoea Batatas* L.) is the seventh most important food crop in the world after wheat, rice, maize, potato, barley, and cassava (FAO, 1993). Sweetpotato is most important in developing tropical countries where it ranks fourth. Although sweetpotato originated in Central and South America, world production is centered in Asia. China accounts for over 84% of the world's volume (Rubatzky and Yamaguchi, 1997). The United States is one of the few developed countries that produce sweetpotatoes. Annual production of sweetpotato ranges from 36500 to 40500 hectares, and it has a gross value of 250 million dollars. Louisiana and North Carolina account for more than half of the total production (LaBonte and Cannon, 1998).

Production statistics show a net reduction in land area dedicated to sweetpotato (Rubatzky and Yamaguchi, 1997). Tonnage however, has remained constant due to higher yielding cultivars (Rubatzky and Yamaguchi, 1997). Production technology, and improved varieties are paramount to maintaining competitiveness of sweetpotato as a major world crop. Varietal maintenance, i.e., virus tested stock and use of elite varietal clones, is a key factor (LaBonte and Cannon, 2000 personal communication).

The occurrence of somatic mutations in sweetpotato is a well-documented phenomenon (Villordon, Jarret, and LaBonte, 2000). The cause of somatic mutations in sweetpotato is unknown. However, plant retrotransposons, which are mobile genetic elements that can alter plant gene expression and effect genome changes, are known to occur in sweetpotatoes (Villordon, Jarret, and LaBonte, 2000).

A rapidly growing body of data from genome characterization, cloning, and sequencing in a variety of organisms is making it increasingly evident that transposable elements have been instrumental in "sculpting" the contemporary genomes of all

organisms (Fedoroff, 1998). “Transposable elements were first discovered in plants because they can have tremendous effects on their genome structure and gene function” (Bennetzen, 2000). Although a few or no elements may be active within a genome at a given time, the genomic alterations they can cause can have a major impact on a species. All major element types appear to be present in all plant species, but their quantitative and qualitative contributions are enormously variable even between closely related species. In some large-genome plants, e.g., corn, these mobile DNA’s make up the majority of the nuclear genome. They can rearrange genomes and alter individual gene structure and regulation through any of the activities they promote: transposition, insertion, excision, chromosome breakage, and recombination (Bennetzen, 2000). An example of the simplest case of insertional mutagenesis is the insertion of an element in the coding region of a functional gene, causing the loss of its function (Lyubomirskaya and Ilyin, 1999).

Transposable elements are classified according to their sequence similarity and are distinguished by their differing modes of transposition. Class I elements are retroelements that use reverse transcriptase by means of an RNA intermediate. Class II elements transpose directly from DNA to DNA and include transposons such as the Activator-Dissociation (*Ac-Ds*) family in maize (Kidwell and Lisch, 1997).

Retrotransposons, which belong to Class I elements, were first characterized in animal and yeast genomes, but evidence has accumulated in recent years to show that they are present in all plant genomes and can constitute a very large part of them. Recently, polymerase chain reaction (PCR)-based techniques were used to study plant retrotransposons systematically (Hirochika, 1997). Retrotransposons, resembling the

integrated copies of retroviruses, are flanked by long terminal direct repeats (LTR's) and contain an internal domain that encodes *Gag* and *Pol* polyproteins. These LTR's carry the regulatory sequences required for transcription, which is the first step in the transposition. The *Pol* protein has conserved domains, which are also a characteristic of integrase, reverse transcriptase, and RNase. According to the sequence and the order of these domains, retrotransposons can be divided into two groups, the *Ty1-copia* and *Ty3-gypsy* group (Hirochika, 1997).

Ty1-copia-like elements have been detected in 56 of 57 plant species (Flavell et al., 1992b). In another survey *Ty1-copia*-like elements were detected in 64 representative species from nine out of ten plant divisions. The presence of these elements in plant, insect, as well as in fungal species, suggests that this super family might be a universal component of eukaryotic genomes (Voytas et al., 1992). Pearce, Kumar and Flavell (1996) have reported activation of the *Ty1-copia*-like elements in *Solanum tuberosum* during protoplast isolation. The majority of the activated *Ty1-copia* sequences were similar to elements, which were transcriptionally induced under the same conditions in *Nicotiana tabacum* (Pearce, Kumar and Flavell, 1996).

An increasing body of evidence shows that the activity of these retrotransposons is tightly controlled, and that abiotic and biotic stresses play a major role in their transcriptional and transpositional activation (Grandbastien, 1998). Grandbastien (1998) also reports that the activation of stress related genes might be a result of cell wall hydrolysis or of pathogenic compounds present in fungal extracts or other pathogens. Activation of retrotransposons due to virus infection has been observed in maize. *Barley stripe mosaic virus* (BSMV) infection was proven to be mutagenic to maize whereas

Tobacco mosaic virus (TMV) infection was shown to activate the *Tto1* transcription (Hirochika, 1995). Furthermore, retrotransposons are suspected in 80% of spontaneous mutations in *Drosophila* (Green, 1988).

Despite the detection of retrotransposons activated under stress factors in some plant species, there are no reports that document the activation of retrotransposons in sweetpotatoes under stress at the whole plant level. The sweetpotato as a whole plant represents a novel species to use in retrotransposon studies due to its vegetative propagation, which permits better control of plant material in a way similar to other retrotransposon mobility studies (Cameron, Loh and Davis, 1979).

The objective of this study is to provide a foundation to better understand the role of stress on retrotransposon activation and aid further studies involving the association between retrotransposons and somatic mutations in sweetpotato. Furthermore, this research will strengthen current theories that retrotransposons play an important role in altering the genome of an organism. McClintock (1984) has suggested that stresses of various kinds, including viral stress, may stimulate genome reorganization and the activation of transposable elements. With McClintock's theory of activation as a guideline, this research will specifically explore, at the transcriptional level, the activation of *Ty1-copia* retrotransposons under viral stress using the relative quantification of its transcripts by Real-Time Quantitative PCR (AppliedBiosystems, Foster City, CA).

CHAPTER 2

LITERATURE REVIEW

2.1 Transposable Elements

Transposable elements are discrete segments of DNA that are distinguished by their ability to move and replicate within genomes. They were discovered almost 55 years ago by the Nobel Prize winner Barbara McClintock and have been found to be ubiquitous in most living organisms (McClintock, 1956). They are a major component of the middle repetitive DNA of genomes of animals and plants and are present in copy numbers ranging from just a few to tens or hundreds of thousands per genome. Transposable elements can represent a major fraction of the genome, especially in plants. For example, transposable elements have been estimated to make up more than 50% of the maize genome (SanMiguel et al., 1996; Kidwell and Lisch, 1997).

Transposable elements were first discovered in plants because of the tremendous effects on their genome structure and gene function. Altered plant genes or genome phenotypes are produced at very high frequencies in both germinal and somatic tissues. Although the activity within the plant genome is variable, from no activity to a few active elements, the genomic alterations they produce can have major outcomes for the species (Bennetzen, 2000). Most mobile elements are several thousand nucleotides in length and encode all, or at least some, of the proteins that are required for their transposition. However their molecular analysis was impossible until methods of gene engineering were elaborated in the late seventies (Lyubomirskaya and Ilyin, 1999). Evidence from genome characterization, cloning, and sequencing in a variety of organisms proves that transposable elements are playing a major role in the evolution of genomes of all organisms (Fedoroff, 1998).

All transposable elements share two basic properties. The first is the ability to move from place to place in the genome-hence their designation as mobile DNA's or transposable elements. The second is their ability to increase their copy number through their property of transposition (Bennetzen, 2000). Based on their mode of transposition and structure, transposable elements are classified into two major groups. Class I, and Class II elements. Class II elements transpose directly from DNA to DNA and include elements such as the well characterized Ac/Ds, *Spm* and *Mu* of maize and *Tam* elements of snapdragon (Hirochika, 1995). These elements are responsible for unstable mutations. Class II elements' ability to transpose to heterologous plant species made it possible to extensively study them as molecular genetic tools to clone and analyze genes as well as agents inducing mutations in a wide range of species (Hirochika, 1997).

In contrast to Class II elements, Class I elements or retrotransposons undergo transposition through RNA as an intermediate and induce only stable mutations (Hirochika, 1997). Retrotransposons can be further separated into two subclasses (Fig. 1). Elements of subclass I are bounded by two long terminal repeats (LTRs) and are also termed LTR retrotransposons. Subclass II elements lack LTRs and therefore are termed non-LTR retrotransposons (Capy, 1998). Both subclasses form a DNA daughter copy by reverse transcription of an RNA template. Also their replication cycle involves an intermediate cytoplasmic step. The replicative type of transposition mechanism that retroelements possess suggests that they are potentially very invasive (Grandbastien, 1998).

These ubiquitous, self-replicating DNA elements do not seem to do anything but invade the host's genome. Their replicative type of activity clearly reflects Dawkins'

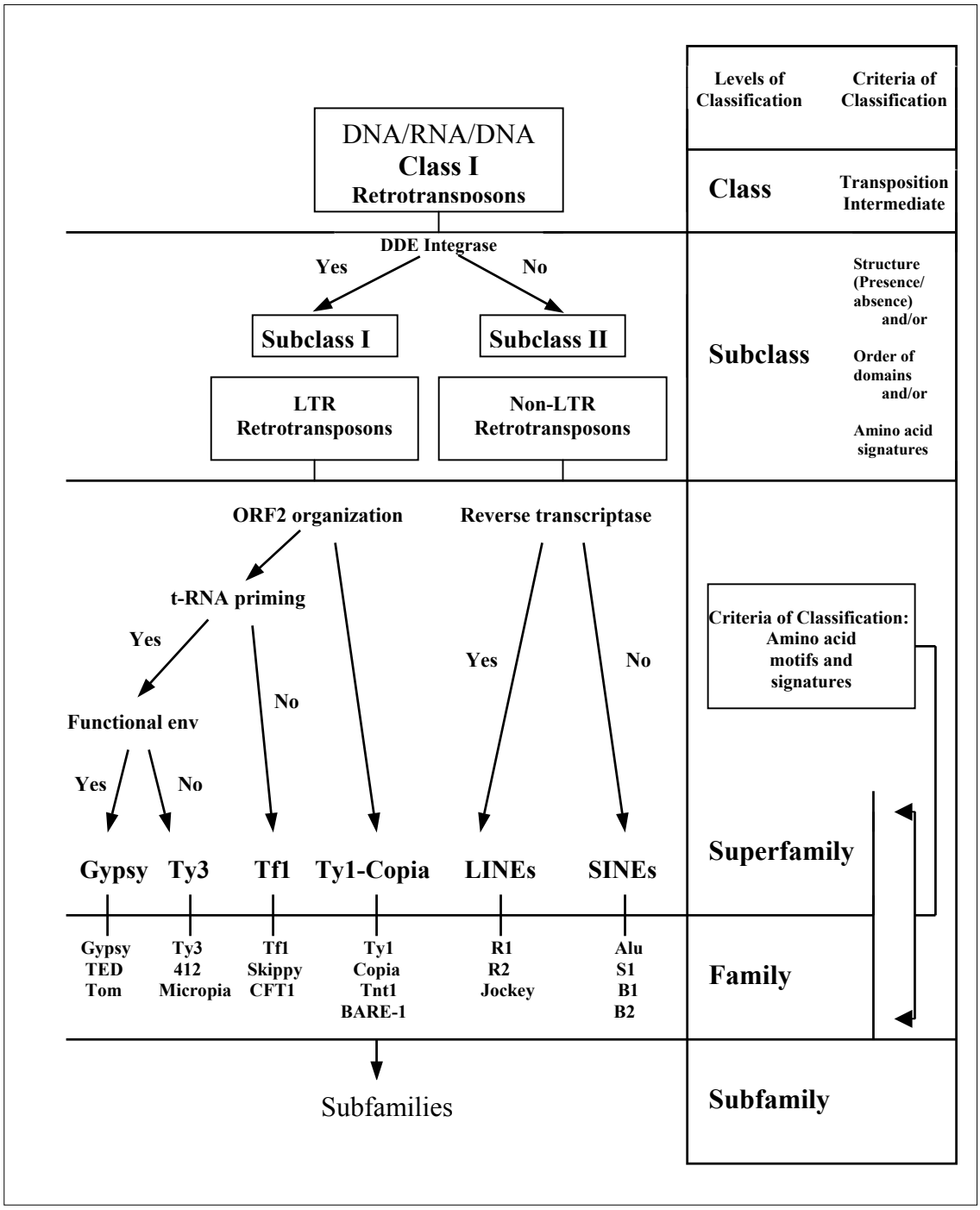


Figure 1. Classification of Class I retrotransposons.

selfish DNA hypothesis that, "the only purpose of DNA is to perpetuate itself" (Rabinowicz, 2000). Mutations caused by the activity of transposable elements may be evolutionarily advantageous, but are more likely to be deleterious for the host organism and even eliminate it from the population. However, insertions of elements that do not disrupt or change the regulation of gene sequences may be perpetuated in the population (Rabinowicz, 2000). With this logic, differences in transposition activity leading to accumulation of multiple repeats, can easily explain the large differences in genome size observed even in closely related organisms (Rabinowicz, 2000).

The impact of transposable element activity is minimized by transcriptional and posttranscriptional mechanisms, inaccurate and incomplete replication, and preferential insertion. Transposons generally have weak promoters and evidence of low translational efficiency is observed in some cases (Fedoroff, 1998). During replication, retrotransposons use an enzymatic mechanism that lacks proofreading and therefore is of relatively low fidelity. Hence, many reinserted copies are mutated. Furthermore, retrotransposon replication frequently is terminated prematurely, giving rise to copies that are "dead" on arrival (Fedoroff, 1998).

Another way by which the host avoids the potentially deleterious effects is by favoring integration sites where the transposed element does not effect the expression of other genes (Curcio and Garfinkel, 1999). Recent evidence indicates accumulation of transposable elements in heterochromatin (Dimitri and Junakovic, 1999). The dynamics of such accumulation, the specificities in targeting and location of different families and roles they might acquire with heterochromatin is at variance with the view of elements being abundant in this region because of the damage they cause in euchromatin. Rather

than mere addition of 'junk DNA' to the genomic 'wasteland', accumulation of transposable elements in heterochromatin might be an aspect of evolutionary interaction between these components of eukaryotic genomes (Dimitri and Junakovic, 1999).

Until the recent identification of a possible DNA deletion mechanism in barley there has been no example of repetitive DNA loss in plants. The return ticket to the one-way trip to genomic obesity was provided when a 42-fold excess of LTRs relative to internal domains of the *BARE-1* retrotransposon was observed (Rabinowicz, 2000). These results suggested that intra- or inter-element recombinations mediated by their LTRs and leading to loss of internal domains is an efficient way to counteract transposon expansion (Rabinowicz, 2000).

As mentioned above, the uncontrolled movement of transposons threatens the genome function of many organisms. However, hosts have evolved specific mechanisms to minimize the level of transposition. A recent literature review by Curcio and Garfinkel (1999) illustrated another concept of host defense against the deleterious effects of element transposition. Conserved cellular functions, such as those involved in differentiation and DNA repair/recombination, have been adapted by the host as a defensive mechanism against rampant retrotransposition (Curcio and Garfinkel, 1999).

The presence of transposable elements in all living organisms suggests an early origin of these mobile DNAs (Bennetzen, 2000). However, mobility makes transposable elements likely candidates for horizontal transmission and therefore the specific mechanisms of their origin are not clear. It seems likely, however, that DNA transposable elements and retroelements are derivatives of independent evolutionary creations. The concept of selfish or parasitic DNA suggests that any sequence amplified in the genome

would be selected, as long as it did not significantly decrease the fitness of the host's genome, and therefore would give rise to such elements through multiple independent origins (Bennetzen, 2000).

2.2 Retrotransposons

Retrotransposons were first characterized in animal and yeast genomes, but evidence in recent years shows clearly that they are present in all plant genomes and can constitute a very large part of some of them (Grandbastien, 1998). Using the PCR method, the ubiquity of retrotransposons has been assessed based on the conservation of certain amino acid sequences in the reverse transcriptase domain among them. In extensive studies involving more than 100 plant species belonging to 9 divisions including bryophytes, lycopods, ferns, gymnosperms and angiosperms, the presence of retrotransposons was surveyed. Retrotransposon sequences were detected in all 100 species examined, indicating that retrotransposons are ubiquitous in higher plants (Hirochika, 1995).

Previous surveys in *Arabidopsis thaliana* and rice indicated that 13 and 20 different families of retrotransposons were present in their genomes respectively (Hirochika, 1995). Further detailed studies revealed the presence of 22 new families in *Arabidopsis* and 12 in rice (Hirochika and Hirochika, 1993). The estimated copy numbers of retrotransposons in *Arabidopsis* and rice were found to be 200 and 1000, respectively (Table 1) (Hirochika et al., 1992). Furthermore, DNA sequence analysis near the *Arabidopsis* AB13 gene revealed the presence of a new non-LTR retrotransposon, designated Ta*11-1* (Voytas et al., 1996). Noma et al. (1999) showed that long

Table 1. Main families and copy numbers per haploid genome of retrotransposons isolated from plants (adapted from Grandbastien, 1992; Grandbastien et al., 1994).

Species	**Superfamily**	**Family**	**Copy number per haploid genome**
Maize	Unknown	*Bs1*	2 to 3
	Unknown	*Cin1*	1,000
	LINE	*Cin4*	50 to 100
	MITEs	*Zm4*	>1,000
	MITEs	*Zm1*	>10,000
Tobacco	SINE	*Ts*	50,000
	Ty1-copia	*Tto1*	30
	Ty1-copia	*Tnt1*	>100
Lily	LINE	*del2*	250,000
	Ty3	*del1*	>13,000
Potatoes	*Ty1-copia*	*Tst1*	1
Pea	Unknown	*PDR1*	50
Pine	*Ty3*	*IFG7*	10,000
Rice	*Ty1-copia*	*Tos*	1,000
Barley	*Ty1-copia*	*BARE-1*	5,000
Wheat	*Ty1-copia*	*Wis-2*	200
A. thaliana	*Ty1-copia*	*Ta1*	2 to 3
Colza	SINE	*S1*	500

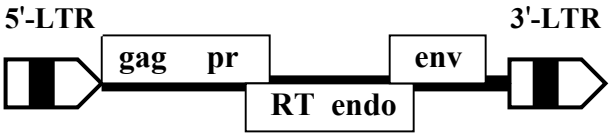
interspersed nuclear elements (LINEs), a superfamily of non-LTR retrotransposons, are ubiquitous in the plant kingdom (Noma et al., 1999).

Many of the world's most important citrus cultivars have arisen through somatic mutations (Asins et al., 1999). The occurrence of this phenomenon in the various species and varieties of the citrus genus is fairly frequent. Using a PCR assay designed to detect copia-like reverse transcriptase sequences, Asins et al. (1999) found that multiple copies of copia-like retrotransposons are integrated throughout the citrus genome. In another study, a partially characterized sequence from banana (*Musa* sp.) showed significant homology to *gypsy*-like LTR retroelements from other species. The element was named *monkey* and showed homology to *RNase H* and integrase genes of retrotransposons from plants, fungi and yeast (Balint-Kurti et al., 2000). These data clearly indicate that retrotransposons are the major class of transposable elements in plants.

2.2.1 Overall Organization

Elements of the subclass I have an overall organization similar to retroviruses (Fig. 2). The conserved enzyme reverse transcriptase, which copies the RNA genome into cDNA, is fundamental to retrotransposition and links the mechanism of transposition of all classes of retrotransposons to that of retroviral replication. In fact LTR-retrotransposons bear so many similarities to retroviruses that the line that separates them has become blurred (Curcio and Garfinkel, 1999). The long terminal repeats (LTRs) of the subclass I elements contain signals for initiation and termination of transcription. Their lengths range from as short as 77 bp (*Bombyx mori* mag element) to 2200 bp (*Drosophila Ulyces* element) (Lauermann et al., 1997). These elements carry one of

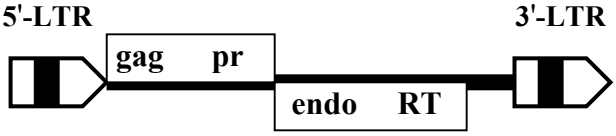
RETROVIRUSES



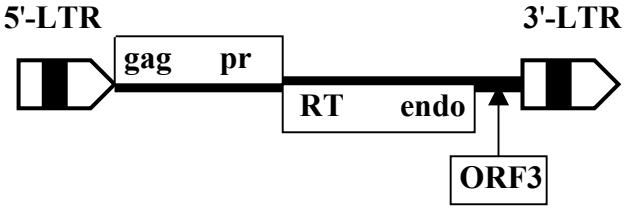
RETROTRANSPOSONS

Subclass I: LTR retrotransposons

Superfamily: *Ty1-copia*



Superfamily: *Ty3-gypsy*



Subclass II: Non-LTR retrotransposons

Superfamily: LINEs

Superfamily: SINEs

Figure 2. Overall organization of the different types of retrotransposons compared to retroviruses (adopted from Grandbastien, 1998).

several open reading frames (ORFs) with coding potential for the structural and enzymatic proteins needed for the transposition cycle (Grandbastien, 1998).

Both retroviruses and LTR-retrotransposons contain two distinct multifunctional domains known as *gag* and *pol* (Curcio and Garfinkel, 1999). The *gag* domain encodes proteins responsible for the formation of the nucleocapsid core. The *pol* domain includes other domains such as the protease (*pr*) domain encoding proteins involved in the maturation of the different proteins, the reverse transcriptase (*RT*) domain encoding the enzymes responsible for the creation of a DNA copy from the RNA template, and the integrase (*IN*) or endonuclease (*endo*) domain encoding proteins necessary for the integration of the daughter DNA copy into the host (Grandbastien, 1998).

Historically, retroviruses were distinguished from retrotransposons by the presence of a third open reading frame, *env*, considered responsible for the formation of the extracellular infectious virion (Curcio and Garfinkel, 1999). However several LTR-retrotransposons of invertebrates and more recently of plants, members of the *Ty3-gypsy* superfamily, also contain an additional open reading frame (ORF3), which sometimes encodes an *env*-like gene (Grandbastien, 1998).

Subclass II elements or non-LTR retrotransposons are not bounded by long terminal repeats. Instead, these elements are terminated by an A-rich tail ([A]n) as shown in Figure 2. Long interspersed nuclear elements (LINEs), which belong to this subclass, generally contain two ORFs, one showing similarities to the *RT* domain and the other encoding a putative *gag*-type nucleic-acid-binding protein ('*gag*'). Although LINEs do not contain a recognizable *endo* domain, some elements might contain a putative nuclease domain (*N*). Short interspersed nuclear elements (SINEs) also belong to the non-

LTR retrotransposon subclass. These elements have no coding capacity and are thought to use foreign *RT* domains to achieve their life cycle (Finnegan, 1997).

A hallmark of retroelements is their genetic heterogeneity (Preston, 1996). This was first documented for retroviruses that have been shown to exist as complex mixtures of genetically heterogeneous ever-changing virions (Katz and Skalka, 1990). The frequency of genetic variants within a population of retroelements is influenced by many factors, including mutation rate, number of transposition cycles, selection, and size of the population (Domingo and Holland, 1994). The mutation rate of *Ty1-copia* elements is high compared to that of cellular DNA. However, in the case of retroviruses and other distantly related RNA viruses, the mutation rate is comparable to that of *Ty1*, suggesting that error-prone replication is not exclusive to infectious viruses and that *Ty1* elements are also spontaneous mutators (Preston, 1996). This is interesting in that there is no obvious benefit for a retrotransposon to mutate at high rates in the absence of an immune system or an antagonist (Preston, 1996).

2.2.2 Distribution of Retrotransposons

Many transposable elements appear to be non-randomly distributed. To date no general distribution pattern of retrotransposons has been found in plants (Miller et al., 1998). The *BIS1* element of barley and the ZLRS element of maize are distributed along chromosomal arms but reduced or missing from heterochromatic centromeres, telomeres, and nucleolus organizing regions (NORs) (Moore et al., 1991). *Ty1-copia* elements in ferns, gymnosperms and angiosperms were found to be distributed throughout the chromosomes but their presence in NORs and centromeric regions was reduced (Brandes et al., 1997). However, in *Arabidopsis thaliana* and *Cicer arietinum* the *Ty1-copia*-like

elements were clustered in paracentromeric heterochromatin (Pelissier et al., 1996). Several retrotransposon fragments are also integrated in the mitochondrial genome of *Arabidopsis thaliana*. These insertions were shown to be derived from all three classes of nuclear retrotransposons, the *Ty1-copia*, *Ty3-gypsy*, and the non-LTR/LINE families (Knoop et al., 1996).

A *Ty1-copia*-related DNA sequence isolated from a sorghum centromere was found to be distributed throughout the sorghum chromosome. However, *Ty3-gypsy*-related sequences had a restricted distribution to the centromeric regions (Jiang et al., 1996b). Chromosomal localization by fluorescent *in situ* hybridization in Banana indicated that a *gypsy*-like LTR retroelement, named *monkey*, was concentrated in NORs. Other copies of the *monkey* appeared to be dispersed throughout the genome (Balint-Kurti et al., 2000). The preferential distribution of *Ty* elements to "silent" chromosomal regions is well known and may be a strategy adopted by a number of retrotransposon families (Langdon, 2000). Favorable refuges for retrotransposons include the subtelomeric and telomeric regions of a chromosome. Retroelement adaptation to these niches may have been efficient enough to modify or even replace conventional telomerase activity (Pardue et al., 1996).

The insertion of retrotransposons into the host genome is independent of the homology between the ends of the elements and the sites of insertion. At the insertion site, a short host segment usually referred as target sequence, is duplicated and therefore surrounds the inserted element. Integration site preferences seem to be influenced by functional or structural states of the target DNA rather than its sequence specificity (Sandmeyer et al., 1990). The Drosophila *gypsy*-like retrotransposons insert at

TAYATA, which may either represent sequence specificity, or a functional specificity for the TATA box in front of the Drosophila genes (Tanda et al., 1998). Analyses of *Ty* insertions indicate a preference for insertion into the promoter or 5' end of the coding sequence. Liebman and Newnam (1993) investigated the effect of mutations in the gene encoding the ubiquitin-conjugating enzyme RAD6, on target site preferences of the yeast *Ty1* and *Ty2*. *Ty1* and *Ty2* insertions isolated from an isogenic *rad6* deletion were random and did not show the promoter insertion preference observed with the non-mutated gene, suggesting that the ubiquitin-conjugating enzyme, RAD6, alters the integration site preferences of *Ty1* retrotransposons (Liebman and Newnam, 1993).

The distributional features of *copia*-like retrotransposons found in the rice genome indicated that most of the loci detected were located in one arm of each chromosome and that one fragment usually detected several loci that are mapped to similar locations of different chromosomes. Furthermore, retrotransposons sharing high nucleotide sequence similarity were usually assigned to similar locations of the chromosomes. The distributional features of the *copia*-like elements in the rice genome suggest the existence of possible lineages among rice chromosomes, which in turn suggest that chromosome duplication and diversification may be a mechanism for the evolution of the rice chromosomes (Wang et al., 1999).

2.2.3 Transposition Cycle

The transposition cycle of long terminal repeat retrotransposons begins with the transcription of a full-length RNA bounded by the redundant R domain. Transcription is initiated in the 5'-LTR, at the boundary between the U3 and R domains, and is terminated in the 3'-LTR, at the boundary between the R and U5 domains (Fig. 3). The full-length

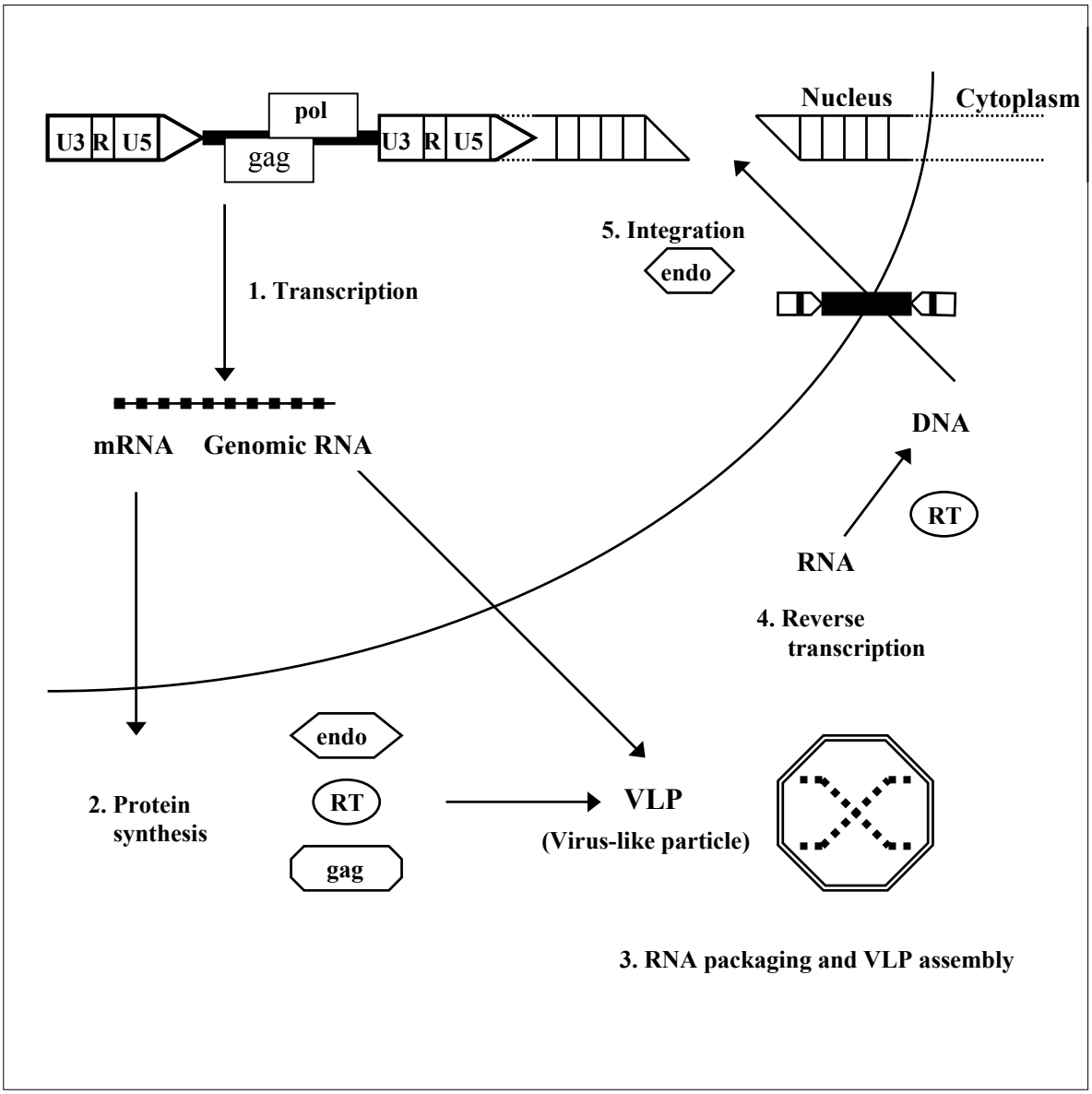


Figure 3. The transposition cycle of long terminal repeat (LTR) retrotransposons (adopted from Grandbastien, 1998)

RNA produced, serves both as a mRNA template for production of proteins needed for the completion of the transposition cycle, and as a template for reverse transcription (Grandbastien, 1998). The second step in the transposition cycle involves the synthesis of proteins, encoded by the full-length RNA, acting as a mRNA template. The structural *gag* protein is produced in large quantities because large quantities are required for the assembly of the virus-like particle (VLP). RNA packaging and VLP assembly is the step following protein synthesis. It involves activation of the protease, which catalyzes the maturation of the *gag-pol* polyproteins, thus ensuring the incorporation of functional proteins into the VLP. The mature protein products of *gag* and *pol* domains are then assembled into a virus-like particle, which packages the RNA transcript (Grandbastien, 1998). The fourth step in the transposition cycle involves the reverse transcription of the RNA transcript and synthesis of a double-stranded cDNA bounded by two LTRs identical to the mother ones. The last step, catalyzed by the *endo*-encoded protein, involves the integration and joining of the daughter retrotransposon into the cleaved host DNA. The mechanism by which the daughter retrotransposon is transported from the cytoplasm to the nucleus is not well understood (Grandbastien, 1998).

LTR retrotransposons cannot normally transfer themselves between adjacent cells, from one animal to another and even more certainly they cannot transfer horizontally from one species into another (Flavell, 1999). However, it has been postulated that horizontal transfer may be one effective strategy that transposable elements and other selfish genes implement to escape the host-mediated silencing mechanisms over the evolutionary time (Jordan et al., 1999). The most definitive evidence that transposable elements can transfer horizontally between species comes

from class II or DNA-type elements. The best examples of these are the *P* and *mariner* transposable elements of insects and other animals including *Drosophila* (Robertson and Lampe, 1995; Burke et al., 1998).

Jordan et al. (1999) reported that a *copia* LTR retrotransposon isolated from *Drosophila willistoni* was virtually identical in sequence to *copia* retrotransposons present within *Drosophila melanogaster* despite the fact that these two species have been separated from a common ancestor for about 50 million years. Total conservation of a retrotransposon sequence for a period 50 million years is not logical (Flavell, 1999) and therefore a recent jump between the two species is the most likely explanation for the above results (Jordan et al., 1999; Flavell, 1999). The mechanism by which horizontal transfers occur remains unclear; however, circumstantial evidence suggests that parasitic mites may be involved in vectoring DNA between the *Drosophila* species (Jordan et al., 1999). The most plausible candidate is the *Proctolaelaps regalis*, a mite with a broad host range that feeds on *Drosophila* eggs without necessarily killing them. The feeding habit of this organism suggests that small contents of one egg can be transferred to another. Such a procedure could easily induce the transfer of transposable elements from one species to another (Flavell, 1999).

2.3 *Ty1-Copia* Elements

The best understood group of LTR retrotransposons is the *Ty1-copia* group. This group was named after the most studied elements isolated from *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Finnegan, 1989). *Ty1-copia* elements are also the best-characterized group of retrotransposons in plants (Flavell et al., 1992b). A number of these elements have been fully sequenced, including *Ta1* of *Arabidopsis*

thaliana (Voytas and Ausubel, 1988), *Tnt1* of tobacco (Grandbastien et al., 1989), *Tst1* of potato (Camirand et al., 1990), and BARE-1 of barley (Mannienen and Schulman, 1993).

The *Ty1-copia* elements structurally resemble retroviral proviruses. They are characterized by long terminal repeats that flank their coding regions. These flanking sequences contain critical *cis*-acting signals. These signals include transcriptional promoters and 3' end formation signals as well as the sequences at the tip of the elements that are recognized by the transposon-encoded *IN* protein for the insertion of the *Ty* DNA into the host genome (Lauermann et al., 1997). Lauermann et al. (1997) investigated the effects and importance of LTR length on *Ty1* transposition and reported that transposition was dramatically inhibited when the total LTR length exceeded 500 base pairs (bp). In addition to that, once the LTR length reached 1000 bp there was a dramatic decrease in the efficiency with which the modified element was inserted into the genome. These results indicated that when the LTR length reached a value between 500 and 1000 bp the modified element dramatically lost its fitness.

Estimates of copy number of *Ty1-copia* elements within plant species have shown that the number of elements is highly variable between species (Pearce et. al., 1996). An in-depth study of the *Ty1-copia* group in the plant genus *Vicia*, which contains species with different genome sizes, indicated that the copy number of these elements is not related to genome size (Pearce et al., 1996). Wang et al. (1999), found that the copy number of *copia*-like elements in rice was as low as 100 per haploid genome compared to larger numbers that Grandbastien (1992 and 1994) reported. The restricted distribution

along with the low copy number, suggested that *copia*-like retrotransposons in rice were relatively inactive during its evolution compared with those in other plants (Wang et al., 1999).

The presence of *copia*-like retroelements among plants, insects, fungi, and protists suggests that this superfamily of LTR retrotransposons might be a universal component of eukaryotic genomes (Voytas et al., 1992). Using a polymerase chain reaction assay designed to detect the *RT* domains of *copia*-like elements, Voytas et al. (1992) was able to identify *copia*-like retrotransposons in 64 plant species. The plant species included representatives from 9 of 10 plant divisions. DNA sequence analysis of the cloned PCR products and of a maize retrotransposon cDNA confirmed the identity of the sequences investigated as *copia*-like *RT* domains (Voytas et al., 1992).

In a similar study, fragments of the *Ty1-copia* group retrotransposons were detected in a wide variety of members of the higher plant kingdom. 56 out of 57 species tested generated an amplified fragment of the size expected for the RT fragments of the *Ty1-copia* group (Flavell et al., 1992). The ubiquity of *copia*-like retrotransposons throughout the plant kingdom may be explained by assuming that the genome of the ancestor of plants also included similar retrotransposons and that during speciation events these elements were transmitted vertically along with other components of the genome. The presence of *copia*-like retrotransposons in all eukaryotic kingdoms supports this assumption and implies that this group of retrotransposons is very ancient (Voytas et al., 1992).

The *Ty1-copia* retrotransposon populations of barley (*Hordeum vulgare*) and bread wheat (*Triticum aestivum*) have also been characterized by sequence analysis of

fragments of the *RT* domains obtained by degenerate PCR. Both populations of the *Ty1-copia* group were found to be comprised of highly heterogeneous set of retrotransposons. The results of this study suggested that the ancestral Gramineae genome also contained a heterogeneous population of *Ty1-copia*, which proliferated to a different degree in the present species (Gribbon et al., 1999). Pearce et al. (1996) showed that the high sequence heterogeneity of plant *Ty1-copia* group is in contrast with that of *S. cerevisiae* and *D. melanogaster* and that the observed heterogeneity is directly correlated with element copy number.

Ty1-copia like sequences were also PCR amplified from sweetpotato [*Ipomoea batatas* (L.) Lam.] genomic DNA samples using *Ty1-copia* reverse transcriptase-specific primers (Villordon et al., 2000). After isolation, cloning, and sequencing of PCR fragments within the expected size range of *Ty1-copia* reverse transcriptase sequences, it was concluded that the amplified fragments were highly homologous to *Ty1-copia* like reverse transcriptase sequences in the GenBank database. Furthermore, PCR amplification from sweetpotato leaf cDNA using the same primers also yielded a *Ty1-copia* like fragment highly homologous to sequences in the GenBank (Villordon et al., 2000). The reported results by Villordon et al. (2000) were the first to prove the presence of *Ty1-copia* reverse transcriptase sequences in *I. batatas*.

2.4 Stress Activation

Evidence that transposable elements are ubiquitous in plants and that genetic instability may provide means for evolutionary and regulatory adaptation has been accumulating in recent years. McClintock (1984) postulated that genomic as well as other forms of stress might stimulate genome reorganization and the activation of transposable

elements, based on the hypothesis that transposition could increase the genetic variability necessary for organisms to adapt to different environmental conditions. The actual words from her 1983 Nobel acceptance speech, which was published a year later in Nature, were: “These elements are probably involved in the genome response to environmental challenges”. One possible source of such stress is the systemic virus infection in plants. Although viral symptoms are generally not heritable, several studies in the past have suggested that virally stressed plants may suffer unusual levels of genetic alterations (Mottinger et al., 1984b).

Descendants of virus-infected maize plants are characterized by an increased incidence of genetic instability, including higher frequencies of spontaneous mutations than uninfected control lines (Mottinger et al., 1984b). Genetic tests on mutations recovered from maize plants that were descendants of plants infected with *Barley stripe mosaic virus* (BSMV) and *Wheat streak mosaic virus* (WSMV) revealed that the causal agent of these mutations were inserted transposable elements. However, the relationship of transposition activity and genomic instability with virus infection and stress was not fully established (Dellaporta et al., 1984). To better understand the induction of mutations on the molecular level, Hirochika (1995) surveyed retrotransposons that are activated under viral stress. The study revealed that the *Tobacco mosaic virus* (TMV) activated the transcription of the *Tto1* element in tobacco. Furthermore, the activation of this retrotransposon was mediated by salicylic acid, which was also induced during the viral infection. When tobacco leaf sections were treated with salicylic acid, activation of the *Tto1* was observed in a dose-dependent manner. However, when leaves on the intact plant were sprayed with salicylic acid without wounding, no activation was observed.

These results suggested that, in addition to viral infection, wounding is necessary for the induction of salicylic acid and the activation of the *Tto1* element (Hirochika, 1995).

The most well characterized plant retrotransposons are activated by stress and environmental growth conditions (Grandbastien, 1998). They are particularly affected by protoplast isolation or *in vitro* cell or tissue culture (Table 2). The first direct evidence of stress activated retrotransposons comes from the discovery that the *Tnt1A* element of tobacco was highly active during protoplast isolation (Pouteau et al., 1991). The tobacco *Tto1* is also one of the few active LTR-retrotransposons in plants. Transposition of this element is activated by tissue culture and is primarily regulated at the transcriptional level. Transcription of the *Tto1* can also be activated by various stresses including viral infection, wounding, and treatment with methyl jasmonate, a signal molecule of plant defense response (Hirochika 1993; Hirochika 1995). Takeda (1999) showed that the *Tto1* LTR promoter is responsible for the high level of expression in cultured tissues of transgenic tobacco plants. In the same study Takeda also demonstrated that a 13 base pair repeat motif in the LTR functions as a *cis*-regulatory element that links the responsiveness to tissue culture, wounding, and methyl jasmonate treatments. In a similar study, Vernhettes et al., (1997) reported that the LTR U3 region of the *Tnt1* element was sufficient to mediate transcriptional activation by biotic and abiotic factors. Analysis of the U3 region revealed that a tandemly repeated sequence, named BII box is involved in the transcriptional activation of this element (Vernhettes et al., 1997).

Protoplast isolation as well as cell and callus culture cause major changes in the metabolism and gene expression of an organism. Conditions like these can also induce the expression of growth and defense related genes. The activation of a variety of plant

Table 2. Activity of *Ty1-copia* LTR retrotransposons (adapted from Grandbastien, 1998).

Classification	Species	Evidence of recent mobility	Evidence for transcripts
Subclass I: LTR retrotransposons Superfamily: **Ty1-copia**			
BARE-1	Barley	None	In leaves and callus
Hopscotch	Maize	Transposition into the *waxy* gene	None
Ji	Maize	None	In roots, leaves and tassels
Opie	Maize	None	In roots, leaves and tassels
Osser	Volvox carteri	Copy with identical LTRs	None
PREM-2	Maize	None	In early microspores
Prt1 and Prt3	Potato	None	In protoplasts
Prt4	Potato	None	In protoplasts
Prt5	Potato	None	In protoplasts
Prt6	Potato	None	In protoplasts
R9	Rye	None	In seedlings
SIRE-1	Soybean	None	In seedlings and leaf tissues
Stonor	Maize	Transposition into the *waxy* gene	None
Tnp2/Tnt1B	Nicotiana plumbaginifolia	Transposition into the *nia* gene in protoplast cultures	None
Tnt1A	Tobacco	Transposition into the *nia* gene in protoplast cultures; increase in copy number in cell culture	In roots, in protoplasts, and after wounding and pathogen attacks
Tos10	Rice	Copy number increase in cell cultures	In cell cultures
Tos17	Rice	Copy number increase and active transposition during cell and tissue culture	In cell cultures
Tos19	Rice	Small increase in copy number in cell cultures	In cell cultures
Tto1	Tobacco	Copy number increase during cell and tissue culture	In protoplasts, cell and tissue cultures, and after wounding and viral attacks
Tto2	Tobacco	Small increase in copy number in cell cultures	In protoplasts
Tto3	Tobacco	None	In protoplasts
Tto5	Tobacco	None	After viral infection
Wis-2	Wheat	Polymorphism in regenerated plants	In protoplasts

retrotransposons in these particular conditions may be linked to the activation of the cell division program or stress responses or both (Grandbastien, 1998). In a recent study, Melayah et al., (2001) reported that the mobility and insertion of the tobacco *Tnt1* element are directly linked to its activation by fungal factors, utilized in protoplast isolation. Studies with *Tnt1A* and *Tto1*, which are activated under a range of stress conditions including protoplast isolation, microbial compounds, wounding, as well as viral, fungal and bacterial infections, revealed that these elements are expressed in the same biotic and abiotic conditions that elicit the plant defense responses. However, the link between retrotransposon activation and plant defense response is still not fully established (Grandbastien, 1998).

The potato (*Solanum tuberosum*) genome contains a highly heterogeneous population of *Ty1-copia* retrotransposons (Pearce et al., 1996). These elements are not transcribed under normal growth conditions in potato. Fungal cell wall hydrolases used in protoplast isolation and a range of other microbial agents isolated from pathogenic fungi and bacteria have been shown to trigger the activation of these retroelements (Pearce et al., 1996).

Tissue culture of plants is an important means to propagate genetically identical individuals asexually, and also to produce transgenic plants (Hirochika et al., 1996). Sometimes, however undesired genetic modifications are induced during tissue culture. Recent work on transposable elements showed that these elements can be activated by tissue culture. More specifically, three retrotransposons of rice (*Tos10*, *Tos17*, and *Tos19*), which appeared to be inactive or almost inactive under normal growth conditions, were activated during tissue culture (Hirochika et al., 1996). Furthermore, the *Tos17*

element, which was the most active of the three, was found responsible for inducing high frequency mutations observed during tissue culture (Hirochika et al., 1996; Hirochika 1997). Hirochika (1993) has also shown that the *Tnt1* retrotransposon of tobacco, which is also activated by protoplast isolation, was activated during tissue culture.

Most eukaryotic genomes contain highly repetitive short interspersed elements (SINEs). SINEs, like other retrotransposons, transpose via an RNA intermediate. The *Alu* family of sequences is present in some genomes in an extraordinarily large copy number (Tzu-Huey Li et al., 1999). Despite the huge transcriptional potential of these elements, their expression in mammalian cultured cells is very low. However various cellular stresses, including heat shock and viral infection, can greatly increase the level of *Alu* RNA. Experiments conducted in mouse, which is an attractive model to examine the SINE RNA stress response *in vivo*, revealed that expression was significantly increased following hyperthermic shock treatments, in all tissue-types tested (Tzu-Huey Li et al., 1999). Similar results were also observed with the Silk worm *Bm1* SINE RNA, which was significantly increased following cellular insults such as heat shock (Kimura et al., 1999).

Abiotic stimuli associated with cell death or cell injury such as physical damages or chemical treatments also activate the expression of retrotransposons (Mhiri et al., 1997). Mhiri et al., (1997) showed that abiotic factors known to induce the plant defense response, including salicylic acid, $CuCl_2$, oxidative stress, and wounding, activated the transcription of the *Tnt1* element in tomato and *Arabidopsis thaliana*. In another study, the steady-state level of transcription of a repetitive element from *Nicotiana alata* with sequence similarity to *gypsy*-type retrotransposon was shown to significantly increase

following gentle touching of the style with a plastic rod. This element termed *Tna1-1* was a chimeric gene formed by the insertion of the *Tna1* element integrase domain into a gene expressed in response to pollination (Royo et al., 1995).

Growth of the plant pathogen *Fusarium oxysporum* in the presence of the toxic compound potassium chlorate caused the rearrangement of its retrotransposons (Anaya and Roncero, 1996). These elements, termed *Skippy*, contain all the conserved features of the *gypsy*-like superfamily of retrotransposons. Genetic analysis of the genomic DNA revealed amplification of the *Skippy* element yielding tandem arrays. In addition to that, the genetic analysis showed generation as well as deletion of the *Skippy* elements (Anaya and Roncero, 1996).

Viral infections can stimulate the synthesis of stress proteins that may act as components of proteolytic systems. Particular associations between viral and stress proteins have been documented (Menees and Sandmayer, 1996). Menees and Sandmayer (1996) examined the effects of the induction of cellular stress response on the transposition of the *Ty3* retrotransposon of *Saccaromyces cerevisiae.* The results clearly indicated that *Ty3* transposition was inhibited under stress conditions. Further detailed studies revealed that *Ty3* DNA was not synthesized and virus-like particles (VLP) did not accumulate. These results suggested that cellular stress proteins destroyed *Ty3* VLPs and therefore transposition was inhibited via that mechanism. The findings of Menees and Sandmayer (1996) compared to those of Melayah et al., (2001), which show direct linkage between stress activation and increased transposition, reveal that different stress treatments can have the exact opposite effects on the transposition of elements that belong to the same subclass.

Looking from the point of view of plant evolution, the relative inactivity of transposable elements during normal growth conditions and their activation by stresses is consistent with the genome-restructuring role that McClintock envisioned for these elements. Such a role is demonstrated by the *Tos17* element that after stress activation was found to be preferentially inserted into single copy sequences (Hirochika et al., 1996). Furthermore, detected retrotransposon sequences in regulatory regions of normal plant genes suggest that retrotransposons, such as *copia*-like elements, may have played this role in the past (Wessler, 1996).

2.5 Transposon Induced Mutations

Transposable elements can generate a significant portion of the spontaneous mutations observed in a number of organisms. The extensive history of genetic studies in morning glories can be a useful tool in better understanding the role of transposable elements as mutagenic agents. Inagaki et al., (1994) reported that a mutable phenotype in flowers of Japanese morning glory (*Ipomoea nil*), named *flecked*, was due to the excision of the *Tpn1* element from one of the anthocyanin biosynthesis genes, called *DFR*. These mutable phenotypes were shown to be transmitted to their progeny (Inagaki et al., 1996). Furthermore, sequences homologous to *Tpn1* were present in multiple copies in the genome of Japanese morning glory plants, suggesting that these particular elements may play an important role in the numerous other types of mutations observed (Inagaki et al., 1996).

Floricultural trait mutations observed in the common morning glory (*Ipomoea purpurea*), a close relative to the Japanese morning glory, result from both insertions and excisions of transposable elements from flower color and other related genes (Epperson

and Clegg, 1992). A recent literature review on the relation between floricultural traits of common morning glories and transposable elements by Iida et al., (1999) revealed that variants in flower color at three mutable alleles, termed *flecked*, *speckled*, and *flaked*, were caused by the integration of class II transposable elements into these genes. The *Tpn1* and *Tpn2* elements were identified as inserts in the *flecked* and *speckled* alleles respectively, whereas the *Tip100* element was identified in the *flaked* allele. The above-mentioned elements belong to the *En*/*Spm* family and are present in multiple copies in the genome of these plants (Iida et al., 1999).

A wide variety of mutant phenotypes, including embryo lethality, chlorophyll defects, defective seedlings, reduced fertility, altered leaf morphology, and aberrant flower or shoot, were detected in *Arabidopsis thaliana* (Altmann et al., 1995). To test whether these mutant phenotypes were the results of insertions or excisions of transposable elements, Southern blot experiments were performed on plants exhibiting the mutant phenotypes as well as on plants exhibiting the wild-type phenotypes. Analysis of the results revealed that all mutants tested carried transposon excision/transposition events (Altmann et al., 1995). Michel et al., (1995) reported that six mutations at the maize *Opaque2* (*O2*) locus were caused by the insertion of a transposon belonging to the *En*/*Spm* family, whereas two other mutations examined were the result of the activity of elements of the *Bergamo* (*Bg*) family.

In similar studies, six mutations of the *Scarlet* (*St*) locus of *Drosophila melanogaster*, two of which arose in laboratory populations and the rest from natural populations, were caused by the insertion of retroelements, some of which were identified as being members of the LINE family (ten Have et al., 1995). Interestingly, in two of the

mutants, the same transposable element was found to be inserted at exactly the same site within the *St* locus (ten Have et al., 1995). Juni et al., (1996) showed that *Optic morphology* mutations in *Drosophila ananassae*, which cause overgrowth of the eyes, are a group of retrotransposon-induced mutations. The retrotransposons involved in those mutations belonged to the *Tom* family.

The presence of transposable elements in humans has tremendous effects on genome function, its structure, and its evolution. The transposition or "reshuffling" of these elements can be the origin of genome reorganization but also the origin of a wide range of mutations (Deragon and Capy, 2000). The Human Genome Project has been an amazing source of information regarding the dynamics of transposable elements and their role in mutations. Deragon and Capy (2000) recently reviewed the literature related to the impact of transposable elements on the human genome. The implication of transposons in a variety of human diseases is profound. These elements, most of which belong to the *Alu* family, are involved in diseases such as breast and ovarian cancer, x-linked immunodeficiency, syndromes such as Alport's, as well as different types of muscular dystrophies. Furthermore, a list of other human diseases can also result from transposon-mediated recombination (Deragon and Capy, 2000).

Mutations in sweetpotato, which are poorly understood, are implicated as a component in cultivar decline. This phenomenon involves the gradual decline of the "fitness" of the cultivars, resulting in significant reduction of yield, poor resistance to pathogens and insect predators, and other undesirable traits (LaBonte et al., 2000). In sweetpotato, mutations are also likely to result from the activity of transposable elements. Sometimes, different DNA fingerprints of the same cultivar are observed, suggesting

among other things probable "reshuffling" of transposable elements. Extensive investigations of the relation between transposons and mutations observed in close relatives of the sweetpotato, such as the Japanese and common morning glory, preface the understanding of transposon-induced mutations in this particular organism (LaBonte et al., 2000).

2.6 Virus Diseases of Sweetpotato

Virus diseases in sweetpotato are a common form of stress. They are present wherever the crop is grown since it is vegetatively propagated and are implicated, as one of the main causal agents, in the phenomenon of cultivar decline (Salazar and Fuentes, 2000). Data show that viral infections can cause a significant decline in yield. Experiments with Beauregard, which has been the dominant cultivar in the U.S for more than ten years now, revealed that yields from virus-tested plants were significantly higher than from plants grafted with scions from farmer's plants. Even though the specific viruses responsible for the reduction were not determined, viral infection is directly implicated (Clark, 2002 personal communication). Viruses in sweetpotato can be transmitted mechanically through vegetative propagation. Furthermore, insects such as whiteflies and aphids have been shown to transmit the virus from plant to plant (Clark and Valverde, 2000). In the United States, the main but probably not the only important virus in sweetpotato is the *Sweet potato feathery mottle virus* (SPFMV). This virus has been shown to be present almost everywhere the crop is vegetatively propagated and grown. SPFMV shares biological characteristics similar to those of other potyviruses including its flexuous shape and its size (Salazar and Fuentes, 2000).

Several other viruses have been reported to infect sweetpotato throughout the world (Table 3). Due to poor communication between scientists some of the viruses, which are physiologically different, have the same name or were assigned to the same family (Salazar and Fuentes, 2000). Some of the most important viral diseases in sweetpotato from around the world involve viruses such as the *Sweet potato latent virus* (SPLV) reported from Taiwan, the *Sweet potato mild mottle virus* (SPMMV) from East Africa, the *Sweet potato leaf curl virus* (SPLCV) from Taiwan and Japan, and the *Sweet potato chlorotic stunt virus* (SPCSV) from Africa, Asia, and America (Salazar and Fuentes, 2000). The severity of symptoms on sweetpotato plants is dramatically increased with combined viral infections. The synergistic effect of SPFMV and SPCSV is well documented (Fig. 4). The symptoms from this specific viral combination are very severe, suggesting complex interactions between the two viruses against the plants defense response. However, the symptomatology seems to differ from one virus complex to another (Salazar and Fuentes, 2000).

Sweet potato feathery mottle (SPFMV) is a Potyvirus. It is 850nm long and has a flexuous shape. This aphid-transmitted virus is distributed worldwide and affects storage root yield and skin color (Salazar and Fuentes, 2000). *Sweet potato leaf curl virus* (SPLCV), which has been reported only from Japan and the United States, is a Bandavirus, has a flexuous shape, and is transmitted by whiteflies. (Salazar and Fuentes, 2000). It causes upward curling of leaves (not on 'Beauregard'), darker sweetpotato skin color, and up to 30% reduction in yields. Anecdotal evidence suggests that Sweet potato leaf curl virus also causes grooving on storage roots (Clark, 2000, personal communication). BWFT-3 is an isolate of *Sweet potato chlorotic stunt virus* (SPCSV). It

Table 3. Recognized viruses of sweetpotato (adapted from Salazar and Fuentes, 2000).

Virus	Shape and Size	Vector	Distribution
SPFMV Potyvirus	Flexuous 850 nm	Aphid	Worldwide
SPVMV Potyvirus	Flexuous 760 nm	Aphid	Argentina
SPV-II Potyvirus	Flexuous 750 nm	Aphid	Taiwan
SPMSV Potyvirus	Flexuous 800 nm	Aphid	Argentina, Peru, Indonesia, Philippines
SPLSV Luteovirus	Isometric 30 nm	Aphid	Peru, Cuba
SPMMV Ipomovirus	Flexuous 950 nm	Whitefly	Africa, Indonesia, Papua New Guinea, Philippines, India, Egypt
SPYDV Ipomovirus	Flexuous 750 nm	Whitefly	Taiwan
SPLCV Bandavirus	Bacilliform 130x30 nm	Whitefly	Taiwan, Japan, Egypt
SPLCV Geminivirus	Geminate	Whitefly	USA
SPCSV Crinivirus	Flexuous 850-950 nm	Whitefly	Africa, Asia, America
SPCSV? Potyvirus	Flexuous 850-900 nm	Unknown	Caribbean region, Kenya, Puerto Rico, Zimbabwe
SPCFV Potyvirus	Flexuous 750-800 nm	Unknown	Peru, Japan, Brazil, China, Cuba, Panama, Colombia, Bolivia, Indonesia, Philippines
SPVG Potyvirus	Flexuous	Unknown	Uganda, Egypt, India, China
SPCaLV Caulimovirus	Isometric 50 nm	Unknown	Puerto Rico, Madeira, Salomon Islands, Australia, Papua New Guinea
SPRSV Nepovirus	Isometric 25 nm	Unknown	Papua New Guinea
Reo-like	Isometric 70 nm	Unknown	Asia
Ilar-like	Isometric 30 nm	Unknown	Guatemala
C-6 Potyvirus	Flexuous 750-800 nm	Unknown	Uganda, Indonesia, Philippines, Peru

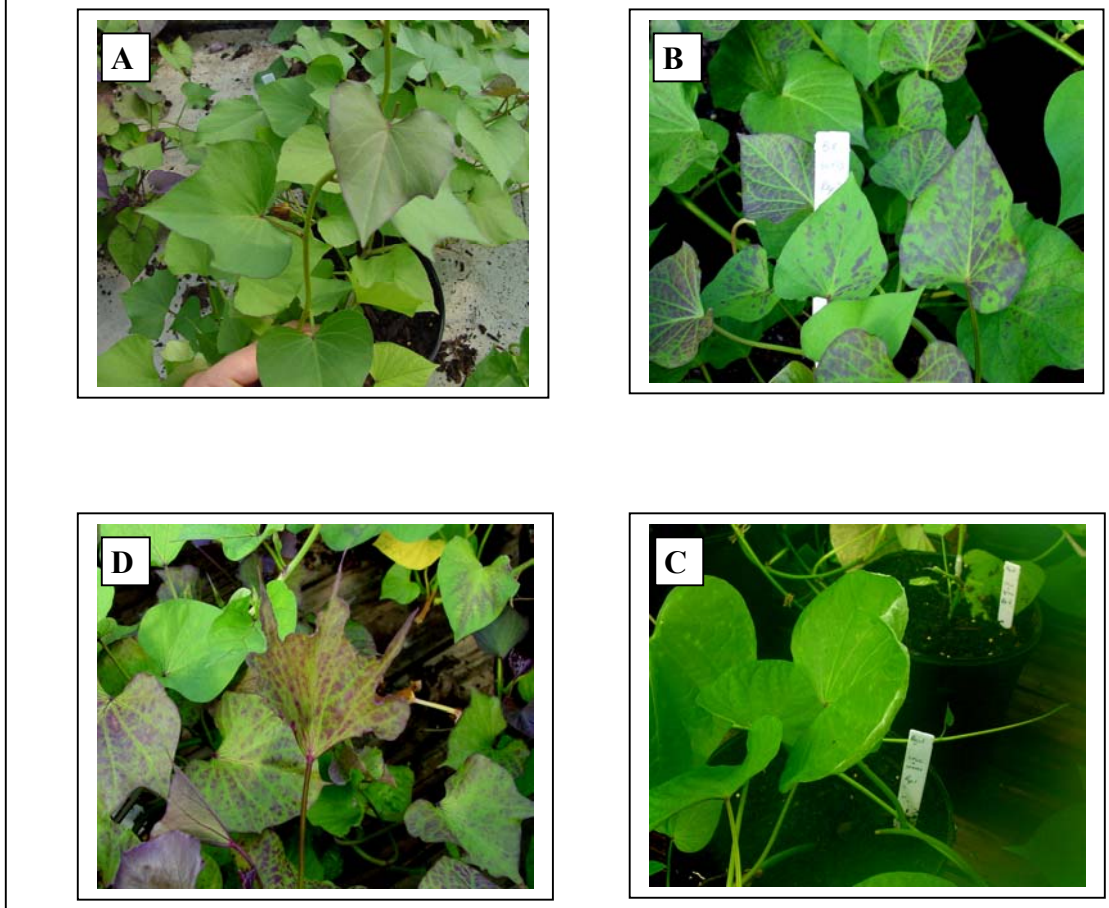


Figure 4. Symptoms of viral diseases on sweetpotato cultivars compared to virus-free plants. (A)Virus-free "Beauregard", (B) SPCSV on "Beauregard", (C) Synergistic effects of SPCSV + SPFMV on "94-96", (D) SPLCV + SPFMV on "Regal".

is a Crinivirus and is transmitted by whiteflies. It is found in countries of Africa, Asia, and South America (Salazar and Fuentes, 2000). SPCSV can cause significant yield reduction. However, in combination with SPFMV yields are drastically reduced as they also are with the SPFMV and SPCSV complex (Salazar and Fuentes, 2000).

2.7 Real-Time Quantitative PCR

The invention, more than a decade ago, of a revolutionary technique in molecular biology, called polymerase chain reaction (PCR), made lab work much faster and easier. Among other things, scientists were able to utilize this technology to detect, amplify and analyze even trace amounts of nucleic acids. A few years ago, in 1996, a new technology, the "predecessor" of PCR as it was characterized by some, was for the first time available to scientists (Zubritsky, 1999). This molecular technique, termed Real-Time Quantitative PCR, enabled researchers not only to detect and amplify a specific nucleic acid sequence but most importantly to quantify the starting amount of it. Since then Real-Time Quantitative PCR has been embraced by researchers and clinicians for gene quantitation (Leutenegger et al., 1999) e.g., in cancer research, pathogen detection (Zhang et al., 1999; Belgrader et al., 1999) e.g., in HIV therapy (Lewin et al., 1999), and even for pharmaceuticals applications such as process validation of different products (Zubritsky, 1999).

The development of Real-Time Quantitative PCR has also eliminated the variability in yield traditionally associated with PCR, by quantifying a PCR product during the cycles when it is first detected rather than the amount of the PCR product accumulated after a fixed number of cycles. This "real-time" system includes a fluorogenic probe in each amplification reaction (Fig. 5), an adapted thermal cycler to

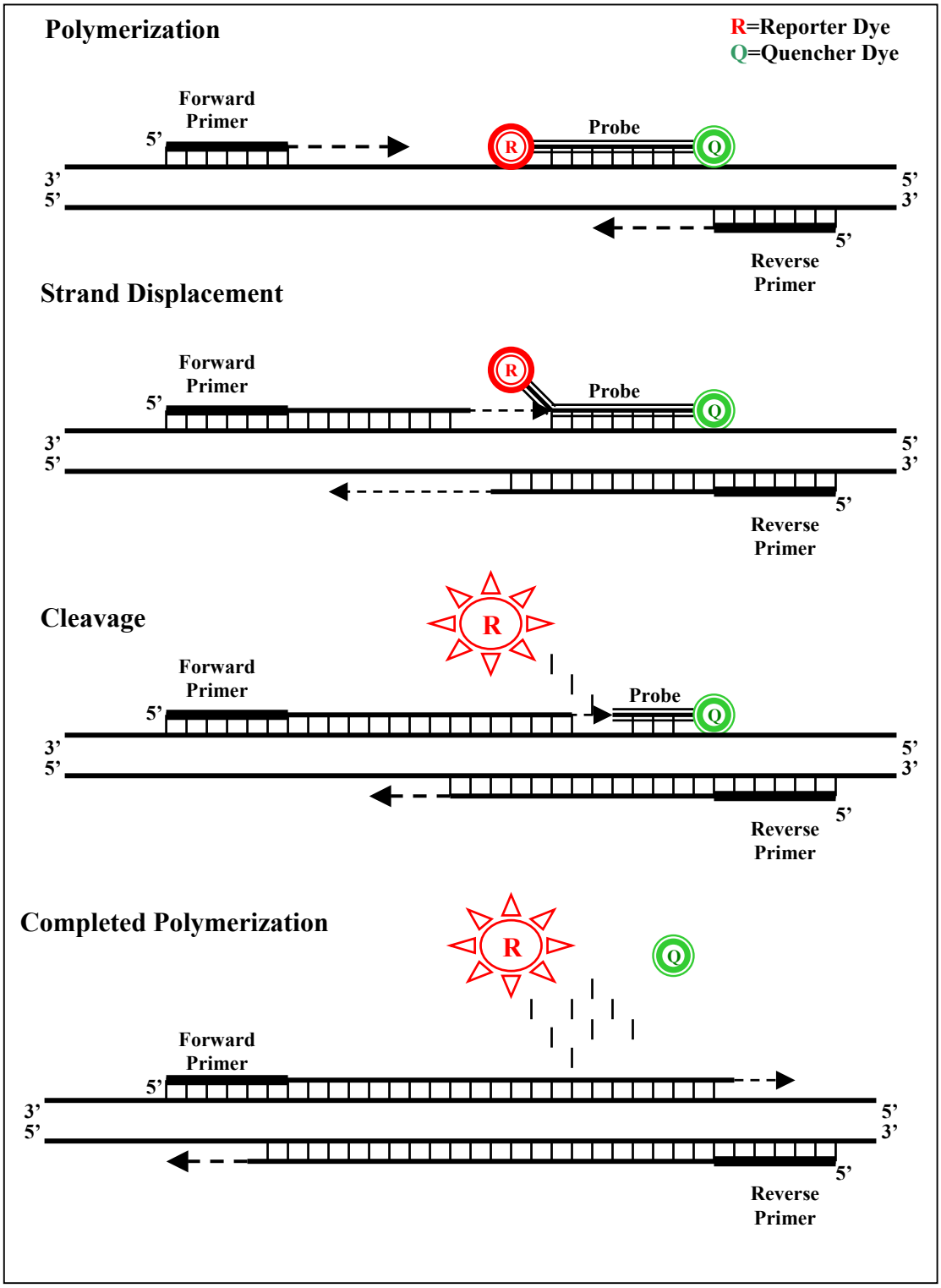


Figure 5. The 5'Nuclease activity and TaqMan® fluorogenic probe chemistry (adopted from AppliedBiosystems, Foster City, CA).

irradiate the samples with ultraviolet light, and a computer-controlled camera for the detection of the resulting fluorescence (AppliedBiosystems, Foster City, CA). As the amplification process produces increasing amounts of double-stranded DNA, the probe binds to the DNA strands resulting in an increase in fluorescence signal.

Figure 5 diagrammatically shows what happens to the TaqMan® fluorogenic probe during the extension phase of PCR. If the target sequence is present, the probe, which bears a reporter and a quencher dye on its 5′ and 3′ ends respectively, anneals downstream from one of the primer sites. The 5′ nuclease activity of *Taq* DNA polymerase will cleave the probe as the primer is extended. The cleavage and displacement of the probe from the target strand disrupts the proximity between the two dyes resulting in a fluorescence signal from the reported dye. Additional reporter dye molecules are cleaved from their respective probes with each PCR cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon (target) produced (AppliedBiosystems, Foster City, CA). By plotting the increase in fluorescence (ΔR_n) versus the PCR cycle number (C_T), the system produces plots that provide a more complete picture of the PCR process. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. Finally, the use of a pre-designed internal positive control reagent (i.e., 18S rRNA "household" gene) in parallel reactions to the target reactions allows the normalization of DNA/RNA extraction variations between samples (AppliedBiosystems, Foster City, CA).

CHAPTER 3

MATERIAL AND METHODS

3.1 Plant Material and Viral Inoculations

Virus tested 'Beauregard', and '94-96' were nodally propagated to generate the plant material used in this experiment. Clones of each genotype were subjected to the following treatments:

1. Control treatment composed of virus-free clones of each genotype [In this study, a virus-free plant is defined as a plant that has been tested three times via a scion graft onto *Ipomoea setosa* and that no symptoms appear on the indicator plant. This does not preclude the existence of unknown viruses that go undetected by *Ipomoea setosa*. Yet, current international guidelines document that graft-indexing successfully reveals most viruses (Moyer et al., 1989)].
2. Sweetpotato leaf curl virus (SPLCV), isolate SWFT-1, was graft inoculated on clones of each genotype.
3. Sweetpotato chlorotic stunt virus (SPCSV), isolate BWFT-3, was graft inoculated on clones of each genotype.
4. Sweetpotato feathery mottle virus (SPFMV), isolate 95-2, was graft inoculated on clones of each genotype.
5. SPFMV and SPCSV as a combined viral infection, graft inoculated on clones of each genotype.
6. SPLCV and SPFMV as a combined viral infection, graft inoculated on clones of each genotype.

Each treatment was replicated four times for each genotype. Cuttings from the two genotypes were planted in 15cm plastic pots filled with 3 pine bark: 1 peat moss: 1 sand (by volume) pot-plant media. Osmocote® Plus 15N-4P-10K slow release fertilizer

(The Scotts Company, Marysville, OH) as well as dolomitic lime was incorporated during the media preparation at a rate of 6kg and 4.8kg per cubic meter respectively. The plant samples were grown under standard greenhouse conditions. Daytime temperatures were held at approximately 30°C using a fan-and-pad evaporative cooling system, whereas a unit heater system was used to avoid temperatures below 18°C during cold nights. All treatments were subjected to the same watering regime. Plants were watered once a day to maintain adequate soil moisture. Weekly applications with the contact insecticide Avid® [active ingredient (%): Abamectin (1.9%)] (Novartis Inc, Greensboro, NC) and a monthly application with the systemic insecticide Merid® 0.5G [active ingredient (%): Imadacloprid (0.5%)] (Bayer Corporation, Kansas City, MO) were performed as a control measure against insect-vectors (whiteflies and aphids) which could transmit the virus from plant to plant treatment, confounding results. All insecticide applications were based on manufacturer's directions.

3.2 Viral Detection and Sample Collection

Virus infected sweetpotato may or may not produce symptoms on a given genotype, complicating detection and identification (Clark, 2000, personal communication). All plants were assessed for viruses using scions grafted onto *Ipomoea setosa* (Moyer et al., 1989). This species, which develops distinctive symptoms when infected with most sweetpotato viruses, was used to test for virus contamination in controls and to confirm successful infection following virus inoculations. Scions were removed from plants in the study for grafting onto *Ipomoea setosa* nine weeks after the initial viral graft inoculations. *Ipomoea setosa* was evaluated for virus symptoms three weeks later.

Twelve weeks after the initial graft inoculation, several leaves per treatment, including younger and older leaves, were harvested. The collection included leaves that were fully opened, from different points on the plant (top 5-8 nodes of each vine). This ensured that the harvested material included leaves that they were stressed due to viral infection. The leaf tissue was immediately frozen in liquid nitrogen to avoid any RNA degradation, and was later stored at -80°C until the RNA extractions were performed.

3.3 Plant RNA Extractions

Plant tissue, frozen in liquid nitrogen, was ground to a fine powder and total RNA was extracted using Qiagen's RNeasy Plant Mini Kit® (P/N 74904) according to the manufacturer's directions (Qiagen Inc, Valencia, CA). For this experiment, ~75mg of finely grounded leaf tissue was used to extract total RNA. To eliminate all possible residual DNA contamination, the RNA samples were on-column treated with DNase I using the RNase-Free DNase Set (P/N 79254) (Qiagen Inc, Valencia, CA). The DNase treatment was performed as an optional step during the RNA extraction based on manufacturer's directions (Qiagen Inc, Valencia, CA). Prior to construction and quantification of cDNA, using the Real-Time Quantitative PCR method, the following preliminary steps took place:

1. DNA contamination was assessed using *Ty1- copia* RT region primers (Tanaka et al 2001) in a specific PCR protocol (Villordon *et al*., 2000).
2. Samples were retreated with DNaseI until amplified fragments were no longer detected when separated by electrophoresis on an agarose gel.
3. An RNA sample was serially ten-fold diluted five times and the dilutions generated were used throughout the experiment to construct the standard curves,

used for the relative quantification and normalization of the samples. Each of five ten-fold diluted samples was given an arbitrary number (input ng amount). These numbers were: 10^6, 10^5, 10^4, 10^3, and 10^2 (from undiluted to most diluted sample).

3.4 PCR Primers and Methods for DNA Contamination Assessment

A set of primers, including a 5' and a 3' primer, (Invitrogen, Carlsbad, CA) was synthesized from a partial sequence of the RT region of a *Ty1-copia* retroelement, called Tib27 (AF223341), which was found to be present only in genomic DNA (Tanaka et al 2001). The primer sequences (forward and reverse respectively) used in PCR amplifications were: 5' GGGCTTTGAGGGAGAAAAAC 3' and 5' ACAAGAACGGCAATGAAAGA 3', also referred to as Tib27FWD and Tib27REV respectively. The Tib27 primers generate a ~230bp fragment. This fragment was cloned, sequenced, and confirmed to be a Tib27 partial RT region sequence. PCR amplifications were performed in 50µl reaction mixtures with 1 to 2µl template RNA, reaction buffer F (Epicenter Technologies, Madison, WI) that included 400µM of both dNTP and 7nM $MgCl_2$, 50pmol of each primer, and 1.5U of *Taq* DNA polymerase (Sigma, St. Louis, MO). Temperature cycling was performed on a Perkin-Elmer GeneAmp PCR System model 9600 (Perkin-Elmer, Norwalk, CT). The following PCR cycling parameters were used: initial denaturation at 94°C for 1 minute, followed by 30-35 cycles of denaturation at 94°C for 1 minute, annealing at 55-60°C for 1 minute, extension at 72°C for 2 minutes, followed by final extension setup at 72°C for 7 minutes. Amplified products were separated on 2% TAE agarose gels. DNA contamination in the form of the presence of a 230bp fragment signaled the initiation of repeated treatments with DNase I. A positive control containing a DNA template was included in all assessments. All RNA extracts

were assessed for DNA contamination. The elimination of any DNA contaminant in the total RNA samples was essential to prevent false positives and inaccurate quantification. However, no method is currently known to completely eliminate DNA contamination in RNA samples.

3.5 Real-Time Quantitative PCR

The relative quantification, at the transcriptional level, of the specific *Ty1-copia* target-retrotransposon was performed using the ABI PRISM® 7700 Sequence Detection System (AppliedBiosystems, Foster City, CA). For the amplification and relative quantification of the target, a set of primers (including a forward and a reverse primer) and a fluoregenic probe (MGB TaqMan® probe, P/N 4316094) were designed from a partial sequence of the RT region of another *Ty1-copia* retrotransposon, called Tib11 (AF223319) (Tanaka et al., 2001), using the Primer Express™ software for primer/probe design (AppliedBiosystems, Foster City, CA). Tib11 was found to be present in genomic DNA as well as a mRNA transcript in sweetpotato (Tanaka et al., 2001). The primer and probe sequences used in the real-time quantitative PCR were the following:

Forward Primer: GGTGAGCATTTGGTTTGCAA
Anneals between residues 71 and 90 with a Tm of 58°

Reverse Primer: GGCGGGAGGCTTGTTTTAAT
Anneals between residues 131 and 112 with a Tm of 59°

MGB TaqMan® Probe: 6-FAM-CTCAAGAAGTCCATTTAC-MGBNFQ
Anneals between residues 92 and 109 with a Tm of 69°

Every time a real-time PCR plate was constructed, parallel to the reactions designed for the relative quantification of the target in unknown samples using the above primers and probe, the same samples were used in reactions (on the same plate) using a pre-developed TaqMan® primer/probe internal positive control. The endogenous control,

an RNA gene that is always transcriptionally present, was used as an active reference for the normalization of quantitation differences of the messenger RNA target due to extraction variations. For this experiment, human 18S rRNA in the form of a 20x primer/probe mix (AppliedBiosystems, Foster City, CA) (P/N 4310893E) was used as an endogenous control for the normalization process. The probe had a VIC dye (Reporter dye) on its 5' and a TAMRA dye (Quencher dye) on its 3' end. The same procedure was also followed for the reactions involving the samples used for the construction of the two standard curves ("gene of interest" and "household gene" standard curves) (User Bulletin #2, AppliedBiosystems, Foster City, CA).

Real-time PCR quantifications of unknown and standard curve construction samples were performed in 50µl reaction mixtures with 5µl template, 900nM of each primer, 200nM of the MGB TaqMan® probe, 25µl of the 2x Master Mix without UNG (AppliedBiosystems, Foster City, CA), and 1.25µl the 40x MultiScribe™ and RNase inhibitor mix (AppliedBiosystems, Foster City, CA). The 2x and 40x mixes mentioned above were the components of the TaqMan® One Step PCR Master Mix Reagents kit (P/N 4309169) (AppliedBiosystems, Foster City, CA). The same protocol was used for the endogenous control reactions, except for the substitution of the primers and probe designed for the target with 2.5µl of the 18S rRNA pre-developed primer/probe mix.

The following real-time PCR thermal cycler conditions were used: 48°C for 30 minutes (cDNA construction), 95°C for 10 minutes (AmpliTaq Gold® activation), followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. These thermal cycling parameters were optimized for use with the TaqMan® One Step PCR Master Mix Reagents kit (AppliedBiosystems, Foster City, CA).

All reactions were performed in MicroAmp® optical tubes (P/N N801-0933) sealed with Optical Caps® (P/N 4323032) and placed on a MicroAmp® 96-well tray/retainer set (P/N 403081) (AppliedBiosystems, Foster City, CA). To minimize any errors due to pipetting differences between samples, duplicates of each sample were performed on each plate, and their obtained C_T values were averaged during data analysis.

3.6 Data Collection and Analysis

All experimental samples were normalized to the endogenous control using standard curves constructed for both the target and the endogenous reference. For each experimental sample, the amount of the target and the endogenous reference was determined from the appropriate standard curve. Next, the target amount was divided by the endogenous reference amount and a normalized target value (relative quantity) was obtained. Due to the fact that the ABI PRISM® 7700 Sequence Detection System (AppliedBiosystems, Foster City, CA) is not designed to construct two standard curves on the same plate, to analyze the raw data, "Results" from the "Export" option in the "File" menu was selected and data were exported to an excel spreadsheet. The exported file contained columns with the sample well number, sample description, standard deviation of the baseline, ΔR_n, and C_T value. The most important parameter for quantitation was the C_T value. The parameter C_T (threshold cycle) is defined as the functional cycle number at which the fluorescence passes the fixed threshold (Fig. 6).

For the construction of the standard curves from the data (Fig. 7), the following steps in Excel were performed (User Bulletin #2, AppliedBiosystems, Foster City, CA):

1. Using the Excel 2000 "Chart Wizard", an XY (scatter) plot was drawn with the Log input amount as the X values and average C_T as the Y values.

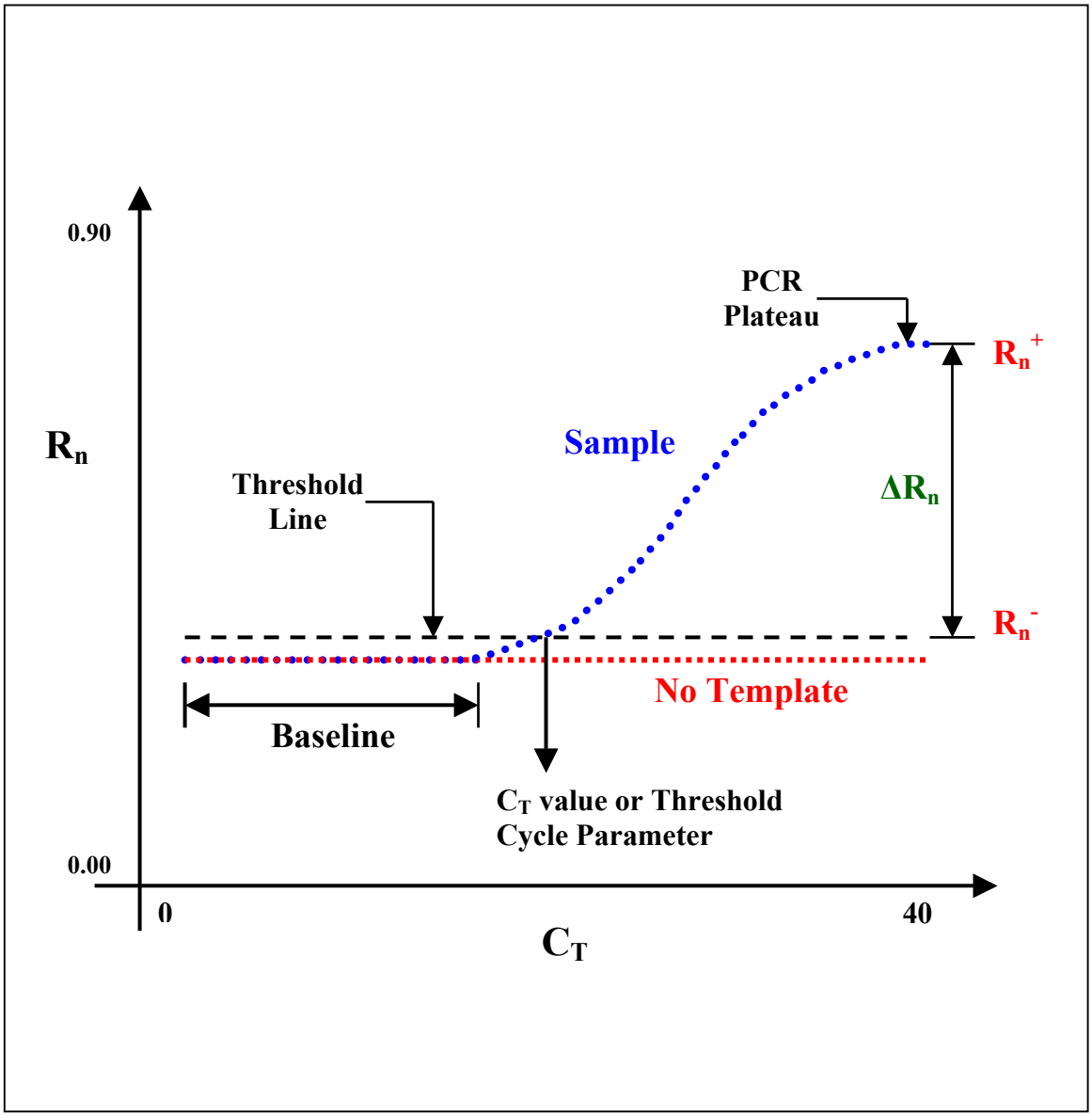


Figure 6. Model of a single amplification plot, showing terms commonly used in real-time PCR (Adopted from User Bulletin #2, AppliedBiosystems, Foster City, CA)

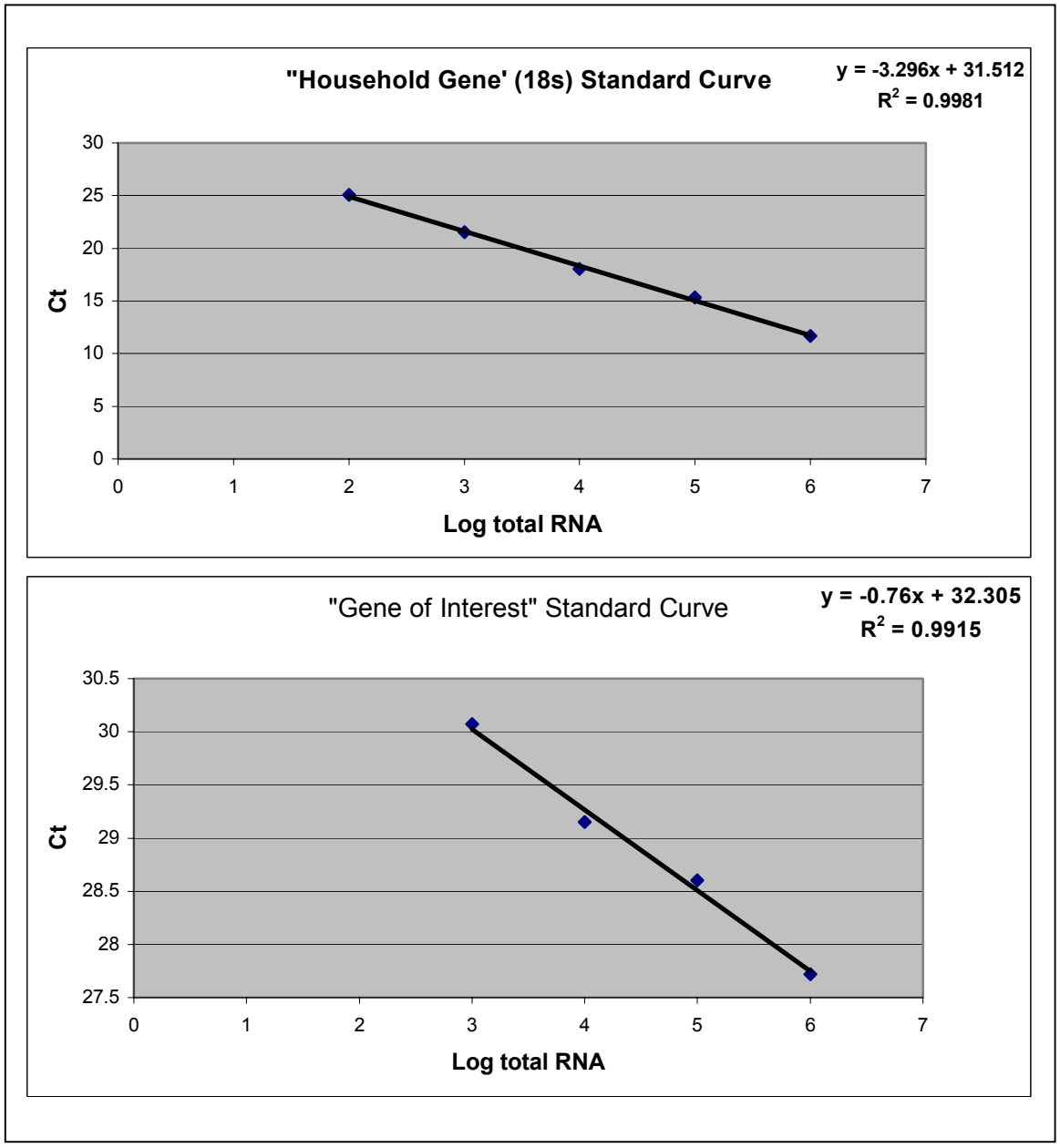


Figure 7. Representative standard curves for the "Household Gene" (18s rRNA) and the "Gene of Interest" (Tib11 retrotransposon).

2. Data points that appeared in graphical view were selected, the "Insert" menu was opened, and "Trendline" was selected to plot a line through the data points.
3. The "Type" page was accessed and "Linear regression" was selected.
4. The final step for the construction of the standard curves included the access of the "Options" page and the selection of the "Display Equation on Chart" and "Display R-squared Value on Chart" boxes.

After the construction of the two standard curves, the relative input amount for the unknown samples was calculated. By entering the slope and intercept values from the equation of the standard curve line, into the following formula the Log input was calculated (User Bulletin #2, AppliedBiosystems, Foster City, CA):

$$\textbf{Log input}=[(C_T \textbf{ value of unknown sample})-(\textbf{intercept})]/(\textbf{slope})$$

The Log input value was then used in the following formula to calculate the input amount for the unknown samples:

$$\textbf{Input amount}=10^{(\textbf{Log input amount})}$$

The units of the calculated relative amount were the same as the units used to construct the standard curve, which were termed nanograms. Once the same procedure was followed for the endogenous reference (18s rRNA), and both input amounts of the target and the endogenous reference were available for each sample, the normalized value of a sample was calculated by dividing the input amount for the target with the input amount of the endogenous reference (User Bulletin #2, AppliedBiosystems, Foster City, CA). To assess any significant difference in input amounts between samples of different treatments, the LSD statistical method (SAS Institute Inc, Cary, NC) was performed.

CHAPTER 4

RESULTS

4.1 Single Viral Infection Treatments

A total of three single viral infection treatments were assessed for their ability to induce transcription of the *RT* domain of the *Ty1-copia* retrotransposon Tib11 (Tanaka et al., 2001). Symptoms generated on plants of both inoculated cultivars were generally "milder" compared to those of combination inoculations. For both cultivars tested, "Beauregard" and "94-96", the three single viral inoculation treatments SPFMV, SPCSV, and SPLCV did not induce increased transcriptional activity of the Tib11 element. Relative amounts of transcripts from plants of the three single viral infection treatments, determined by Real-Time PCR, were statistically compared to relative amounts of transcripts from virus-free plants of the control treatment. A LSD T-test at the $p \leq 0.05$ level of significance showed that there was no significant difference between the single viral inoculation treatments and the control treatment (Table 4). Furthermore, statistical analysis revealed no significant difference in relative transcript amounts among the three viral treatments (SPFMV, SPCSV, and SPLCV). No significant difference was observed in relative transcript amounts for the two cultivars inoculated with the same virus, i.e., no significant treatment vs. cultivar effect (Table 4).

4.2 Combination Viral Infection Treatments

SPLCV + SPFMV and SPLCV + SPFMV were the two combination virus inoculation treatments used in this study to examine the synergistic effect of viruses on the transcriptional activation of the Tib11 retroelement. The symptoms generated by the SPCSV + SPFMV treatment were severe on both cultivars. They included symptoms such as discolored and severely misshapen leaves as well as symptoms of stunted growth.

Table 4. Effect of virus stress on relative Tib11 retrotransposon transcription levels in sweetpotato.

Treatment[1]	Replications[2]	Tib11[3]	Std Dev	LSD t-Grouping[4]
Control (Virus-free Plants)	8	0.0	±0.01	B
SPFMV	8	0.1	±0.18	B
SPLCV	8	0.1	±0.13	B
SPLCV+SPFMV	8	0.1	±0.11	B
BWFT-3 (SPCSV isolate)	8	7.1	±19.89	B
BWFT-3+SPFMV	8	1220.0	±2193.83	A

[1]Treatment abbreviations: Control=Virus-free plants; SPFMV=Sweet Potato Feathery Mottle Virus; SPLCV=Sweet Potato Leaf Curl Virus; SPCSV=Sweet Potato Chlorotic Stunt Virus.
[2]No significant cultivar vs. treatment effect. Means and Standard Deviations take in account replications for both cultivars inoculated with the same virus.
[3]Mean relative amounts of Tib11 transcripts
[4]Means with the same letter are not significantly different.

On the other hand, the second combination virus inoculation treatment, SPLCV + SPFMV, generated "milder" symptoms than those of the SPCSV + SPFMV treatment. Relative amounts of Tib11 transcripts from plants of the two combination viral infection treatments were statistically compared to those of the control and single viral infection treatments. Results from the LSD test performed at $p \leq 0.05$ level of significance showed that the relative amount of Tib11 transcripts from the treatment SPCSV + SPFMV was significantly greater in comparison to all other viral treatments and controls (Table 4). Finally, the combination viral infection treatment of SPLCV + SPFMV had no significant difference when statistically compared to all other treatments.

4.3 Statistical Analysis of Sub-samples

In this study all RNA samples were run as duplicates every time a Real-Time PCR run was performed. To assess any variation in cycle numbers (C_T) between a duplicated sample due to potential pippeting errors, the standard deviation of the C_T values for every duplicated sampled was calculated. The standard deviation for the 18S rRNA duplicated C_T values ranged from 0 to 0.431335 (18s rRNA C_T values ranged from 12.69 to 21.49). For the target (Tib11), the standard deviation for each pair of C_T values ranged from 0 to 2.56 (Tib11 C_T values ranged from 23.3 to 36.18). The standard deviations calculated for the 18S rRNA and Tib11 C_T values indicate that pippeting variation between duplicated samples was not a major variable in this experiment. Yet, when C_T values are converted to relative quantities, e.g., SPCSV + SPFMV replicates, logarithmic-scaled differences between sub-samples approached 100% in some replicates.

CHAPTER 5

DISCUSSION

McClintock (1984) suggested that transposable elements are "involved" in the genome response to environmental challenges. The genome response that McClintock talked about involves among other mechanisms, the transcriptional and transpositional activation of these elements. The majority of the transpositional activation of plant retrotransposons that has been characterized to date is stress-induced. Stress agents include viral infections, a common form of stress in plants. Even though virus-induced symptoms are not heritable, plants stressed by virus infection have been shown to suffer genetic alterations (Mottinger, 1984b). Hirochika (1995) revealed that *Tobacco mosaic virus* (TMV) activated the transposition of the *Tto1* element in tobacco. It was shown that salicylic acid, produced in response to virus infection, was involved in the activation of the particular retroelement. It is important to note that increased transposition of the *Tto1* element was dose dependent. Fungal cell wall hydrolasates as well as a range of microbial compounds, isolated from plant pathogenic organisms, also have been shown to trigger the transposition of various retroelements in a similar dose dependent fashion (Pearce et al., 1996)

In contrast, abiotic stresses, including physical and chemical wounding, protoplast isolation, and tissue culture, have been shown to have a different "effect" on retrotransposon activation. Transcriptional and transpositional activation under abiotic stresses is viewed as an "on/off" process rather than a dose dependent one (Mhiri, 1997; Anaya and Roncero, 1996). The Tib11 retrotransposon is an element that has been found to be active during normal cell conditions (in sweetpotato this element was detected in genomic DNA as well as a mRNA transcript) (Tanaka et al., 2001). Any observed increase in transcript amounts due to virus-induced stress, which is a biotic form of stress,

suggests that this element is more likely to be activated in a dose dependent manner. The word "dose" can be defined as either the amount of viral particles present in a particular treatment or the amount of secondary compounds induced by a particular viral presence.

This study showed that the combination viral infection treatment of SPCSV + SPFMV, which generated the most severe symptoms on both infected genotypes, induced a statistically significant increase in Tib11 transcription in sweetpotato compared to virus-free plants of the control treatment. Relative transcript amounts from the other treatments were not significantly different from transcript amounts of the control treatment. These results indicated that the transcriptional activation of the Tib11 retroelement was potentially associated with the stress severity observed on the plants. Supportive evidence comes from one of the eight repetitions of the SPCSV + SPFMV treatment. This particular repetition did not produce the severe symptoms that were characteristic of the other replicates, and it had lower relative transcript amounts. The high standard deviation for this combination viral inoculation treatment included the replicate with the "mild" symptoms and corresponding low relative transcription rate. Experiment wise, the high standard deviations are possibly a function of stress induction in individual plants. Yet, both cultivars behaved similarly showing no cultivar vs. treatment effect. To date, no mechanism exists to inoculate plants with a known amount of viral particles and therefore each of the repetitions likely received a different "dose" of viral particles. Hirochika (1995) found that the transposition of the *Tto1* element was dose dependent on salicylic acid, suggesting a "dose dependent" response to stress rather than an "on/off" activation response. The SPCSV + SPFMV replicate with the "mild"

symptoms and low transcription levels suggests "dose dependency" but cannot be substantiated. Only by quantifying both transposon transcription rates and virus titer can a correlated assessment be made.

Unlike previous research on retrotransposons, which focused on the assessment of stress-activation at the transpositional level, this research is unique in the sense that activation was assessed at the transcriptional level. Hirochika (1993, and 1995) assessed activation at the transpositional level by detecting and quantifying transposons before and after stress was applied. By using the Real-Time PCR, this study directly quantified relative amounts of transcripts from various viral stress treatments and compared them to those of virus-free, unstressed plants. Our results showed a low level of transcription in controls and most viral treatments and a high rate in the BWFT-3 + SPFMV treatment. In total, this data supports an "on/off" response to stress. Yet, transcription was always evident, but at a low level. This study was not designed to disclose dose dependent transcriptional activation. Variation in transcription among the "mild" and "severe" viral treatments was anticipated and not found. This study also did not examine the changes in transcription rates over time. It is not known how transcript rates change once a stress is applied. It is viewed (transposition) as a one time only event in the literature (Hirochika, 1993; Vernhettes, 1997; Mhiri, 1997).

In this study the assessment of the transcriptional activation of the Tib11 retroelement, does not necessarily suggest transpositional activation. This is supported by the fact that during replication retrotransposons use an enzymatic mechanism that lacks proofreading and therefore is of low fidelity. Furthermore, retrotransposon replication frequently is terminated prematurely, giving rise to copies that are "dead" on arrival

(Fedoroff, 1998). Melayah (2001) showed that the mobility of the tobacco *Tnt1* retrotransposon does correlate with its stress-induced transcriptional activation. Again, as with studies by Hirochika (1993 and 1995) no direct quantification of transcripts was taken. In the case of the Tib11 retroelement further research is required to show any relationship between its transcriptional activation and mobility in the sweetpotato genome.

CHAPTER 6

SUMMARY AND PROSPECTUS

Stress-induced transposon activation assessment represents an important and emerging approach to better understand mutations and the critical genetic mechanisms behind them. The current research showed, for the first time, the utility of using Real-Time PCR and TaqMan® chemistry for the relative quantification of retrotransposon transcripts and assessment of transcriptional activation in sweetpotato. These results showed that the combination viral infection treatment of SPCSV and SPFMV induced a significant increase in the transcriptional activity of the Tib11 retrotransposon in both sweetpotato cultivars tested. Other viral infection treatments tested had no significant difference in relative amounts of Tib11 transcripts when compared to those of virus-tested, nonstressed plants. Furthermore, our results showed no significant treatment vs. cultivar effect. The findings of this research will aid further studies aiming to better understand the genetic mechanisms behind the phenomenon of sweetpotato cultivar decline, i.e., viral stress may increase transposon transcription and subsequent transposition.

REFERENCES

Altmann, T., G. Felix, A. Jessop, A. Kauschmann, U. Uwer, H. Pena-Cortes, and L.Willmitzer. 1995. *Ac*/*Ds* transposon mutagenesis in *Arabidopsis thaliana*: mutant spectrum and frequency of *Ds* insertion mutants. Mol. Gen. Genet. 247:646-652.

Anaya, N., and M. I. G. Roncero. 1996. Stress-induced rearrangement of *Fusarium* retrotransposon sequences. Mol. Gen. Genet. 253:89-94.

Asins, M. J., A. J. Monforte, P. F. Mestre, and E. A. Carbonell. 1999. *Citrus* and *Prunus copia*-like retrotransposons. TAG 99:503-510.

Balint-Kurti, P. J., S. K. Clendennen, M. Dolezelova, M. Valarik, J. Dolezel, P. R. Beetham, and G. D. May. 2000. Identification and chromosomal localization of the *monkey* retrotransposon in *Musa sp.* Mol. Gen. Genet. 263:908-915.

Belgrader, P., W. Benett, D. Hadley, J. Richards, P. Stratton, R. Mariella Jr., and F. Milanovich. 1999. PCR detection of bacteria in seven minutes. Science 284:449-450.

Bennetzen, J. L. 2000. Transposable element contributions to plant gene and genome evolution. Plant Mol. Biol. 42: 251-269.

Brandes, A., J. S. Heslop-Harrison, A. Kamm, S. Kubis, and R. L. Doudrick. 1997. Comparative analysis of the chromosomal and genomic organization of *Ty1-copia*-like retrotransposon in pteridophytes, gymnosperms, and angiosperms. Plant Mol. Biol. 33: 11-21.

Burke, D. W., H. S. Malik, W. C. Lathe III, and T. H. Eickbush. 1999. Are retrotransposons long-term hitchhikers? Nature 392:141-142.

Cameron, J. R., E. Y. Loh, and R. W. Davis. 1979. Evidence for transposition of dispersed repetitive DNA families in yeast. Cell 16:739.

Camirand, A., B. St Pierre, C. Martineau, and N. Brisson. 1990. Occurrence of a copia-like transposable element in one of the introns of the potato starch phosphorylase gene. Mol. Gen. Genet. 224:33-39.

Capy, P., C. Bazin, D. Hiquet, and T. Langin. 1998. Dynamics of transposable elements. In: P. Capy, C. Bazin, D. Hiquet, and T. Langin (Eds) Dynamics and evolution of transposable elements. Landes Bioscience, Austin, TX.

Clark, C. A., and R. A. Valverde. 2000. Viruses and sweetpotato cultivar decline in Louisiana, USA. International Workshop on Sweetpotato Cultivar Decline Study. Miyakonojo, Japan.

Cribbon, B. M., S. R. Pearce, R. Kalendar, A. H. Schulman, L. Paulin, P. Jack, A. Kumar, and A. J. Flavell. 1999. Phylogeny and transpositional activity of *Ty1-copia* group retrotransposons in cereal genomes. Mol. Gen. Genet. 261:883-891.

Curcio, M. J., and D. J. Garfinkel. 1999. New lines of host defense: inhibition of *Ty1* retrotransposition by *Fus3p* and *NER/TF11H*. TIG 15:43-45.

Dellaporta, D. L., P. S. Chomet, J. P. Mottinger, J. A. Wood, S. M. Yu, and J. B. Hicks. 1984. Endogenous transposable elements associated with virus infection in maize. Cold Springs Harbor Symp. Quant. Biol. 43:321-328.

Deragon, J. M., and P. Capy. 2000. Impact of transposable elements on the human genome. Ann. Med. 32:264-273.

Dimitri, P., and N. Junakovic. 1999. Revising the selfish DNA hypothesis. TIG 15:123-124.

Domingo, E., and J. J. Holland. 1994. The evolutionary biology of viruses. ed. S. S. Morse Raven, New York. pp 161-184.

Eperson, B. K., and M. T. Clegg. 1992. Unstable white flower color genes and their derivatives in the morning glory. Journal of Heredity 83:405-409.

Federoff, N. 1989. Transposable elements as molecular evolutionary force. Annals New York Academy of Sciences. pp 251-559.

Finnegan, D. J. 1989. Eukaryotic transposable elements and genome evolution. TIG 5:103-107.

Finnegan, D. J. 1997. How non-LTR retrotransposons do it. Current Biology 7:R245-R248.

Flavell, A. J., E. Dubar, R. Anderson, S. R. Pearce, R. Hartley, and A. Kumar. 1992. *Ty1*-copia group retrotransposons are ubiquitous and heterogeneous in higher plants. Nucleic Acids Res. 20:3639-3644.

Food and Agricultural Organization. 1993. 1992 Production Yearbook. Food and Agricultural Organization statistics series, vol. 46, No. 112. Rome.

Grandbastien, M. A., A. Spielmann, and M. Caboche. 1989. *Tnt1*, a mobile retroviral-like transposable element of tobacco isolated by plant cell genetics. Nature 337: 376-380.

Grandbastien, M. A. 1992. Retroelements in higher plants. TIG. 8:103-108.

Grandbastien, M. A., C. Andeon, J. M. Casacuberta, P. Crappin, H. Lucas, C. Moreau, and S. Pouteau. 1994. Functional analysis of the tobacco *Tnt1* retrotransposon. Genetica 93:181-189.

Grandbastien, M. A. 1998. Activation of plant retrotransposons under stress conditions. Trends in Plant Science: Reviews 3:181-187.

Green, M. M. 1998. Mobile DNA elements and spontaneous gene mutation. Gold Spring Harbor Laboratory Press, New York. pp 41-50.

Hirochika, H., A. Fukuchi, and F. Kikuchi. 1992. Retrotransposon families in rice. Mol. Gen. Genet. 233:209-216.

Hirochika, H. 1993. Activation of tobacco retrotransposons during tissue culture. EMBO J. 12:2521-2528.

Hirochika, H., and R. Hirochika. 1993. *Ty1-copia* group retrotransposons as ubiquitous components of plant genomes. Jpn. J. Genet. 68:35-46.

Hirochika, H. 1995. Regulation of plant retrotransposons and their use for genome analysis. Gamma Field Symposia No. 34. Institute of Radiation Breeding, NIAR, MAFF.

Hirochika, H., K. Sugimoto, Y. Otsuki, H. Tsugawa, and M. Kanda. 1996. Retrotransposons of rice involved in mutations induced by tissue culture. Proc. Natl. Acad. Sci. USA. 93:7783-7788.

Hirochika, H. 1997. Retrotransposons of rice: their regulation and use for genome analysis. Plant Mol. Biol. 35: 231-240.

Iida, S., A. Hoshiro, Y. Johzuka-Hisatomi, Y. Habu, and Y. Inagaki. 1999. Floricultural traits and transposable elements in the Japanese and common morning glories. Annals New York Academy of Sciences. 870:265-274.

Inagaki, Y., Y. Hisatomi, T. Suzuki, K. Kasahara, and S. Iida. 1994. Isolation of a Suppressor-Mutator/Enhancer-like transposable element, *Tpn1*, from Japanese morning glory bearing variegated flowers. The Plant Cell 6:375-383.

Inagaki, Y., Y. Hisatomi, and S. Iida. 1996. Somatic mutations caused by excision of the transposable element, *Tpn1*, from the *DFR* gene for pigmentation in sub-epidermal layer of periclinal chimeric flowers of Japanese morning glory and their germinal transmission of their progeny. TAG 92:499-504.

Jiang, J., S. Nasuda, F. Dong, C. W. Scherrer, and S. Woo. 1996b. A conserved repetitive DNA element located in the centromeres of cereal chromosomes. Proc. Natl. Acad. Sci. USA. 93:14210-14213.

Jordan, K., L. V. Matyunina, and J. F. McDonald. 1999. Evidence for recent horizontal transfer of long terminal repeat retrotransposons. PNAS 96:12621-12625.

Juni, N., T. Awasaki, K. Yoshida, and S. H. Hori. 1996. The *Om* (*1E*) mutation in *Drosophila ananassae* causes compound eye overgrowth due to *tom* retrotransposon-driven overexpression of a novel gene. Genetics 143:1257-1270.

Katz, R. A., and A. M. Skalka. 1990. Control of retroviral RNA splicing through maintenance of suboptimal processing signals. Molecular and Cellular Biology 10:696-704.

Kidwell, M. G., and D. Lisch. 1997. Transposable elements as sources of variation in animals and plants. Proc. Natl. Acad. Sci. USA. 94:7704-7711.

Kimura, R. H., P. V. Choudary, and C. W. Schmid. 1999. Silk worm Bm1 SINE RNA increases following cellular insults. Nucleic Acids Res. 27:3380-3387.

LaBonte, D. R., and J. M. Cannon. 1997. Production and utilization of sweetpotato in the United States. International Workshop on Sweetpotato Production System toward the 21st Century. Miyakonojo, Japan.

LaBonte, D. R., A. Q. Vollordon, and D. S. Fajardo. 2000. Mutations in sweetpotato. International Workshop on Sweetpotato Cultivar Decline Study. Miyakonojo, Japan.

Langdon, T., C. Seago, M. Mende, M. Leggett, H. Thomas, J. W. Foster, H. Thomas, R. N. Jones, and G. Jenkins. 2000. Retrotransposon evolution in diverse plant genomes. Genetics 156:313-325.

Lauermann, V., M. Hermankova, and J. D. Boeke. 1997. Increased length of long terminal repeats inhibits *Ty1* transposition and leads to formation of tandem multimers. Genetics 145:911-922.

Leutenegger, C. M., C. N. Mislin, B. Sigrist, M. U. Ehrengruber, R. Hofmann-Lehmann, and H. Lutz. 1999. Quantitative real-time PCR for the measurement of feline cytokine mRNA. Veterinary Immunology and Immunopathology 71:291-305.

Lewin, S. R., M. Vesanen, L. Kostrikis, A. Hurley, M. Duran, L. Zhang, D. D. Ho, and M. Markowitz. 1999. Use of real-time PCR and molecular beacons to detect virus replication in human immunodeficiency virus Type1-infected individuals on prolonged effective antiretroviral therapy. Journal of Virology 73:6099-6103.

Li, T. H., J. Spearow, C. M. Rubin, and C. W. Schmid. 1999. Physiological stresses increase mouse short interspersed element (SINE) expression *in vivo*. Gene 239:367-372.

Liebman, S. W., and G. Newman. 1993. A ubiquitin-conjugating enzyme, RAD6, affects the distribution of *Ty1* retrotransposon integration positions. Genetics 133:499-508.

Lyubomirskaya, N. V., and Y. V. Ilyin. 1999. Eukaryotic mobile genetic elements: the Past, the Present, and the Future. Molecular Biology 33:846-853.

Mannienen, I., A. H. Schulman. 1993. *Bare-1*, *copia*-like retroelement in barley (*Hordeum vulgare*). Plant Mol. Biol. 22:829-864.

McClintock, B. 1956. Mutations in maize. Carnegie Inst. Wash. Year Book. 55:323-332.

McClintock, B. 1984. The significance of responses of the genome to challenge. Science 226:792-801.

Melayah, D., E. Bonnivard, B. Chalhoup, C. Andeon, and M. A. Grandbastien. 2001. The mobility of the tobacco *Tnt1* retrotransposon correlates with its transcriptional activation by fungal factors. The Plant Journal 28:159-168.

Menees, T. M., and S. Sandmeyer. 1996. Cellular stress inhibits transposition of the yeast retrovirus-like element *Ty3* by a ubiquitin-dependent block of virus-like particle formation. Proc. Natl. Acad. Sci. USA. 93:5629-5634.

Mhihri, C., J. B. Morel, S. Vernhettes, J. M. Casacuberta, H. Lucas, and M. A. Grandbastien. 1997. The promoter of the tobacco *Tnt1* retrotransposon is induced by wounding and by abiotic stress. Plant Mol. Biol. 33:257-266.

Michel, D., H. Hartings, S. Lunzini, M. Michel, and M. Motto. 1995. Insertion mutations at the maize *Opaque2* locus induced by transposable element families *Ac*, *En/Spm* and *Bg*. Mol. Gen. Genet. 248:287-292.

Miller, J. T., F. Dong, S. A. Jackson, J. Song, and J. Jiang. 1998. Retrotransposon-related DNA sequences in the centromeres of grass chromosomes. Genetics 150:1615-1623.

Moore, G., W. Cheung, T. Schwarzacher, and R. Flavel. 1991. *BIS1*, a major component of the cereal genome and a tool for studying genomic organization. Genomics 10:469-476.

Mottinger, J. P., M. A. Johns, and M. Freeling. 1984b. Mutations of the Adh1 gene in maize following infection with barley stripe mosaic virus. Mol. Gen. Genet. 195:367-369.

Moyer, J. W., G. V. H. Jackson, and E. A. Frison. 1989. FAO/IBPGR technical guidelines for the safe movement of sweetpotato germplasm. FAO/IBPGR. Food and Agriculture Organization of the United Nations, Rome/ International Board for Plant Genetic Resources, Rome.

Noma, K., E. Ohtsubo, and H. Ohtsubo. 1999. Non-LTR retrotransposons (LINEs) on ubiquitous components of plant genomes. Mol. Gen. Genet. 261:71-79.

Pardue, M. L., O. N. Danilerskaya, K. Lowenhaupt, F. Slot, and K. L. Traverse. 1996. Drosophila telomeres: new views on chromosome evolution. TIG 12:48-52.

Pearce, S. R., A. Kumar, and A. J. Flavell. 1996. Activation of the *Ty1*-copia group retrotransposons of potato (*Solanum tuberosum*) during protoplast isolation. Plant Cell Reports 15:949-953.

Pearce, S. R., G. Harrison, D. Li, J. S. Heslop-Harrison, A. Kumar, and A. J. Flavell. 1996. The *Ty1-copia* group retrotransposons in *Vicia* species: copy number, sequence heterogeneity and chromosomal localization. Mol. Gen. Genet. 250:305-315.

Pelissier, T., S. Tutois, S. Tourmente, J. M. Deragon, and G. Picard. 1996. DNA regions flunking the major *Arabidopsis thaliana* satellite are principally enriched in *Athila* retroelement sequences. Genetica 97:141-151.

Pouteau, S., S. Huttner, M. A. Grandbastien, and M. Caboche. 1991. Specific expression of the tobacco *Tnt1* retrotransposon in protoplasts. EMBO J. 10:1911-1918.

Preston, B. 1996. Error prone retrotransposition: Rime of the ancient mutators. Proc. Natl. Acad. Sci. USA. 93:7427-7431.

Rabinowicz, P. D. 2000. Are obese plant genomes on a diet? Genome Research 10:893-894.

Robertson, H. M., and D. J. Lampe. 1995. Distribution of transposable elements in anthropods. Annual Reviews 40:333-357.

Royo J., N. Nass, D. P. Matton, S. Okamoto, A. E. Clarke, and E. Newbigin. 1997. A retrotransposon-like sequence linked to the S-locus of *Nicotiana alata* is expressed in styles in response to touch. Mol. Cen. Genet. 250:180-188.

Rubatzky, V. E., and M. Yamaguchi. 1997. World vegetables: principles, production, and nutritive values. 2nd ed. Chapman and Hall, New York.

Salazar, L. F., and S. Fuentes. 2000. Current knowledge on major virus diseases of sweetpotatoes. In: Proceedings of International Workshop on Sweetpotato Cultivar Decline, Miyakonojo, Miyazaki, Japan.

Sandmeyer, S. B., L. T. Hansen, and D. L. Chalter. 1990. Integration specificity of retrotransposons and retroviruses. Annu. Rev. Genet. 24:491-518.

SanMiguel, P., A. Tikhonov, Y. K. Jin, N. Motchoulskaia, D. Zakharov, A. Melake-Berham, P. S. Springer, K. J. Edwards, M. Lee, Z. Avramova, and J. L. Bennetzen. 1996. Nested retrotransposons in the intergenic regions of the maize genome. Science 274:765-768.

Takeda, S., K. Sugimoto, H. Otsuki, and H. Hirochika. 1999. A 13-bp *cis*-regulatory element in the LTR promoter of the tobacco retrotransposon *Tto1* is involved in responsiveness to tissue culture, wounding, methyl jasmonate and fungal elicitors. The Plant Journal 18:383-393.

Tanaka, M., M. Yoshinaga, Y. Nakazawa, and M. Nakatani. 2001. Identification of a novel SINE-like sequence in retrotransposon fragments of sweetpotato. Plant Biotechnology 18:157-161.

Tanda, S., A. E. Shrimpton, C. Ling-Ling, H. Itayama, H. Matsubayashi, K. Saiga, Y. N. Tobari, and C. H. Langley. 1988. Retrovirus-like features and site specific insertions of a transposable element, *Tom* in *Drosophila ananassae*. Mol. Gen. Genet. 214:405-411.

tenHave, J. F. M., M. M. Green, and A. J. Howells. 1995. Molecular characterization of spontaneous mutations at the *scarlet* locus of *Drosophila melanogaster*. Mol. Gen. Genet. 249:673-681.

Vernhettes, S., M. A. Grandbastien, and J. M. Casacuberta. 1997. *In vivo* characterization of transcriptional regulatory sequences involved in the defense-associated expression of the tobacco retrotransposon *Tnt1*. Plant Mol. Biol. 35:673-679.

Villordon, A. Q., R. L. Jarret, and D. R. LaBonte. 2000. Detection of *Ty1-copia*-like reverse transcriptase sequences in *Ipomoea batatas* (L.) Poir. Plant Cell Reports 19:1219-1225.

Voytas, D. F., and F. M. Ausubel. 1988. A *copia*-like transposable element family in *Arabidopsis thaliana*. Nature 336:342-344.

Voytas, D. F., M. P. Cummings, A. Konieczny, F. M. Ausubel and S. R. Rodermel. 1992. *Ty1*-copia like retrotransposons are ubiquitous among plants. Proc. Natl. Acad. Sci. USA. 89:7124-7128.

Wallace, H. L., C. A. Clark, and D. R. LaBonte. 1999. Effect of viral infection and clonal variability on sweetpotato yield and quality. HortScience 34:528.

Wang, S., N. Liu, K. Peng, and Q. Zhang. 1999. The distribution and copy number of *copia*-like retrotransposons in rice (*Oryza sativa* L.) and their implications in the organization and evolution of the rice genome. Proc. Natl. Acad. Sci. USA. 96:6824-6828.

Wessler, S. R., 1996. Plant retrotransposons: Turned on by stress. Current Biology 6:959-961.

Wright, D. A., N. Ke, J. Smalle, B. M. Hauge, H. M. Goodman, and D. F. Voytas. 1996. Multible non-LTR retrotransposons in the genome of *Arabidopsis thaliana*. Genetics 142:569-578.

Zhang, A. W., G. L. Hartman, B. Curio-Penny, W. L. Pedersen, and K. B. Becker. 1999. Molecular detection of *Diaporthe phaseolorum* and *Phomopsis longicola* from soybean seeds. Phytopathology 890:796-804.

Zubritsky, E. 1999. Pinning down PCR. Analytical Chemistry News and Features 3:191A-195A.

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