BIOTECHNOLOGY AND ITS USES IN IMPROVEMENT OF CANKER RESISTANCE IN CITRUS TREES

By

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Dedication

To the memory of my mother who I will never ever forget her undying love and encouragement during all the steps of my life.

To the memory of my brother Ragab who taught me that dreams are to be pursued and challenges to be met.

To my father and my family who give me unconditional support and encouragement during all the steps of my life.

To my wife Azza

To my beloved daughter whose smile gives me a reason to wake up everyday.

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BIOTECHNOLGY AND ITS USES IN IMPROVEMENT OF CANKER RESISTANCE IN CITRUS TREES

By

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'Hamlin' sweet orange (*Citrus sinensis* (L.) Osbeck) is one of the leading commercial cultivars in Florida because of its high yield potential and early maturity. 'Hamlin' also has a high regeneration capacity from protoplasts and is often used in transformation experiments. Citrus canker disease caused by the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* is becoming a worldwide problem. The *Xa21* gene is a member of the *Xa21* gene family of rice that provides broad spectrum *Xanthomonas* resistance in rice. Since the citrus canker pathogen is in the same genus, this gene may have the potential to function against canker in citrus. The wild-type *Xa21* gene contains an intron, and there is some question as to whether dicot plants can process genes containing monocot introns (the cDNA is intron free). The development of canker resistant citrus has become an important research objective.

Genetic transformation has become a widespread tool in both basic research and commercial plant breeding programs for disease resistance. Plasmid DNA (pARS108)

encoding the non-destructive selectable marker Green Fluorescent Protein (*GFP*) gene, and the plasmid cDNA of the *Xa21* gene (pCR506-mtaq) were co-transformed into 'Hamlin' orange protoplasts using polyethylene glycol. Also, plasmid DNA (pAO3), encoding the *GFP* gene and the cDNA of the *Xa21* gene, was transformed into 'Hamlin' orange protoplasts. Following protoplast culture in liquid medium and transfer to solid medium, transformed colonies were microscopically selected via expression of *GFP*, physically separated from non-transformed tissue, and cultured on somatic embryogenesis induction medium. More than 150 transgenic embryoids were recovered. Over a thousand transgenic plantlets were regenerated from about 80 independent transformation events. PCR analysis revealed the presence of the cDNA of the *Xa21* and the *GFP* genes in some of the transgenic plantlets. The recovery of multiple transgenic plants was expedited by in vitro grafting. The transgenic plants have shown normal growth and stable *GFP* expression for over a year in the greenhouse. Transgenic greenhouse plants include 400 growing on different rootstocks and over 200 plants on their own roots. This is the first time to report a large population of transgenic 'Hamlin' sweet orange plants using protoplast/*GFP* transformation system. PCR analysis revealed the presence of the cDNA of the *Xa21* and the *GFP* genes in the transgenic greenhouse plants. Some of the plants have only *GFP*. Southern analysis shows integration of the cDNA into different sites ranging from 1-5 sites per plant. Real-Time PCR shows integration of the cDNA into different sites in citrus genome ranging from 1-4 copies per plant. Western analysis shows the expression of the cDNA of the *Xa21* gene in the transgenic citrus plants. This is the first time that a gene from rice has been stably integrated and expressed in citrus plants.

CHAPTER 1
INTRODUCTION

The importance of emerging biotechnologies in the agriculture industry and the desire to apply science to benefit society were among the goals of this study. The significance of the potential benefits biotechnology-based science offers can be appreciated by considering the tremendous progress in plant improvement that has been made in recent years. Recent advances in molecular genetics, informatics, and genomics research have created many new possibilities for applying biotechnology in agriculture. The promise of biotechnology as an instrument of development lies in its capacity to improve the quantity and quality of plants quickly and effectively. The application of biotechnology holds great potential for creating plants that are more drought resistant, more tolerant to acidic and saline soils, more resistant to pests (thereby reducing pesticide use), and more resistance to biotic stresses including viral, fungal, and bacterial diseases. Using biotechnology tools in the agriculture industry can help to supply the world, especially the developing countries with enough high quality food and innovative pharmaceutical products, goals that are difficult to achieve by classical methods.

Genetic engineering of agronomically and horticulturally important species is creating a new era in agriculture with the first generation of achievements in herbaceous plants already in commercial use and greater prospects on the horizon. The potential of genetic improvement is of great interest to growers of woody fruit plants. Most commercial varieties are propagated vegetatively and are hybrids of unknown origin or budsports that have been selected by growers and essentially cannot easily be improved

by traditional breeding methods due to the aspects of their biology including high heterozygosity. Even when traditional breeding may be effective for crop improvement, there is always a long period of time between generations. Genetic engineering allows the insertion of specific genes into the genome of currently successful varieties, theoretically adding desirable traits without otherwise altering cultivar integrity.

Citrus fruits taste good and are a well-known source of vitamin C and antioxidants. Moreover, several epidemiological studies have shown that citrus fruits and their components are protective against a variety of human cancers (Tian et al., 2001; Manthey and Guthrie, 2002; Rafter, 2002; Kim et al., 2005). Processed citrus peel and pulp is a valuable, high energy by-product that can partly replace cereal grains in animal diets without adverse effect on their products (i.e., milk in terms of yield or composition) (Fegeros et al., 1995). Because of its nutritional relevance, citrus is an important industry worldwide, raising economies at macro and local levels by supporting social development directly with jobs and secondary industries and services. Citrus fruits are produced in many countries around the world, although production shows geographical concentration in certain areas, primarily within tropical and subtropical regions (within 40° north-south latitude). Mediterranean countries are the leading producers for the international fresh market. The total production has systematically increased in the last four decades, and, more recently reached more than one hundred million tons yearly (Table 1-1) (Anonymous, 2005). It is not only the quantities of citrus that are important, but also the quality of the fruit. Implementation of modern technology in citrus production has improved efficiency, flexibility, and resulted in high quality standards. As a result of

trade liberalization and technological advances in fruit transport and storage, the citrus fruit industry has become more global in scope.

The exact center of origin of citrus is not clearly identified, although most researchers place it in South-East Asia, at least 4000 years BC (Davies and Albrigo, 1994). The spread of citrus fruits from Asia to Europe was initially slow. The Arabs introduced the citron, the sour orange, the lemon, and the shaddock into Spain and the countries of North Africa (Reuther et al., 1967). Probably after the fall of the Roman Empire, citrus was introduced to the South of Europe, and its availability expanded in the Middle Ages. Citrus was first brought to the new world by the Spanish and Portuguese explorers at the beginning of the fifteen century (Allen, 2000). By the second half of the nineteen century, the fresh fruit companies had been established, and the frozen concentrated technology, developed in the 1940s in Florida (Lewandowski, 2000), increased the demand for citrus juices.

To achieve and maintain an adequate level of quality, many changes in production systems were necessary to meet the ongoing needs of growing markets and the demands of new challenges, such as changes in biological concerns, unexpected drought and cold stresses, outbreaks of pests and diseases, and establishment of economic and political barriers. Consequently, production constraints have been overcome by the use of grafted plants to replace seedlings, changes in rootstocks, and selection of new cultivars. The application of biotechnology tools such as somatic hybridization, somaclonal variation, embryo rescue, cytology, and genetic transformation should accelerate the production of improved varieties. Such varieties are expected to help the industry overcome barriers to production and to help create new marketing opportunities.

Available literature suggests that the need for innovative research and the use of biotechnology tools in citriculture are not recent events. In fact, despite frequent new challenges in the last decade, citrus production has exceeded other important fruit crops such as bananas, apples, and grapes according to FAO (Anonymous, 2005).

Egypt produces a significant amount of high quality citrus fruits in the world, producing approximately 2.56 million tons in 2004 (ranking number ten in citrus production (Table 1-1)). The breakdown of citrus production in Egypt is as follows: sweet oranges 68%, tangerine and mandarin 20%, lemons and limes 12%, and grapefruit and pummelos less than 1%. The citrus-planted area has expanded over the last three decades to reach about 143,883 Ha (Anonymous, 2005).

Table 1-1. Total production of citrus fruit (Mt)

Country	Production (Mt) Metric ton
Brazil	20,594,000
United States of America	14,907,660
China	14,654,875
Mexico	6,475,411
Spain	6,206,800
India	4,750,000
Islamic Republic of Iran	3,825,000
Nigeria	3,250,000
Argentina	2,690,000
Egypt	2,561,600
Others	28,620,142
Total (The World)	108,535,488

CHAPTER 2
REVIEW OF LITERATURE

Citrus Canker

Asiatic citrus canker (ACC) is one of the most economically damaging plant diseases affecting citrus worldwide. ACC is caused by the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* (*Xac*) (syn. *X. citri*, *X. campestris* pv. *citri*). The citrus canker pathogen can affect the majority of the commercial citrus varieties and close relatives of citrus in the family *Rutaceae* worldwide where moist, subtropical to tropical climates occur. All above ground tissues of citrus are susceptible to *Xac* when they are young, and maximum susceptibility occurs during the last half of the expansion phase of growth (Gottwald and Graham, 1992).

The disease symptoms appear as distinctive necrotic raised lesions on leaves, stems, and fruits. Severe infections can cause defoliation, blemished fruit, premature fruit drop, twig dieback, and general tree decline (Schubert et al., 2001). Stall et al. (1982) reported that once leaves, twigs and fruit reach mature size and begin to harden off physiologically, they become more resistant to natural stomatal infection but may be subject to wound infection (Schubert et al., 2001). The disease spreads rapidly in situations in which high wind, rain, and warm temperatures occur at the same time as new shoots and fruit emerge (Gabriel, 2001).

Among citrus cultivars, grapefruit is the most susceptible to citrus canker followed by sweet oranges ‘Hamlin’, ‘Pineapple’ and ‘Navels’, ‘Mexican’ (Key) lime, and the hybrids of trifoliate orange used as rootstocks (Gottwald et al., 2002). These cultivars

have proven to be very difficult to grow profitably in the presence of the citrus canker pathogen in moist subtropical and tropical climates due to high production costs linked with windbreaks and additional foliar sprays of copper bactericides (Graham, 2001; Gottwald et al., 2002).

There are different forms of citrus bacterial canker (CBC) based on different pathogenicities of the bacteria (Cubero and Graham, 2002). The Asiatic type of canker (A type), caused by a group of strains originally found in Asia, is by far the most widespread and severe form of the disease. This is the group of *X. axonopodis* pv. *citri* strains that causes the disease most referred to as Asiatic citrus canker (Gottwald et al., 2002). It is the most virulent form and affects the majority of susceptible hosts. The CBC B and C types are caused by *X. axonopodis* pv. *aurantifolii*. Pathotype B strains are most severe on lemons (*Citrus limon* (L.) Burm f.), and are found only in Argentina, Uruguay, and Paraguay. Pathotype C strains have been found in Sao Paulo, Brazil on 'Key' lime (*Citrus aurantifolii* (Christm.) Swingle) (Schubert et al., 2001). Vernière et al. (1998) isolated and characterized a new strain of *X. axonopodis* pv. *citri* designated as A* from southwest Asia. This strain can infect only 'Key' lime and is closely related to type A strains. A similar strain has been isolated from Florida and designated as A^w (Sun et al., 2000). This strain has a restricted host range that includes 'Key' lime and alemow (*Citrus macrophylla* Wester).

Replacing the susceptible varieties by field resistant cultivars seems to be the best long-term solution. Even though classical selection or breeding for canker resistance is a promising solution, it is excessively time consuming. Genetic transformation could be a

useful alternative, since the resistance gene could be introduced into the susceptible cultivars without otherwise altering cultivar integrity.

Methods of Transformation

Plant genetic transformation is a powerful application used to study gene expression in plants. It has contributed substantially to the understanding of gene function and the regulation of physiological and developmental processes, in the generation of transgenic organisms for widespread usage in agriculture, and has increased the potential uses of plants for industrial and pharmaceutical purposes. The powerful combination of genetic engineering and conventional breeding programs permits the introduction of useful traits encoded by transgenes into commercial crops within an economically viable time frame. There is great potential for genetic manipulation of crops to enhance productivity through increasing resistance to diseases, pests, and environmental stress (Hansen and Wright, 1999). Advances in tissue culture, combined with improvements in transformation technology, have resulted in increased transformation efficiencies.

Successful plant transformation systems require that certain criteria be met. Among the requirements for transformation are target tissues capable of propagation or regeneration, an efficient DNA delivery method, agents to select for transgenic tissues, the ability to recover transgenic plants at a reasonable frequency, a simple, efficient, reproducible, genotype-independent and cost-effective process, and a short time frame in culture to avoid somaclonal variation and possible sterility (Hansen and Wright, 1999). There are three techniques that appear to meet these criteria: (1) *Agrobacterium*-mediated transformation, (2) biolistics or microprojectile bombardment, and (3) protoplast transformation.

Arobacterium tumefaciens-Mediated Transformation

About three decades ago, the concept of using *Agrobacterium tumefaciencs* as a vector to create transgenic plants was considered as a prospect and a wish. Today, many agronomically and horticulturally important species are routinely transformed using this bacterium. *Agrobacterium tumefaciencs* is the pathogen that causes crown gall disease in many plant species. The infection cycle of *Agrobacterium* is very complex, involving a number of signals emitted by both host and the pathogen (Gelvin, 2003). The virulent strains of *A. tumefaciencs* contain large plasmids that are responsible for the DNA transfer that subsequently causes the gall formation. This plasmids have been termed tumor-inducing or Ti plasmids (Schell et al., 1979; Chilton et al., 1980).

The transformed DNA (T-DNA) is referred to as the T-region that is located on the Ti plasmid. T-regions on native Ti are approximately 10 to 30 kbp in size (Barker et al., 1983; Suzuki et al., 2000). Thus, T-regions generally represent less than 10% of the Ti plasmid. Some plasmids contain one T-region, whereas the others contain multiple T-regions (Barker et al., 1983). T-regions are specified by T-DNA border sequences. These borders are 25 bp in length and highly homologous in sequence (Yadav et al., 1982). In general, the T-DNA borders specify the T-DNA, because these sequences are targets of the *VirD1*/*VirD2* border specific endonuclease that processes the T-DNA from the Ti plasmid (Gelvin, 2003). There are many proteins encoded by *vir* genes that play essential roles in the *Agrobacterium*-mediated transformation process (Christie, 1997; Gelvin, 2000; Zupan et al., 2000; Tzfira and Citovsky, 2002).

The T-DNA becomes covalently integrated into plant nuclear DNA. T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes

encoding for the synthesis of opines. These compounds, produced by condensation between amino acids and sugars, are synthesized and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Located outside the T-DNA are genes for opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer (de la Riva et al., 1998). The process of gene transfer from *Agrobacterium tumefaciens* to plant cells could be summarized as follows: (1) bacterial colonization, (2) induction of bacterial virulence system, (3) generation of T-DNA transfer complex, (4) T-DNA transfer, and (5) integration of T-DNA into plant genome (de la Riva et al., 1998). The T-DNA transfer is mediated by products encoded by the 30-40 kb *vir* region of the Ti plasmid. This region is composed of at least six essential operons (*virA, virB, virC, virD, virE, virG*) and two non-essential (*virF, virH*) (Iuchi, 1993). The activation of *vir* genes produces the generation of single-stranded (ss) molecules representing the copy of the bottom T-DNA strand. Any DNA placed between T-DNA borders will be transferred to the plant cell as single strand DNA and integrated into the plant genome. These are the only *cis* acting elements of the T-DNA transfer system. The proteins *VirD1* and *VirD2* play a key role in this step, recognizing the T-DNA border sequences and nicking (endonuclease activity) the bottom strand at each border (Zupan and Zambryski, 1995; Christie, 1997).

Inside the plant cell, the ssT-DNA complex is targeted to the nucleus crossing the nuclear membrane. Among the *vir* proteins, two have been found to be important in this step *VirD2* and *VirE2* (the most important); and a third, *VirF*, that probably has a minor contribution to this process (Hooykaas and Shilperoort, 1992). The final step of T-DNA

transfer is its integration into the plant genome. The mechanism involved in the T-DNA integration has not been completely characterized. It is considered that the integration occurs by illegitimate recombination (Lehman et al., 1994; Puchta, 1998).

To use the *Agrobacterium*-mediated system, the optimization of *Agrobacterium tumefaciens*-plant interaction is probably the most important feature to be considered. This interaction could include the wholeness of the bacterial strain, its correct manipulation, and the study of reaction in wounded plant tissue, which may develop in a necrotic process in the wounded tissue or affect the interaction and release of inducers or repressors of the *Agrobacterium* virulence system. The type of explant is also an important fact and it must be suitable for regeneration allowing the recovery of whole transgenic plants. The establishment of a method for the efficient regeneration of one particular species is all-important for its transformation.

The *Agrobacterium* system is attractive because of the ease of the protocol coupled with minimal equipment costs. Moreover, transgenic plants obtained by this method often contain single copy insertions (Hansen and Wright, 1999). At present, many species both monocotyledonous and dicotyledonous have been transformed using *Agrobacterium*-mediated transformation system. In citrus, many researchers reported using the system to transfer different genes into different citrus cultivars. Peña et al. (1995b) used *A. tumefaciens* EHA105 carrying the binary vector p35SGUSINT that encodes β-glucuronidase (*GUS*) gene as a reporter and the neomycin phosphotransferase II (*NPT II*) gene for resistance to kanamycin as a selectable marker to transform Carrizo citrange stem segments. Embryogenic calluses of ‘Ponkan’ mandarin has been transformed with a ribonuclease gene using the *Agrobacteriun*-mediated system (Li et al., 2002). Many

transgenic citrus plants have been obtained by *Agrobacterium tumefaciens*-mediated transformation system**,** among them grapefruit (Luth and Moore, 1999), Swingle citrumelo (*Citrus paradise* Macf. X *Poncirus trifoliate* L. Raf.) (Molinari et al., 2004), 'Washington' navel orange (Bond and Roose, 1998), sour orange (*Citrus aurantium* L.) (Gutiérrez et al., 1997), sweet orange (Peña et al., 1995a), lime (*Citrus aurantifolia* Swing.) (Peña et al., 1997), and 'Hamlin' orange (Mendes et al., 2002; Yu et al., 2002; Boscario et al., 2003). Carrizo citrange has been transformed via *Agrobacterium*-mediated with the halo-tolerance gene *HAL2*, originally isolated from yeast and implicated in salt tolerance mechanisms (Cervera et al., 2000). Thus, *Agrobacterium*-mediated transformation has been established for many citrus cultivars by using either embryogenic cell suspension cultures or stem explants.

Although *Agrobacterium*-mediated transformation has been a reliable and efficient system for transforming many dicotyledonous species, citrus seems to be less amenable to *Agrobacterium*-mediated gene transfer. Moore et al. (1992) suggested that the limiting step in the production of transgenic citrus shoots was the low DNA transfer efficiency; only 4 to 8% of the inoculated explants contained *GUS*-positive sectors. By comparison, a much higher transfer efficiency has been achieved with fruit crops, including apple 80% (Yao et al., 1995) and kiwifruit 66% (Janssen and Gardner, 1993). So, it may be useful to explore other DNA transfer systems for certain citrus cultivars.

Biolistics or Microprojectile Bombardment

The ability to deliver foreign DNA directly into regenerable cells, tissues, or organs was one of the best methods to achieve truly genotype-independent transformation in many agronomic crops, bypassing *Agrobacterium* host-specificity and tissue culture-related regeneration difficulties. Microprojectile bombardment employs

high-velocity metal particles (tungsten or gold) to deliver biologically active DNA into plant cells (Sanford, 1988). Klein et al. (1987) observed that tungsten particles could be used to introduce macromolecules into epidermal cells of onion with subsequent transient expression of enzymes encoded by these compounds. Christou et al. (1988) demonstrated that this process could be used to deliver biologically active DNA into living cells and produce stable transformants. Moreover, bombardments can even be performed using desiccated bacteria (as microprojectiles) containing the gene to be transferred (Rasmunssen et al., 1994). Combining the relative ease of DNA introduction into plant cells with an efficient regeneration protocol not requiring protoplast or suspension cultures, particle bombardment is the optimum system for transformation in many cereals (in which protoplast culture is difficult), as well as dicots which can be recalcitrant to *Agrobacterium* infection. Gray and Finer (1993) described several advantages for particle bombardment over *Agrobacterium*-mediated transformation: (1) non-hosts of *Agrobacterium*, such as monocots, can be transformed, (2) plasmid construction does not require insertion of the sequences essential for DNA replication and transfer in *Agrobacterium*, (3) the introduction of multiple plasmids, Co-transformation, is possible with particle bombardment, (4) false positives resulting from growth of the *Agrobacterium* in host cells are eliminated, and (5) the transformation protocols are easy by eliminating the complex bacteria-plant interaction present with the *Agrobacterium* system. On the negative side, plants regenerated from bombarded plant tissues are usually chimeric in terms of introduced foreign genes due to random bombardment of a small number of cells in a multiple cell system (Sanford, 1990). Researchers have overcome

this problem by using different selectable marker genes to sort out transformants that are stabilized in their progenies (Lowe et al., 1995).

Particle bombardment has made it possible to transfer foreign DNA into organelles. Watsona et al. (2004)) reported on the introduction and transient expression of foreign genes in suspension cell-derived chloroplasts of tobacco. Chloroplast transformation systems offer unique advantages in biotechnology, including a high level of foreign gene expression, maternal inheritance, and polycistronic expression (Kang et al., 2004).

In citrus, particle bombardment has been used to transform tangelo (*Citrus reticulata* x *Citrus paradisa*) embrogenic cells. Although fifteen transgenic embryo lines were reported, no transgenic plants were obtained (Yao et al., 1996). The bombardment of epicotyl thin explants has been used by Filho et al. (2003) for stable transformation of Carrizo citrange and sweet orange.

Protoplast Transformation

Protoplasts are cells (plant, fungal, or bacterial) that have had their cell walls removed. This can be done either by a mechanical or an enzymatic process. The “naked” cells are surrounded only by a cell membrane and can be used in a variety of ways. This results in the production of a suspension containing millions of individual cells and therefore offers the advantage of probable single cell targets. Protoplasts are frequently obtained from an established suspension cell line of callus initiated from immature embryos, immature inflorescences, mesocotyls, immature leaf bases and anthers (Maheshwari et al., 1995). Protoplasts can either be transformed by *Agrobacterium* or by direct DNA uptake methods, facilitated by polyethylene glycol treatment, electroporation or liposomes (Shillito, 1999). DNA uptake into protoplasts is now a routine and universally accepted procedure in plant biotechnology for introducing and evaluating

both short-term (transient) and long-term (stable) expression of genes in cells and regenerated plants (Davey et al., 2005b). Moreover, direct DNA uptake into plant cells has been especially important in transforming plants that are not amenable to other methods of gene delivery, particularly, *Agrobacterium*-mediated transformation (Rakoczy-Trojanowska, 2002). Protoplasts are ideal candidate cells for direct DNA uptake and the subsequent selection of transgenic events. DNA could be delivered into protoplast cells by either chemical (Polyethylene glycol (PEG)-mediated) or electroporation. Protoplasts have been transformed with Ti plasmid from *Agrobacterium tumefaciens*, and genes carried on a simple *E. coli*-based cloning vector which confirmed that Ti-DNA borders were not important for DNA integration into the plant genome (Davey et al., 1989). Songstad et al. (1995) reported that the efficiency for recovery of transgenic events is higher because cross feeding and chimerism between transgenic and wild-type cells are minimized in comparison to transformation systems based on multicellular tissues. However, Davey et al. (2005a) stated that protoplast transformation frequencies remain low (one in 10^4 protoplast giving stably transformed tissues), and protoplast-to-plant systems with efficient selection need to be improved to recover transformed cells and tissues. Protoplasts can be co-transformed with more than one gene carried on the same or separate plasmids. There are many factors that influence protoplasts transformation, with the stage of the cell cycle probably being the most important factor. Some treatments could enhance transformation frequency, such as heat shock treatment and irradiation of recipient protoplasts, probably by increasing the recombination of genomic DNA with incoming foreign DNA, or the initiation of repair mechanisms that favor integration. Carrier DNA and the nature of the plant genome also

affect transformation (Davey et al., 2000). DNA fragment size can influence stable transformation frequencies (Fleming et al., 1995). Since plants regenerated from protoplast come from a single cell, all cells in the transgenic plant are expected to contain inserted gene(s) of interest.

There are many studies utilizing protoplast procedures for efficient delivery of plasmids into suspension culture-derived protoplasts and optimization of protoplast-to-plant systems. Many such studies focused on cereals, particularly rice (Davey et al., 2005a). Polyethylene glycol (PEG) has been used to induce DNA uptake into protoplasts isolated from tobacco (*Nicotiana tabacum*) (Uchimiya et al., 1986; Jarl and Rietveld, 1996), *Arabidopsis thaliana* (Damm et al., 1989), *Datura inoxia* (Schmidt-Rogge et al., 1993), wheat (*Triticum aestivum* L.) (Marsan et al., 1993), rice (Alam et al., 1995; Chair et al., 1996), barley (Nobre et al., 2000), sugarbeet (Dovzhenko and Koop, 2003), apple (Maddumage et al., 2002), sweet potato (Garcia et al., 1998; Winfield et al., 2001), and citrus (Vardi et al., 1990; Fleming et al., 2000; Guo et al., 2005). The direct-gene-mediated method results in more multiple and rearranged copy number of the transgene in transgenic plants than does *Agrobacterium* (Dong et al., 1996; Krasnyanski et al., 1999) . DNA can be delivered into protoplasts isolated from different plant species using several different techniques (Table 2-1).

As nucleases may block DNA uptake into isolated protoplasts, experiments have been undertaken to reduce DNA damage during transformation. Folling et al. (1998) studied PEG-mediated DNA transfer into protoplasts of *Lolium perenne* and reported that plasmids were protected by a combination of high pH (9.0) and reduced temperature (0 °C), since such conditions suppressed DNA nicking and improved transformation

efficiency. The same authors showed that two nucleases usually associated with isolated protoplasts were involved with DNA degradation, with one being released into the medium and the other localized to the plasma membrane.

Electroporation is used to produce stable genetic transformants using protoplasts as target cells (Table 2-1). Electroporation conditions were optimized for transfection of protoplasts isolated from an embryogenic line of 'Hamlin' orange (*Citrus sinensis* (L.) Osbeck) and to obtain stable transgenic plants (Niedz et al., 2003).

There are several advantages from using transformation technology as a tool to generate new cultivars. First, transgenic lines expressing variable levels of resistance can be selected. For example, some *Xa21* transgenic rice lines have shown increased resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) as compared with the donor line (Song et al., 1995). Second, transformation techniques can be used to improve valuable cultivars currently containing some quantitative or qualitative resistance to disease leading to increased durability (Wang et al., 1994). Third, transformation results in the modification of a few well-defined genetic elements, whereas sexual recombination might lead to the introgression of undesirable genes involved in the production of allergens or toxins, if these genes are linked to the traits of interest (Breiteneder and Radauer, 2004). Finally, intervarietal, interspecific, or intergeneric gene transfer would bypass sterility problems common to interspecific hybridization (Ronald, 1997).

Plant Resistance (R) Proteins

More than 30 resistance genes have been cloned from both monocotyledonous and dicotyledonous plants (Nurnberger and Scheel, 2001). The majority of resistance (R) proteins that are activated upon effector recognition are classified into five different classes based primarily upon their combination of a limited number of structural motifs

(Table 2-2) (Martin et al., 2003). Class 1 consists of just one member, *Pto* from tomato, which has a serine/threonine kinase catalytic region and a myristylation motif at its N terminus. Class 2 contains a large number of proteins having a region of leucine rich repeats (LRRs), a putative nucleotide binding site (NBS), and an N-terminal putative leucine-zipper (LZ) or other coiled-coil (CC) sequence. Class 3 is similar to class 2, but instead of the CC sequence, these proteins have a region with similarity to the N terminus of the Toll and Interleukin 1 receptor (IL-1R) proteins (therefore referred to as the TIR region). The R proteins belonging to the first three classes lack transmembrane (TM) domains and all are thought to be localized intracellularly. The *Cf* proteins from tomato form class 4. They lack an NBS and instead have a TM and an extracellular LRR, and a small putatively cytoplasmic tail without obvious motifs. Finally, class 5 consists of just the *Xa21* protein from rice that in addition to an extracellular LRR and a TM, has a cytoplasmic serine/threonine kinase region. The *Xa21* is the only known resistance gene that encodes three structural features found in various combinations in other resistance gene products (Century et al., 1999). Thus, R proteins in the five major classes rely on a limited number of structural and functional domains, of which the LRR appears to play a central role. A few R proteins do not fit into these five classes and form class 6 (Table 2-2). Figure (2-1) shows schematic representation of different genetically defined plant resistance proteins. The structural similarity of different R genes could suggest a common or limited number of resistance pathways in plants. Resistance genes from monocots and dicots are highly conserved, suggesting that they share common functional domains (Song et al., 1995). This suggests the possibility of using monocot R genes to control dicot diseases.

Table 2-1. Examples of plant protoplast transformation experiments and success of transgenic plant recovery.

Plant	Transformation Method	Transgenic plant obtained	References
Tobacco	Liposome-mediated	Yes	(Deshayes et al., 1985)
Tobacco (*Nicotiana tabacum)*	PEG[a]	Yes	(Uchimiya et al., 1986)
Tomato (*Lycopersicon esculentum*)	Calcium phosphate/ PEG[a] or PVA[b]	No	(Jongsma et al., 1987)
Rassica campestris	*Agrobacterium*	No	(Ohlsson and Eriksson, 1988)
Arabidopsis thaliana	PEG[a]	Yes	(Damm et al., 1989)
Solanum-dulcamara	Electroporation/ Agrobacterium	Yes	(Chand et al., 1989)
Tobacco	Liposomes	Yes	(Zhu et al., 1990)
Citrus	PEG[a]	Yes	(Vardi et al., 1990; Fleming et al., 2000)
Tobacco (*N. tabacum*, *N. debneyi* and *N. rustica)*	*Agrobacterium tumefaciens*	Yes	(Dijak et al., 1991)
Sugarcane	Electroporation	Yes	(Rathus and Birch, 1992)
Pea	Electroporation	No	(Puonti Kaerlas et al., 1992)
Datura inoxia	PEG[a]	Yes	(Schmidt-Rogge et al., 1993)
Maize (*Zea mays* L.)	Electroporation	Yes	(Sukhapinda et al., 1993)
Wheat (*Triticum aestivum* L.)	PEG[a]	Yes	(Marsan et al., 1993)
Citrus	Electroporation	No	(Hidaka and Omura, 1993)
Wheat (*Triticum aestivum* L.)	Electroporation	Yes	(He et al., 1994)
Citrus	Electroporation	No	(Niedz et al., 1995)
Indica rice	PEG[a]	Yes	(Alam et al., 1995)
Tobacco (*Nicotiana tabacum)*	PEG[a] / Electroporation	Yes	(Jarl and Rietveld, 1996)
Rice (*Oryza sativa* L.)	PEG[a]	Yes	(Chair et al., 1996)
Creeping bentgrass (*Agrostis palustris* Huds.)	PEG[a]	Yes	(Lee et al., 1996)
Linum usitatissimum and *L. suffruticosum*	PEG[a] / *Agrobacterium*	Yes	(Ling and Binding, 1997)
Peucedanum terebinthaceum	PEG[a]	Yes	(Wang et al., 1999)
Barley	PEG[a]	Yes	(Nobre et al., 2000)
Barley	Microinjection	Yes	(Holm et al., 2000)
Barley *(Hordeum vulgare L.)*	PEG[a]	No	(Tiwari et al., 2001)
Apple (*Malus domestica* Borkh.)	PEG[a]	No	(Maddumage et al., 2002)

Table 2-1. Continued

Plant	Transformation Method	Transgenic plant obtained	References
Festuca arundinacea	PEG[a]	Yes	(Bettany et al., 2002)
Cauliflower (*Brassica oleracea* var. botrytis)	PEG[a]	Yes	(Radchuk et al., 2002)
Citrus	Electroporation	Yes	(Niedz et al., 2003)
Brassica napus	*Agrobacterium*	Yes	(Wang et al., 2005)
Citrus	PEG[a]	Yes	(Guo et al., 2005)

[a] PEG = Poly-Ethylene Glycol
[b] PVA = Poly-Vinyl Alcohol

Among the cloned plant resistance genes, only three have shown a physical interaction between a resistance protein and the corresponding avirulence (*Avr*) gene product, *Pto* in tomato (Tang et al., 1996), *Pi-ta* in rice (Jia et al., 2000), and *RRS1* in *Arabidopsis* (Deslandes et al., 2003).

Mode of Action of the *Xa21*

The *Xa21* gene is a member of a multigene family containing seven members, grouped into two classes based on sequence similarity (Song et al., 1997). The *Xa21* class contains members *Xa21*, D and F; and class A2 contains A1, A2, C and E. The identity of nucleotide sequence within each class is very high (98% for *Xa21* class and 95.2% for A2 class), but only 63.5% of the identity was observed between the two classes (Song et al., 1997). The *Xa21* gene is located on rice chromosome 11 (Ronald et al., 1992; Song et al., 1995). The *Xa21* confers resistance to over 30 distinct strains of the bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which causes leaf blight in rice (Wang et al., 1996; Hammond-Kosack and Jones, 1997). The *Xa21* encodes a 1025-amino acid protein that revealed a novel class of plant disease resistance gene products with several regions exhibiting similarity to known protein domains (Table 1-2 and Figure 2-1). The amino terminus of the *Xa21* protein encodes 23 hydrophobic residues characteristic of a signal

peptide. The central core of the *Xa21* contains 23 imperfect copies of a 24 amino acid (extracytoplasmic LRRs) with numerous potential gycosylation sites. The LRRs are followed by a 26 amino acid hydrophobic stretch that likely forms a membrane spanning helix tarnsmembrane domain (TM). The carboxyl terminal sequence encodes a putative interacellular serine/threonine kinase (STK) domain. This region carries the 11 subdomains and all 15 invariant amino acid diagnostic of protein kinases (Song et al., 1995). Thus, compared with the proteins encoded by other cloned plant disease-resistance genes, the structure of *Xa21* protein is unique because it contains the extracellular receptor LRR domain and the intracellular kinase domain.

Based on models of mammalian receptor kinases (RKs), (Ronald, 1997; Wang et al., 1998) proposed a model for *Xa21*-mediated resistance as follows: first the LRR domain binds a polypeptide produced by the pathogen or plant cell. This specific binding would cause receptor dimerization, activation of the intracellular kinase domain, and subsequent phosphorylation on specific serine or threonine residues. Liu et al. (2002) demonstrated that the intracellular domain encoded by the rice disease resistance gene *Xa21* is an active serine/threonine kinase capable of autophosphorylation. The same authors suggested that *Xa21* can initiate multiple defense responses by the binding of distinct signaling proteins with specific phosphorylated residues onto *Xa21* kinase. Phosphorylated residues may then serve as binding sites for proteins that can initiate downstream responses. This reaction may lead to phosphorylation of transcription factors. Upon phosphorylation, the transcription factors can move into the nucleus from the cytosol.

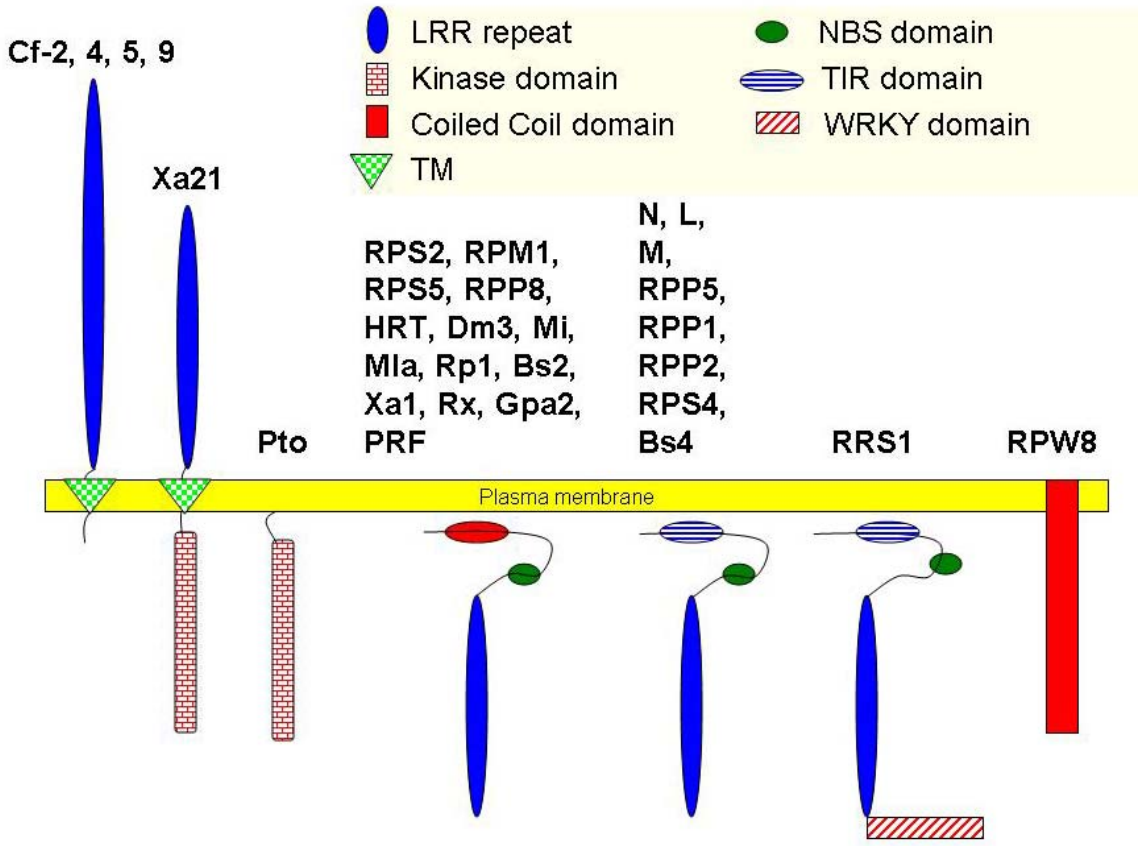

Figure 2-1. Schematic representation of different genetically defined plant resistance proteins. Protein structures named above are examples of R proteins in their respective class. Proteins are shown in relation to the plant plasma membrane. LRR, leucine-rich repeat; Kinase domain, serine/threonine kinase catalytic core; NBS, nucleotide bibdibg site; TM, transmembrane domain; TIR, Toll/Interleuken 1-receptor-like; WRKY, W-box DNA binding domain (Nimchuk et al., 2003)

Table 2-2. Plant disease resistance (R) proteins and their predicted structure (Hulbert et al., 2001; Martin et al., 2003)

Class/*R Protein		Predicted protein structure	Plant	Pathogen(s) or Pest(s)	Effector(s)
1	*Pto*	Protein Kinase	Tomato	*Pseudomonas syringae* (B)	*AvrPto, AvrPtoB*
2	*Bs2*	NBS-LRR	Pepper	*Xanthomonas campestris* (B)	*AvrBs2*
	Dm3	NBS-LRR	Lettuce	*Bremia lactucae* (F)	
	Gpa2	NBS-LRR	Potato	*Globodera pallida* (N)	
	Hero	NBS-LRR	Potato	*G. rostochiensis*, *G. pallida* (N)	
	HRT	NBS-LRR	*Arabidopsis*	Turnip Crinkle Virus	Coat Protein
	I2	NBS-LRR	Tomato	*Fusarium oxysporum* (F)	
	Mi	NBS-LRR	Tomato	*Meloidogyne incognita* (N)	
	Mi	NBS-LRR	Tomato	*Macrosiphum euphorbiae* (I)	
	Mla	NBS-LRR	Barley	*Blumeria graminis* (F)	
	Pib	NBS-LRR	Rice	*Magnaporthe grisea* (F)	
	Pi-ta	NBS-LRR	Rice	*M. grisea* (F)	*AVR-Pita*
	R1	NBS-LRR	Potato	*Phytophthora infestans* (O)	
	Rp1	NBS-LRR	Maize	*Puccinia sorghi* (F)	
	RPM1	NBS-LRR	*Arabidopsis*	*P. syringae* (B)	*AvrRpm1*
	RPP8	NBS-LRR	*Arabidopsis*	*Peronospora parasitica* (O)	
	RPP13	NBS-LRR	*Arabidopsis*	*P. parasitica* (O)	
	RPS2	NBS-LRR	*Arabidopsis*	*P. syringae* (B)	*AvrRpt2*
	RPS5	NBS-LRR	*Arabidopsis*	*P. syringae* (B)	*AvrPphB*
	Rx1	NBS-LRR	Potato	Potato Virus X	Coat Protein
	Rx2	NBS-LRR	Potato	Potato Virus X	Coat Protein
	Sw-5	NBS-LRR	Tomato	Tomato Spotted Wilt Virus	
	Xa1	NBS-LRR	Rice	*X. oryzae* (B)	
3	*L*	TIR-NBS-LRR	Flax	*Melampsora lini* (F)	
	M	TIR-NBS-LRR	Flax	*M. lini* (F)	
	N	TIR-NBS-LRR	Tobacco	Tobacco Mosaic Virus	Helicase
	P	TIR-NBS-LRR	Flax	*M. lini* (F)	
	RPP1	TIR-NBS-LRR	*Arabidopsis*	*P. parasitica* (O)	
	RPP4	TIR-NBS-LRR	*Arabidopsis*	*P. parasitica* (O)	
	RPP5	TIR-NBS-LRR	*Arabidopsis*	*P. parasitica* (O)	
	RPS4	TIR-NBS-LRR	*Arabidopsis*	*P. syringae* (B)	*AvrRps4*
4	*Cf-2*	LRR-TM	Tomato	Cladosporium fulvum (F)	*Avr2*
	Cf-4	LRR-TM	Tomato	*C. fulvum* (F)	*Avr4*
	Cf-5	LRR-TM	Tomato	*C. fulvum* (F)	
	Cf-9	LRR-TM	Tomato	*C. fulvum* (F)	*Avr9*
5	*Xa21*	LRR-TM-Kinase	Rice	*Xanthomonas oryzae* (B)	
6	*Hm1*	Toxin reductase	Maize	*Cochliobolus carbonum* (F)	
	HS1	Unique	Beet	*Heterodera schachtii* (N)	
	mlo	Membrane prot.	Barley	*B. graminis* (F)	
	Rpg1		Barley	*Puccinia graminis* (F)	
	RPW8	Unique	*Arabidopsis*	*Erisyphe chicoracearum* (F)	

Table 2-2. Continued

Class/*R Protein	Predicted protein structure	Plant	Pathogen(s) or Pest(s)	Effector(s)
RRS1-R		*Arabidopsis*	*Ralstonia solanacearum* (B)	
RTM1		*Arabidopsis*	Tobacco Etch Virus	
RTM2		*Arabidopsis*	Tobacco Etch Virus	
Ve1, Ve2		Tomato	*Verticillium alboatrum* (F)	

* R protein = Resistance protein
Abbreviated as: B, bacterium; F, fungus; I, insect; N, nematode, O, oomycete.
NBS, nucleotide binding site; LRR, leucine-rich repeat; TIR, domain with homology to the *Toll* gene of *Drosophila* and the *Interleukin-1* receptor of mammals; TM, transmembrane domain.
Domains are listed as they appear in the proteins from N to C terminal end (Hulbert et al., 2001)

The *avrXa21*-derived ligand might have a novel molecular identity, because *Xanthomonas oryzae* pv. *oryzae* is predominantly a xylem vessel colonizing bacterium. Conceivably, it is delivered extracellularly, unlike other bacterial *avr* products, in which case the *Xa21* LRRs might be involved in the recognition (Hammond-Kosack and Jones, 1997). The LRR is involved in protein-protein interactions. *Xa21D* is a *Xa21* family member that lacks the transmembrane and kinase domains, but encodes a receptor-like protein carrying LRR motifs in the presumed extracellular domain (Wang et al., 1998). In transgenic rice plants, *Xa21D* conferred partial resistance to *Xoo* at an intermediate level compared with that of *Xa21* but showed the same spectrum of resistance as *Xa21*. However, other members (A1, A2, C, E, F) did not confer any resistance in transgenic plants (Wang et al., 1998). These results suggest that the extracellular LRR domain of *Xa21D* is involved in pathogen recognition.

It was observed that several defense responses were initiated in transgenic rice cells expressing a fusion gene composed of the extracellular LRR and transmembrane domains of the *Arabidopsis* receptor kinase *BRI1* and the serine/threonine kinase of *Xa21* upon treatment with brassinosteroids, which is the ligand for the *BRI1*-encoded protein kinase

(He et al., 2000). These results indicated that the extracellular LRR domain of the *Xa21* protein functions in recognition of the *Xoo avr* proteins and its interacelluar serine/threonine kinase domain transmits the signal to activate the defense mechanism.

The cloning of two plant resistance genes encoding serine threonine kinase supports a central role for protein phosphorylation in gene-for-gene mediated disease resistance (Martin et al., 1993; Song et al., 1995). The serine/threonine kinase capacity possessed by *Pto* and *Xa21* could clearly facilitate downstream signaling by a distinct mechansim. A lysine residue is conserved in all protein kinases and is important for phosphor-transfer for both *Pto* and *Xa21* (Andaya and Ronald, 2003). The kinase domain of the rice *Xa21* gene product is the most homologous to that of the *Arabidopsis* protein *RLK5*. When *RLK5* was used in an interaction cloning system, a type 2C phosphatase was identified (Stone et al., 1994). Moreover, for many gene-mediated resistances, the addition of either kinase or phosphatase inhibitors significantly blocked the induction of rapid defense responses (Levine et al., 1994; Dunigan and Madlener, 1995). It appears likely that both kinases and phosphatases are involved in downstream R protein-mediated signaling events.

The kinase domain of *Xa21* is functional serine/threonine kinase (STKs) (Liu et al., 2002). The same authors confirmed the serine/threonine specificity of *Xa21* kinase by phosphoamino acid assays. In these assays, serine and threonine residues were phosphorylated, whereas no detectable tyrosine residues were marked. Also, they showed that the autophophorylated *Xa21* kinase can be dephosphorylated by the serine/threonine phosphatase PP1. These results indicated that *Xa21* kinase carries serine/threonine specificity. By phosphopeptide mapping approaches, Liu et al. (2002) demonstrated that

at least 20 of 27 phosphospots on the GST-Xa21K (Glutathions S-transferase-Xa21K) peptide map were because of autophosphorylation of *Xa21* kinase. These observations strongly suggest that multiple residues on *Xa21* kinase were phosphorylated, which suggest that *Xa21* can initiate multiple defense responses by binding of different signaling proteins with specific phosphorylated residues on *Xa21* kinase (Liu et al., 2002). The kinase activity of the *Xa21* is very important for full resistance (Andaya and Ronald, 2003).

Objectives

The major objective of this study was to develop citrus canker resistance in one of the most commercially important citrus cultivars, 'Hamlin' sweet orange [*Citrus sinensis* (L.) Osbeck], by introducing the cDNA of the *Xa21* gene from rice that confers broad spectrum resistance to *Xanthomonas oryzae* (rice bacterial blight) in rice.

The specific goals were to:

- Clone the cDNA of the *Xa21* gene into citrus a transformation vector.
- Produce transgenic 'Hamlin' sweet orange plants with potential ACC resistance genes from the *Xa21* gene family via a protoplast transformation/*GFP* system.
- Characterize the transgenic plants for gene expression and stability of the transgene in the citrus genome.
- Developed a real-time PCR based method to accelerate characterization of the transgenic plants.
- Assay transgenic plants for resistance to Asiatic Citrus Canker.

CHAPTER 3
TRANSFER OF THE *XA21 XANTHOMONAS* RESISTANCE GENE FROM RICE INTO 'HAMLIN' SWEET ORANGE [*CITRUS SINENSIS* (L.) OSBECK] USING A PROTOPLAST/*GFP* CO-TRANSFORMATION SYSTEM

Introduction

Citrus is the most extensively grown fruit crop worldwide. Sweet orange (*Citrus sinensis* L. Osbeck) is the most important citrus species. It accounts for approximately 60% of the world citrus production according to FAO (Anonymous, 2005). 'Hamlin' sweet orange is one of the leading commercial cultivars in Florida because of its high yield potential and early maturity. 'Hamlin' orange also has a high regeneration capacity from protoplasts and is often used in transformation studies.

Sweet orange improvement via conventional breeding programs has been hampered by large plant size, nucellar polyembryony, high levels of heterozygosity, and long juvenility (Grosser and Gmitter, 1990). Most of the commercially cultivated sweet orange cultivars have been developed either from chance seedling selection or from a mutation in a particular seedling cultivar (Hodgson, 1968), rather than from organized breeding programs. Nowadays, biotechnology tools, including tissue-culture based tools and genetic engineering, are being used to improve sweet orange (Grosser et al., 1996a; Grosser et al., 1996b).

Genetic engineering has opened new avenues to modify crops and provided new solutions to solve specific problems (Estruch et al., 1997). Citrus genetic transformation has become more attractive to biotechnology-based citrus improvement programs because of the exciting possibility of adding a desirable trait to an already established

cultivar without altering its integrity (Bond and Roose, 1998). The powerful combination of genetic engineering and conventional breeding programs permits introduction of useful traits encoded by transgenes into commercial crops within an economically viable time frame (Hansen and Wright, 1999). There is great potential for genetic engineering of citrus to enhance productivity through increasing resistance to diseases and environmental stress.

Since late 1980s, researchers started to report transgenic citrus (Kobayashi and Uchimaya, 1989; Hidaka et al., 1990; Vardi et al., 1990; Moore et al., 1992; Hidaka and Omura, 1993; Peña et al., 1995a; Gutiérrez et al., 1997; Peña et al., 1997; Cervera et al., 2000; Fleming et al., 2000; Mendes et al., 2002; Yu et al., 2002; Niedz et al., 2003; Guo et al., 2005). Most of those researchers obtained transgenic citrus via an *Agrobacterium*-mediated transformation system, with the selection of transgenic tissue based on antibiotic resistance and the tissue-destructive *GUS* reporter-gene system. In the beginning, the transformation efficiency was low, but over time it has been improved due to continued-improvements in the *Agrobacterium*-mediated system (Peña et al., 1995b; Bond and Roose, 1998). More recently, researchers reported transgenic citrus without antibiotic resistance genes and used Green Fluorescent Protein (*GFP*) as the reporter-gene instead of *GUS* (Fleming et al., 2000; Niedz et al., 2003; Guo et al., 2005).

Since many important citrus cultivars, including some sweet orange clones, are commercially seedless (zero to five seeds per fruit) or totally seedless, it can be difficult or impossible to obtain adequate nucellar seedling explants for *Agrobacterium*-mediated transformation. The protoplast transformation could be a promising alternative (Fleming et al., 2000). Protoplast techniques are well established in citrus (Grosser and Gmitter,

1990; Grosser et al., 2000), and progress has been made regarding the development of a competitive transformation system. Citrus protoplast transformation, selection of transformants via *GFP* and regeneration of transgenic plants via somatic embryogenesis have recently been reported (Fleming et al., 2000; Niedz et al., 2003; Guo et al., 2005). On the other hand, protoplast/*GFP*-based citrus-transformation system does not utilize antibiotic resistance selection or *Agrobacterium*. Protoplast/*GFP* transformation system utilizes non-destructive *GFP* selection, instead of destructive *GUS* selection and therefore requires no antibiotics. Since these markers are currently not legally accepted for marketing particularly in Europe (Stewart, 2001; Puchta, 2003; Schaart et al., 2004), using the protoplast/*GFP* system may be an advantage over the *Agrobacterium*-mediated transformation system.

Asiatic citrus canker (ACC) is one of the most economically damaging problems affecting citrus production worldwide. ACC is caused by the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* (*Xac*). Canker pathogen can affect the majority of the commercial citrus varieties and close relatives of citrus in the family *Rutaceae* grown in moist, subtropical to tropical climates. All aboveground tissues of citrus are susceptible to *Xac* when they are young, and the maximum susceptibility occurs during the last half of expansion phase of growth (Gottwald and Graham, 1992). The pathogen causes distinctive necrotic raised lesions on leaves, stems, and fruits. Severe infections can cause defoliation, blemished fruit, premature fruit, twig dieback, and general tree decline. Citrus canker is not systemic, it causes local lesions only (Schubert et al., 2001). There is no highly effective method for citrus canker control. Moreover, chemical control is expensive and could cause problems in the environment.

Citrus canker eradication programs (CCEP) are very expensive, and many times unsuccessful (Brown, 2001). Nowadays, the state of Florida is spending millions of dollars annually in an effort to eradicate citrus canker. There is no an accurate estimate for how long and how much it will cost to eradicate citrus canker. Moreover, there is a real possibility that the eradication program may fail or become too costly to both state and federal government. Therefore, replacing susceptible varieties with field resistance ones appears to be the best long-term solution. Although classical selection or breeding for canker resistance is a promising solution (Viloria et al., 2004), it is a very long-term solution. Genetic transformation could be a useful alternative, since the resistance gene could be introduced into the susceptible varieties without otherwise altering cultivar integrity.

The cloned *Xa21* gene from rice has been shown to confer resistance to over 30 distinct strains of the bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) isolated from eight different countries, which causes leaf blight in rice (Wang et al., 1996; Hammond-Kosack and Jones, 1997). The wild-type *Xa21* gene contains an intron, and there is some question as to whether dicot plants can process genes containing monocot introns (the cDNA is intron free). Since the citrus canker pathogen is in the same genus, this gene may have the potential to confer resistance against canker in citrus. The development of canker resistant citrus has become an important research objective. A similar approach was successful with tomato resistance genes to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Oldroyd and Staskawicz, 1998; Tang et al., 1999).

To achieve this objective, the protoplast isolated from a relatively new embryogenic suspension culture line of ‘Hamlin’ sweet orange were co-transformed with

GFP for selection and the cDNA of the *Xa21* gene. Transformed clones were screened for *GFP* expression using blue light. Polymerase chain reaction (PCR) was performed to identify the co-transformed clones. Transgenic plants were regenerated from co-transformed clones via somatic embryogenesis and organogenesis. Transgenic plants containing the cDNA of the *Xa21* gene were assayed by southern blot analysis and western blot analysis to determine if the gene is integrating into the citrus genome and functioning to produce RNA and subsequently protein.

Materials and Methods

Plasmid Construction

The plasmid pARS108 with the *GFP* endoplasmic reticulum (ER)-targeting gene was kindly provided by Dr. R. P. Niedz (USDA, Agriculture Research Service, US Horticultural Research Laboratory, Ft. Pierce, FL, USA). Construction of pARS108 has been described previously (Niedz et al., 2003). Plasmid pARS108 contains the Enhanced Green Fluorescent Protein (*EGFP)* coding sequence (GenBank accession #U55761) placed under the control of the double 35S cauliflower mosaic virus (35S-35S CaMV) promoter with the alfalfa mosaic virus (AMV) untranslated leader sequence and the *nos* terminator. An *Arabidopsis* signal sequence (SS) is included in the 5′ end to target the protein to the endoplasmic reticulum (ER), and the codon sequence of the HDEL amino acid was included in the 3′ end for retention of the protein in the lumen of the ER. The SS-EGFP-HDEL sequence was cloned into pBI524 as a *NcoI/BamHI* cassette, placing it under the control of the double 35S promoter with the AMV untranslated leader sequence and the *nos* terminator (Figure 3-1).

The plasmid p524EGFP.1 containing the *EGFP* reporter gene was constructed and described by Fleming et al. (2000). This version of the *GFP* is targeted to the cytoplasm. Plasmid p524*EGFP*.1 contains the *EGFP* coding sequence (GenBank accession #U55761) placed under the control of the double 35S CaMV promoter with the AMV untranslated leader sequence and the SV40 poly A terminator. The fragment containing the double 35S cauliflower mosaic virus (35-35S CaMV) promoter sequence followed by the alfalfa mosaic virus (AMV) enhance sequence was cloned into the *HindIII*/*BamHI* site on the pEGFP.1 from clontech (Palo Alto, CA, USA) (Figure 3-2).

Vector pCR504 was kindly provided by Dr. Pamela Ronald, University of California-Davis (Figure 3-3). It contains a 3.1 kb *BamHI* fragment encoding the entire cDNA of the *Xa21* gene from rice. Plasmid pCR504 was a promoterless plasmid containing the cDNA of the *Xa21* gene. Methods used for plasmid construction were similar to those described by Sambrook and Russell (2001), or according to the manufacturer. To construct the plasmid used in the co-transformation experiment, the fragment containing the cDNA of the *Xa21* gene was excised from pCR504 as a *BamHI* fragment and ligated into the *BamHI* site on the pBHU-SalI (provided by Dr. W. Y. Song, Plant Pathology Department, University of Florida, Gainesville, FL, USA) (Figure 3-4). The re-ligated plasmid was designated pBHU-XacDNA. The *NotI*/*HindIII* fragment from pBHU-XacDNA has been replaced by the *NotI*/*HindIII* fragment from pLitmus28-F (provided by Dr. W. Y. Song, Plant Pathology Department, University of Florida, Ganisville, FL), which contains the c-myc tag sequence (mtag) in the 5′ end. The resulting plasmid is referred to as pBHU-XacDNA-mtag. The *BamHI* fragment from pBHU-XacDNA-mtag was excised and ligated into *BamHI* site on pJIT vector to place

the cDNA-mtag under the control of the 35S cauliflower mosaic virus (35S CaMV) promoter and CaMV poly-A. The final construction was designated pXa21-mtag and used in co-transformation experiments (Figure 3-4).

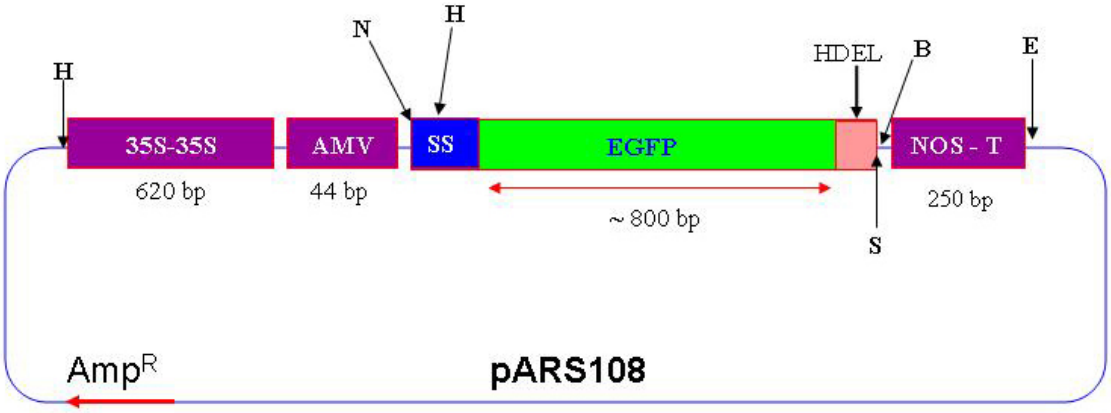

Figure 3-1. Schematic diagram of pARS108 ER targeting. Restriction enzyme (H, *HindIII*; N, *NcoI*; S, *SacI*; B, *BamHI*; E, *EcoRI*). HDEL = Histidine, Aspartic acid, Glutamic acid, and Leucine, respectively.

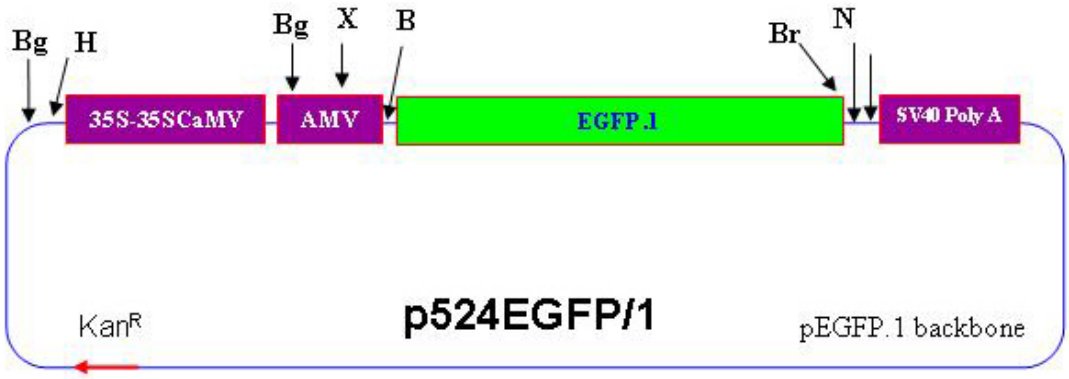

Figure 3-2. Schematic diagram of p524EGFP Cyt-targeting. Restriction enzyme (Bg, *BglII*; H, *HindIII*; X, *XbaI*; B, *BamHI*; Br, *Brs GI*; N, *NotI*).

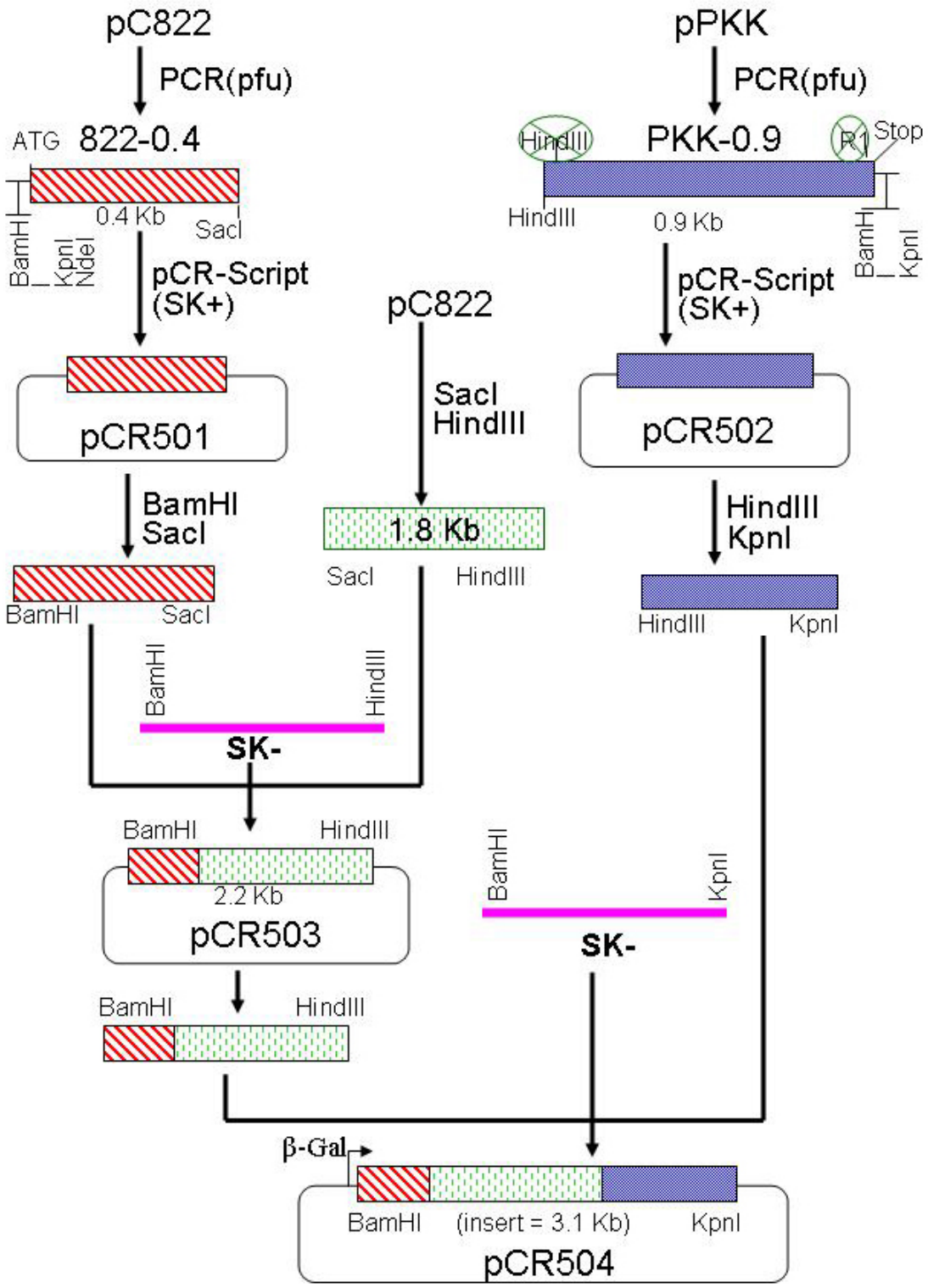

Figure 3-3. Schematic diagram of the construction of *Xa21* cDNA clone pCR504.

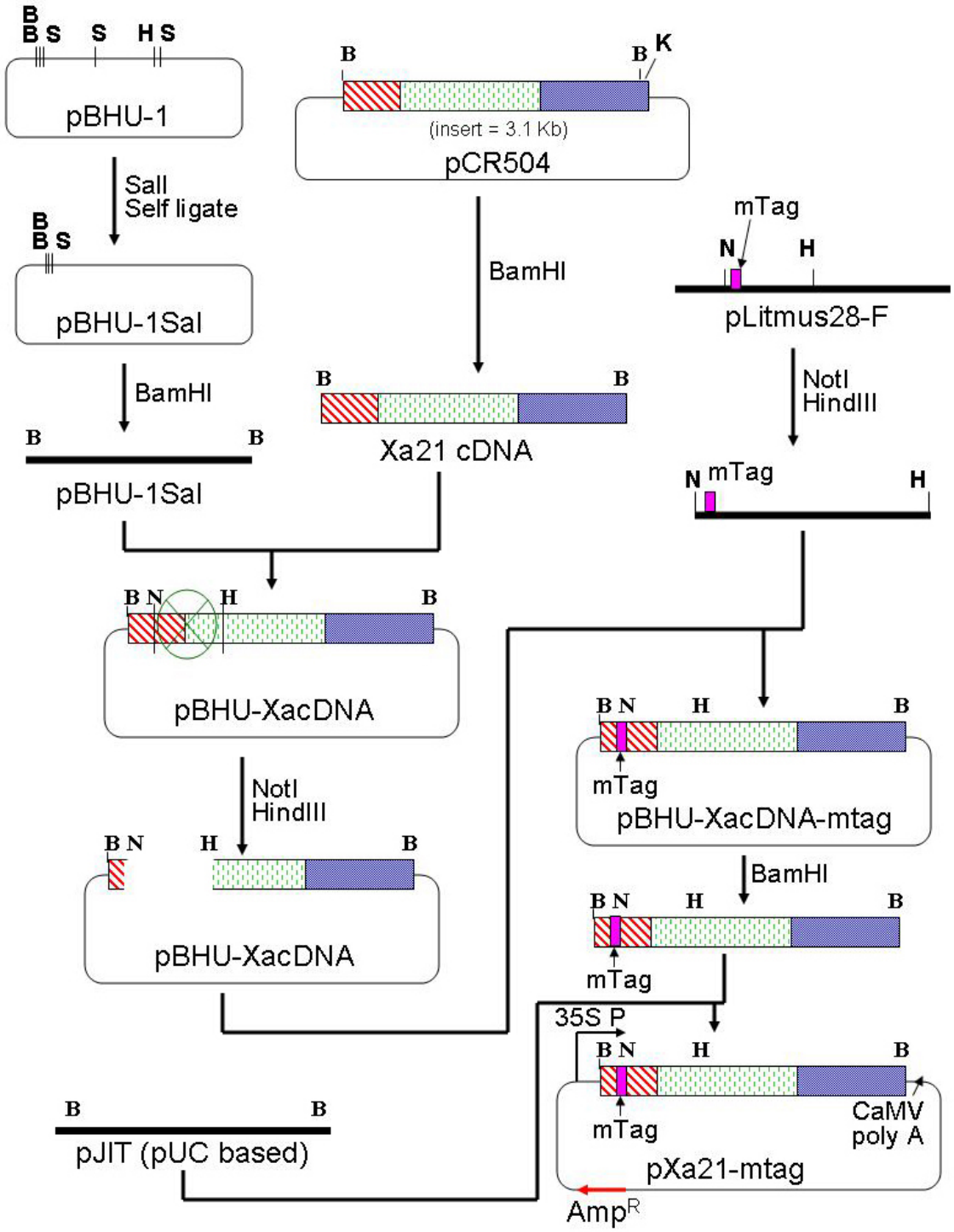

Figure 3-4. Schematic diagram of the construction of pXa21-mTag plasmid. Restriction enzyme (B, *BamHI*; K, *KpnI*; S, *SalI*; N, *NotI*).

Plasmid Multiplication

Transformation of *E. coli*

Before a large scale plasmid preparation can be performed, the plasmid must first be transformed into competent *E. coli* cells. Max efficiency® DH5α™ competent cells were obtained from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA, Cat. No. 18258-012). The transformation procedure was followed exactly as described in the product manual.

Quick miniprep plasmid preparation and confirmation of insert orientation

Plasmid DNA was extracted from several recovered bacterial colonies and compared to the DNA from the known plasmid stock by restriction analysis using agarose gel electrophorosis.

Using a sterile bacterial loop, single colonies were selected from the plated culture and transferred to a bacterial culture tube containing 2 ml LB liquid media (10 g/L Bacto-tryptone, 5 g/L yeast extract, and 10 g/L NaCl) and the appropriate antibiotic. The bacteria were placed on the shaker at 225 RPM and left to grow overnight at 37º C. The DNA was extracted from the overnight culture bacteria using QIAprep® Spin Miniprep kit (QIAGEN Inc., Valencia, CA, USA, Cat. No. 27104).

A restriction digest was then performed on the extracted DNA to test for the presence of the cDNA of the *Xa21* gene. Reaction volume of 20 µl was performed with (4 µl DNA, 2 µl 10X of the restriction enzyme buffer (Promega), 0.2 µl of the restriction enzyme (Promega), and 13.8 µl ddH_2O). The reaction mixture was incubated at 37º C for at least 2 hours. The restriction enzyme was deactivated by heating the samples at 65º C for 10 minutes. Indicator dye (4 µl of 6X loading buffer) was added and the gel was loaded. The gel was run for 90 minutes at 100 Volts in TAE buffer (Tris-Acetate-EDTA).

The gel was removed and stained with 10 µl of ethidium bromide (10 mg/ml) in 100 ml TAE buffer with gentle shaking for 15-20 minutes and observed and photographed on a UV Transilluminator. Samples which contained the band corresponding to the band for the cDNA of the *Xa21* gene were saved to use for the large scale isolation.

Large scale isolation and plasmid preparation

To obtain a large amount of the DNA for the further use, two 1-liter flasks containing 500 ml LB media plus the appropriate antibiotic were inoculated with the transformed *E. coli* and the cultures were incubated overnight at 37º C on a shaker at 225 RPM. The DNA was extracted from the overnight cultured bacteria using a Wizard® PlusMaxiprep DNA Purification System from Promega (Promega Corporation, Madison, WI, USA, Cat. No. A7270). To determine the concentration of plasmid DNA, an absorbance reading at $O.D._{260}$ was obtained, (an absorbance reading of 1.0 corresponds to 50 µg of plasmid DNA per ml) (Sambrook and Russell, 2001).

Plant Material, Protoplast Transformation, and Culture

Establishment and maintenance of suspension cultures

The 'Hamlin' sweet orange (*Citrus sinensis* L. Osbeck) embryogenic callus line was induced from unfertilized ovules taken from mature fruit and maintained on Murashige and Tucker's (MT) basal medium (Murashige and Tucker, 1969). Suspension cultures were initiated and maintained according to Grosser and Gmitter (1990) in 40-50 ml of H+H liquid media (see appendix A) in 125 ml erlenmeyer flasks covered with aluminum foil and sealed with masking tape. Cultures were maintained on a shaker at 125 RPM. Suspensions were subcultured every 2 weeks by splitting the contents between two sterile flasks and adding 25 ml of fresh culture medium to each flask. Suspension cells used for protoplast isolation were taken 4-10 days after subculture.

Transformation and culture of citrus protoplasts

The protoplast transformation protocol was modified from the PEG-mediated protoplast fusion protocol developed by Grosser and Gmitter (1990) for citrus somatic hybridization with slight modification. Suspension culture tissue was digested overnight in the enzyme mixture (see appendix A), and the protoplasts were purified by centrifugation on a sucrose-mannitol gradient. The protoplast pellet was resuspended in 3 ml 0.6 M BH3 protoplast culture medium (see appendix A; MT basal medium containing 8-P multivitamins, organic acids, and sugar/alcohol additives (Kao and Michayluk, 1975), at a concentration of 2×10^6 cells per ml, as described by Grosser and Gmitter (1990). The plasmids DNA pXa21-mtag and pARS108 or p524EGFP.1 were added (25 µg DNA/100 µl protoplast suspension) and mixed well using a pasteur pipet. Four drops of the resuspend mixture were pipeted to the center of each Petri dish (60 × 15 mm). Immediately, four drops of a 40% PEG solution were added (see appendix A) to each Petri dish and incubated 25-30 minutes at room temperature. Following incubation in PEG, four drops of 9:1 A:B elution solution as described by Grosser and Gmitter (1990) (see appendix A) were added to each Petri dish. The A+B solution was mixed immediately prior to use to avoid precipitation. After another incubation period of 25-30 minutes, approximately 20 drops of fresh BH3 medium were added around the periphery of the protoplasts. Ten minutes later, the PEG, A+B solution, and the medium were carefully removed with a Pasteur pipet, and immediately replaced with 30 drops of fresh BH3 medium. After 10-15 minutes, the BH3 medium was removed and replaced with another 30 drops of fresh BH3 medium. The last wash was repeated twice. Following the final wash, the protoplasts were cultured directly in the same Petri dish by adding 15-20 drops of either fresh BH3, EME, (see appendix A) or a 1:1 (v:v) mixture of both. After

plating, Petri dishes were sealed efficiently with Nescofilm. Protoplasts were incubated in sealable plastic boxes at 25-27º C under low light for 4-6 weeks in which transgenic tissue could be recovered for the subsequent regeneration of transgenic plants.

Regeneration and selection of transformed protoplasts

The *GFP* expression was detected in the transgenic protoplasts illuminated with blue light within 24 h after transformation using a Zeiss SV11 epifluorescence stereomicroscope equipped with a 100 W mercury bulb light source and an FITC/GFP filter set with a 480/30 nm excitation filter and a 515 nm longpass emission filter (Chroma Technology Corp., Brattleboro, VT, USA). Continued expression of the *GFP* protein was monitored occasionally for the next few weeks. Four weeks following transformation, cultures were supplemented with fresh medium containing reduced osmoticum. This was accomplished by adding 10-12 drops of a 1:2 (v:v) mixture of 0.6 M BH3 medium and 0.146 M EME medium (Grosser and Gmitter, 1990). After two more weeks, another reduction in osmoticum was made by adding 10-12 drops of a 1:1:1 (v:v:v) of 0.6 M BH3 : 0.146 M EME : 0.6 M EME media. At this point, vigorous colonies from each Petri dish were transferred to larger plates containing solid medium by pouring over 100 × 20 mm^2 Petri dishes containing agar-solidified EME with 0.146 M maltose, substituted for sucrose to promote embryogenesis. Four weeks following plating the culture on solid medium, transgenic calli and embryoids were identified based on the blue-green fluorescence expression of the *GFP* protein and were physically separated in vitro from non-transgenic tissues. Selected transgenic embryoids and calli were transferred to new plates of solid EME-maltose overlayed with a thin layer of fresh liquid 0.146 M maltose EME medium to promote continued embryo initiation and development. Regenerated transgenic small embryoids were then cultured on 0.22 μm cellulose acetate

membrane filters, which were layed on fresh 1500 medium (MT basal medium containing 1.5 g/L malt extract) (see appendix A), to normalize and enlarge the embryoids (Niedz et al., 2002). The cultures were put on fresh 1500 medium for 4 weeks and then transferred onto B+ medium [MT basal medium containing 3.0 mM gibberellic acid and 0.11 mM naphthaleneacetic acid (NAA)] (see appendix A) to encourage embryo conversion (Grosser and Gmitter, 1990). Enlarged transgenic embryos were cultured on DBA3 medium [MT basal medium containing 13.3 mM 6-benzyl-aminopurine and 0.045 mM 2,4-dichlorophenoxy-acetic acid (2,4-D)] for adventitious shoot induction (Deng et al., 1992). Transgenic shoots obtained were transferred to RMAN rooting medium (half-strength MT basal medium containing 0.11 mM NAA and 0.5 g/L activated charcoal) (see appendix A) for root induction (Grosser and Gmitter, 1990). Transgenic shoot tips were also micrografted onto Carrizo citrange or sour orange nucellar seedlings according to Navarro (1992) to expedite transgenic plant recovery.

Comparison of different *GFP*-containing constructs at the whole plant level

Mature leaves from transgenic plants obtained from p524EGFP.1 and pARS108 were scanned using a confocal laser scanner (Leica TCS SL, Exton, PA, USA) with settings for three different fluorescent wavelengths, green, red and blue, at the same time with a 488, 543, and 633 nm excitation filter and a 500-543, 610-630, and 675-750 tunable spectral window, respectively.

Molecular Analysis of Transgenic Tissue

Presence of the *GFP* gene and the cDNA of the *Xa21* gene in the selected tissue were confirmed by PCR amplification of the transgenes. The copy number and integration pattern of the transgenes were determined by Southern blot analysis. The expression of the cDNA of the *Xa21* gene was determined by western blot analysis.

Polymerase chain reaction (PCR) for detection of transformants

The PCR was used initially to screen regenerated 'Hamlin' sweet orange plants obtained via co-transformation. Genomic DNA was extracted from about 100 mg of young leaf tissue of putative transgenic citrus plants using a GenElute™ Plant Genomic DNA Miniprep kit (Sigma, Inc. St. Louis, MO, USA, Cat. No. G2N350). To confirm the presence of the *GFP* gene and the cDNA of the *Xa21* gene, multiple PCR experiments were performed using two different pairs of primers for *GFP* and the cDNA at the same time. Primers for PCR amplification matching the coding sequence within the *GFP* gene were 108F, 5′-GAATTCGTGAGCAAGGGCGA-3′ and 108R, 5′-GGATCCTTAGAGTTCGTCGTG-3′. Primers for PCR amplification matching the coding sequence within the cDNA of the *Xa21* gene were XaL, 5′-AATCCCTAACACGCTTGGTG-3′ and XaR, 5′-CACACACTGGAAACAATCCG-3′. All primers were synthesized by Qiagen Operon (Alameda, CA, USA). The PCR was performed in 25 µl reaction volume containing 12.5 µl of 2X GoTaq® Green Master Mix (Promega, Cat. No. M7123), 1.5 µl of 5 µM each primer, 2.5 µl genomic DNA (stock 100 ng/µl) and 4 µl Nuclease-Free Water (Promega). The PCR amplification program was 1) 95º C/2 minutes, 2) 95º C/30 seconds, 3) 59º C/30 seconds, 4) 72º C/1 minute, 5) 30 cycle from 2-4, and 6) 72º C/10 minutes(see appendix B). The amplified DNA products were electrophoretically separated in 1% agarose gel that contained TAE buffer (Tris-Acetate-EDTA) and 1 µl/100 ml ethidium bromide (10 µg/ml).

Southern blot analysis

Southern blot analyses were performed to confirm stable integration of the cDNA of the *Xa21* gene in the transgenic plants and to determine the number of integrated

copies of the gene in transgenic clones. Prior to the isolation of the genomic DNA, all labware was sterilized by autoclaving for one hour at 15 psi and dried in an oven. All buffers and solutions were sterilized by autoclaving. Gloves were used to handle all glassware during isolation.

Genomic DNA was extracted from the leaves of transgenic and non-transgenic plants using the modified CTAB method as described by Dr. T. E. Mirkov (personal communication). Twenty µg of DNA was digested with *SphI* and *ApaLI* restriction enzymes. Standard protocols for gel electrophoresis, denaturation, and neutralization of the gel were performed as described by Sambrook and Russell (2001). After electrophoresis, gels were treated with 0.25 M HCl for 10 minutes, and then washed with 0.4 M NaOH for 10 minutes. DNA was transferred to positively charged nylon membranes (Immobilon^{TM}-Ny+; Millipore Corporation, Billerica, MA, USA) by capillary transfer using 20X SSC buffer overnight according to Sambrook and Russell (2001). Probe DNA for the cDNA of the *Xa21* gene was prepared from the *XhoI* fragment, 1.49-kb, of the pXa21-mtag plasmid. Probe was labeled with digoxigenin-dUTP using a random primer labeling kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Applied Science, Cat No. 11 585 614 910). The probe labeling was performed according to manufacturer's manual. Fixation of the DNA to the membrane, prehybridizaton, hybridization, and immunological detection were performed as described in the instruction manual of DIG High Prime DNA Labeling and Detection Starter Kit II.

Western blot analysis

To carry out western blot analysis, total protein was extracted from transgenic and non-transgenic citrus leaves. About 0.3 g leaf tissue from transgenic and non-transgenic

'Hamlin' sweet orange plants was collected and ground in liquid nitrogen. To isolate the soluble fractions, the ground tissue was thawed in extraction buffer [50 mM Tris-HCl, pH 6.5; 1 mM EDTA; 150 mM NaCl; 0.1% Triton X-100]. Prior to use of the extraction buffer immediately, the following protease inhibitors were added: 2 µg/ml Antipain; 2 µg/ml Leupeptin; 2 µg/ml Aprotinin; 1 mM of 4-[2-aminoethyl]-benzenesulfonyl fluoride (AEBSF) Sigma; and 5% of 2- mercaptoethanol. Samples were incubated at 4º C for 30 minutes with shaking and then centrifuged at 10,000 RPM for 10 minutes at 4º C. The supernatant was recentrifuged at 13,000 RPM for 10 minutes at 4º C. The supernatant was either immediately subjected to protein blot analyses or stored at -80º C after determining the concentration of the protein by the method of Bradford (Bradford, 1979). After adding the loading dye and boiling the samples for 5 minutes, total protein (35 µg) for each sample was separated on 5% stacking and 6.5% separation gels in a Mini-Protean III cell (Bio-Rad) according to Laemmli (1970) using Tris-glycine as the SDS-PAGE electrophoresis buffer.

Proteins were electrophoretically transferred to a PVDF membrane (Immobilon^{TM}-P; Millipore Corporation, Bedford, MA, USA, Cat. No. IPVH 000 10) using a Trans-Blot Cell (BioRad). The non-specific binding sites on the membrane were then blocked with Blotto [5% non-fat dried milk in TTBS (100 mM Tris-HCl, pH 7.9; 150 mM NaCl; 0.1% Tween 20) (see appendix C)] for one hour at room temperature. The membrane was incubated with primary antibody (anti-c-myc, 1:700) in 3% bovine serum albumin (BSA) in TTBS overnight at 4º C. After three 10-minute washes in TTBS, membranes were incubated with secondary antibody (C-myc: Anti-mice IgG), (Amersham Biosciences Corp, Piscataway, NJ, USA, Cat. No. NXA931), for one hour at

room temperature, followed by three 10-minute washes in TTBS. Detection of the protein band on blots was carried out according to ECL Plus Western Blotting Detection Kit (Amersham Biosciences Corp, Piscataway, NJ, USA, Cat. No. RPN2133). Membranes were exposed to X-ray film.

Results and Discussion

Plasmid Preparation

The cDNA of the *Xa21* gene was inserted into plasmid pJIT (pUC based) as necessary to be under the control of the 35S CaMV promoter and CaMV poly A terminator. A c-myc epitope sequence was tagged to the 5′ end of the cDNA. This plasmid was designated pXa21-mtag. Figure (3-4) shows a schematic diagram of the construction of pXa21-mtag plasmid. Plasmid was transformed to *E. coli* strain DH5α™ using a chemical protocol according to the manufacture's instructions. Selection for positive colonies was performed using PCR with a specific primer set for the cDNA. Testing for correct orientation was performed using restriction enzymes according to Sambrook and Russell (2001) prior to large scale preparation of the plasmid. The positive bacterial colonies were replated and single colonies were selected to begin large scale plasmid preparation as described previously. Preps with DNA concentration of 0.5 µg/µl and above were used in co-transformation experiments. Plasmid DNA was stored at -20º C for short term use and at -80º C for long-term use.

Transformation of Citrus Protoplasts and Plant Regeneration

Isolation and transformation of citrus protoplast and the ultimate regeneration of plants are controlled by several factors. The two most important factors are the quality of the starting material (cell suspension) and quality of the protoplasts after isolation (high yields of viable protoplasts with little or no debris). The cell suspension line used was

chosen from ‘Hamlin’ sweet orange new embryogenic cells because they were exhibiting good regeneration capacity. The best protoplasts were obtained from suspensions 4-10 days after subculture when isolated according to Grosser and Gmitter (1990). Using the sucrose-mannitol gradient allows for the removal of the non-viable protoplasts and cellular debris which could interfere with the uptake of the plasmid by the viable protoplasts, or the regeneration in the following stages. Moreover, starting with pure and viable protoplasts could possibly increase the transformation efficiency.

Comparison of different *GFP*-containing constructs

To develop a *GFP* construct suitable for the identification of citrus transformants by visualization of green fluorescence, two plasmid constructs containing either the targeted *GFP*-ER (Endoplasmic reticulum targeting and retention sequence) for improved fluorescence (Haseloff et al., 1997) or nontargeted *GFP* (Cytoplasmic targeting) were compared in order to assay stable expression of the protein in the transformed citrus tissue. Both versions of the *GFP* were cloned under the control of the double 35S cauliflower mosaic virus promoter with the alfalfa mosaic virus (AMV) untranslated leader sequence (Figure 3-1 and 3-2). The double 35S-AMV promoter was reported by Datla et al. (1993) to increase expression up to 20-fold relative to the 35S promoter. Although both of these constructs gave transient expression at the protoplast level, nontargeted *GFP* gave less stable expression than targeted *GFP* at the colony or plant level. The most likely explanation for the lack of stable expression from nontargeted *GFP* construct may be due to insufficient accumulation of protein for detection because of its degradation by the proteases in the cytoplasm. Citrus callus tissue sometimes autofluoresces yellow when illuminated with blue light. To assure transformant identification without doubt, strong and stable *GFP* expression is critical. Thus, the

combination of insufficient protein accumulation and possible pale yellow autofluorescence from callus tissue could have prevented identification of transformed colonies with the nontargeted version of the *GFP*, even though expression at the protoplast level was sufficient for transient detection.

Transient and stable transformation frequencies using *GFP* as a selectable marker

Transient expression of *GFP* was visible at the protoplast level as early as 6 hours after transformation in some pARS108 experiments. Transient expression of green fluorescence was intense 16-24 hours after transformation in both p524EGFP.1 and pARS108 (Figure 3-7B). Visually, there was no difference in fluorescence intensity between p524EGFP.1 and pARS108 treated protoplasts. The difference between the two constructs became particularly evident by first week when the cells had started dividing. This expression persisted for 2-3 weeks, declining more significantly in p524EGFP.1 experiments. The average transient transformation frequency varied from one experiment to another, even when using the same *GFP* construct. This could be influenced by the culture cycle stage and the cultivar of the culture used as source of protoplasts. Using a 2-week subculture cycle, protoplasts isolated from 4- to 10-day-old cell suspension cultures show the best transient expression and division capacity. A similar result was reported by Fleming et al. (2000).

Stable transformation frequencies, based on the number of transformed protoplasts and not corrected for plating efficiency, were in the range of 1×10^{-5} protoplasts. These results are consistent with others in the literature. Davey et al. (2005a) reported that stable protoplast transformation frequencies remain low (one per 10^4 protoplasts), indicating a need to improvement as necessary for efficient selection and recovery of transformed cells and tissue. Transgenic tissue was selected and separated from non-transformed

tissue and transferred to solid medium approximately 2 months after the transformation procedure. Using the FITC/*GFP* filter set, detection of transgenic calli by exposing regenerated tissue under blue light was clear and direct by expression of a bright blue-green color (Figure 3-7C and D). Autofluorescence was generally not detected from non-transformed calli (Figure 3-7C), with the occasional expression of a pale yellow fluorescence in older cultures. There was little visual difference in fluorescence at this stage between p524EGFP.1 and pARS108. Visual selection of transgenic citrus is particularly effective at this stage as the colonies are small enough (250-500 mm) so that a single plate containing thousands of colonies can be rapidly screened, but large enough to be easily rescued and cultured individually. Aside from the expression of *GFP* detectable with blue light, the transgenic tissue appeared normal and could not be distinguished from non-transformed tissue under white light. At the embryo stage, it was easy to distinguish between transformants (green) and non-transformants (red) (Figure 3-7D).

Elliott et al. (1999) tested the efficiency of visual selection by *GFP* with no additional selection and concluded that without an additional selective agent, preferential growth of *GFP*-positive tissue is difficult to maintain. However, when *GFP*-positive tissue can be identified, selectively cultured, and plants regenerated, *GFP* has been successfully used as a visual screenable marker (Fleming et al., 2000; Niedz et al., 2003; Guo et al., 2005). Protoplasts form colonies or embryoids directly from single cells, making the selection and regeneration of transgenic individuals an efficient process, limited only by the efficiency of the particular protoplast system. Fluorescent protoplast-

derived colonies were regenerated into plants and maintained as cell lines. Green fluorescent colonies were not observed in any of the control plates.

At the whole plant level, there were distinct fluorescence differences between shoots transformed with the two *GFP* constructs. Shoots transgenic for the ER-targeted *GFP* (pARS108) were generally brighter than those derived from the nontargeted construct (p524EGFP.1), and exhibited a uniform green fluorescence with minimal red fluorescence from chlorophyll (Figure 3-5 and 3-6). However, the fluorescence intensity varied among different parts of the plant, being higher in new expanding leaves. This could be due to lower metabolic activity and chlorophyll accumulation that partially masks the green fluorescence provided by the *GFP* in old tissue. Haseloff et al. (1997) reported that they could consistently regenerate intensely fluorescent *Arabidopsis* plants when *GFP* was targeted to the ER. They also reported difficulty in regenerating plants from the brightest nontargeted transformants. Both results are similar to our observations in citrus. Other than the differences discussed above, development and regeneration responses appeared similar between plants transgenic for either p524EGFP.1 or pARS108.

Plant regeneration

The newly obtained ‘Hamlin’ orange embryogenic suspension culture line used in these experiments was selected because it was rapidly proliferating and routinely provided reasonable yields of good quality protoplasts and high regenerating efficiencies under the optimum condition. However, due to many factors in the procedure, it was difficult to optimize conditions. Embryogenic callus and particularly suspension cultures of some citrus cultivars have high rates of mutations and cytological aberrations that diminish their capacity for whole-plant recovery (Grosser and Gmitter, 1990). ‘Hamlin’

has proven to be a highly stable variety in culture. The high performance characteristics, high regeneration capacity of the 'Hamlin' suspension line merited its use in these experiments. Furthermore, 'Hamlin' is one of the more highly susceptible varieties to the citrus canker pathogen. For many years J. W. Grosser at CREC, Lake Alfred has been comparing several hundred 'Hamlin' and 'Valencia' orange somaclones regenerated from embryogenic calli, embryogenic suspension culture-derived protoplasts, and from nucellar seedling stem internodes via organogenesis. Although useful somaclonal variation was found in all of these populations, the large majority (80%) of the somaclones from all sources are not significantly different from the original clones (Grosser et al., 1996a). These results suggest that it should not be difficult to recover true-to-type transgenic plants using the protoplast-transformation system (Fleming et al., 2000).

Generally, the colonies selected for *GFP* expression were transferred to EME-maltose 4-8 weeks after transformation. Shortly thereafter, embryos began to form. Transformed embryogenic callus develops normal, bipolar, heart shaped embryos when cultured on cellulose acetate membranes overlaid on solid EME-maltose medium (Figure 3-7E and F). The mechanism of membrane induced embryo normalization remains unclear (Niedz et al., 2002). These embryos were removed and transferred to another fresh plate containing EME-maltose medium, and allowed to proliferate. Embryos were then transferred to 1500 medium for further growth.

Efforts to regenerate transgenic plants were therefore focused on these cultures. More than 500 transgenic somatic embryos recovered from several experiments were cultured on B+ germination medium for two passages. The majority of these embryos

became larger, but did not convert to plantlets. However, as is often the case with citrus somatic embryos, multiple shoots were regenerated following culture on DBA3 shoot induction medium (Deng et al., 1992; Grosser et al., 2000) (Figure 3-7G and H). Approximately 200 embryos produced shoots, but a few of them produced both shoots and roots. These plants were transferred to soil but were not successfully acclimated, probably due to poor root quality. Shoots from the other germinated embryos were cultured on RMAN root-induction medium, and most of them successfully produced roots (Figure 3-7I). These plants were also transferred to soil but again most of them were not successfully acclimated. Potting soil contamination was by a fungus determined to be at least partially responsible for this result (about 200 plants were recovered out of more than 1200 plantlets transferred to the soil) (Figure 3-7J and K).

As mentioned, the non-converted transgenic embryos were sectioned and cultured on DBA3 shoot-induction medium for two to three passages. Some of them produced multiple shoots and others responded poorly. Many clusters of healthy transgenic shoots were recovered. Shoots were cultured on RMAN root-induction medium, and again most of them successfully produced roots (>75%). Transgenic plants growing on their own roots exhibited a high level of *GFP* expression in the root, attributed to the absence of chlorophyll in root tissue (Data not shown).

There are several reasons for the low regeneration efficiency in protoplast transformation experiments. First, problems related to the culturing of the protoplasts, or the condition of the cell suspension cultures used for protoplast isolation could be influential. If large numbers of microcalli are produced (i.e., high plating efficiency), there is a tendency for callus to be formed at the expense of embryo induction. If

embryos do form, there is often an overgrowth of those embryos by the callus, which inhibits further embryo development. Other reasons for the failure of plant regeneration include contamination of the media, the condition of the protoplasts after isolation and transformation, and possibly other unknown factors.

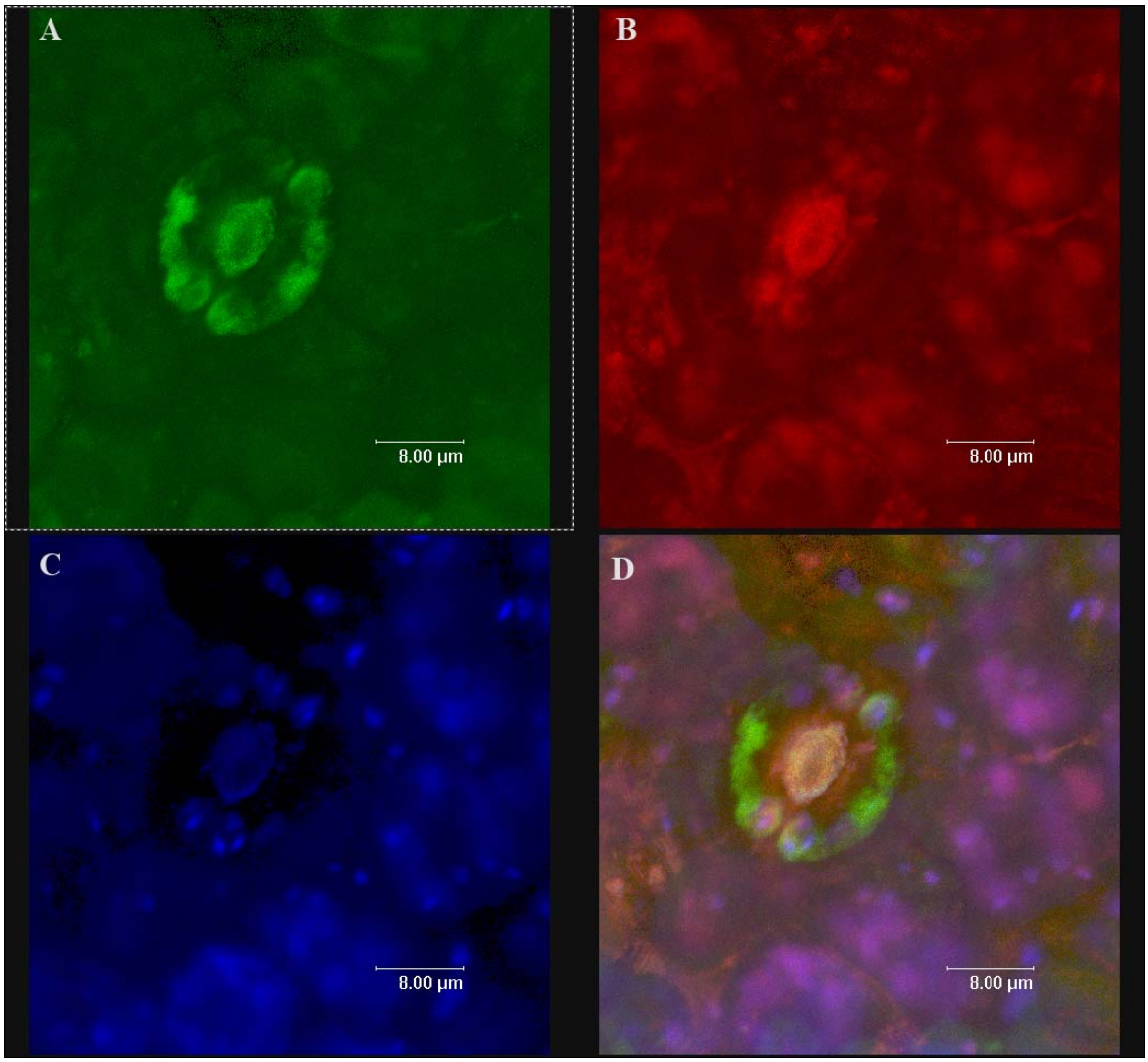

Figure 3-5. Cytoplasmic targeting of *GFP* expression in a mature citrus leaf. Mature leaves from transgenic plant obtained from p524EGFP.1 were scanned using confocal laser scanner with a setting for three different fluorescent wavelengths, green, red, and blue, at the same time with a 488, 543, and 633 nm excitation filter and a 500-543, 610-630, and 675-750 tunable spectral window, respectively. (A) green, (B) red, (C) blue, and (D) overlay of three filters together.

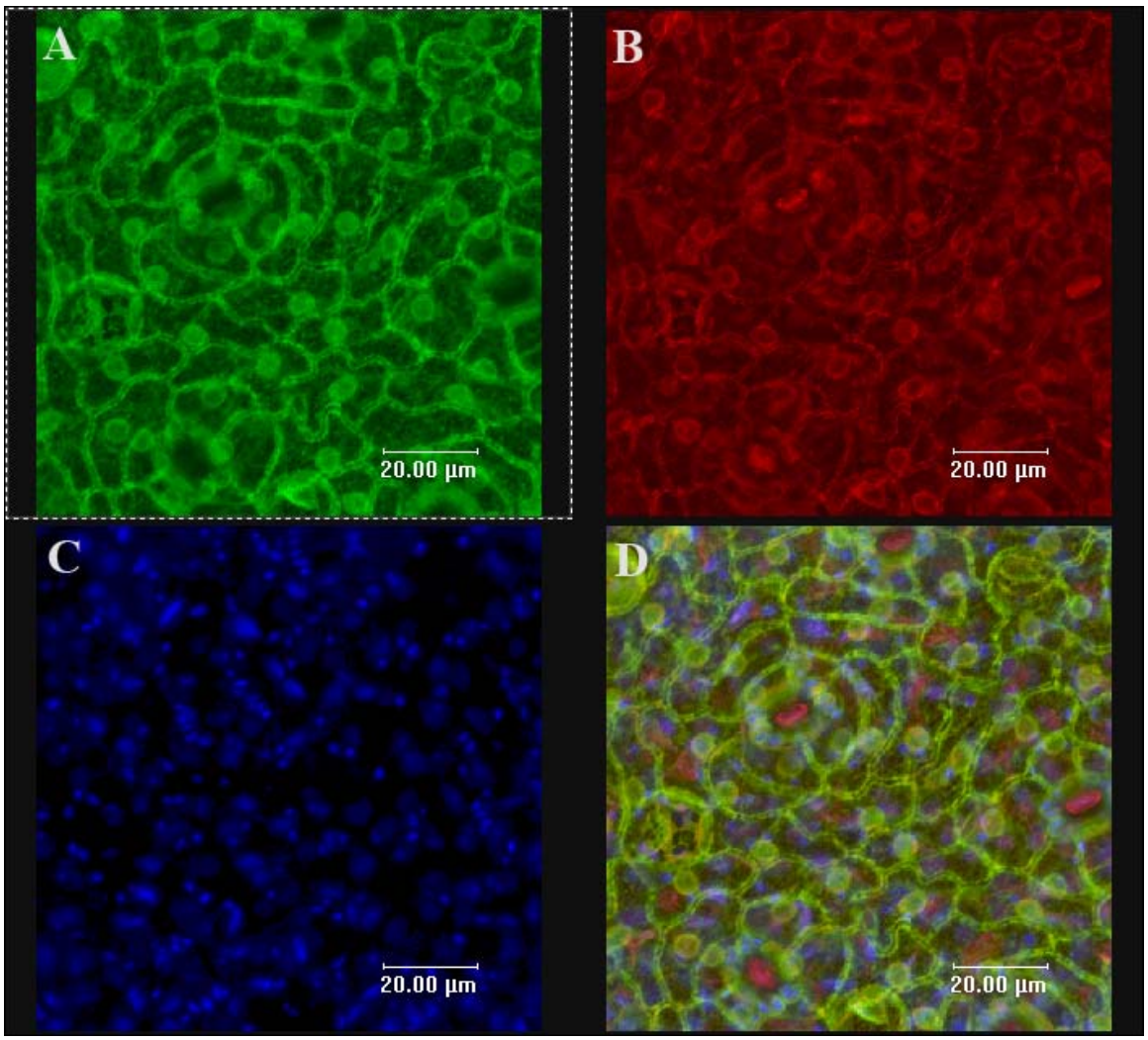

Figure 3-6. ER targeting of *GFP* expression in a mature citrus leaf. Mature leaves from a transgenic plant obtained from pARS108 were scanned using confocal laser scanner with setting for three different fluorescent wavelengths, green, red, and blue, at the same time with a 488, 543, and 633 nm excitation filter and a 500-543, 610-630, and 675-750 tunable spectral window, respectively. (A) green, (B) red, (C) blue, and (D) overlay of three filters together.

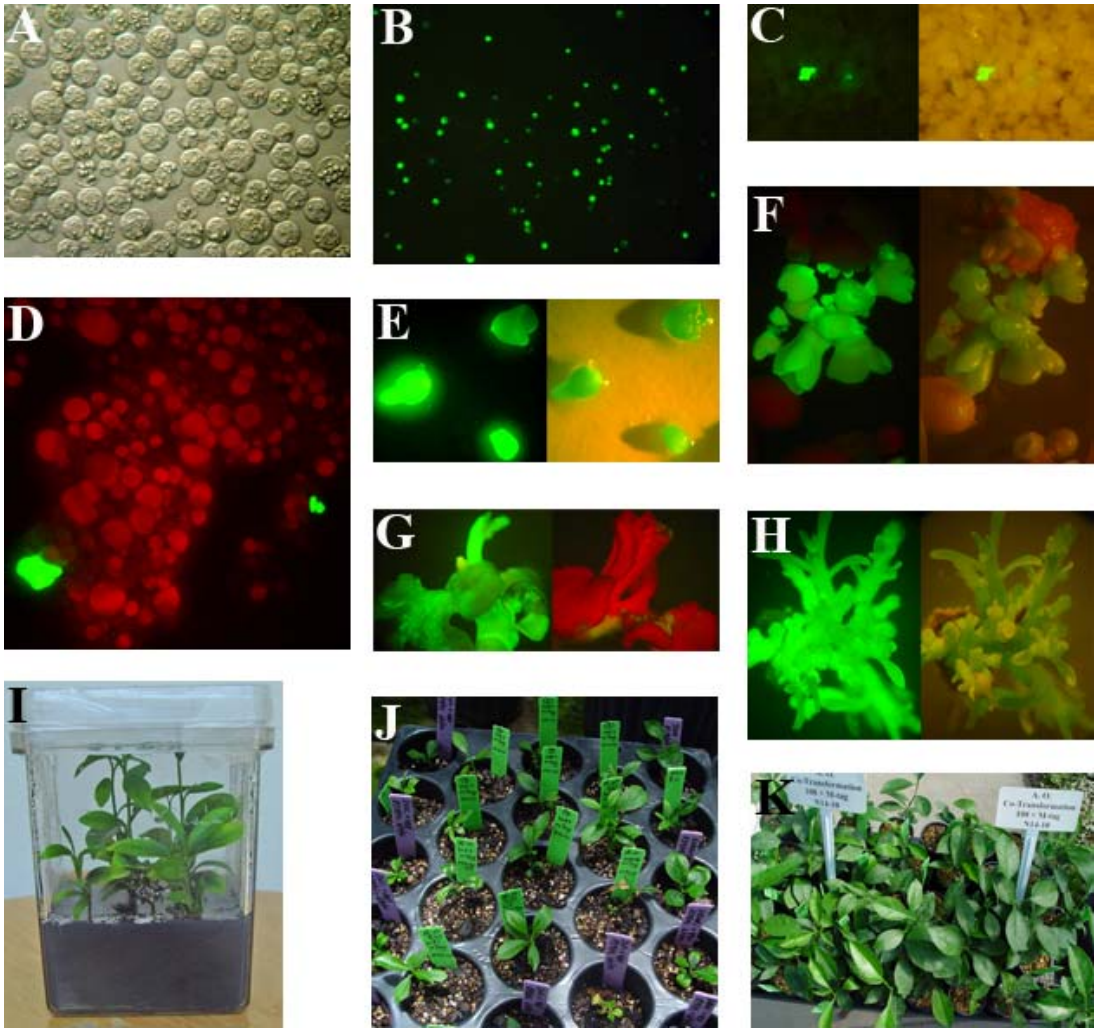

Figure 3-7. Citrus transgenic plant regeneration and *GFP* monitoring from protoplast to plant. (A) protoplasts after isolation from the ring between sucrose 25% and mannitol 13%; (B) protoplasts after 24 hours from co-transformation with pARS108/p524EGFP.1 and pXa21-mtag by direct DNA uptake protocol using Polyethylene Glycol (PEG); (C) protoplast-derived calli (transformed and non-transformed) on EME-maltose solid medium 36 days after transformation, (Left) blue light; (Right) white light; (D) transgenic (green) and non-transgenic (red) somatic embryos growing on EME medium 6 to 8 weeks after transformation; (E) transgenic somatic embryos growing on cellulose acetate membranes laid on 1500 medium 2-3 months after transformation; (F) transgenic (green) and non-transgenic (red) somatic embryos growing on 1500 media 3-4 months after transformation; (G) embryo-derived transformed (green) and non-transformed (red) shoots on B+ medium 5-6 months after transformation; (H) embryo-derived transformed shoots on DBA3 medium 6 months after transformation; (I) *in vitro* rooted transgenic citrus plants constitutively expressing the *GFP* gene; (J and K) transgenic 'Hamlin' sweet orange plants in soil.

Shoots failing to produce roots were either *in vitro* grafted to nucellar seedlings of Carrizo citrange (Figure 3-8) or shoot-tip grafted onto Carrizo citrange and/or sour orange in the greenhouse (Figure 3-9). The grafting technique was chosen based on the shoot condition. Small and soft shoots were selected for *in vitro* micrografting, whereas larger and more hardened ones were chosen for shoot-tip grafting in the greenhouse. Over 95% of the *in vitro* micrografts were successful, producing more vigorous plantlets that were, acclimated with a 75% success rate.

Shoot-tip grafting has been used successfully to recover transgenic shoots regenerated from citrus epicotyls treated with *Agrobacterium* (Peña et al., 1995a; 1997; Bond and Roose, 1998; Cervera et al., 2000). Grafting of small shoots (<1 cm) as they arise overcomes rooting difficulties that can occur (Moore et al., 1992). Rooting protoplast derived sweet orange plants can take up to 3 months before roots emerge. Shoot-tip grafting greatly accelerates plant recovery. Over 500 transgenic plants were obtained from co-transformation experiments using shoot tip grafting. The typical morphology of the regenerated transgenic 'Hamlin' orange plants was similar to that of the non-transformed plants recovered from the control cultures.

The time frame from initial protoplast isolation to plant establishment in soil varied greatly from one experiment to another. Several factors influence this chain of events. Delays in the osmoticum reduction process and culture transfer slowed down calli and embryo growth. In some experiments, rapidly developing embryos could be transferred directly from EME to B+ medium, skipping the transfer to 1500 medium. In others, embryo growth was slower, requiring an additional subculture on EME, 1500, or B+

media. However, if all the stages went smoothly, the plantlets could be established in soil in as little as 5 to 6 months.

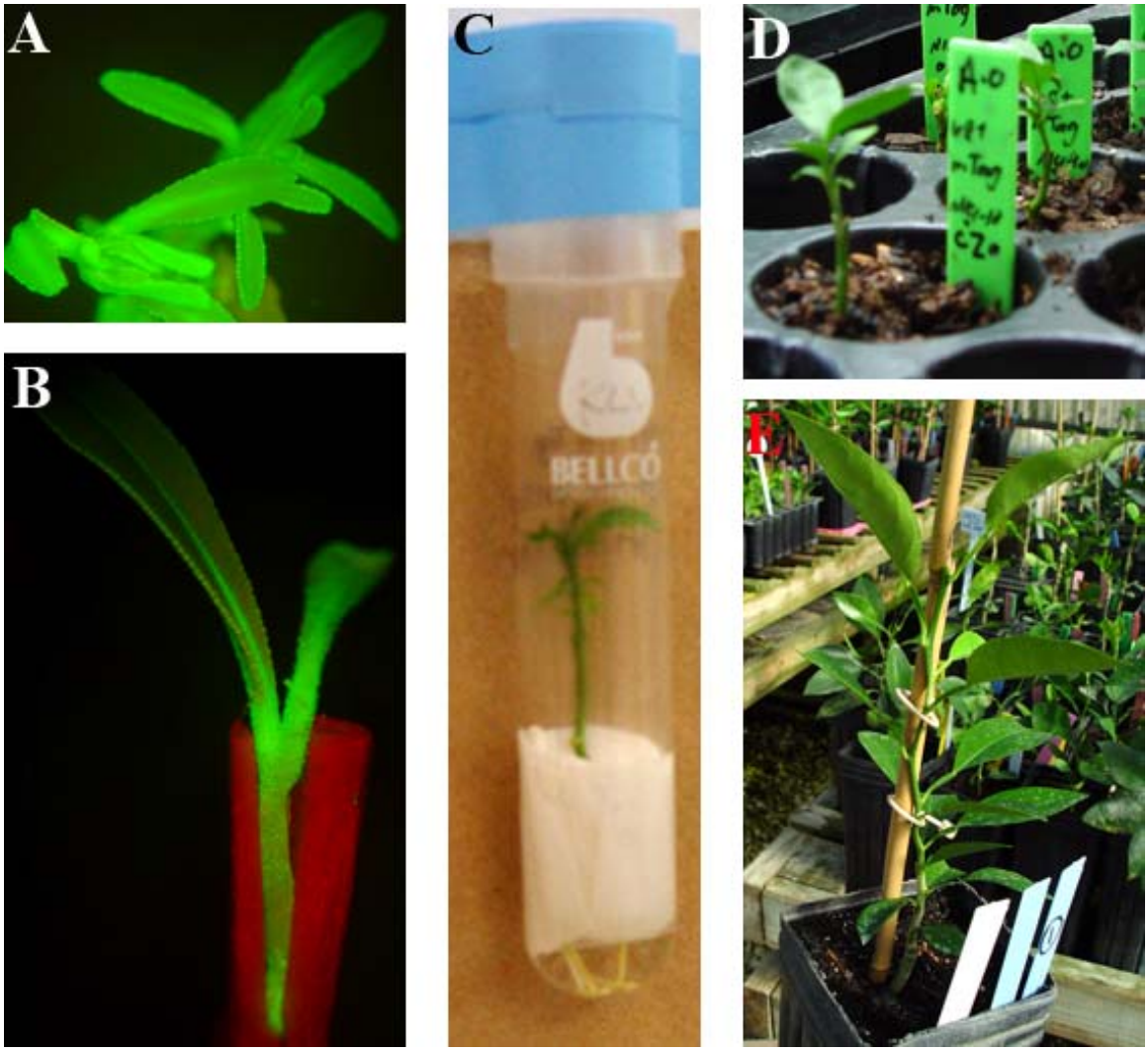

Figure 3-8. *in vitro* grafting of transgenic 'Hamlin' sweet orange onto nucellar seedlings of Carrizo citrange. (A) shoots failing to root *in vitro*; (B) grafted transgenic scion (green) onto Carrizo seedling (red); (C) micrografted transgenic 'Hamlin' sweet orange onto Carrizo plant *in vitro*; (D) transgenic 'Hamlin' sweet orange growing on Carrizo rootstock in soil 6 weeks after grafting; (E) transgenic plant more than a year in soil under the greenhouse condition.

From more than 15 co-transformation experiments with p524EGFP.1 and pXa21-mtag, only one transgenic event was recovered and 7 transgenic plants were successfully propagated using grafting techniques. From more than 30 co-transformation

experiments with pARS108 and pXa21-mtag, 75 transgenic events were recovered and more than 500 transgenic plants were regenerated and transferred to the greenhouse. According to the literature, this is the first time to report a large population of normal transgenic ‘Hamlin’ sweet orange plants using the protoplast/*GFP* transformation system.

Figure 3-9. Shoot-tip grafting of transgenic ‘Hamlin’ sweet orange onto Carrizo citrange and/or sour orange. (A) shoot failing to root *in vitro*; (B) shoot tip grafting of transgenic ‘Hamlin’ sweet orange onto Carrizo plant in soil (under the tip); (C) transgenic ‘Hamlin’ sweet orange growing onto Carrizo rootstock in soil 2-3 weeks after removing the tip; (D) transgenic scion growing onto Carrizo rootstock in soil 6-8 weeks after removing the tip; (E) transgenic plants after more than a year in soil.

Molecular Analysis

Polymerase chain reaction (PCR) for screening transformants

When putative transgenic scions grafted onto Carrizo citrange seedlings had developed several leaves, a leaf was removed from each plant, excited with blue light to confirm *GFP* expression. The polymerase chain reaction was used initially to screen

regenerated ‘Hamlin’ orange plants obtained via co-transformation. The PCR analysis was performed using DNA from leaves of regenerated plants, transgenic and non-transgenic. To confirm the presence of the *GFP* gene and the cDNA of the *Xa21* gene in the transgenic plant genomes, multiple PCR experiments were performed using two different pairs of primers for *GFP* and the cDNA at the same time. A predicted internal fragment of about 780 nucleotides was amplified in all DNA samples from green fluorescent leaves (Figure 3-10). No amplification was detected in DNA samples from non-transgenic regenerated control plants, which emitted red fluorescence under blue light. A predicted internal fragment of about 1400 nucleotides was amplified in 35% of the DNA samples from green fluorescent leaves, which corresponds to the cDNA of the *Xa21* gene (Figure 3-10).

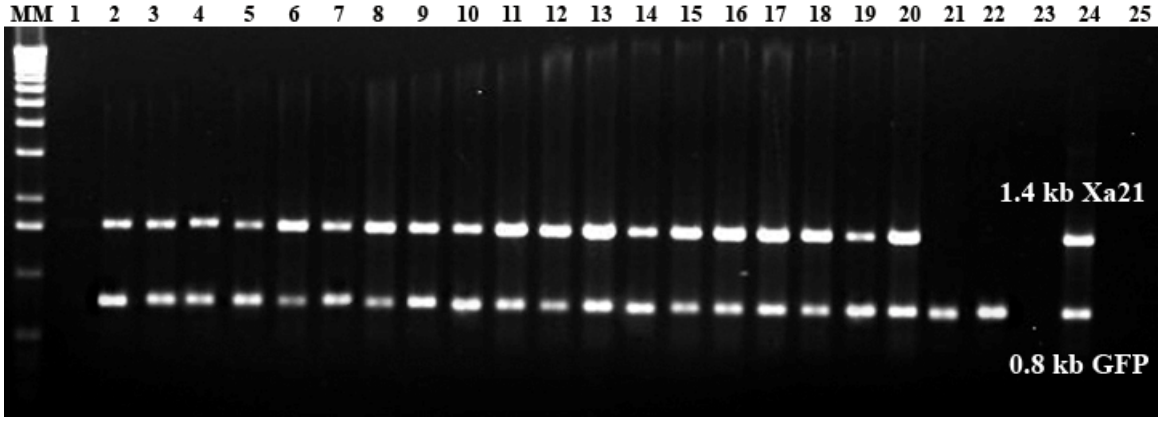

Figure 3-10. Multiple PCR analysis to detect the presence of the *GFP* (0.8 kb) and the cDNA of the *Xa21* (1.4 kb) genes in transgenic citrus plants. Lane 24 is a positive control for both genes (DNA plasmid). Products have shown in lanes 2-20 the presence of *GFP* gene and the cDNA of the *Xa21* gene in the transgenic plants. Lanes 21 and 22 are positive for *GFP* only. Lane 23 is negative for both genes. Lane 1 is a non-transgenic control, and lane 25 is negative control (H_2O), MM = 1 kb DNA ladder.

Screening for transformants using southern blot analysis

When the grafted plants measured 20-30 cm in height, southern analysis was performed to confirm the stable integration of the cDNA of *Xa21* coding sequence in the transgenic plants genome and to provide some information regarding their integration patterns. Eight randomly chosen putative transgenic plants (grown in the green house for more than 12 months), which were PCR positive for *GFP* and *Xa21* were analyzed. Genomic DNA was digested with *SphI* that has a unique restriction site within the 5′ end of the integrated sequence of the cDNA of the *Xa21*, and *ApaLI* that does not cut in the cDNA. Therefore, bands hybridizing to the probe result from one site within the vector and one site within the flanking genomic DNA. Hybridization patterns with multiple bands were observed (Figure 3-11). In general, the direct-gene-mediated method results in more multiple and rearranged fragment in transgenic plants than do plants transformed by the *Agrobacterium*-method (Dong et al., 1996; Krasnyanski et al., 1999), and this was the case for this study on citrus. Even though different hybridization patterns were observed, the results of southern blot analysis provided molecular evidence confirming the presence of the introduced cDNA of the *Xa21* gene in the citrus genome.

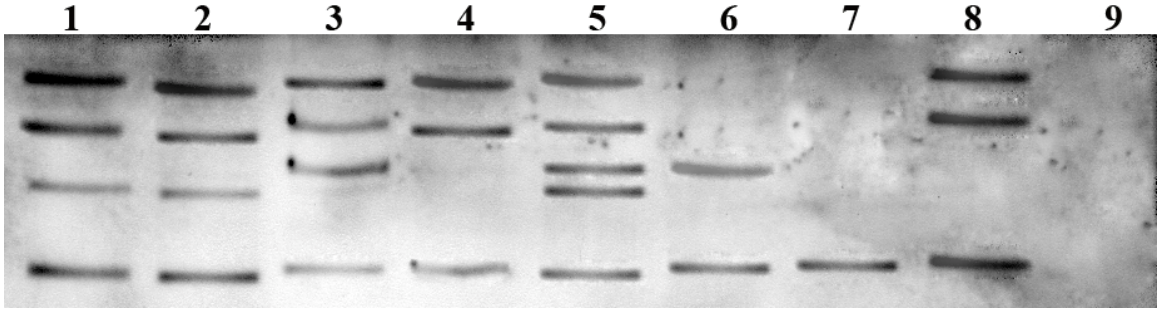

Figure 3-11. Southern hybridization analysis of ‘Hamlin’ sweet orange plants with the cDNA of the *Xa21* gene. The probe was a 1641-bp *Nco*I855 → *Nco*I2496 fragment of the cDNA of the *Xa21* gene isolated from plasmid pAO3. Lanes 1-9 *Genomic* DNAs were digested with *SphI* and *ApaLI*. Lanes 1-8 transgenic plants and lane 9 non-transgenic plant.

Confirmation of transformation using western blot analysis

Leaf tissue from transgenic plants was used to obtain crude protein extracts for Western analyses. The extracted proteins (35 µg per lane) were separated subsequently by SDS/PAGE on 5% stacking and 6.5% separation gels and transferred to Immobilon-PVDF membranes for immunostaining. The Western blots were developed with anti-c-myc as primary antibody followed by anti-mice immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase as a secondary antibody as described in the material and methods. Detection of immune complexes was achieved by enhanced chemiluminescence according to ECL Plus Western Blotting Detection Kit. Finally, expression of the *Xa21* was detected on the membrane to verify that the gene was integrated and expressed in the transgenic plants. In this assay, a protein product of 120 kDa corresponding to the cDNA of the *Xa21* gene was immunoreactive with the monoclonal antibody in 22 out of 34 tested transgenic plants (Figure 3-12). In this assay soluble extracts of the transgenic rice expressing the wild-type *Xa21* gene and non-transgenic rice were used as positive and negative controls. As shown in Figure (3-12), there is a non-specific band appearing in all the samples which is larger than the *Xa21* corresponding band. Some of the transgenic plants were PCR and southern analysis positive, but did not have the corresponding band for the *Xa21* protein. The most likely explanation for this phenomenon is that even though the DNA had been transferred and integrated into the plant genome, it was not transcribed to RNA and subsequently protein. As shown, most Southern-positive plants transformed with cDNA of the *Xa21* express *Xa21* protein to detectable levels. According to the literature, this is the first time that a gene from rice has been stably integrated and expressed in citrus plants. After more than

12 months growing in the greenhouse, all transgenic plants showed a normal phenotype, identical to that of control non-transformed ‘Hamlin’ orange plants.

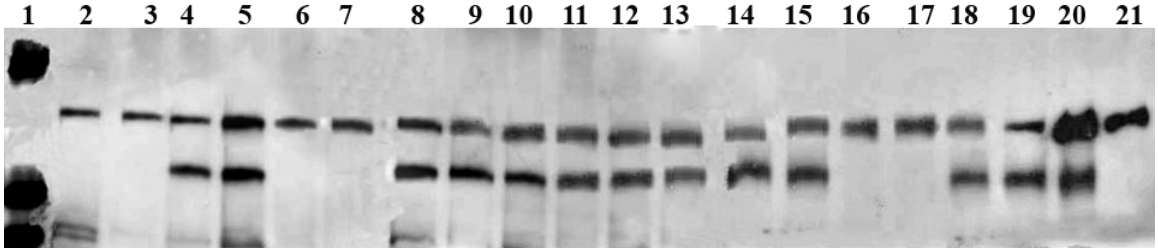

Figure 3-12. Western blotting analysis of transgenic ‘Hamlin’ sweet orange. The extracted proteins (35 µg per lane) were separated subsequently by SDS/PAGE on 5% stacking and 6.5% separation gels and transferred to polyvinylidene difluoride membranes for immunostaining. Soluble extracts of the transgenic rice expressing the wild-type *Xa21* gene (lane 20), non-transgenic rice (lane 21), transgenic citrus expressing the *GFP* gene only (lane 1), non-transgenic citrus (lane 2), transgenic citrus positive for *GFP* and *Xa21* genes (lanes 4-19) were subjected to Western blotting with reference to molecular mass markers (indicated in lane 1). The Western blots were developed with anti-c-Myc as primary antibody and c-Myc: Anti-mice IgG as a secondary antibody as described in the material and methods.

CHAPTER 4
TRANSGENIC 'Hamlin' SWEET ORANGE PLANTS CONTAINING A RICE *XA21* cDNA *XANTHOMONAS* RESISTANCE GENE OBTAINED BY PROTOPLAST/*GFP* TRANSFORMATION SYSTEM

Introduction

'Hamlin' sweet orange (*Citrus sinensis* (L.) Osbeck) is one of the leading commercial cultivars in Florida because of its high yield potential and early maturity. 'Hamlin' also has a high regeneration capacity from protoplasts and is often used in transformation experiments. Citrus canker disease caused by the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* is becoming a worldwide problem. The *Xa21* is a member of the *Xa21* gene family of rice which provides broad spectrum *Xanthomonas* resistance in rice. Since the citrus canker pathogen is in the same genus, this gene may impart resistance to canker in citrus. The wild-type *Xa21* gene contains an intron, and there are some questions whether dicot plants can process genes containing monocot introns (the cDNA is intron free).

In the last decade, improvements of plant transformation vectors and methodologies have increased the efficiency of plant transformation and the ability to stably express transgenes in plants. DNA has been delivered into the plant cell using a wide range of tools such as *Agrobacterium*-mediated transformation, microprojectile bombardment, chemical (PEG) treatment of protoplasts and electroporation of protoplasts. Using chemical (PEG) treatment of protoplasts to transfer the gene of interest can be done by either transformation or co-transformation system. In co-transformation experiments, researchers used two different plasmids, one encodes the reporter gene and

the other encodes the gene of interest. This is very laborious, costly, and time consuming. In addition, the final transformation efficiency will be low because it is not guaranteed that in all the cases both plasmids will be transferred. A transformation system using a single plasmid, which encodes both the reporter and the gene of interest, is an alternative. Producing transgenic plants containing the plasmid pAO3, which encodes the *GFP* endoplasmic reticulum (ER)-targeting and the cDNA of the *Xa21* gene from the *Xa21* gene family of rice into one construction, was among the objectives of this study.

To achieve this objective, protoplasts isolated from a new embryogenic callus line of 'Hamlin' sweet orange were transformed with plasmid DNA (pAO3) using polyethylene glycol. Transformed clones were screened for *GFP* expression using blue light. Polymerase chain reactions (PCR) were performed to identify the transformed clones. Transgenic plants were regenerated from transformed clones via somatic embryogenesis and organogenesis. Transgenic plants containing the cDNA of the *Xa21* gene were assayed by southern blot analysis and western blot analysis to determine if the gene is integrated into the citrus genome and functioning to produce RNA and subsequently protein.

Materials and Methods

Plasmid Construction

The plasmid pARS108 containing the *GFP* ER-targeting gene was kindly provided by Dr. R. P. Niedz (USDA, Agriculture Research Service, US Horticultural Research Laboratory, Ft. Pierce, FL, USA). Construction of pARS108 has been described previously by Niedz et al. (2003). Plasmid pARS108 contains the *EGFP* coding sequence (GenBank accession #U55761) placed under the control of the double 35S cauliflower mosaic virus (35S-35S CaMV) promoter with the alfalfa mosaic virus (AMV)

untranslated leader sequence and the *nos* terminator. An *Arabidopsis* signal sequence (SS) is included in the 5′ end to target the protein to the ER, and the codon sequence for the amino acids HDEL were included at the 3′ end for retention of the protein in the lumen of the ER (Figure 3-1 in chapter 3).

Vector pCR504 was kindly provided by Dr. Pamela Ronald, University of California-Davis (Figure 3-3 in chapter 3). It contains a 3.1 kb *BamHI* fragment containing the entire cDNA of the *Xa21* gene from rice. Plasmid pCR504 is a promoterless plasmid containing the cDNA of the *Xa21* gene. Methods used for plasmid construction were similar to those described by Sambrook and Russell (2001). Plasmid pXa21-mtag has been constructed as described in chapter 3 and used to construct a new DNA plasmid pAO3.

To construct a new plasmid which contains ER-targeted *GFP* and the cDNA of *Xa21* in one construction, first the cDNA needed to be cloned into a separate plasmid with specific restriction enzymes. Plasmid pRTL2.vec was kindly provided by Dr. T. E. Clemente (Plant Science Initiative, University of Nebraska, Lincoln, NE, USA). This plasmid contains the 35S CaMV promoter and terminator cascade. To construct the plasmid used in the transformation experiment, the *BamHI* fragment containing the cDNA of *Xa21* with the c-myc sequence was excised from pXa21-mtag and ligated into *BamHI* site of pRTL2.vec (Figure 4-1). The re-ligated plasmid contained the cDNA of *Xa21* gene under the control of E35S promoter and T35S terminator was designated pAORTL. This plasmid could be used in co-transformation experiments. The *PstI* fragment from pAORTL (E35S-TEV-Xa21 cDNA-T35S) was excised and inserted into the *PstI* site of pARS108. The resulting plasmid is referred to as pAO3. The new plasmid

(pAO3) has both of the cDNA-mtag under the control of the 35S cauliflower mosaic virus (35S CaMV) promoter and T35S terminator and the ER-targeted *GFP* (Figure 4-1).

Plasmid Multiplication

Transformation of *E. coli*

Before a large scale plasmid preparation can be performed, the plasmid must first be transformed into competent *E. coli* cells. Max efficiency® DH5α™ competent cells were obtained from Invitrogen (Invitrogen Coporation, Carlasbad, CA, USA, Cat. No. 18258-012). The transformation procedure was followed exactly as described in the product manual.

Quick miniprep plasmid preparation and confirmation of the orientation of the insert

Plasmid DNA was extracted from several recovered bacterial colonies and compared to the DNA from the known plasmid stock by restriction analysis using agarose gel electrophorosis as described previously in chapter 3. To confirm the orientation of the insert, a restriction digest was then performed on the extracted DNA to test for the presence of the cDNA of the *Xa21* gene and it orientation as described in previously in chapter 3.

Large scale preparation of plasmid DNA

To obtain a large amount of the DNA, two 1-liter flasks containing 500 ml LB media plus the appropriate antibiotic were inoculated with the transformed *E. coli* and the cultures were incubated overnight at 37º C on a shaker at 225 RPM. The DNA was extracted from the overnight culture bacteria using Wizard® PlusMaxipreps DNA Purification System (Promega Corporation, Madison, WI, USA, Cat. No. A7270). To determine the concentration of plasmid DNA, an absorbance reading at an $O.D._{260}$ was

obtained, (an absorbance reading of 1.0 corresponds to 50 µg of plasmid DNA per ml) (Sambrook and Russell, 2001).

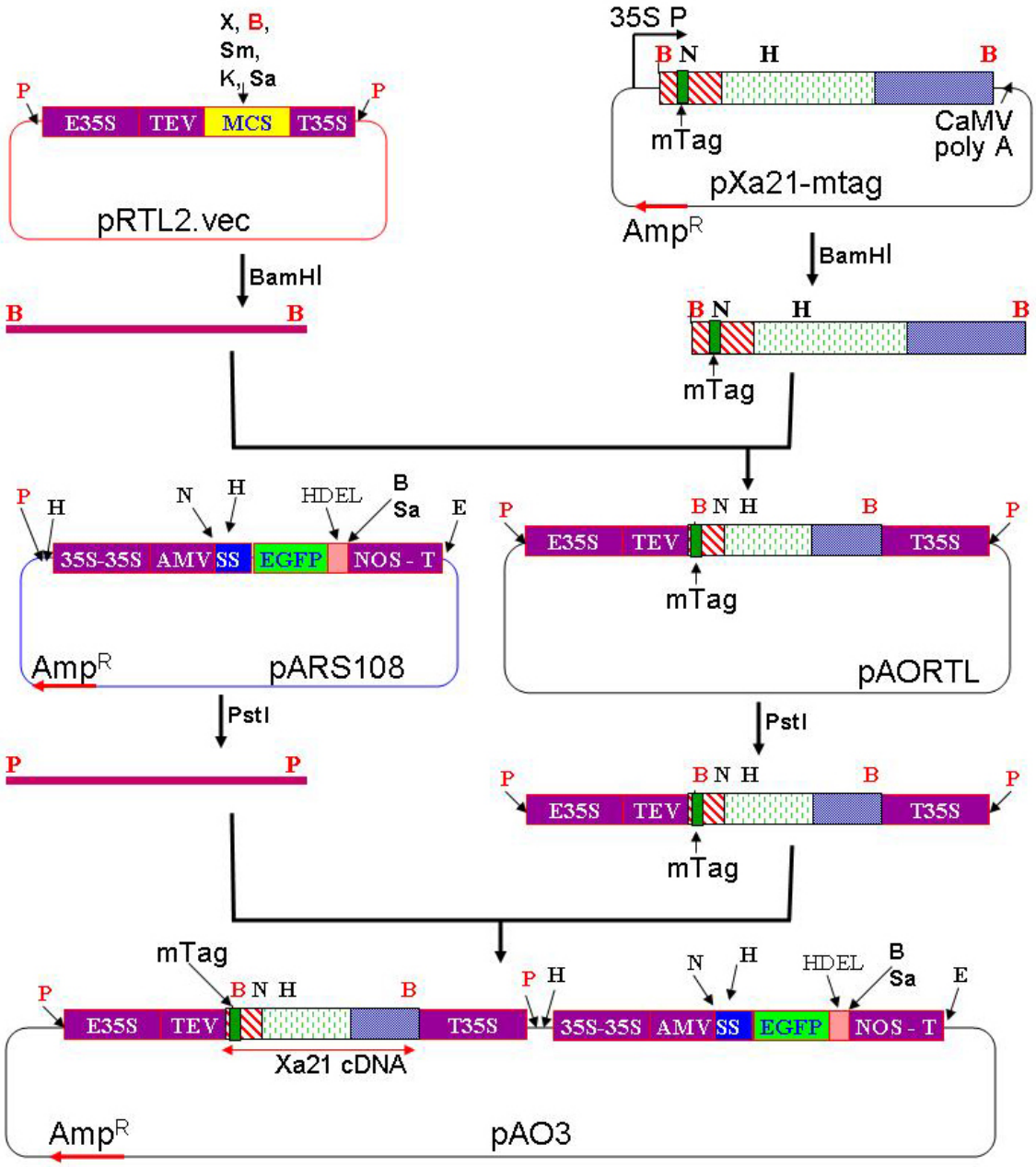

Figure 4-1. Schematic diagram of pAO3 plasmid. Restriction enzyme (B, *BamHI*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; N, *NcoI*; P, *PstI*; Sa, *SacI*; Sm, *SmaI*; X, *XbaI*). HDEL = Histidine, Aspartic acid, Glutamic acid, and Leucine, respectively.

Plant Material, Protoplast Transformation, and Culture

Establishment and maintenance of suspension cultures

A 'Hamlin' sweet orange (*Citrus sinensis* L. Osbeck) embryogenic callus line was induced from unfertilized ovules taken from mature fruit and maintained on Murashige and Tucker's (MT) basal medium (Murashige and Tucker, 1969). Suspensions cultures were maintained according to Grosser and Gmitter (1990) and subcultured every two weeks by splitting the contents between two sterile flasks and adding a 25 ml of fresh culture medium to each flask. The cells were used for protoplast isolation 4-10 days after subculture.

Isolation, transformation, and culture of citrus protoplasts

Protoplasts were isolated from an embryogenic suspension culture of 'Hamlin' sweet orange (*C. sinensis* L. Osbeck) and maintained on a 2-week subculture cycle according to Grosser and Gmitter (1990). Protoplasts were isolated from suspension culture 4-10 days after subculture as described in detail previously in chapter 3.

The protoplast transformation protocol was modified from the PEG-mediated protoplast fusion developed by Grosser and Gmitter (1990) for citrus somatic hybridization with slight modification as described previously in chapter 3.

Regeneration and selection of transformed protoplasts

The *GFP* expression was detected in the transgenic protoplasts illuminated with blue light within 24 hours after transformation using a Zeiss SV11 epifluorescence stereomicroscope equipped with a 100 W mercury bulb light source and an FITC/GFP filter set with a 480/30 nm excitation filter and a 515 nm longpass emission filter (Chroma Technology Corp., Brattleboro, VT, USA). Continued expression of the *GFP* protein was monitored occasionally for the next few weeks. Cultures were treated exactly

as described previously in chapter 3 to obtain transgenic plantlets. Transgenic shoot tips were micrografted onto Carrizo citrange or sour orange nucellar seedlings according to Navarro (1992) to expedite transgenic plant recovery.

Molecular Analysis of Transgenic Plants

Presence of the *GFP* gene and the cDNA of *Xa21* in the selected tissue were confirmed by PCR amplification of the transgenes. The copy number and integration pattern of the transgene were determined by Southern blot analysis. The expression of the cDNA of the *Xa21* gene was determined by western blot analysis.

Polymerase chain reaction (PCR) for detection of transformants

The PCR was used initially to screen regenerated 'Hamlin' sweet orange plant obtained via protoplast/*GFP* transformation system. Genomic DNA was extracted from young leaf tissue of transgenic and non-transgenic citrus plants using a GenElute^{TM} Plant Genomic DNA Miniprep kit (Sigma, Inc., St. Louis, MO, USA, Cat. No. G2N350). To confirm the presence of *GFP* and the cDNA of *Xa21*, multiple PCR experiments were performed using two different pairs of primers for *GFP* and for the cDNA simultaneously as described previously in chapter 3.

Southern blot analysis

The genomic DNA samples used for southern blot analysis were extracted and purified from young leaves of transgenic and non-transgenic plants using the CTAB method (Sambrook and Russell, 2001) as modified by T. E. Mirkov (personal communication). Twenty µg of DNA was digested with *SphI* and *ApaLI* restriction enzymes. Standard protocol for gel electrophoresis, denaturation, and neutralization of the gel were described by Sambrook and Russell (2001). After electrophoresis, the gels were treated with 0.25 N HCl for 10 minutes, and then washed with 0.4 N NaOH for

10 minutes. DNA was transferred to a positively charged nylon membrane (Immobilon^{TM}-Ny+; Millipore Corporation, Billerica, MA, USA) by capillary transfer using 20X SSC buffer overnight according to Sambrook and Russell (2001). Probe DNA for the cDNA of the *Xa21* gene was prepared from an *XhoI* fragment (1.49-kb) of the pXa21-mtag plasmid. Probe was labeled with digoxigenin-dUTP using a random primer labeling kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Applied Science, Cat No. 11 585 614 910). The probe labeling was performed according to the manufacturer's manual. Fixation the DNA to the membrane, prehybridizaton, hybridization, and immunological detection were performed as described in the instruction manual of DIG High Prime DNA Labeling and Detection Starter Kit II.

Western blot analysis

To carry out western blot analysis, total protein was extracted from transgenic and non-transgenic plants. About 0.3 g leaf tissue from transgenic and non-transgenic 'Hamlin' sweet orange plants was collected and ground in liquid nitrogen. To isolate the soluble fractions, the ground tissue was thawed in extraction buffer [50 mM Tris-HCl, pH 6.5; 1 mM EDTA; 150 mM NaCl; 0.1% Triton X-100]. Prior to use the extraction buffer immediately, the following protease inhibitors were added: 2 µg/ml Antipain; 2 µg/ml Leupeptin; 2 µg/ml Aprotinin; 1 mM of 4-[2-aminoethyl]-benzenesulfonyl fluoride (AEBSF) Sigma; and 5% of 2- mercaptoethanol. Samples were incubated at 4º C for 30 minutes with shaking and then centrifuged at 10,000 RPM for 10 minutes at 4º C. The supernatant was recentrifuged at 13,000 RPM for 10 minutes at 4º C. The supernatant was either immediately subjected to protein blot analyses or stored at -80º C after determinating the concentration of the protein by the method of Bradford (Bradford, 1979). After adding the loading dye and boiling the samples for 5 minutes, total protein

(35 μg) for each sample was separated on 5% stacking and 6.5% separation gels in a Mini- Protean III cell (Bio-Rad) according to Laemmli (1970) using Tris-glycine as the SDS-PAGE electrophoresis buffer.

Proteins were electrophoretically transferred to a PVDF membrane (Immobilon^{TM}-P; Millipore Corporation, Bedford, MA, USA, Cat. No. IPVH 000 10) using Trans-Blot Cell (BioRad). The non-specific binding sites on the membrane were then blocked with Blotto [5% non-fat dried milk in TTBS (100 mM Tris-HCl, pH 7.9; 150 mM NaCl; 0.1% Tween 20) (see appendix C)] for one hour at room temperature. The membrane was incubated with primary antibody (anti-c-myc, 1:700) in 3% bovine serum albumin (BSA) Fwashes in TTBS, the membrane was incubated with secondary antibody (C-myc: Anti-mice IgG) (Amersham Biosciences Corp, Piscataway, NJ, USA, Cat. No. NXA931) for one hour at room temperature, followed by three 10-minute washes in TTBS. Detection of the protein band on the blot was carried out according to the ECL Plus Western Blotting Detection Kit instruction (Amersham Biosciences Corp, Piscataway, NJ, USA, Cat. No. RPN2133). The membrane was exposed to X-ray film.

Results and Discussion

Plasmid Preparation

The cDNA of the *Xa21* gene was inserted into plasmid pRTL2.vec under the control of 35S CaMV promoter and CaMV poly A terminator. A c-myc epitope sequence was tagged to the 5′ end of the cDNA as described previously in chapter 3. This plasmid was designated pAORTL. The *PstI* fragment from pAORTL (E35S-TEV-Xa21 cDNA-T35S) was excised and inserted into *PstI* site on pARS108. The new construction was referred to as pAO3. Figure (4-1) shows a schematic diagram of the construction of pAO3 plasmid. Chemically competent *E. coli* strain DH5α^{TM} was transformed with

pAO3 plasmid according to the manufacture's instructions. Colony selection was performed using PCR with gene specific primers. Testing for the orientation of the insert was performed using restriction enzymes according to Sambrook and Russell (2001) prior to large scale preparation of the plasmid DNA. The positive bacterial colonies were replated and single colonies were selected to begin large scale plasmid DNA preparation as described previously. Preps with DNA concentration of 0.5 µg/µl and above were used in transformation experiments. Plasmid DNA was stored at -20º C for short-term use and at -80º C for long-term use.

Transformation of Citrus Protoplasts and Plant Regeneration

Isolation and transformation of protoplast and the ultimate regeneration of plants are controlled by several factors. The two most important factors are the quality of the starting material (cell suspension) and quality of the protoplast after isolation (high yields of viable protoplasts with little or no debris). The cell suspension line used was chosen from 'Hamlin' sweet orange new embryogenic cells because they were exhibiting a good regeneration capacity. The best protoplasts were obtained from suspensions 4-10 days after subculture when isolated according to Grosser and Gmitter (1990). Using the sucrose-mannitol gradient allows for the removal of the non-viable protoplasts and cellular debris which could interfere with the uptake of the plasmid by the viable protoplasts, or the regeneration in the following stages. Moreover, starting with pure and viable protoplasts could possibly increase the transformation efficiency.

Transient and stable transformation frequencies using *GFP* as selectable marker

Transient expression of *GFP* was visible at the protoplast level as early as 6-12 hours after transformation with pAO3 plasmid. Transient expression of green fluorescence was intense 16-24 hours after transformation (Figure 4-2 B). The average

transient transformation frequency varied between experiments. This may have been influenced by the culture cycle stage and/or the cultivar used as source of protoplasts. Using a 2-week subculture cycle, protoplast isolated from 4- to 10-day-old cell suspension culture showed the best transient expression and division capacity. A similar results was reported by Fleming et al. (2000).

Stable transformation frequencies, based on the number of transformed protoplasts and not corrected for plating efficiencies, were in the range of $1X10^{-5}$ protoplasts. These results are consistent with others in the literature. Davey et al. (2005a) reported that protoplast transformation frequencies remain low (one in 10^4 protoplasts giving stably transformed tissues) which requirers protoplast-to-plant system with efficient selection to recover transformed cells and tissue. Transgenic tissue was selected from non-transformed tissue and transferred to solid medium approximately 2 months after the transformation procedure. Using the FITC/GFP filter set, detection of transgenic calli by exposing regenerated tissue with blue light was detected by expression of a bright blue-green color (Figure 4-2 C and D). Autofluorescence was generally not detected from non-transformed calli (Figure 4-2 C), with the occasional expression of a pale yellow fluorescence in older cultures. Visual selection of transgenic citrus is particularly effective at this stage as the colonies are small enough (250-500 mm) so that a single plate containing thousands of colonies can be rapidly screened, but large enough to be easily rescued and cultured individually. Aside from the expression of *GFP* detectable with blue light, the transgenic tissue appeared normal and could not be distinguished from non-transformed tissue under white light. At the embryo stage, it is very easy to

distinguish between transformant (green) and non-transformant once (red) (Figure 4-2 D).

Elliott et al. (1999) tested the efficiency of visual selection by *GFP* with no additional selection and concluded that without an additional selective agent, preferential growth of *GFP*-positive tissue is difficult to maintain. However, when *GFP*-positive tissue can be identified, selectively cultured, and plants regenerated, *GFP* has been successfully used as a visual screenable marker (Fleming et al., 2000; Niedz et al., 2003; Guo et al., 2005). Protoplasts form colonies or embryoids directly from single cells, making the selection and regeneration of transgenic individuals an efficient process limited only by the efficiency of the particular protoplast system. Fluorescent protoplast-derived colonies were regenerated into plants and maintained as cell lines. Non-transformed green fluorescent colonies were not observed in any of the subculture control plates.

Plant regeneration

The newly obtained ‘Hamlin’ sweet orange embryogenic suspension culture line used in these experiments was selected because it was rapidly proliferating and routinely provided reasonable yields of good quality protoplasts and high regenerating efficiencies under the optimum condition. However, due to many factors that influence the procedure, it was difficult to optimize the condition. Embryogenic callus and particularly suspension cultures of some cultivars have high a rate of mutations and cytological aberrations that diminish the capacity for whole-plant recovery (Grosser and Gmitter, 1990). The high performance characteristics and high regeneration capacity of the ‘Hamlin’ callus line merited its use in these experiments. Moreover, ‘Hamlin’ is one of the highly susceptible varieties to citrus canker pathogen.

Generally, the colonies selected for *GFP* expression were transferred to EME-maltose medium 4-8 weeks after transformation. Shortly thereafter, embryos began to form. Transformed embryogenic callus developed normal, bipolar, heart shaped embryos when cultured on cellulose acetate membranes overlaid on solid EME-maltose medium (Figure 4-2 D). The mechanism of membrane induced embryo normalization remains unclear (Niedz et al., 2002). These embryos were removed and transferred to another fresh plate containing EME-maltose medium, and allowed to proliferate. Embryos were then transferred to 1500 medium for further growth.

Efforts to regenerate transgenic plants were focused on these cultures. More than 200 transgenic somatic embryos recovered from several experiments were cultured on B+ germination medium for two passages. The majority of these embryos became larger, but did not convert to plantlets. However, as is often the case with citrus somatic embryos, multiple shoots were regenerated following culture on DBA3 (Deng et al., 1992). Only eight embryos produced shoots, but three of them produced both shoots and roots. Half of the plants were transferred to soil but were not successfully acclimated, probably due to poor root quality. The other half and shoots from the other germinated embryos were cultured on RMAN root-induction medium, and most of them successfully produced roots (Figure 4-2E). Some of these plants were transferred to soil, but again all of them were not successfully acclimated, probably due to poor root quality. Also, a fungal potting soil contamination was determined to at least partially responsible.

As mentioned, the non-converted transgenic embryos were sectioned and cultured on DBA3 shoot-induction medium for five to seven passages, but none of them produced shoots. Shoots recovered from the eight lines were cultured on RMAN root-induction

medium, and again most of them successfully produced roots (>90%). Transgenic plants growing on their own root are showing a high level of *GFP* expression in the root, attributed to the absence of chlorophyll in root tissue (data not shown).

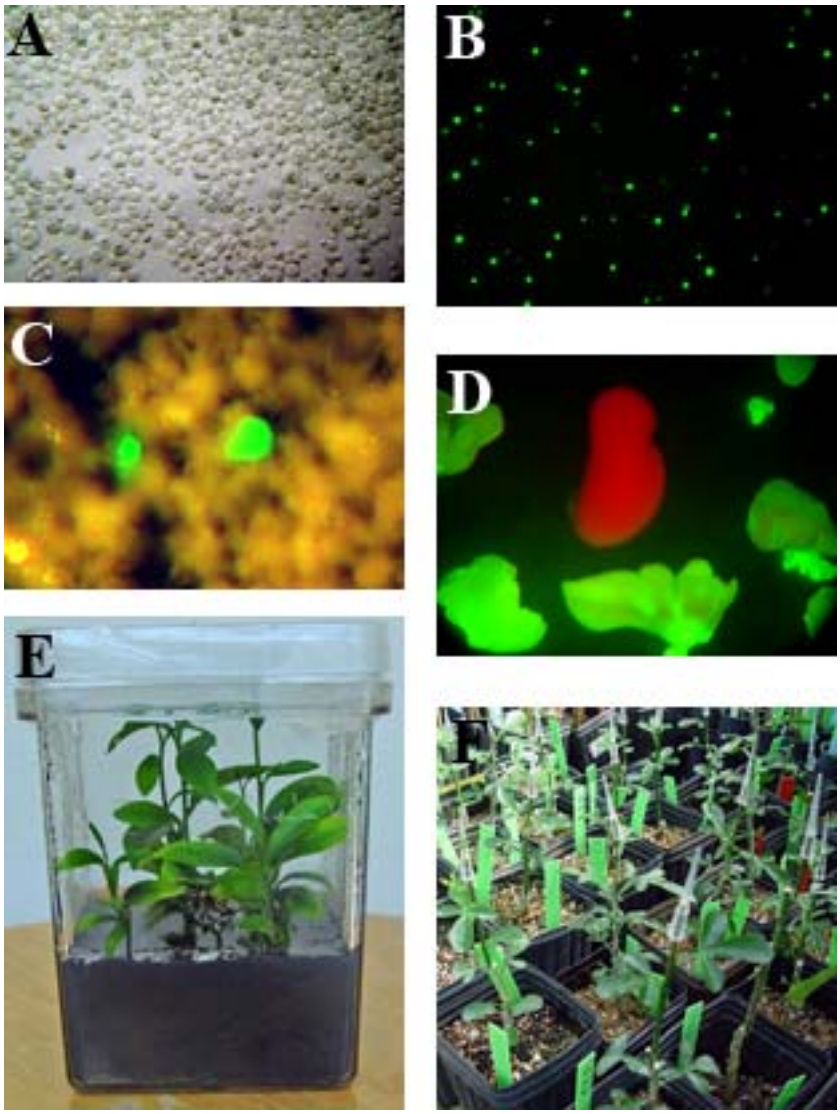

Figure 4-2. Citrus transgenic plant regeneration and *GFP* monitoring from protoplast to plant. (A) protoplasts after isolation from the ring between sucrose 25% and mannitol 13%; (B) protoplasts after 24 hours from transformation with pAO3 by direct DNA uptake protocol using Polyethylene Glycol (PEG); (C) protoplast-derived calli (transformed and non-transformed) on EME-maltose solid medium 36 days after transformation; (D) transgenic (green) and non-transgenic (red) somatic embryos growing on EME medium 6 to 8 weeks after transformation; (E) *in vitro* rooted transgenic citrus plants constitutively expressing the *GFP* gene; (F) shoot tip grafting of transgenic 'Hamlin' sweet orange onto Carrizo plant in soil (under the tip).

There are several reasons for the low regeneration efficiency in protoplast transformation experiments. First, problems related to the culturing of the protoplast, or the condition of the cell suspension cultures used for protoplast isolation could be influential. If large numbers of microcalli are produced (i.e., high plating efficiency), there is a tendency for callus to be formed at the expense of embryo induction. If embryos do form, there is often an overgrowth of those embryos by the callus, which inhibits further embryo development. Other reasons for the failure of plant regeneration include contamination of the media, the condition of the protoplast after isolation and transformation, and possibly other unknown factors.

Shoots failing to produce roots were either in vitro grafted to nucellar seedlings of Carrizo citrange or shoot-tip grafted onto Carrizo citrange and/or sour orange in the green house. Since small plantlets did not pass the acclimation stage in soil, they have been used as a source of grafting material. The grafting technique was chosen based on the shoot condition. Small and soft shoots were chosen for *in vitro* micrografting, whereas larger and more hardened ones were chosen for shoot-tip grafting in the greenhouse. One hundred percent of the i*n vitro* micrografts were successful, producing more vigorous plantlets that were acclimated with 90% success rate.

Shoot-tip grafting has been used successfully to recover transgenic shoots regenerated from citrus epicotyls treated with *Agrobacterium* (Peña et al., 1995a; Peña et al., 1997; Bond and Roose, 1998; Cervera et al., 2000). Grafting of small shoots (<1 cm) as they arise overcomes rooting difficulties that can occur (Moore et al., 1992). Rooting protoplast derived sweet orange plants can take up to 3 month before roots emerge. Shoot-tip grafting greatly accelerates the recovery of plants. About 72 transgenic plants

were obtained by using shoot tip grafting from eight different transgenic events. The typical morphology of the regenerated transgenic 'Hamlin' plants was similar to that of the non-transformed plants recovered from the control cultures.

The time frame from initial protoplast isolation to plant establishment in soil varied greatly from one experiment to another. Several factors influence this chain of events. Delays in the osmoticum reduction process and transferring slowed down calli and embryo growth. In some experiments, rapidly developing embryos could be transferred directly from EME to B+ medium, skipping the transfer to 1500 medium. In others, they required an additional subculture on EME, 1500, or B+ media. However, if all the stages went smoothly, the plantlets were established in soil in as little as 5 to 6 months.

From over 20 transformation experiments with pAO3, only eight transgenic events were recovered and 72 transgenic plants were propagated using grafting techniques and transferred to the greenhouse.

GFP expression at the mature stage of the transgenic plants

At the mature plants stage, the fluorescence intensity varied among different parts of the plant, being higher in new growing leaves than in old ones (Figure 4-3 and 4-4). That could be due to lower metabolic activity and chlorophyll accumulation that partially masked the green fluorescence provided by the *GFP* in old tissue. Haseloff et al. (1997) reported that they could consistently regenerate intensely fluorescent *Arabidopsis* plants when *GFP* was targeted to the ER. They also reported difficulty in regenerating plants from the brightest nontargeted transformants. Both results are similar to our observations in citrus. Other than the differences discussed above, developmental and regeneration responses appeared similar between transgenic and non-transgenic plants.

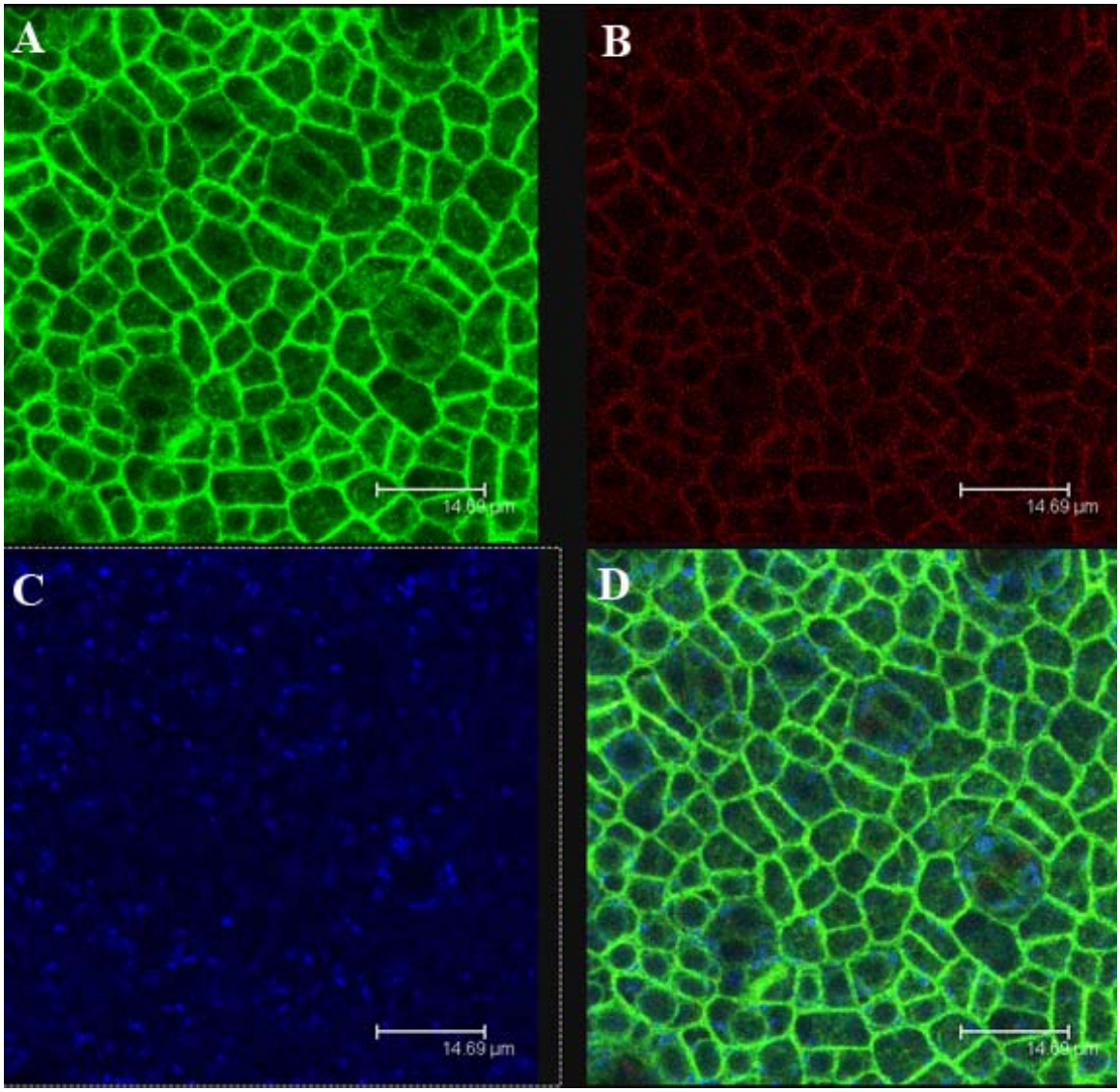

Figure 4-3. ER targeting of *GFP* expression in a mature citrus leaf. New growing leaf from a transgenic plant obtained from pAO3 were scanned using confocal laser scanner with setting for three different fluorescent wavelengths, green, red, and blue, simultaneously with a 488, 543, and 633 nm excitation filter and a 500-543, 610-630, and 675-750 tunable spectral window, respectively. (A) green, (B) red, (C) blue, and (D) overlay of three filters together.

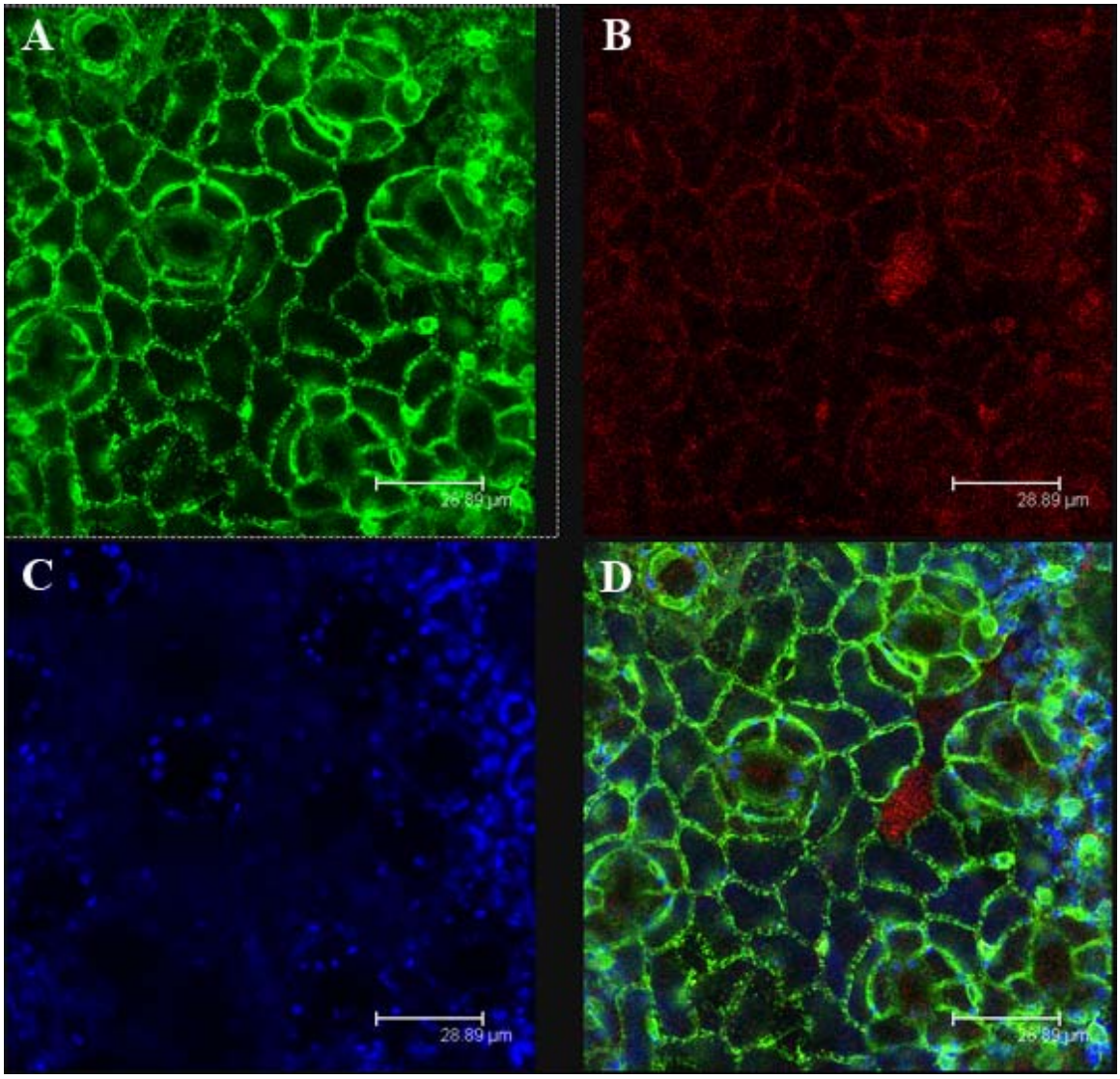

Figure 4-4. ER targeting of *GFP* expression in a mature citrus leaf. Old growing leaf from a transgenic plant obtained from pAO3 were scanned using confocal laser scanner with setting for three different fluorescent wavelengths, green, red, and blue, at the same time with a 488, 543, and 633 nm excitation filter and a 500-543, 610-630, and 675-750 tunable spectral window, respectively. (A) green, (B) red, (C) blue, and (D) overlay of three filters together.

Molecular Analysis

Polymerase chain reaction for selection of transformants

When putative transgenic scions grafted on Carrizo citrange seedlings had developed several leaves, a leaf was removed from each plant, excited with blue light to confirm *GFP* expression. PCR was used initially to screen regenerated 'Hamlin' sweet orange plant obtained via transformation. PCR analysis was performed using DNA from leaves of regenerated plants, both transgenic and non-transgenic. To confirm the presence of the *GFP* gene and the cDNA of the *Xa21* gene in the transgenic plant genome, multiple PCR experiments were performed using two different pairs of primers for *GFP* and *Xa21* simultaneously. A predicted internal fragment of about 780 nucleotides was amplified in all DNA samples from green fluorescent leaves (Figure 4-5). No amplification was detected in DNA samples from non-transgenic regenerated control plants, which emitted red fluorescence under blue light. A predicted internal fragment of about 1400 nucleotides was amplified in 100% of the DNA samples from green fluorescent leaves, which corresponds to *Xa21* (Figure 4-5). This is compared with protoplast/*GFP* co-transformation system in which just 35% of the DNA samples from green fluorescent leaves had the corresponded band to the cDNA of the *Xa21* gene. Thus, these results indicated that the final transformation efficiency for protoplast/*GFP* transformation is higher than for co-transformation.

Screening for transformants using southern blot analysis

When the grafted plants measured 20-30 cm in height, southern analysis was performed to confirm the stable integration of the cDNA of *Xa21* coding sequence in the transgenic plants genome and provide some information related to their integration pattern. Five chosen putative transgenic lines (grown in the greenhouse for more than

12 months), which were PCR positive for *GFP* and *Xa21*, were analyzed. Genomic DNA was digested with *SphI* a unique restriction site within the 5′ end of the integrated sequence of the cDNA of the *Xa21*, and *ApaLI* that does not cut in *Xa21*. Therefore, bands hybridizing to the probe result from one site within the vector and one site within the flanking genomic DNA. Hybridization patterns with 1-3 bands/copy numbers were observed (Figure 4-6). In general, the direct-gene-mediated method results in more multiple insertions and rearranged fragments in transgenic plants than *Agrobacterium*-mediated transformation (Dong et al., 1996; Krasnyanski et al., 1999), and that was the case for this study on citrus. Even though different hybridization patterns were observed, the results of southern blot analysis provided molecular evidence confirming the presence of the introduced cDNA of the *Xa21* gene in the 'Hamlin' sweet orange genome. Many researchers reported that transgenic plants with multiple copies of the integrated DNA into one or more chromosomal location have been shown to be more likely to exhibit transgene silencing (Iyer et al., 2000; James et al., 2002) by affecting the level and stability of gene expression. However in a recent study, Craig et al. (2005) reported that there is no correlation between the number of gene insertions and gene expression level, suggesting that multiple insertions may have little or no effect on transgene expression.

Confirmation of transformation using western blot analysis

Leaf tissue from transgenic plants was used to obtain crude protein extracts for Western analyses. The extracted proteins (35 µg per lane) were separated by SDS/PAGE on 5% stacking and 6.5% separation gels and transferred to Immobilon-PVDF membranes for immunostaining. The western blots were developed with anti-c-myc as the primary antibody followed by anti-mice immunoglobulin G (IgG) antibody

conjugated with horseradish peroxidase as a secondary antibody as described previously. Detection of immune complexes was achieved by enhanced chemiluminescence according to ECL Plus Western Blotting Detection Kit. Finally, to verify that the gene was integrated and expressed into the transgenic plants, expression of *Xa21* was detected on the membrane. In this assay, a protein product of 120 kDa corresponding to the expressed *Xa21* was seen in seven out of eight tested transgenic plants (Figure 4-7). In this assay, soluble extracts of the transgenic rice expressing the wild-type *Xa21* gene and non-transgenic rice were used as positive and negative controls. As shown in Figure (4-7), a non-specific band appeared in all the samples which is larger than the *Xa21* corresponding band. One transgenic plant was PCR and southern analyses positive, but did not have the corresponded band for the *Xa21* protein. The most likely explanation for this phenomenon, even though the DNA has been transferred and integrated into the plant genome, it was not transcribed or expressed. As shown, all but one Southern-positive plant transformed with cDNA of the *Xa21* expressed *Xa21* protein at detectable levels. According to the literature, this is the first time that a gene from rice has been stably integrated and expressed in citrus plants. After more than 12 months growing in the greenhouse, all transgenic plants showed a normal phenotype, identical to that of control non-transformed ‘Hamlin’ orange plants.

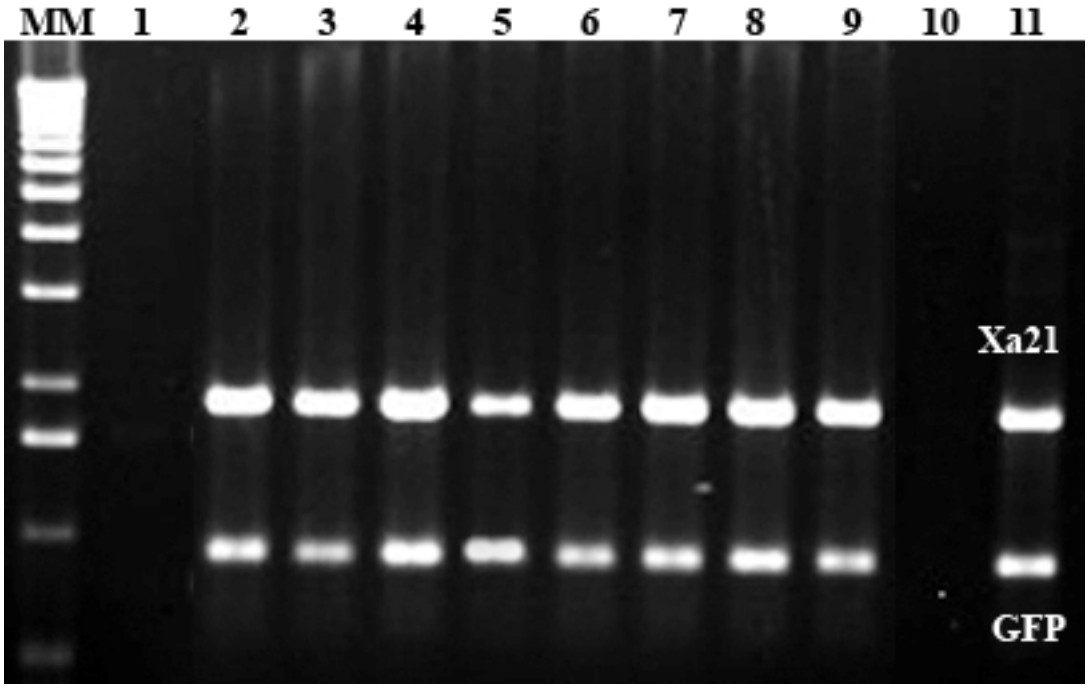

Figure 4-5. Multiple PCR analysis to detect the presence of the *GFP* (0.8 kb) and the cDNA of the *Xa21* (1.4 kb) genes in transgenic citrus plants. Lane 11 is a positive control for both genes (pAO3 plasmid). Products are seen in lanes 2-9 indicate the presence of *GFP* gene and the cDNA of the *Xa21* gene in the transgenic plants. Lane 1 is a non-transgenic control, and lane 10 is negative control (H_2O), MM = 1 kb DNA ladder.

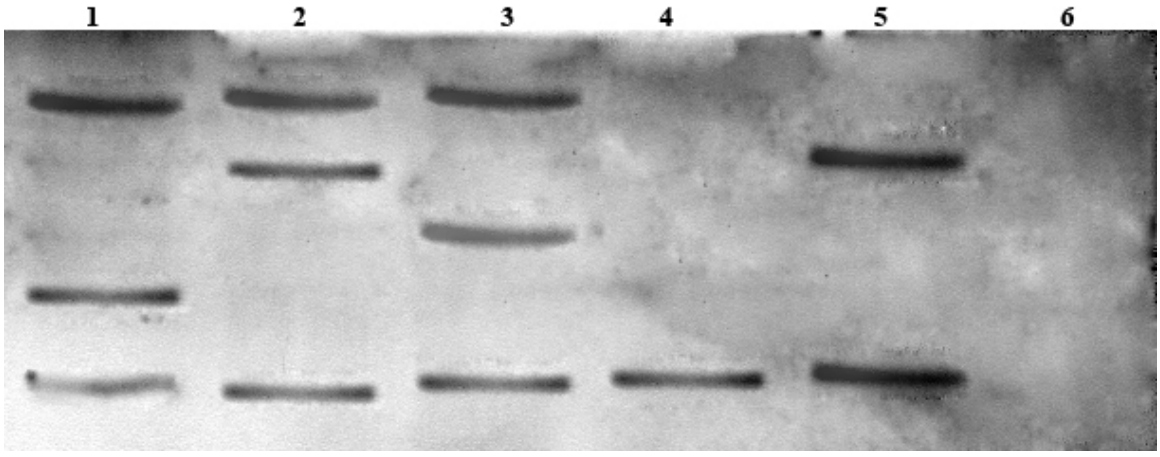

Figure 4-6. Southern hybridization analysis of 'Hamlin' sweet orange plants with the cDNA of the *Xa21* gene. The probe was a 1641-bp *Nco*I855 → *Nco*I2496 fragment of the cDNA of the *Xa21* gene isolated from plasmid pAO3. Lanes 1-6 Genomic DNAs were digested with *SphI* and *ApaLI*. Lanes 1-5 transgenic plants and lane *6* non-transgenic plant.

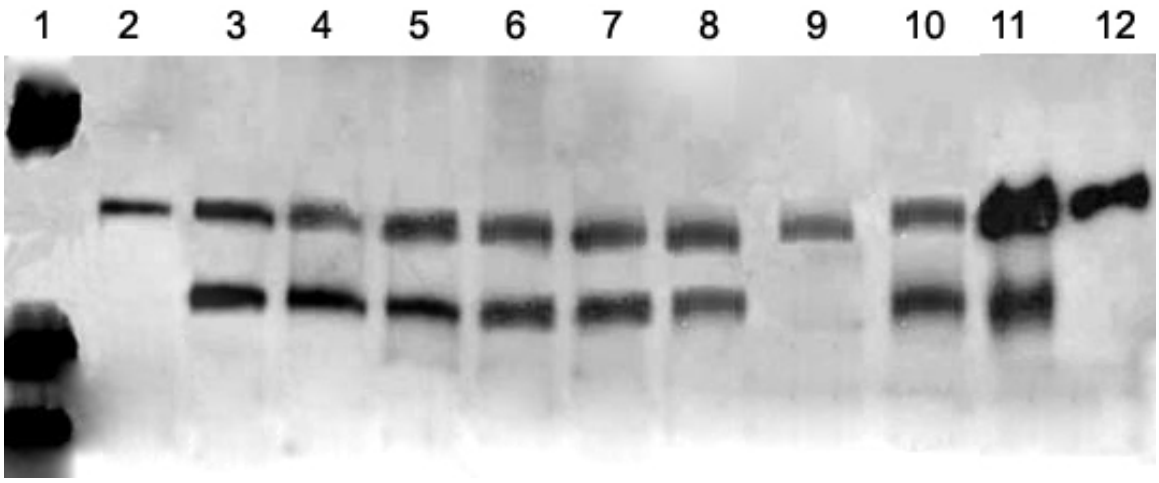

Figure 4-7. Western blotting analysis of transgenic 'Hamlin' sweet orange. The extracted proteins (35 µg per lane) were separated by SDS/PAGE on 5% stacking and 6.5% separation gels and transferred to polyvinylidene difluoride membranes for immunostaining. Soluble extracts transgenic rice expressing the wild-type *Xa21* gene (lane 11), non-transgenic rice (lane 12), non-transgenic citrus (lane 2), transgenic citrus positive for *GFP* and *Xa21* genes by PCR and Southern (lanes 3-10) were subjected to western blotting. Molecular markers indicated in lane 1. The western blots were developed with anti-c-Myc as primary antibody and c-Myc: Anti-mice IgG as a secondary antibody as described in the material and methods.

CHAPTER 5
ESTIMATING THE COPY NUMBER OF TRANSGENES IN TRANSFORMED CITRUS PLANTS BY QUANTITATIVE REAL-TIME PCR

Introduction

Genetic transformation has become a widespread tool in both basic research and commercial plant breeding programs. Its application requires that transgenes be stably integrated and expressed in the plant genome. During the last decade, the area under cultivation with transgenic crops has increased worldwide from 1.7 million hectares in 1996 to 81 million hectares in 2004 (Figure 5-1), and were grown mostly in the U.S. (47.6 million ha), Argentina (16.2 million ha), Canada (5.4 million ha), Brazil (5.0 million ha), China (3.7 million ha) and Paraguay (1.2 million ha) (James, 2004).

When new transgenic plants are developed, two early and essential questions are which plants contain the transgene and in how many copies. Thus, transgenic plants must be characterized at the molecular level because the new DNA is randomly inserted into the plant genome. While multiple copies of the transgene are useful for over-expression experiments, transformants that carry multiple copies of the integrated DNA into one or more chromosomal locations have been shown to be more likely to exhibit transgene silencing (Iyer et al., 2000; James et al., 2002), by affecting the level and stability of gene expression. However, in recent study Craig et al. (2005) reported that there is no correlation between the number of gene insertions and gene expression level, suggesting that multiple insertions may have little or no effect on transgene expression. Thus, estimating transgene copy number is critical to the selection and cultivation of the

transgenic plants. Most of the transgenic plants obtained via direct DNA delivery methods such as particle bombardment, electroporation, PEG, etc., may consist of complex patterns of transgene integrations, i.e., multiple copies and/or partial insertion of the integrated DNA (Dong et al., 1996; Kohli et al., 1998; Krasnyanski et al., 1999; Srivastava et al., 1999). Less than 20% of the transgenic events generated using direct DNA delivery display low-copy integration (three copies or less) (Song et al., 2002). To date, there is no transformation method that can completely control the number of transgene integrations into the plant genome. Therefore, transgenic events should be screened as early in the transformation process as possible to identify multiple copy transformants, allowing for continued focus on low copy number transformants. Such screening can be very difficult to handle, especially when the number of independent transformed events is sufficiently large.

Southern blot analysis, in which a blot of digested genomic plant DNA is hybridized with a labeled DNA probe corresponding to the transgene to produce an instructive band pattern, has been used to estimate transgene copy number. This method has become a routine procedure in many labs around the world because of its highly reliable results under the optimum condition. However, it is quite costly in terms of reagents, labor, and time, and also requires a considerable amount of DNA from fresh or frozen material. Furthermore, Southern blot analysis may not be accurate enough to determine a copy number greater than two (Honda et al., 2002), although multiple integrations are often found with transgenic plants (Dong et al., 1996; Kohli et al., 1998; Krasnyanski et al., 1999; Srivastava et al., 1999). Some other methods could be used for the same purpose including comparative genomic hybridization (Larramendy et al.,

1998), fluorescence in situ hybridization (Kallioniemi et al., 1996), multiplex amplification probe hybridization (Armour et al., 2000) and microarray (Li et al., 2002). Unfortunately, all of those methods share the same limitations as southern blot analysis.

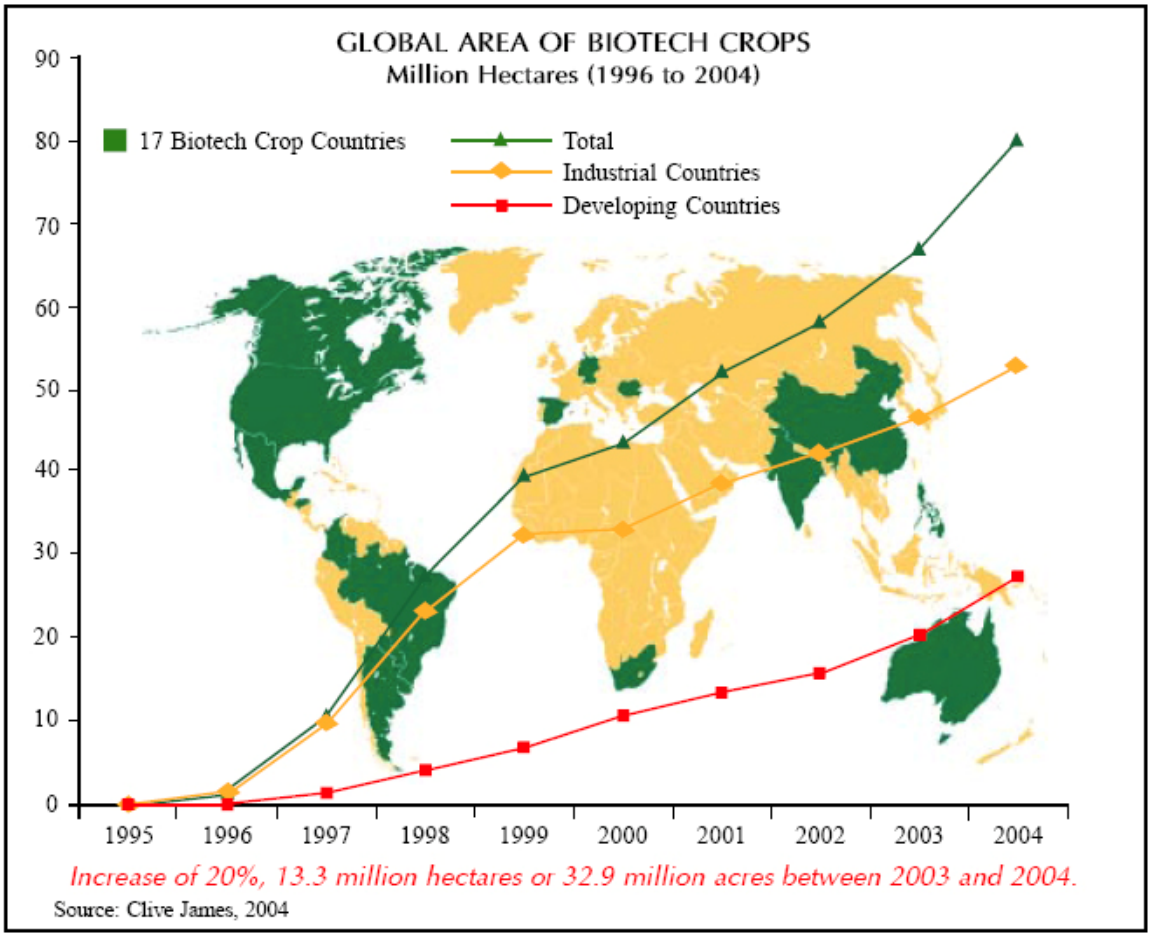

Figure 5-1. Global area of biotech crops (James, 2004).

The polymerase chain reaction (PCR) is one of the most sensitive techniques for detecting the integrated gene in the transgenic plant genome, and thus it can reduce the amount of DNA required for analysis (Wong and Medrano, 2005). Advances in PCR instrumentation and fluorescence chemistry have made the precise quantification of specific amplification products possible without the need for post-PCR analyses. In contrast to conventional PCR where only the amount of end product is determined

(Freeman et al., 1999), real-time PCR allows researchers to track the changes of PCR product during the reaction. Quantitative real-time PCR technology relies on the ability to progressively monitor fluorescence emitted from nonspecific double-stranded DNA binding dyes (SYPR® Green I) or fluorophore-labeled specific probes (TaqMan®) that hybridize with target sequences during the exponential phase of the PCR reaction. The probe is labeled at the 5′ end with a fluorescent molecule and at the 3′ end with a quencher molecule. In an intact probe, the 5′ and 3′ labels are in close proximity, and the quencher silences the fluorescent signal that would otherwise be generated by the 5′ fluorescent label (Livak et al., 1995). In the TaqMan assay, the degradation of target-specific probe molecules by the 5′-3′ exonuclease activity of the Taq DNA polymerase liberates the fluorescent label, which subsequently produces a fluorescent signal during each cycle of the amplification. For a high fluorescent signal, the probe must bind tightly to the template, enabling Taq polymerase to cleave nucleotides from the 5′ end of the probe (Bubner and Baldwin, 2004). This fluorescent signal is proportional to the accumulation of PCR product generated which is proportional to the quantity of initial DNA template in the sample (Livak et al., 1995). Fluorescence levels are detected during each cycle of amplification by specialized instrumentation. During the early cycles of amplification, the fluorescence level is low, but at a critical point fluorescence accumulates to a significant level perceived by the instrument's detection system. This point, which is called the threshold cycle (Ct) depends primarily on the starting amount of nucleic acid (Heid et al., 1996). The higher the initial amount of nucleic acid in the reaction, the smaller the Ct values. In practice, there is a linear relationship between the log of the starting quantity of the template and its Ct value during real-time PCR reaction.

Accordingly, the Ct is defined as the cycle at which the reaction's fluorescence reaches the threshold line. Quantitative real-time PCR (qTR-PCR) techniques have been reported for determining transgene copy number in transformed plants (Ingham et al., 2001; Mason et al., 2002; Song et al., 2002; Yang et al., 2005).

To date, the use of qRT-PCR for estimating transgene copy number in citrus has not yet been investigated. In this chapter, we will describe a quantitative RT-PCR assay for fast and accurate estimation of exogenous *Xa21* gene copy numbers in transgenic citrus by comparison with the citrus endogenous reference gene coding for Lipid Transfer Protein (*LTP*) (Wu and Burns, 2003). We used quantitative multiplex TaqMan assay, in which two TaqMan probes were simultaneously used within the same reaction to identify the copy number of the transgene. One TaqMan probe was designed to identify the transgene (*Xa21*) while the other identified an endogenous reference gene (*LTP*). We have chosen the citrus lipid transfer protein (*LTP*) gene (Wu and Burns, 2003), a known two-copy gene, as a reference gene. For the purpose of choosing a gene to use as an endogenous control in a comparative Ct multiplexed PCR reactions, any gene, regardless of how many homologous sequences may exist in the genome, may successfully be used (Schmidt and Parrott, 2001). Since the proportion of the reference endogenous gene remains constant relative to the total genomic DNA, it was possible to normalize differences in the amount of DNA in each reaction. Moreover, real-time PCR results can be subjected to statistical analysis.

Materials and Methods

Transgenic Plants

Plasmid pXa21-mtag and pAO3 containing cDNA of the *Xa21* gene under the control of 35S CaMV promoter (described previously in chapters 3 and 4, respectively)

were introduced into ‘Hamlin’ sweet orange (*Citrus sinensis* L. Osbeck) by means of protoplast/GFP transformation or co-transformation system. Transgenic plants were obtained as described previously in chapters 3 and 4.

DNA Extraction

For the events generated containing the plasmid DNA pXa21-mtag based on the conventional PCR results, genomic DNA samples from 34 transgenic plants were used for the quantitative RT-PCR. Genomic DNA was extracted and purified from about 100 mg of young leaf tissue using a GenElute^{TM} Plant Genomic DNA Miniprep kit (Sigma, Inc. St. Louis, MO, USA, Cat. No. G2N350). For the PCR analysis, genomic DNA was diluted to 10 ng/µl in free nuclease water (Promega). The citrus genomic DNA samples used for Southern blot analysis were extracted and purified from fresh leaves of transgenic and non-transgenic plants according to the CTAB method (Sambrook and Russell, 2001) and modified by T. E. Mirkov (personal communication). Genomic DNA was measured by UV absorption at 260 nm, while DNA purity was evaluated based on the UV absorption ratio at 260/280 nm. Twenty µg of DNA was digested with *SphI* and *ApaLI* restriction enzymes. Standard protocol for southern blot analysis including gel electrophoresis, denaturation, neutralization, fixation, prehybridization, hybridization, and immunological detection was performed as described previously in chapter 3.

Quantitative Real-time Polymerase Chain Reaction (qRt-PCR)

Primers and probes

The oligonucleotide primers and TaqMan probe used in this study (Table 5-1) were designed with the Primer Express 2.0 software (Applied Biosystems-Perkin-Elmer, Foster City, CA, USA). All primers and probes were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). The internal oligonucleotide probe specific for

the *Xa21* gene (Song et al., 1995) was 5′ end-labeled with the fluorescent reporter dye FAM (6-carboxy-fluoroscein, excitation wavelength = 494 nm, emission wavelength = 521nm), whereas the probe specific for the endogenous gene, lipid transfer protein (*LTP*) (Wu and Burns, 2003), was labeled with the fluorescent reporter dye VIC (excitation wavelength = 535 nm, emission wavelength = 555 nm, a proprietary dye by ABI, Foster City, CA, USA) on the 5′ end. The 3′ end of both probes was labeled with the fluorescent quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) and phosphate-blocked to prevent extension during PCR cycling. The Xa21-mtag-2823F/Xa21-mtag-2889R primer pair combined with the Xa21-mtag-2847T probe and LTP-178F/LTP-236R primer pair with the LTP-196T probe were employed for *Xa21* and *LTP* quantitative real-time PCR yielding amplicons of 67 bp and 86 bp, respectively. The *LTP* gene was chosen as low-copy number endogenous control for the citrus samples in the comparative Ct method.

Table 5-1. Primers pairs and probes of *LTP*[a] and *Xa21*[b] genes used for quantitative real-time PCR assays

Name	Orientation	Sequence (5′-3′)	Length (bp)	Position (bp)
LTP-178F	Forward primer	GCTGCCGCCAGAACCA	16	178-194
LTP-236R	Reverse primer	GCGGCTTGCTTCAAGCA	17	236-253
LTP-196T	Forward probe	CCTGACCGCCAAACTGCATGC	21	196-217
Xa21-mtag-2823F	Forward prime	CGGCCAACTGACAGTACATTCA	22	2823-2845
Xa21-mtag-2889R	Reverse primer	CATGTAGGCCCAGTTCAACGT	21	2889-2910
Xa21-mtag-2847T	Forward probe	CCCGATTTGGGCCTCCGTCAG	21	2847-2868

[a] *LTP* gene from GenBank acc. No. AF369931
[b] cDNA of *Xa21* gene from GenBank acc. No. U37133

Real-time PCR reactions and conditions

Quantitative multiplex real-time PCR (qRT-PCR) assays were carried out in a fluorometric thermal cycler (ABI PRISM^{TM} 7000 Sequence Detection System, Applied Biosystems-Perkin-Elmer, Foster City, CA, USA) in 96-well plate microtubes using a TaqMan system in a final volume of 25 µl. The reaction mixture contained 1x TaqMan Universal PCR Master Mix (2X) (Applied Biosystems), 25 ng DNA sample and an

optimal concentration of each transgene-specific primer and probe (900 nM each primer and 300 nM each probe, see appendix D). The amplification conditions consisted of one cycle of one 2 minute cycle at 50º C and a 10 minute cycle at 95º C, followed by 40 cycles of 15 seconds at 95º C and 1 minute at 60º C. Fluorescence was monitored during the 60º C annealing step. Each sample, including all the controls and points from the standard curves, were quantified in four replicates. The data were analyzed with ABI PRISM^{TM} 7000 SDS software ver.1.1 provided by P. E. Applied Biosystems.

To generate a standard curve for the endogenous *LTP* and the transgene *Xa21*, genomic DNA from one of the transgenic lines was used (Mason et al., 2002). Standard curves were calibrated using four concentrations of genomic DNA of the chosen transgenic line, i.e., 25, 50, 75, and 100 ng per reaction. These standard curves were used for relative quantification of the endogenous gene and the transgene. A no-template control (NTC) was also prepared as a negative control for the analysis.

Optimization of primer concentrations

The variables most likely to affect PCR efficiency are $MgCl_2$, primer, and probe concentration. It is best to maintain a constant annealing temperature if possible, as all assays can be run under the same PCR cycling condition. A preliminary experiment was performed to optimize the primer concentration. Real-time PCR reactions were run with different concentrations of primer. Primer concentrations were 100, 300, and 900 nM (final concentration) for the endogenous gene and the transgene.

PCR efficiency

PCR amplification efficiency of the reaction is an important factor when using a relative quantification method. The common logarithm of dilution series of DNA was plotted against the Ct values of those dilutions. The PCR efficiency can be calculated

from the equation $E = 10^{-(1/slope)} - 1$ as described by Ginzinger (2002). The ideal slope should be -3.32 for 100% PCR efficiency, which means that the PCR product concentration doubles during every cycle within the exponential phase of the reaction (Gibson et al., 1996). However, using the relative quantification method requires that the PCR efficiencies of all genes be similar and preferably at or over 90% (Ginzinger, 2002).

Calculation of Copy Number and Statistical Analysis

To calculate the *Xa21* copy numbers, we used a relative quantitative method (referred to as comparative Ct) that combined two absolute quantification reactions: one for the target-specific gene and the other for the endogenous reference gene (Schmidt and Parrott, 2001; Mason et al., 2002; Ding et al., 2004; Yang et al., 2005). Therefore, we compared the quantified results of the *Xa21* transgene with those of the *LTP* endogenous gene. Standard curves were prepared for the transgene *Xa21* and the endogenous *LTP* gene; these were compared to the experimentally determined levels in each transgenic citrus sample, and the amount of transgene was divided by the amount of endogenous gene. Each reaction had four replicates.

We followed the calculation method described by Mason et al. (2002), which can be summarized as follows:

Using the generated standard curve to determine the starting quantities for the transgene and the endogenous then the ratio:

$$r_{line} = SQ_{Xa21}/SQ_{LTP} \qquad \text{Eq. 1}$$

where SQ is the starting quantities. The uncertainty (δr_{line}) was propagated from equation No. 2

$$\delta r_{line} = r_{line} [(\delta SQ_{Xa21}/SQ_{Xa21})^2 + (\delta SQ_{LTP}/SQ_{LTP})^2]^{½} \qquad \text{Eq. 2}$$

To determine the copy number for each line, Ingham et al. (2001) and Bubner and Baldwin (2004) chose a transgenic line whose copy number is known to be one copy as a calibrator. The r_{line} ratio for the calibrator (r_{cal}) line would be associated with one copy of the transgene, therefore the copy number for other lines would be determined as r_{line}/r_{cal}. Since such a transgenic line is not available in our case, we will use the virtual calibrator as described by Mason et al. (2002) in which all the available lines are considered. The main idea is to find a value called r_1 corresponding to copy number one, then this value will be used instead of r_{cal} in determining the copy numbers of the transgenic lines. To choose the value of r_1 the following equation should be applied:

$$F(r1) = \Sigma_{lines} [r_{line}/r_1 - N(r_{line}/r_1)]^2 / (\delta r_{line})^2 \qquad \text{Eq. 3}$$

Where $N(r_{line}/r_1)$ is the nearest integer of r_{line}/r_1 (for more information about $F(r_1)$ see Mason et al. 2002). The r_1 value in this study was 0.37 for the transgene *Xa21*. Once r_1 was calaulated, the copy number for each line was determined as r_{line}/r_1. All the calculation steps were presented together with their 95% confidence interval.

Results and Discussion

Transgene *Xa21* Copy Number Estimation in Transgenic Citrus by Comparison to the Endogenous *LTP* Gene

A pair of primers and an internal hybridization fluorogenic TaqMan probe for detecting the endogenous *LTP* and the transgene *Xa21*, present in transgenic citrus plants, were designed for quantitative real time PCR (Table 5-1). In this method, multiplexed PCR reactions were performed whereby the amplification of the transgene of interest (*Xa21*) was compared to that of an endogenous gene (*LTP*). The two genes had different fluorogenic TaqMan probes. By amplifying both genes in the same reaction tube simultaneously, we achieved identical conditions. DNA was prepared from the transgenic

plants as well as the non-transgenic plants (control). Four replicates of samples from each plant were subjected to the multiplex reaction. Standard curves were obtained from serial dilution of a transgenic line using ABI PRISM^{TM} 7000 Sequence Detection System. The correlation coefficients of the standard curves were 0.98 and 0.985 for the endogenous *LTP* and the transgene *Xa21*, respectively (Figure 5-2 and 5-3).

Validation of the Standard Curves and PCR Efficiency

For any quantitative assay, the establishment of a standard curve is one of the key steps in determining the copy number of the transgene. Ideally, a standard curve should be established using one of the transgenic lines in which the copy number had been previously determined by Southern blot analysis. In our case, such a line of transgenic was not available. Therefore, transgene copy number estimations were made in relation to standardized curves obtained from quantitative RT-PCR analysis of serial standard DNA dilutions of the *LTP* and *Xa21* genes. Before using the relative quantitative method to estimate the transgene copy numbers, we needed to validate the curves and demonstrate that the reaction efficiencies for the transgenes and the endogenous reference gene were identical or very close.

The success of the assay depends on the assurance that the endogenous and transgene amplifications occur at approximately equal efficiencies. Thus, determining PCR efficiency is especially important for relative quantification. Serial dilutions of genomic DNA from one of the transgenic plants were assayed in multiplex reactions to determine the reactions' efficiency according to the equation described by Ginzinger (2002). By plotting the common logarithm of dilution series of DNA (25, 50, 75, and 100 ng) against the Ct values of these dilutions, the efficiency can be calculated by the formula $[10^{(1/\text{-slope})} - 1]$.

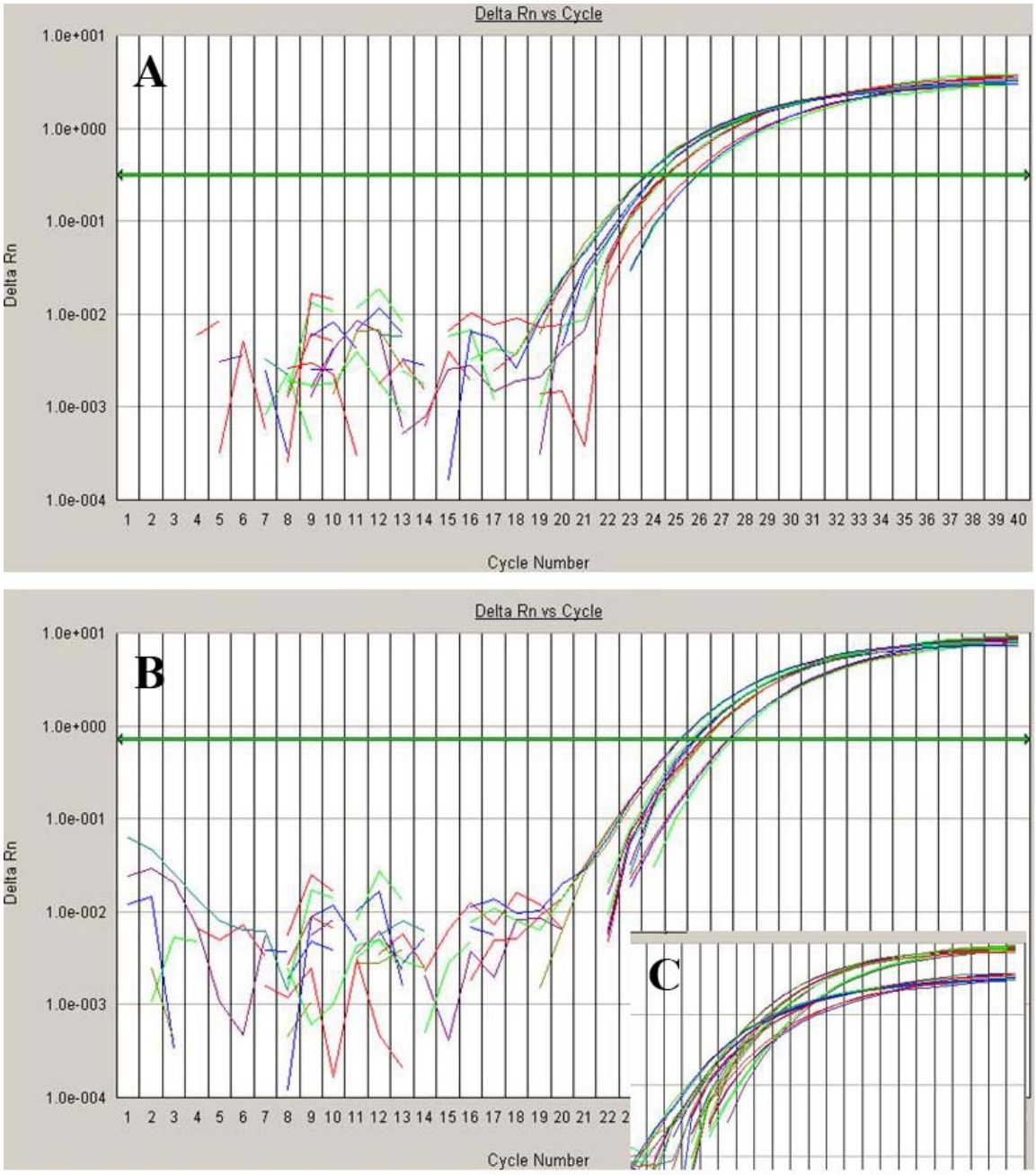

Figure 5-2. Real-time PCR amplification of endogenous *LTP* and transgene *Xa21* genes. (A) real-time PCR logarithmic plot resulting from the amplification of four serial dilutions of a citrus standard DNA using the *LTP*-specific primers and probe; (B) real-time PCR logarithmic plot resulting from the amplification of four serial dilutions of a citrus standard DNA using the *Xa21*-specific primers and probe; (C) multiplex real-time PCR for both genes simultaneously.

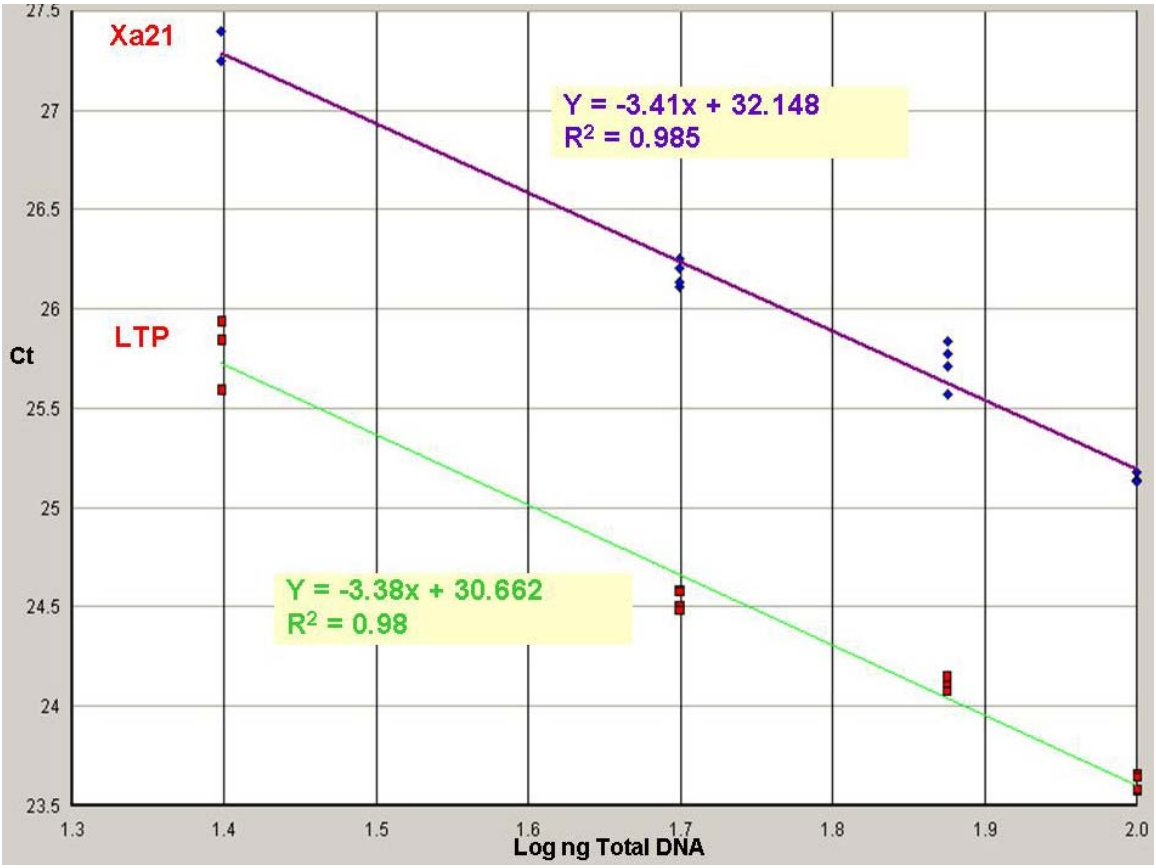

Figure 5-3. Standard curve of endogenous *LTP* and transgene *Xa21* genes. Correlation coefficient and slope values are indicated. The calculated Ct values were plotted versus the log ng total DNA of each starting quantity. Each sample was run in four replicates.

These efficiencies were 98% for endogenous *LTP* and 96% for transgene *Xa21* in the standard dilutions, indicating high and very close efficiency in both reactions. The high R^2 values obtained indicate very low pipeting error in this experiment. The ideal slope should be -3.32 for 100% PCR efficiency (Ginzinger, 2002). Equal efficiencies could be achieved by testing several combinations of primer concentrations of the two PCR reactions and finding the condition whereby one reaction is not competing with the other and thereby consuming the reaction components (Schmidt and Parrott, 2001). The PCR efficiencies obtained in this study were adequately functional and accurate to calculate the starting amount of the unknown samples.

To further validate the quantification accuracy, the standard curve was generated three times. The reproducibility of this quantitative real-time PCR system was estimated with four citrus DNA dilutions, 25, 50, 75, and 100 ng. The Ct values ranged from 23.07 to 26, the coefficient of variation (CV%) values varied from 2.14 to 2.91, and the standard deviation (SD) values ranged from 0.52 to 0.69 for the endogenous *LTP* (Table 5-2). These values for the transgene *Xa21* were Ct (24.06-27.5), CV% (2.66-3.01) and SD (0.71-0.97). These results showed that the CV% and the SD values derived from these tests were relatively small, indicating that this quantitative real-time PCR assay was stable and reliable.

Estimating the Copy Number of Xa21 in the Transgenic Citrus

The copy numbers of *Xa21* were determined by comparing the absolutely quantified *Xa21* transcripts with those of the endogenous *LTP* gene in terms of the standard curves. It follows that the validity of this technique relies on the transgene and reference gene being amplified at approximately equal efficiency, which was the case in this study. Each transgenic citrus DNA sample was tested in four replicates to correct for pipetting errors and the values were averaged to give the starting copy numbers of the transgene or endogenous gene. With the ability to measure the PCR products as they are accumulated or in real time, it is possible to measure the amount of PCR product at a point in which the reaction is still in the exponential phase. It is only during this exponential phase of the PCR reaction that it is possible to extrapolate back to determine the starting amount of template. These values were given with a 95% confidence interval and used to calculate the copy number of the transgene in each transgenic citrus DNA sample (Table 5-3).

Table 5-2. Reproducibility of the Ct measurement of replicate standards 25, 50, 75, and 100 ng citrus genomic DNA for endogenous *LTP* and transgene *Xa21* using multiplex quantitative real-time PCR.

LTP	Ct value for reaction					
DNA amount (ng)	1	2	3	Mean	SD[a]	CV%[b]
25	25.11	24.98	26.00	25.36	0.56	2.19
50	23.94	23.97	24.85	24.25	0.52	2.14
75	23.45	23.48	24.67	23.87	0.69	2.91
100	23.07	23.17	24.26	23.50	0.66	2.80
Xa21	Ct value for reaction					
DNA amount (ng)	1	2	3	Mean	SD[a]	CV%[b]
25	26.09	26.67	27.50	26.75	0.71	2.66
50	24.85	25.71	26.32	25.63	0.74	2.88
75	24.44	25.28	26.01	25.24	0.79	3.12
100	24.06	24.76	25.55	24.79	0.75	3.01

[a] Standard deviation
[b] Coefficient of variation

To estimate the number of the transgene copies in the transgenic plants, the ratio between transgene and endogenous gene (r_{line}) was calculated (Table 5-3). Using the r_{line} values, the virtual calibrator (r_1) was calculated. The virtual calibrator is the value which corresponding to one copy of the transgene (Mason et al., 2002). The r_1 value in this study was 0.37 for the transgene *Xa21*. The copy number for each plant was then determined as r_{line}/r_1 (Table 5-3). Thirty-four transgenic citrus samples were tested, and the results indicated that two samples had one copy, five samples had two copies, 22 samples had three copies, and five samples had four copies of the transgene (Table 5-3).

Comparison of Copy Number Determination by qRT-PCR and Southern Blot Analysis

Correlation analysis revealed that there was a strong relationship between the copy number determined by qRT-PCR and the copy number determined by Southern blot analysis (Figure 5-4). The correlation efficiency was 0.834. However, among the 34 transgenic plants, the copy number determined by quantitative RT-PCR was not always similar to the number detected by Southern blot analysis. However, when qRT-PCR had

been run with different from the same line, the result was always the same. This result indicates again that this qRT-PCR assay was stable and reliable.

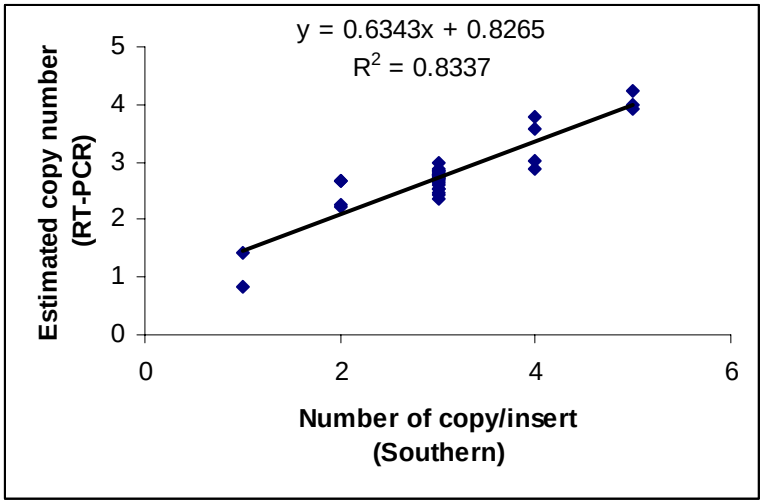

Figure 5-4. Correlation between the copy numbers in transgenic citrus determined by quantitative RT-PCR and the number of copy/inserts detected with southern blot analysis.

Conclusion

The results we obtained from quantitative real-time PCR for quantifying transgene copy number correlated highly with those obtained using the traditional southern blot analysis. In addition, the high sensitivity and efficiency of this technology allowed us to analyze more samples and quantify the transgene copy number more quickly and accurately; up to 96 samples can be prepared and analyzed in approximately 3 hours. In citrus, this assay could have numerous potential applications in genetic engineering and tissue culture, including the expediting of the identification of transgenic tissue and selecting transformation events with low copy number of the transgenes. A distinct benefit of early analysis of regenerating plants is the early identification of escapes as well as multiple-copy transformants that may exhibit gene silencing (Iyer et al., 2000; James et al., 2002) . The prompt identification of low copy number lines would thus

expedite plant transformation and breeding projects, especially those of species that have long generation times, such as citrus. In quantitative real-time PCR, there is no post-PCR analysis of samples, thereby minimizing handling time and lessening the likelihood of contamination that may lead to false positive results. As reported previously, direct DNA delivery transformation methods yield a high proportion of transformants with highly differing numbers of transgenes, and only a small proportion of the primary transformants with low copy numbers (Dong et al., 1996; Kohli et al., 1998; Krasnyanski et al., 1999; Srivastava et al., 1999). Thus, it is important to screen the transformants at an early stage to distinguish transformants with low copy number from those with high copy number. Quantitative real-time PCR provides a fast and reliable method to achieve this goal.

Table 5-3. Real-time PCR estimates of copy number for *Xa21* transgene. Calculated starting quantities (SQ) of the two genes (endogenous and transgene) and calculated copy number for *Xa21* transgene, all data are shown together with their 95% confidence interval.

Line	SQ-*LTP*	SQ-*Xa21*	r_{line} (SQ-*Xa21*/SQ-*LTP*)	$(r_{line}/r_1)^b$	Copy number
NT[a]	70.01 ± 4.37	2.79 ± 0.29	0.04 ± 0.01	0.14 ± 0.03	0
AO1	76.11 ± 14.41	74.50 ± 9.77	0.98 ± 0.06	2.52 ± 0.15	3
AO2	53.79 ± 8.38	51.18 ± 12.53	0.95 ± 0.09	2.43 ± 0.24	2
AO3	73.38 ± 12.52	63.89 ± 6.94	0.87 ± 0.09	2.24 ± 0.24	2
AO4	36.50 ± 3.91	60.14 ± 6.66	1.65 ± 0.03	4.22 ± 0.08	4
AO5	101.07 ± 2.08	107.33 ± 6.10	1.06 ± 0.04	2.72 ± 0.11	3
AO6	57.62 ± 5.12	60.59 ± 5.19	1.05 ± 0.10	2.70 ± 0.27	3
AO7	166.04 ± 6.16	176.67 ± 4.71	1.06 ± 0.02	2.73 ± 0.04	3
AO8	116.09 ± 8.05	125.66 ± 8.52	1.08 ± 0.05	2.78 ± 0.13	3
AO9	81.50 ± 3.45	124.88 ± 5.39	1.53 ± 0.09	3.93 ± 0.24	4
AO10	196.42 ± 13.71	205.09 ± 10.22	1.04 ± 0.02	2.68 ± 0.06	3
AO11	205.15 ± 23.78	229.48 ± 34.23	1.12 ± 0.05	2.87 ± 0.13	3
AO12	252.54 ± 22.98	280.04 ± 20.98	1.11 ± 0.04	2.84 ± 0.09	3
AO13	160.12 ± 11.43	173.92 ± 14.73	1.09 ± 0.02	2.78 ± 0.06	3
AO14	206.72 ± 18.29	238.91 ± 21.39	1.16 ± 0.07	2.97 ± 0.19	3
AO15	211.01 ± 15.23	218.92 ± 18.83	1.04 ± 0.10	2.66 ± 0.24	3
AO16	235.02 ± 8.66	250.23 ± 9.72	1.06 ± 0.03	2.73 ± 0.09	3
AO17	225.55 ± 12.15	251.63 ± 16.23	1.12 ± 0.06	2.86 ± 0.16	3
AO18	92.98 ± 10.59	101.09 ± 10.76	1.09 ± 0.03	2.79 ± 0.08	3
AO19	344.77 ± 18.85	376.22 ± 8.23	1.09 ± 0.05	2.80 ± 0.13	3
AO20	118.92 ± 14.38	103.39 ± 8.37	0.87 ± 0.08	2.23 ± 0.20	2
AO21	20.55 ± 2.30	24.09 ± 2.13	1.17 ± 0.06	3.01 ± 0.16	3
AO22	71.79 ± 7.42	73.28 ± 5.45	1.02 ± 0.04	2.62 ± 0.11	3
AO23	54.77 ± 5.69	56.04 ± 2.56	1.03 ± 0.08	2.63 ± 0.20	3
AO24	40.58 ± 8.60	63.00 ± 7.60	1.56 ± 0.16	4.01 ± 0.41	4
AO25	115.38 ± 9.05	119.51 ± 7.90	1.04 ± 0.07	2.66 ± 0.19	3
AO26	35.50 ± 7.02	32.56 ± 3.31	0.92 ± 0.09	2.37 ± 0.23	2
AO27	27.70 ± 8.53	9.30 ± 4.01	0.33 ± 0.07	0.85 ± 0.18	1
AO28	28.93 ± 7.05	15.88 ± 2.47	0.55 ± 0.08	1.42 ± 0.20	1
AO29	218.81 ± 7.71	211.02 ± 12.38	0.96 ± 0.03	2.47 ± 0.08	2
AO30	200.82 ± 23.92	295.78 ± 40.17	1.47 ± 0.13	3.78 ± 0.32	4
AO31	282.42 ± 30.68	278.87 ± 20.49	0.99 ± 0.06	2.54 ± 0.15	3
AO32	265.74 ± 45.55	370.82 ± 44.35	1.40 ± 0.08	3.59 ± 0.22	4
AO33	311.32 ± 70.61	335.36 ± 61.75	1.08 ± 0.09	2.77 ± 0.23	3
AO34	117.78 ± 11.34	131.68 ± 3.88	1.12 ± 0.10	2.87 ± 0.25	3

[a] NT = non-transgenic

[b] r_{line}/r_1 = estimated copy number

CHAPTER 6
SUMMARY AND CONCLUSIONS

The successful transformation and co-transformation of 'Hamlin' sweet orange with cDNA of the *Xa21 Xanthomonas* resistance gene using PEG-mediated direct DNA uptake were achieved. From over 20 transformation experiments using pAO3, only eight independent transgenic events were recovered and 73 transgenic plants were propagated using grafting techniques. These plants were successfully acclimated and transferred to a greenhouse. From more than 15 co-transformation experiments using p524EGFP.1 and pXa21-mtag, only one independent transgenic event was recovered and 7 transgenic plants were successfully propagated using grafting techniques. From more than 30 co-transformation experiments using pARS108 and pXa21-mtag, 75 transgenic events were recovered and more than 500 transgenic plants were regenerated and transferred to the greenhouse. To accelerate regeneration of the transgenic plants, two different grafting techniques were used: *in vitro* micrografting and standard shoot tip grafting. It is important to note that these transgenic plants were obtained without any antibiotic resistance genes for selection at the cellular level. This is an advantage over the standard citrus transformation methodology using *Agrobacterium*, in which antibiotic resistance genes are used for selection and to kill *Agrobacterium* following transformation. Selection was made first based on expression of the *GFP* (Green Fluorescent Protein) gene at the protoplast, calli, and somatic embryo level. Further verification was achieved using the polymerase chain reaction. Confirmation of stable transgene integration into the 'Hamlin' genome was shown by Southern blot analysis and western blot analysis.

Western analysis showed expression of the *Xa21* gene in the transgenic plants obtained from transformation and co-transformation at 87.5% and 24%, respectively. This is the first time to report a large population of normal transgenic 'Hamlin' sweet orange plants using the protoplast/GFP transformation system. According to the literature, this is the first time that a gene from rice has been stably integrated and expressed in citrus plants.

To accelerate characterization of the transgenic plants, a real-time PCR based method has been developed to determine the copy number of the transgene in recovered transgenic plants. The results obtained from real-time PCR analysis for quantifying transgene copy number correlated highly with those obtained using the traditional Southern blot analysis. In addition, the high sensitivity and efficiency of this technology allowed us to analyze more samples and quantify the transgene copy number more quickly and accurately. Up to 96 samples can be prepared and analyzed in approximately 3 hours. In citrus, this assay could have numerous potential applications in genetic engineering and tissue culture, including the expediting of the identification of transgenic tissue and selection of transformants with low transgene copy numbers. A distinct benefit of the early analysis of transgenic plants is that escapes and multiple-copy transformants that may exhibit gene silencing can be identified (Iyer et al., 2000; James et al., 2002). The prompt identification of low copy number lines would thus expedite plant transformation and breeding projects, especially those of species that have long generation times, such as citrus. Thus, it is important to screen the transformants at an early stage and distinguish transformants with low copy number from those with high copy number. Quantitative multiplex real-time PCR was shown to be a successful

screening tool for estimating transgene copy number in transgenic citrus at a very early stage of the process. This could save up to 6-9 months time in the overall process.

Evidence of somaclonal variation was difficult to ascertain in the populations of transgenic plants. There were differences in growth rate, particularly for the transgenic plants obtained from transformation experiments using pAO3 plasmid DNA and a few events from co-transformation experiments. The reasons for this are difficult to determine. The stunting of the plants could be due to a positional effect of the inserted gene, the effect of the Xa21 gene product, or a combination of these and other metabolic pathways in the regenerated transgenic plants.

The regenerated transgenic plants are now ready to be tested by challenge inoculation with the citrus canker pathogen, *Xanthomonas axonopodis* pv. *citri* (*Xac*), in the greenhouse at the quarantine facility in Gainesville. The results of this test will help to determine whether these transgenic plants have acquired a potential level of resistance against the citrus canker pathogen. If these tests are positive, field-testing can then be conducted at the appropriate location. Positive results in the field would mean that citrus canker-resistant ‘Hamlin’ sweet orange has been achieved. If successful, this technology could be applied to other commercially important scions, including other oranges, grapefruit, and mandarins.

APPENDIX A
CITRUS PROTOPLASTS MEDIA AND SOLUTION

Table A-1. Composition of the EME medium.

Component	mg/L
NH_4NO_3	1,650
KNO_3	1,900
KH_2PO_4	170
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
KI	0.63
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Thiamine.HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Nicotinic acid	5

50 g/L sucrose was added for 0.146 M EME and 205.38 g/L sucrose for 0.6 M EME. For 1500 EME malt extract was added at 1500 mg/L and sucrose at 50 g/L. Solid medium contains 8 g/L agar.

Table A-2. Composition of RMAN medium.

Component	mg/L
NH_4NO_3	825
KNO_3	950
KH_2PO_4	85
$MgSO_4.7H_2O$	185
$CaCl_2.2H_2O$	440
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.H_2O$	11.15
$ZnSO_4.7H_2O$	4.30
H_3BO_3	3.10
KI	0.42
$Na_2MoO_4.2H_2O$	0.13
$CuSO_4.5H_2O$	0.013
$CoCl_2.6H_2O$	0.013
Thiamine.HCl	5
Pyridoxine.HCl	5
Nicotinic acid	0.50
Naphhthalin acetic acid	0.020
Activated charcoal	500
Sucrose	25,000
Agar	8,000

Table A-3. Composition of 0.6 m BH3 nutrient medium.

Component	mg/L
KH_2PO_4	170
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
KCl	1,500
KI	0.63
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Glutamine	3,100
Thiamine.HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Casein hydrolysate	250
Nicotinic acid	1
Mannitol	81,990
Sucrose	51,350 (85,560 for 0.7 M)
Coconut water	20 mL
Fructose	250
Ribose	250
Xylose	250
Mannose	250
Rhamanose	250
Cellobiose	250
Galactose	250
Glucose	250
Sodium pyruvate	20
Citric acid	40
Malic acid40	40
Fumaric acid	40
Vitamin B12	0.02
Calcium pantothene	1
Ascorbic acid	2
Choline chloride	1
p-aminobezoic acid	0.02
Folic acid	0.40
Riboflavin	0.20
Biotin	0.01
Vitamin A (retinol)	0.01
Vitamin D3 (cholecalciferol)	0.01

Table A-4. Composition of sucrose and mannitol solutions (CPW salts).

Component	mg/L
$MgSO_4.7H_2O$	250
KNO_3	100
KH_2PO_4	27.20
KI	0.16
$CuSO_4.5H_2O$	0.00025
$CaCl_2.2H_2O$	150

For CPW-25 sucrose, 25 g/100 mL sucrose was added and for CPW-13 mannitol, 13 g/100 mL mannitol was added. pH of both solutions was 5.8.

Table A-5. Composition of protoplast transformation solutions.

I- Polyethylene Glycol 40%			
40% polyethylene glycol (PEG) (MW = 8000)			
0.3 M Glucose			
66 mM $CaCl_2$			
pH = 6			
II- Solutions A and B			
Solution A		Solution B	
Component	g/100 mL	Component	g/100 mL
Glucose (0.4 M)	7.20	Glycine (0.3 M)	2.2
CaCl2 (66 mM)	0.97		
DMSO	10 mL		
pH 6.0		pH 10.5	

Table A-6. Composition of H+H medium.

Component	mg/L
NH_4NO_3	825
KNO_3	950
KH_2PO_4	170
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
KI	0.63
KCl	750
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Thiamine.HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Nicotinic acid	5
Glutamine	1,550
Sucrose	35,000

Table A-7. Composition of B+ medium.

Component	mg/L
NH_4NO_3	1,650
KNO_3	1,900
KH_2PO_4	170
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
KI	0.63
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Thiamine.HCl	10
Pyridoxine.HCl	10
Myo-inositol	100
Malt extract	500
Nicotinic acid	5
Coconut water	20 mL
Coumarin	14.60
NAA	0.02
GA	1
Sucrose	25,000
Agar	8,000

Table A-8. Composition of DBA3 medium.

Component	mg/L
NH_4NO_3	1,485
KNO_3	1,710
KH_2PO_4	153
$MgSO_4.7H_2O$	333
$CaCl_2.2H_2O$	440
Na_2EDTA	37.30
$FeSO_4.7H_2O$	27.80
$MnSO_4.H_2O$	21.40
$ZnSO_4.7H_2O$	7.70
H_3BO_3	5.58
KI	0.567
$Na_2MoO_4.2H_2O$	0.225
$CuSO_4.5H_2O$	0.0225
$CoCl_2.6H_2O$	0.0225
Thiamine.HCl	9
Pyridoxine.HCl	9
Myo-inositol	90
Nicotinic acid	4.5
Coconut water	20 mL
Malt extract	1,500
2,4 D	0.01
DAP	3
Sucrose	25,000
Agar	8,000

Table A-9. Composition of the enzyme solution used for citrus protoplast isolation.

Component	Concentration
Mannitol	0.7 M
$CaCl_2$	12.0 mM
MES[1] (buffer)	6.0 mM
NaH_2PO_4	1.4 mM
Onozuka RS cellulose	1%
Macerase or macerozyme	1%
Pectolyase Y-23	0.2%
pH = 5.6	
Filter sterilize (Nalgene®, 0.2 μm	

[1]MES = 2[N-morpholino] ethane sulfonic acid.

APPENDIX B
PCR PRIMERS AND PROGRAM

PCR primers

For pARS108

108F 5′-GAATTCGTGAGCAAGGGCGA-3′

108R 5′-GGATCCTTAGAGTTCGTCGTG-3′

For pXa21-mtag

XaL 5′-AATCCCTAACACGCTTGGTG-3′

XaR 5′-CACACACTGGAAACAATCCG-3′

PCR reaction mixture

GoTaq® Green Master Mix 2X	12.5 µl
5 µM 108F primer	1.5 µl
5 µM 108R primer	1.5 µl
5 µM XaL primer	1.5 µl
5 µM XaR primer	1.5 µl
DNA template (100 ng/µl)	2.5 µl
Nuclease-Free Water	4.0 µl

PCR program

Step 1.	2 minutes at 95º C	Denaturation
Step 2.	30 seconds at 95º C	Denaturation
Step 3.	30 seconds at 59º C	Annealing
Step 4.	1 minute at 72º C	Elongation
Step 5.	Repeat steps 2-4 30 times	
Step 6.	10 minutes at 72º C	Elongation
Step 7.	4º C forever	
Step 8.	End	

APPENDIX C
MOLECULAR ANALYSIS SOLUTION

Southern Blot analysis Solution

5 M NaCl

292.2 g NaCl dissolve into 1000 ml H_2O

10 N NaOH

100 g NaOH dissolve into 250 ml H_2O

10% SDS

10 g SDS dissolve into 100 ml H_2O

20X SSC

	1 Litter
NaCl	175.3 g
Sodium Citrate	88.2 g

Adjust pH to 7.0

Autoclave

Standard Hybridization Buffer:

	Stock	1 Litter
5X SSC	20X	250 ml
N-lauroylsarsine 0.1%	10%	10 ml
SDS 0.02%	10%	2 ml
1% Dry milk	-----	10 g

Keep in freezer (-20º C)

Buffer 1 (Maleic acid buffer)

	1 Litter
0.1 M Maleic acid	11.61 g
0.15 M NaCl	8.786 g

Adjust pH to 7.5 w/ solid NaOH (about 7.8 g)

Autoclave

Buffer 2 (Blocking solution)

2% dry milk in maleic acid buffer

Buffer 3 (Detection buffer)

	1 Litter
0.1 M Tris-base	12.1 g
0.1 M NaCl	5.84 g

Adjust pH to 9.5 w/ NaOH or HCl

Autoclave

Western Blot Analysis

Tris Buffered Saline (TBS):

	1 L	4 L	8 L	10 L
Tris base	12.11 g	48.44 g	96.88 g	121.1 g
NaCl	8.775 g	35.1 g	70.2 g	87.75 g

pH = 7.9

Autoclave

Tween-Tris buffered saline (TTBS):

1 L TBS + 1 ml Tween-20

5 x Transfer Buffer:

	1 L	2 L	Final for 1X
Tris base	15.1 g	30.2 g	24.9 mM
Glycine	72.0 g	144.0 g	191.8 mM

5 x Running Buffer:

	1 L
Glycine	72 g
Tris base	15 g
10% SDS	50 ml;

Loading Dye:

	2X 1 ml	4X 1 ml	Final
Tris-HCl pH 6.8	125 µl	250 µl	62.5 mM
Glycerol	200 µl	400 µl	10%
SDS	200 µl of 20%	20 mg	2%
5% β-ME	100 µl	200 µl	0.5%
Bromophenol blue	2 mg	4 mg	0.1%
H_2O	To 1 ml	To 1 ml	

APPENDIX D
QUANTITATIVE REAL TIME-PCR

Primers pairs and probes of LTP[a] and Xa21[b] genes used for quantitative real-time PCR assays.

Name	Orientation	Sequence (5′-3′)	Length (bp)	Position (bp)
LTP-178F	Forward primer	GCTGCCGCCAGAACCA	16	178-194
LTP-236R	Reverse primer	GCGGCTTGCTTCAAGCA	17	236-253
LTP-196T	Forward probe	CCTGACCGCCAAACTGCATG	21	196-217
Xa21-mtag-2823F	Forward prime	CGGCCAACTGACAGTACATT	22	2823-2845
Xa21-mtag-2889R	Reverse primer	CATGTAGGCCCAGTTCAACG	21	2889-2910
Xa21-mtag-2847T	Forward probe	CCCGATTTGGGCCTCCGTCA	21	2847-2868

[a] LTP gene from GenBank acc. No. AF369931
[b] cDNA of Xa21 gene from GenBank acc. No. U37133

Real-time PCR reaction.

Number of reactions	1 (µl)	96 (µl)	Final
TaqMan Universal PCR Master Mix (2X)	12.5	1200	1X
Xa21-mtag-2823F primer (15 mM)	1.5	144	900 nM
2 Xa21-mtag-2889R primer (15 mM)	1.5	144	900 nM
LTP-178F primer (15 mm)	1.5	144	900 nM
LTP-236R primer (15 mm)	1.5	144	900 nM
Xa21-matg-2847 probe (5 mM)	1.5	144	300 nM
LTP-196 probe (5 mM)	1.5	144	300 nM
Free Nuclease Water	1	96	--------
DNA (10 ng/µl)	2.5	240	25 ng
Total	25	2400	_____

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BIOGRAPHICAL SKETCH

Ahmad Al-Sayed Mohamad Omar was born in El-Senblaween, Dakahiliah, Egypt, on February 1, 1971. He graduated form Ahmed Lotfey El-sayed High School in El-Senblaween in 1989. He received a Bachelor of Science in Agricultural from Zagazig University, Egypt, with an honors degree in a major of agriculture chemistry in June 1993. Upon graduation, Ahmad was appointed to a position as a research assistant by Biochemistry Department, Zagazig University, Egypt, where he received the Master of Science in biochemistry in November 1997. During this period, Ahmad conducted biochemical research and taught inorganic chemistry, organic chemistry, and biochemistry courses for undergraduate students at the Faculty of Agriculture, Zagazig University.

Ahmad was awarded a scholarship from the Egyptian Government to pursue his Ph.D. studies abroad. In the spring of 2000, he enrolled in the graduate program of the Plant Pathology Department at University of Florida. In the summer of 2000, he changed the program to the Horticultural Science Department at the same university under the supervision of Dr. Jude W. Grosser, professor of cell genetics at University of Florida. He completed his research at the Citrus Research and Education Center in Lake Alfred, Florida.

After completing his Ph.D. program, Ahmad will continue to work in Dr. J. W. Grosser's program as a postdoctoral researcher to gain more experience in the area of biochemistry and plant biotechnology. After his wife finishes her Ph.D. program, they

will return to Egypt to take their positions as an assistant professor at Biochemistry Department, Faculty of Agriculture, Zagazig University (Ahmad) and Mansoura University (Azza). Ahmad will teach undergraduate and graduate biochemistry and molecular biology courses and conduct research to apply what he has learned to improve the Egyptian citrus industry.

He also is an active member of the Egyptian Student Association North America (ESANA). He is married to Azza H. Mohamed who is pursuing her Ph.D. at the University of Florida, too. They have one daughter, Aala A. Omar. Finally, after more than five years in which life has happened, tears have been shed, and dreams that did not come true exactly the way he expected, HE IS GOING TO HAVE HIS DOCTORATE.