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Citrus Transformation: Challenges and Prospects

Vicente Febres, Latanya Fisher, Abeer Khalaf and Gloria A. Moore
*Horticultural Sciences Department, Plant Molecular and
Cellular Biology Program, University of Florida
USA*

1. Introduction

Citrus is an important commodity worldwide and is produced in tropical and subtropical regions around the world. Annually, the total citrus fruit production is estimated to be more than 124.5 million tonnes worldwide, with China, Brazil, the United States, Mexico and India the main producers (FAO, 2011). Oranges, lemons, tangerines and grapefruits are among the most commonly grown citrus types and they are traded as fresh fruit, juice, or as concentrate. Growers, however, face important challenges for maintaining or improving yield: disease, drought, cold and soil salinity are some of the factors that can limit production and can have an important economic impact on growers. Traditional breeding methods have been used successfully over the years to improve citrus; however this is done with difficulty due to the slow growth and maturation of this crop, incompatibility, polyembryony, parthenocarpy, etc. Because traditional breeding takes such a long time the fast incorporation of desirable traits is not possible. In other instances, certain desirable traits are not present in cultivated citrus types. This has been made more evident in the battle against diseases. Diseases can appear in a region and within a few years spread and become limiting factors for production and have a major economical impact because of yield reduction and/or increased production costs. Therefore, genetic engineering via citrus transformation is an alternative method used to incorporate desirable traits into citrus genotypes.

2. Citrus transformation: generalities

The genetic transformation procedure involves two major processes. The first is the incorporation of the foreign gene of interest into the plant genome while the second entails the regeneration of the transformed cells into whole transgenic plants (Singh & Rajam, 2009). The success of the genetic transformation technique depends on an effective and reliable procedure as efficiencies are often low. Several techniques such as polyethylene glycol (PEG)-mediated direct uptake of DNA by protoplast (Kobayashi & Uchimaya, 1989), particle bombardment (Yao et al., 1996) and *Agrobacterium*-mediated transformations (Hidaka & Omura, 1993) have been developed and used with various *Citrus* spp. However, the latter transformation system is now the most commonly used method because it has been proven most successful with higher transformation efficiencies resulting in the production of transgenic plants (Peña et al., 2007; Singh & Rajam, 2009; Yu et al., 2002).

2.1 Protoplast transformation

Although, *Agrobacterium*-mediated transformation is considered the best overall method, direct uptake of DNA by protoplasts and particle bombardment have their advantages over the former method. Protoplast transformation is mostly used with commercially important citrus genotypes that are either seedless or contain very few seeds, which is required in most *Agrobacterium*-mediated transformation procedures (Fleming et al., 2000). Here, the citrus plant is regenerated from the protoplast via somatic embryogenesis and additionally it can eliminate the need for the use of antibiotics either for plant selection or bacterial inhibition (Fleming et al., 2000). This method also allows the improvement of citrus genotypes that are sexually incompatible by producing superior scion or rootstock somatic hybrids (Fleming et al., 2000; Grosser et al., 1998a; Grosser et al., 1998b). Regeneration using this system has been used with many citrus species, including lemons [*C. limon* (L.) Burm. F.], limes [*C. aurantifolia* (Cristm.) Swingle], mandarins (*C. reticulata* Blanco), grapefruits (*C. paradisi* Macf.), sweet orange (*C. sinensis* Osbeck) and sour orange (*C. aurantium* L.). Although, limited success has previously been reported using protoplast transformation with sweet orange, rough lemon (*C. jambhiri* Lush.) and 'Ponkan' mandarin (Hidaka & Omura, 1993; Kobayashi & Uchimaya, 1989; Vardi et al., 1990). Fleming et al. (2000) have reported success in recovering transgenic sweet orange plantlets by an optimized version of this method.

2.2 Particle bombardment

Particle bombardment involves the direct delivery of DNA coated onto microprojectiles into intact cells or organized tissue via a gene gun or a biolistic particle delivery system (Yao et al., 1996). This method is used alternatively in cases where citrus genotypes are recalcitrant to *Agrobacterium* infection. A reason for this is that citrus is not a natural host for the bacteria (Khan, 2007). A problem that arises from this method is the low regeneration frequency of stably transformed cells from calli as was observed with tangelo (*C. reticulata* x *C. paradisi*) (Yao et al., 1996). Nevertheless, transformation efficiencies of 93%, based on transient expression experiments, have been reported with citrange (*C. sinensis* x *P. trifoliata*) when particle bombardment is carried out using thin epicotyl segments (Bespallhok et al., 2003).

2.3 *Agrobacterium*-mediated transformation

This system uses the ability of the *Agrobacterium*-plant interaction to transfer and integrate genetic information into the plant's genome. The bacteria, depending on the species, contain either a rhizogenic (Ri) or a tumor-inducing (Ti) plasmid which includes a T-region or transferred DNA region (T-DNA). This T-DNA region is manipulated by genetic engineering to include the gene of interest for transfer in the transformation process. The T-DNA movement from *Agrobacterium* occurs only onto wounded plant cells (Gelvin, 2003; Messens et al., 1990). The initiation of this transfer depends on the induction of the virulence (*vir*) region located in the Ti plasmid. There are 6 *vir* genes *virA-virE* and *virG* that make up this 35 kilobase pairs (kb) region between the left and right borders of the T-DNA. Wounded plant cells produce *vir* inducing compounds such as acetosyringone and α -hydroxyacetosyringone that induce the expression of these *vir* genes initiating the T-DNA transfer and thus transformation of the plant cells (Gelvin, 2003; Messens et al., 1990). *Agrobacterium*-mediated transformation experiments have been carried out with numerous hybrids and species of citrus, such as grapefruit, sour orange, sweet orange, trifoliolate orange (*Poncirus trifoliata* Raf.), 'Carrizo' citrange, 'Mexican' lime, 'Swingle' citrumelo (*C. paradisi* x *P. trifoliata*), 'Cleopatra' mandarin, and alemow (*C. macrophylla* Wester) (Dominguez et al.,

2000; Ghorbel et al., 2000; Gutierrez-E et al., 1997; Luth & Moore, 1999; Molinari et al., 2004; Moore et al., 1992; Peña et al., 2004, 2007). Transformation of other economically important citrus cultivars with the existing protocols has not yet been successful.

Generally, transformation efficiencies obtained by using *Agrobacterium* with most citrus cultivars can range from 0 to 45%. This is due to a number of limiting factors that can affect the transformation process. These include: species or cultivar specificity, age and type of explant used, competence of the citrus cells or tissues, *Agrobacterium* strains used and inoculation procedure, co-cultivation and pre-culturing conditions, adequate selection conditions and recovery of transgenic shoots (Bond & Roose, 1998; Costa et al., 2002; Peña et al., 2007; Yu et al., 2002).

2.3.1 Species or cultivar specificity

Data from early studies indicated that the type of citrus species and cultivar used in transformation experiments affect transformation efficiencies. Bond & Roose (1998) showed that when 7 citrus cultivars, 'Washington navel' and 'Olinda Valencia' oranges, 'Lisbon' lemon, 'Rio Red' grapefruit, 'Carrizo' citrange, mandarin and 'Mexican' lime were transformed with *Agrobacterium* only 'Washington navel' and 'Carrizo', resulted in GUS-positive shoots. These results were indicative of the receptiveness of these cultivars to this type of transformation protocol compared to the others. Although very little diversity exists between the sweet orange cultivars, 'Washington navel' and 'Olinda Valencia', the difference that exists was sufficient to affect the transformation efficiency. As a result, different protocols have been developed for different citrus species and cultivars (Bond & Roose, 1998; Costa et al., 2002; Peña et al., 1997).

2.3.2 Age and type of explant used

Studies have also shown that lower transformation efficiencies are obtained with older segments (Moore et al., 1992; Peña et al., 1995a). Transformations of three week old 'Washington navel' orange epicotyl segments resulted in efficiencies of up to 87%, while 5 to 8 week old epicotyl segments gave lower efficiencies of 5 to 40% (Bond & Roose, 1998). This reduction in transformation efficiency is presumed to be the result of older epicotyl segments having a lower number of actively dividing cells and consequently less susceptible to T-DNA integration and the regeneration of shoots (Bond & Roose, 1998; Villemont et al., 1997). In addition, it is regarded that older epicotyl segments have different wound exudates or cell wall components that result in a reduction in bacterial binding or the activation of the virulence genes (Bond & Roose, 1998).

Various types of explants such as, callus, leaf sections, seeds, epicotyl nodal and inter-nodal stem segments are often used, with varying results (Hidaka & Omura, 1993; Kaneyoshi et al., 1994; Moore et al., 1992). For instance, higher transformation efficiencies are obtained from citrus callus of 'Ponkan' mandarin. The advantages of using callus as explants are that a larger number of transgenic plants are produced, there is rapid proliferation and chimeras are rarely observed during the regeneration process (Li et al., 2002). However, drawbacks to using this system are that some citrus varieties do not possess embryogenic potential and the regenerated plants are juvenile, resulting in a long waiting period for the evaluation of the traits of interest and, additionally, it increases the risk of somaclonal variation which results in abnormal plant morphologies (Cervera et al., 2000; Li et al., 2002). *Agrobacterium*-mediated transformation involving epicotyl and internodal stem segments are the

predominantly used explants for regeneration of transgenic citrus plants. These types of explant are the most widely used in citrus transformation experiments and appear to be the most responsive. The disadvantage of using these types of explants is that the process is very laborious and takes a long time. Alternatively, another efficient system uses cotyledons from ungerminated mature seeds, followed by shoot regeneration via direct organogenesis (Khawale et al., 2006; de Oliveira, Fisher and Moore unpublished). The advantage of this method is that it is less time consuming and laborious. It involves the use of mature seeds that are sterilized, subsequently the seed coat is removed and the cotyledons are directly inoculated with the *Agrobacterium* suspension and later transferred to the appropriate selection media. The use of this type of explant eliminates the time required for germination of seedlings to produce epicotyl segments and we have obtained higher transformation and regeneration frequencies with grapefruit and sweet orange. Although GUS expression was observed, we have not yet carried out the evaluation for stable integration of the transgene in the putative transgenic plants generated by this method. However, Khawale et al. (2006) proved the stability of this transformation method in 'Nagpur' mandarin.

2.3.3 Competence of the citrus cells or tissues

Cell division and dedifferentiation of plant cells are responsible for the explants' competent state and result in callus proliferation (Peña et al., 1997, 2004). Observations of transformed citrus inter-nodal and epicotyl segments showed that resulting transgenic cells were localized in callus tissue and are of cambial origin. It is also suggested that certain treatments such as the inclusion of auxins, which promote active cell division and dedifferentiation of plant cells, correlated with higher transformation efficiencies (Peña et al., 2004).

2.3.4 *Agrobacterium* strains used and inoculation procedure

A study involving the use of three different strains of *Agrobacterium* (C58 C1, EHA101-5 and LB4404) to transform seven citrus cultivars showed varying transformation efficiencies (Bond & Roose, 1998). In four separate experiments, strain C58 C1 had the highest transformation efficiency of 45%, while strains EHA101-5 and LB4404 resulted in transformation efficiencies of 29% and 0%, respectively (Bond & Roose, 1998).

The inoculation of the citrus explants with the *Agrobacterium* culture typically requires incubation periods of 1 to 30 minutes (Bond & Roose, 1998; Costa et al., 2002; Luth & Moore, 1999; Peña et al., 1997). However, incubation periods greater than 10 minutes have led to the increase in regeneration of escape shoots and a reduction in transformation efficiency (Costa et al., 2002).

The optimal *Agrobacterium* culture concentrations that have been determined for the effective inoculation and transformation of citrus are 5×10^8 and 4×10^7 cfu/ml, and are dependent on the citrus cultivar being transformed (Bond & Roose, 1998; Cervera et al., 1998b; Costa et al., 2002; Dominguez et al., 2000; Luth & Moore, 1999; Peña et al., 1995a; Yu et al., 2002). A limited source of bacterial cells reduces the frequency of T-DNA transfer while excess bacteria stress the plant cells (Costa et al., 2002; Yu et al., 2002).

2.3.5 Co-cultivation and pre-culturing conditions

Co-cultivation involves incubating both the explants and *Agrobacterium* on media containing no selective agent for the transformed cells or against the bacteria, for a period of time. An increase in the co-cultivation period has been associated with a higher number of

regenerated and transformed shoots (Costa et al., 2002). Transformation frequency increased when the co-cultivation period was increased from 1 to 5 days at which it reached a maximum (Cervera et al., 1998a). However, prolonged co-cultivation periods often lead to an overgrowth of *Agrobacterium* which reduces the regeneration frequency of transformed shoots (Cervera et al., 1998b; Costa et al., 2002). As a result, most transformation protocols routinely use a 2 to 3 days co-cultivation period (Cervera et al., 1998b; Costa et al., 2002; Luth & Moore, 1999; Peña et al., 1997).

The composition of the co-cultivation medium also affects the transformation process. The presence of auxins such as 2,4 dichlorophenoxyacetic acid (2,4-D), in co-cultivation medium has resulted in higher transformation frequencies in comparison to co-cultivation medium containing a filter paper layer, tomato cell suspension or a cell feed layer alone (Cervera et al., 1998b; Costa et al., 2002). The use of tomato cell feeder layers with high auxin concentrations has also improved citrus transformation (Costa et al., 2002).

The principle of pre-culturing the explants on co-cultivation medium before inoculation with *Agrobacterium* is to promote the production of vir-inducing cell components by metabolically active cells, which enhances the transformation process (Costa et al., 2002; Spencer & Towers, 1991). However, some studies have shown that pre-culturing citrus explants has a negative effect on the transformation efficiency (Cervera et al., 1998b; Costa et al., 2002). Explants without pre-culture gave a reported 8.4-fold higher level in transformation efficiency compared to those that were pre-cultured (Costa et al., 2002). Most transformation experiments have bypassed this pre-culturing stage and have instead used acetosyringone (Cervera et al., 1998b). In nature, this phenolic compound is produced in wounded plant cells and is responsible for the activation of the *vir* genes. This has been shown to increase transformation efficiencies when added to the *Agrobacterium* inoculum and the co-cultivation medium by promoting transcription of *A. tumefaciens* virulence genes (Cervera et al., 1998b; Kaneyoshi et al., 1994); however, in our personal experience working with grapefruit the addition of acetosyringone does not have much of an effect on the transformation efficiencies.

2.3.6 Adequate selection conditions

Finding suitable selective agents to recover transformed cells is critical in citrus transformation in order to eliminate the high numbers of chimeras and escapes that can be obtained during the process (Gutierrez-E et al., 1997; Moore et al., 1992; Peña et al., 1995a). Hence, an effective selective agent is required to improve transformation recovery. Selection is usually based on antibiotic or herbicide resistance. Kanamycin is one of the most widely used selective antibiotics in transformation processes and is most effective when used in concentrations of up to 100 mg/L. However, shoot regeneration may be inhibited at this concentration. Other antibiotics such as geneticin and hygromycin have also been used, but are not as effective as kanamycin (Costa et al., 2002; Peña et al., 1997). The selective antibiotic can be ineffective in situations where residual *Agrobacterium* cells are present or neighboring transformed cells result in the break down or neutralization of the antibiotic. Invariably, non-transformed plant cells, i.e. escapes, thrive in the absence of selective pressure (Cervera et al., 1998b). Other non-toxic selective genes, for instance *manA*, which encodes for the enzyme phosphomannose-isomerase (PMI), have been successfully used in the transformation of sweet orange (Boscariol et al., 2003). The principle is based on the ability of the transformed cells to metabolize mannose as a carbon source present in the selective

medium. Additionally, the use of non-metabolizable genes instead of antibiotic and herbicide resistance genes as selective agents provides a suitable alternative and would satisfy public concerns about their dissemination into the environment and potential effect to consumers. This PMI positive selection system has been shown to be more effective than using kanamycin in many plant transformation protocols (Sundar & Sakthivel, 2008) but this did not seem to be the case in citrus.

2.3.7 Recovery of transgenic shoots

Recovering whole transgenic plants from transformation experiments is often difficult. Typically, most regenerated transformed shoots are either placed directly in soil containing rooting hormone or on rooting media containing varying levels (0 to 1.0 mg/l) of the auxin naphthaleneacetic acid (NAA) which promotes root development (de Oliveira et al., 2009; Gutierrez-E et al., 1997; Luth & Moore, 1999; Moore et al., 1992). Some researchers have gotten better results by first transferring the shoots to hormone-free media to eliminate the cytokinin benzyl aminopurine (BA) from the regeneration media before placing on NAA containing media. Different combinations of BA, NAA and another auxin, indole 3-butyric acid (IBA), NAA and IBA only or just IBA and BA in the rooting medium have been tested so as to improve rooting efficiency in citrus cultivars such as mandarin, lemon, 'Troyer' citrange and lime (Al-Bahrany, 2002; Jajoo, 2010; Moreira-Dias et al., 2000; Singh et al., 1994). Again, the concentrations of these phytohormones vary depending on the citrus genotype. High rooting efficiencies of transgenic shoots have been obtained with citrus types, such as grapefruit, 'Carrizo' citrange and *P. trifoliata* (Peña et al., 2007), but with other citrus types, the rooting efficiency is very low. This problem is overcome by shoot-tip micrografting the transgenic shoot onto a decapitated rootstock seedling (Peña et al., 1995a, 1995b).

3. Genetic engineering and disease control in citrus

Recent advances in genomics, both in citrus and other species, have made available an abundance of genes that can be easily cloned and used in transformation. This is particularly useful in the genetic engineering process as characterized gene(s) derived from known sources can be incorporated into the genome of a recipient plant to obtain desirable traits. Because of its economic impact, disease control is often the objective of plant improvement programs. Hence, resistance and defense genes isolated from well studied plant species have been successfully incorporated into other species to generate pathogen-resistant plants. Another successful strategy in the control of diseases has been the transformation of genes derived from pathogens which can also result in resistant plants.

According to the USDA economic research service, genetically engineered (GE) crops have been widely adopted since their introduction in 1996 (USDA, 2010). Herbicide-tolerant genetically engineered soybeans and cotton have been the most extensively and rapidly adopted GE crops in the U.S., followed by insect-resistant cotton and corn (Cao et al., 2010). The positive impact of these GE crops was due to lower labor and production costs, and gains in profitability, in addition to their increased environmental benefits. In the particular case of citrus, although a variety of transgenic types have been reported in the literature, none has reached commercialization. However, field trials, including our own, are underway. Below we describe some recent and relevant cases of transgenics in citrus.

3.1 Pathogen-derived genes

Some of the earliest success stories in the control of diseases by genetic engineering were using pathogen-derived genes from viruses (Abel et al., 1986). When certain viral genes, particularly the capsid protein (CP), were transformed into plants they showed resistance or immunity against closely related viral strains. A well-known case in a perennial species is the control of *Papaya ringspot virus* by the insertion of its CP into the papaya genome. This effort virtually saved this industry in Hawaii (Gonsalves, 1998). The control mechanism that prevents viral replication in the transgenic plants was initially denominated co-suppression but it is currently referred to as RNA interference or RNA silencing.

Several studies have transformed sequences from a variety of economically important viruses into different citrus types to attempt to produce resistant plants. One of such viral diseases is caused by *Citrus tristeza virus* (CTV). Severe strains of CTV can dramatically reduce production and in some instances lead to tree death in a relatively short period of time (Moreno et al., 2008). In some areas of the world CTV is an important or the most important limiting factor in citrus production and incorporation of resistance by traditional breeding techniques is not possible. For this reason many laboratories have tried to genetically engineer different CTV sequences into citrus as a way to control this important pathogen. However, these attempts have never been completely successful. For example, transforming the major CP (p25) into 'Mexican' lime had two types of response to viral challenge. In replicate plants, propagated from the same line (i.e. genetically identical), 10 to 33% were resistant to CTV while the rest developed typical symptoms, despite a significant delay in virus accumulation (Domínguez et al., 2002). Similar results were obtained in 'Duncan' grapefruit when translatable and untranslatable versions of the major CP were transformed (Febres et al., 2003, 2008). Various forms (full length, hairpins) of the p23 gene, located in the 3' end of the viral genome, have also been transformed into citrus genotypes. In 'Mexican' lime expression of the p23 protein produced viral symptoms in some plants (Fagoaga et al., 2005). Lines with normal phenotype (no symptoms) were further propagated and tested for CTV resistance and again the results were mixed with some plants completely immune to the virus while others from the same line had delayed symptom development and virus accumulation (Fagoaga et al., 2006; López et al., 2010). The use of the 3' region of the p23 and the contiguous 3'-untranslated region (UTR), either as a hairpin or as single copy, has also been transformed into 'Duncan', 'Flame', 'Marsh', and 'Ruby Red' grapefruit and alemow plants with similar results as described above in which some plants derived from a particular line were fully resistant and others were not (Ananthakrishnan et al., 2007; Batuman et al., 2006; Febres et al., 2008). Only in one line full resistance was observed (Febres et al., 2008). This line is currently being evaluated in the field for its horticultural value and durability of the resistance under natural conditions. Other CTV genes have been used but either no transgenic plants were regenerated (p20 and minor CP/p27) or they did not show resistance (RdRp gene) (Febres et al., 2003, 2008).

Resistance to another important viral disease, *Citrus psorosis virus* (CPsV), has been reported in transgenic sweet orange plants transformed with intron-hairpin constructs (ihp) corresponding to the viral CP, the 54K or the 24K genes (Reyes et al., 2011). After challenge with the virus, the CP transgenic plants were more effective in controlling the CPsV and consistently showed lower virus levels and no symptom development compared to 54K and 24K transgenic plants. The study reported that the observed CPsV resistance was due to pre-activated RNA silencing rather than the siRNA accumulation levels in the *ihp-CP* transgenic sweet orange plants prior to virus challenge (Reyes et al., 2011).

Pathogen-derived genes have also been used to control bacterial diseases. Citrus canker, caused by *Xanthomonas axonopodis* pv. *citri* is an economically important disease, especially for the fresh fruit market. The *pthA* protein is involved in the pathogenesis and symptom development of this bacterial pathogen and the C-terminus contains three nuclear localizing signals (NLS) critical for the interaction with a host protein, translocation to the nucleus and function (Yang et al., 2011). By using a truncated version of the *pthA* gene, coding only for the C-terminus portion of the protein, it was theorized that the resulting protein would interrupt binding and function of the native bacterial *pthA* during infection and prevent symptom development and pathogen growth. Indeed transgenic sweet orange plants that expressed the truncated protein showed lower disease incidence and symptom development compared to wild type plants, demonstrating a certain degree of resistance (Yang et al., 2011). The authors are currently conducting field experiments to determine the effectiveness of this strategy under natural conditions.

In another strategy, also to control citrus canker, a *hrpN* gene derived from *Erwinia amylovora* was transformed into 'Hamlin' sweet orange plants. The *hrpN* encodes a harpin protein that elicits the hypersensitive response (HR) and systemic acquired resistance (SAR) in plants. The *hrpN* gene was inserted in a construct made up of *gst1*, a pathogen-inducible promoter (so the gene would not be expressed constitutively and hence the SAR response would only be induced in the presence of the pathogen), a signal peptide for protein secretion to the apoplast (the canker bacterium does not penetrate the cell and remains apoplastic). Several of the *hrpN* transgenic lines showed reduction in their susceptibility to citrus canker as compared to wild type plants, and one line in particular displayed very high resistance to the pathogen (up to 79% reduction in disease severity) (Barbosa-Mendes et al., 2009).

Fungal pathogens also affect citrus production. In particular, *Phytophthora* spp can cause root rot and gummosis in mature trees and damping-off in seedlings. For the control of *Phytophthora nicotianae* Azevedo et al (2006) used a bacterio-opsin (*bO*) gene to transform 'Rangpur' lime. The *bO* gene is derived from *Halobacterium halobium* and can spontaneously activate programmed cell death and enhance broad-spectrum disease resistance accompanied by pathogenesis-related (PR) protein accumulation. In two of the transgenic lines, higher levels of tolerance to this pathogen with significantly smaller lesions were observed; however, these lines also exhibited HR-like lesions in the absence of pathogen (Azevedo et al., 2006). It remains to be seen if this strategy will work under field conditions given the fact that the transgenic plants develop spontaneous lesions.

3.2 Plant defense genes

Upon recognition of a potential pathogen plants naturally respond by triggering defense mechanisms that can, in some instances, halt pathogen colonization. One such defense mechanism is SAR, a form of inducible defense in which infection by a pathogen leads to an enhanced defense state that is durable and provides resistance or tolerance to a wide range of pathogens in subsequent challenges (Durrant & Dong, 2004).

A gene that has been identified as critical in the establishment of SAR is the *NON-EXPRESSOR OF PATHOGENESIS RELATED 1* (*NPR1*). *NPR1* is a transcription co-activator and plays a key role in regulating defense gene transcription and signal transduction pathways that lead to SAR (Despres et al., 2000; Zhang et al., 1999). Under normal conditions the *NPR1* protein does not induce SAR, however in the presence of a pathogen and increased levels of salicylic acid (SA) *NPR1* is translocated to the nucleus where it

interacts with transcription factors that ultimately induce the expression of SAR-associated genes (Kinkema et al., 2000).

A number of studies have demonstrated that the over-expression of the *Arabidopsis* NPR1 provides a broad-spectrum enhanced resistance to various pathogens (Cao et al., 1998; Lin et al., 2004). Our laboratory and others have invested a considerable amount of time and effort investigating the nature of SAR in citrus and the full length sequences of five citrus NPR1-like genes has been cloned and sequenced. Their expression levels are differentially affected by pathogen and other treatments (Febres and Khalaf, unpublished results).

Zhang et al. (2010) reported transforming the *Arabidopsis* NPR1 gene into 'Duncan' grapefruit and 'Hamlin' sweet orange. The over-expression of this gene increased resistance to citrus canker and the observed resistance correlated with the expression levels of the transgene. Our results of transgenic 'Carrizo' citrange plants, also transformed with the *Arabidopsis* NPR1 gene indicated that the transgenic lines were as well more tolerant to citrus canker (slower lesion development) and had higher levels of pathogenesis-related (PR) genes than wild type plants (Febres, unpublished).

3.3 Additional strategies

As mentioned above, attempts to use pathogen-derived sequences for the control of CTV have not rendered consistent results. A different approach has recently been tested (Cervera et al., 2010) by using single-chain variable fragments (scFv) from two monoclonal antibodies that in combination seem to detect the major CP from most CTV isolates. 'Mexican' lime plants were transformed with each scFv either individually or in combination. Essentially all constructs conferred some level of protection when the plants were challenged with a severe strain of CTV. Between 40 to 60% of the plants tested did not get infected, compared to 95% infection in control plants. In addition a delay and attenuation in symptom development was also observed. Although complete resistance was not observed in this case either it is still a promising approach that needs further investigation.

4. Emerging technologies

The production of new varieties via transformation in citrus and many other woody perennials poses a challenge not found in the breeding of annuals and other fast-growing plants. Due to combinations of long juvenile periods, biological barriers to crossing, and the difficulty of reconstituting favored types, such as the complex hybrids sweet orange and grapefruit in citrus, new cultivars will probably have to be selected from T0 transformants. There are several implications to this, discussed below.

One of the greatest challenges of producing and testing transgenic *Citrus* plants is the long juvenile periods observed in this genus. As discussed above, most citrus transformation techniques utilize explants derived from juvenile tissue, and the transgenic plants must be grown for many years, in most cases, for their horticultural attributes to be evaluated. Two approaches are being investigated to overcome this problem. The first is efforts to decrease the juvenile periods of transgenic plants. There are both historical work and ongoing efforts to use horticultural methods to bring citrus plants into bearing earlier. Another alternative for shortening the juvenile period is to produce transgenic plants that over-express a flower meristem identity gene that causes them to flower earlier. The *Arabidopsis* LEAFY and APETALA1 genes have been over-expressed in 'Carrizo' citrange (Peña et al., 2001) and transgenic *Poncirus* plants over-expressing a citrus orthologue of *Arabidopsis* FLOWERING

LOCUS T (FT) have been produced (Endo et al., 2005; Nishikawa et al., 2010). However, in most cases, the expression of these genes, while dramatically reducing time to flowering, also conferred deleterious morphological phenotypes to the transgenic plants. Thus this approach may benefit citrus breeding efforts and early testing of traits designed to be evinced in fruit, it may not produce T0 transgenic plants that could directly be used in production. However, all possibilities of this approach have not been explored. For instance, citrus genomes contain at least three orthologues of *FT* that produce quite different phenotypes when overexpressed in transgenic tobacco (Kamps and Moore, unpublished). Also, Carrizo plants transformed with *APETALA1* displayed normal morphology (Peña & Séguin, 2001).

The second approach for overcoming juvenility is to use explants from mature plants for transformation. However, taking explants directly from mature trees is not likely to be successful due to the low regeneration potential of such explants and perhaps also of lower competence for transformation. Success has been achieved by reinvigorating mature citrus types by grafting mature buds on vigorous juvenile rootstocks and using the first flushes for *Agrobacterium*-mediated transformation (Cervera et al., 1998a, 2005). However, this is a technically demanding approach. The plant material must be in excellent condition, which is particularly difficult to achieve in humid climates, where the pathogen load on tissue, even when grown under greenhouse conditions, may make disinfection of explants difficult. Even then, only a relatively small number of explants can be obtained from the first flush or two of the grafted plant. In some genotypes, a lack of bud uniformity in sprouting and morphology is problematic (Cervera et al., 2008). In other cases, culture requirements for regeneration may be quite different for even closely related citrus types (Almeida et al., 2003; Rodríguez et al., 2008). Kobayashi (2003) circumvented some of these problems by using already grafted 'Pera' sweet orange nursery plants for harvest of explants and by thin segments (1 to 2 mm) of stems as explants. In all cases, transgenic plants in the greenhouse began to flower after 14 months or less after micrografting the transgenic scions on rootstock. Experiments are underway in several laboratories to improve still further on the production of transgenic plants from mature tissue. The importance of the cambium in producing transgenic tissue in many of the above reports and the recent description of the cambium cells of several plants as analogous to vascular stem cells (Lee et al., 2010) suggest that one research direction could be exploration of other types of explants where the cambium cells are maximally exposed to *Agrobacterium* and subsequent growth hormones in the culture medium.

Another problem with using T0 plants is that the gene insertion site(s) is unknown. This can affect the expression of the transgene and could lead to altered morphology that was not intended. However, genomic changes that are not selected for also may happen during conventional breeding due to, for instance, transposon activity or irradiation and mutation breeding.

Of course there are also advantages to utilizing T0 transformants in perennials. With the explosion in genomic information, the functions of more and more genes are being elucidated (Talon & Gmitter Jr, 2008), so choosing a transgene that will impart a particular trait should be more targeted in the future. It has also been found in both conventional and molecular breeding that valuable genes or alleles are found in plant relatives or wild species. In such cases using T0 transgenics circumvents the problem of linkage drag that may result from the transfer of unknown and undesirable genes that are linked to the desirable gene or allele from the donor parent. It might also be possible to "stack" valuable genes or alleles in

a desirable citrus type via multiple transformations or multiple genes inserted in a single transformation.

Another important area of research has been to increasing the cold hardiness of citrus. This could potentially extend production areas to new regions where pathogens or other limiting factors are not present. As in the case with disease resistance there are some citrus relatives that can endure freezing temperatures. While most commercially important citrus varieties are susceptible to freezing, *P. trifoliata* for instance can tolerate temperatures well below freezing if cold acclimated prior to the exposure (Talon & Gmitter Jr, 2008).

Genes associated with cold acclimation have been identified in citrus as an initial milestone in a multistep approach to ultimately incorporate some of these genes in the genome of selected citrus varieties that are naturally susceptible to freezing. Our laboratory and others have studied the effect of cold stress or freezing on gene expression. For instance, in an attempt to minimize the chilling injury during citrus fruits storage, a genome-wide transcriptional profiling analysis was performed (Maul et al., 2008). Grapefruit flavedo RNA was used to study the responses of citrus fruit to low temperatures. The study applied a pre-storage conditioning treatment of 16°C for 7 days and utilized an Affymetrix Citrus GeneChip microarray. While the applied treatment seemed to have halted the expression of general cellular metabolic activity, it induced changes in the expression of transcripts related to membranes, lipid, sterol and carbohydrate metabolism, stress stimuli, hormone biosynthesis, and modifications in DNA binding and transcription factors.

Our laboratory provided the first evidence of an association in citrus between C-repeat binding factors (CBF) expression levels and the extent of cold tolerance (Champ et al., 2007). CBFs have been identified in many species and they function as transcriptional activators regulating the expression levels of a number of genes that impart cold and stress tolerance. *P. trifoliata*, a *Citrus* relative, can survive freezes of -20°C when fully cold acclimated. On the other hand, grapefruit cannot withstand temperatures lower than 0°C. In *P. trifoliata* transcripts of *CBF1* and *CORc115* (a cold-induced group II LEA gene, and a likely target of *CBF1*) accumulate both earlier and to higher levels than in grapefruit when exposed to cold temperatures. Additionally, using subtractive hybridization we identified a number of new, differentially cold-regulated genes from *P. trifoliata* (Sahin-Cevik & Moore, 2006). Although several of the genes identified were unique sequences, many were homologous to cold and environmental stress-induced genes from other species. Taken together, our results indicate that similar pathways are present and activated during cold acclimation in diverse plant species.

In a more recent study (Crifo et al., 2011) performed a transcriptome analysis based on subtractive hybridization to study cold stress response of pigmented sweet oranges (blood oranges) in order to study the overall induction in gene expression after the exposure to low temperatures. On the whole, the expression of transcripts related to defense, oxidative damage, osmo-regulation, lipid desaturation and primary and secondary metabolism were induced. In addition, cold stress induced flavonoid biosynthesis, including those reactions involved in anthocyanin biosynthesis and metabolic pathways supplying it. Several transcription factors were identified for the first time as cold responsive genes in plants.

In summary, cold stress has been linked to signaling pathways where gene expression can further interrelate with additional stress related pathways. The entire signaling network throughout the plant affects its response(s) to biotic or abiotic stress. Along with the mentioned gene annotations, additional functional analyses are crucial to study the nature of the expected phenotype before we can introduce new genes into the *Citrus* genome using transformation techniques.

Antimicrobial peptides (AMPs) are currently the subject of intense research for the control of diseases in citrus, particularly canker and huanglongbing (HLB) or citrus greening. There is no known resistance in *Citrus* to HLB (caused by *Candidatus Liberibacter* spp); however, it can have devastating effects by reducing overall production. Infected trees have smaller fruits with less juice, the flavor of the juice is changed and it eventually leads to micronutrient deficiencies, defoliation and tree death. It has been known for years that AMPs play a vital role in plant defense. Plant AMPs are monomer or oligomer building units that have mostly three-dimensional or tertiary structures of either amphipathic or amphiphilic nature (Sitaram & Nagaraj, 1999, 2002). The latter characteristic and folding are essential for the peptides antibacterial activity (Epanand & Vogel, 1999). Different scenarios for their function have been suggested but they all agree on the fact that these AMPs operate by the formation of membrane pores that ultimately cause the disruption of the membrane and subsequently cell death through ion and metabolite leakage (Yeaman & Yount, 2003). A number of studies have confirmed the inhibitory effect of these peptides to fungal and bacterial pathogens when expressed in different plant species such as rice, wheat, and tomato fruits (Jha & Chattopadhyay, 2010; Jha et al., 2009; Ramamoorthy et al., 2007). In a recent study, two AMP genes, *Shiva A* and *Cecropin B*, were transformed into 'Jincheng' and 'Newhall' sweet orange. Subsequently, the transgenic plants were challenged with *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus canker. In both greenhouse and field experiments with artificial or natural inoculation, respectively, some transgenic lines were highly resistant to canker and either did not develop canker lesions or the number of lesions was significantly reduced compared to wild types. The plants were also phenotypically normal, flowered after two years (grafted on *Poncirus*), borne fruit and the juice was no different in solid and sugar content and acidity from non-transgenic plants (He et al., 2011).

4.1 Transformation vs. transient expression

Transient expression systems are beneficial for some purposes, such as rapidly and easily assaying promoter function or gene expression under some conditions. Although it has been surprisingly difficult to implement transient expression in citrus leaves it has been possible to transiently express genes in the fruit, particularly young fruit (Ahmad & Mirza, 2005; Spolaore et al., 2001).

Finally, a vector based on CTV has been developed (Folimonov et al., 2007). Such vectors have been used in herbaceous plants to study gene function, expression, and silencing, but have not been available for woody plants. This can be seen as a hybrid strategy between transient expression and stable transformation. Although the virus vector nucleic acid is not incorporated into the genome of the citrus host, Folimonov et al. (2007) reported that expression of GFP continued for up to four years after introduction of the scorable marker into CTV vectors.

5. Conclusions: The future of citrus transformation

Ultimately the use of genetic engineering is just another tool in the improvement of citrus. Genetic transformation has the advantage of potentially reducing breeding time, particularly important in the case of a perennial crop such as citrus with a long juvenile period, and also facilitating the introduction of traits not readily available in the particular species. Breeding programs take into consideration the needs of both farmers and

consumers. Production of genetically modified citrus should also take into consideration the needs of both; however, genetically modified organisms (GMOs) tend to be more controversial and subjected to more public scrutiny than traditionally produced varieties. For instance, a recent European survey indicated that among respondents GMOs were considered unnatural (70%), made them feel uneasy (61%), harmed the environment (59%) and were unsafe for people's health (59%) (European Commission, 2010). Regardless of whether these concerns are just perceived or real they will have to be addressed in order to fully implement the benefits of genetic engineering in solving real and important problems for citrus farmers and at the same time delivering desirable products to consumers.

Two major concerns regarding GMOs are: 1) impact to the environment, in the form of the transgene 'escaping' and transferring to wild species and thus eroding the biodiversity of wild relatives of the crop or, on the other hand, creating 'super weeds' of species that acquire the transgene and become better fitted and difficult to control (Azevedo & Araujo, 2003; Parrott, 2010; Sweet, 2009); and 2) impact to human health by a potentially toxic or allergenic transgenic protein (Domingo & Gine Bordonaba, 2011).

In the particular case of citrus there are ways to mitigate these concerns. Essentially all presently grown GMOs are *transgenic* in nature, with "trans" referring to genetic sequences that come from organisms that are not crossable with the plant in question, such as sequences from viruses or bacteria or even from a plant species that is not crossable, for instance the insertion of an *Arabidopsis* gene into a citrus plant. This has led to many countries and groups being resistant to the growth and consumption of GMOs. Thus, there are proponents of producing GMOs that are *cisgenic*, where all of the inserted genetic material comes from the original plant or a crossable type (Jacobsen & Schouten, 2008). Such genes could be perceived by the public as more "natural" and could potentially be less likely to be toxic or allergenic (although this would have to be tested experimentally on a case by case basis). Plants transformed this way do not appear to raise the fear and ethical concerns that the production of transgenic plants inspires (Conner et al., 2006; Rommens et al., 2007). However, this approach would rule out the use of most commonly used selectable and scorable marker genes, as well as the most commonly used promoters and termination sequences and the necessary T-DNA borders for *Agrobacterium*-mediated transformation. A cisgene consists of a native gene with its native promoter and termination. In these discussions, there is also mention of *intragenes* in which gene parts can originate from different genes as long as the donor is a crossable type (Jacobsen & Schouten, 2008). Many laboratories are now looking for plant DNA sequences that are homologous to the bacterial sequences present in T-DNA borders and for methods to produce genetically modified plants where selectable and scorable genes can be either removed after transformation or are of plant origin (Rommens et al., 2007).

There has been a small amount of research of this kind in citrus. Fleming et al. (2000) transformed sweet orange protoplasts with a construct containing the GFP scorable gene using a PEG method. Transformed regenerating somatic embryos were identified by their GFP expression and physically separated from nontransformed tissues, resulting in transgenic plants. No *Agrobacterium* was involved and there was no selective agent applied. Ballester et al. (2008) compared the most common citrus transformation and selection system, using kanamycin selection and scorable GUS staining to three methods that did not utilize antibiotic selection, in 'Carrizo' citrange and 'Pineapple' sweet orange. The alternative methods included scoring for GUS staining without applying selection, transforming explants with a multi-autotransformation (MAT) vector, combining an

inducible recombinase-specific recombination system (R/RS) with transgenic-shoot selection through expression of isopentenyl transferase (*ipt*) and indoleacetamide hydrolase/tryptophan monooxygenase (*iaaM/H*) marker genes, and selection with the PMI/mannose conditional positive selection system (Boscariol et al., 2003). Transgenic plants were obtained from all treatments, but selection for *nptII* expression was by far the most efficient. The authors preferred the MAT vector, because with it they could obtain transformed plants where the selectable marker would recombine out (Ballester et al., 2007). However, all of the transgenic plants still contained some sequences of bacterial origin.

Another approach is the use of promoters that do not express the transgene in the edible parts (fruits). Again this would potentially reduce the possibility of becoming harmful to human health. Several groups are actively searching for such promoters in citrus, including inducible promoters that would be turned on at will by chemical application, etc. As explained before the genomic information currently available should facilitate this endeavor. A third strategy we are exploring is the use of transgenic rootstocks that could confer the desired trait to the wild type (non transgenic) scion, without the need of incorporating and expressing transgenes in the scion and edible parts of the plant. This would prevent or at least reduce the chances of spreading transgenic pollen into the wild. There is evidence for the transfer of genetic material between rootstock and scion but this seems to be limited to the graft union region (Stegemann & Bock, 2009). However, it is unlikely that this grafting approach would work with all transgenes since not all expressed proteins are translocated and/or have a systemic effect. One case in which it could work in citrus is the reduction of juvenility using the FT protein. Transgenic FT is capable of inducing flowering through graft unions (Notaguchi et al., 2008; Notaguchi et al., 2009). Induction of pathogen defense could potentially be tackled this way as well since some of the proteins activate systemic signaling (Xia et al., 2004).

These approaches take into consideration consumer's perception about GMOs, educated concerns about the release of GMOs and the needs of citrus farmers for better, disease resistant crops. Citrus production faces important challenges due to climate change and disease and genetic engineering has the potential, as has been the case in other crops, of becoming an important weapon in the arsenal against these major challenges.

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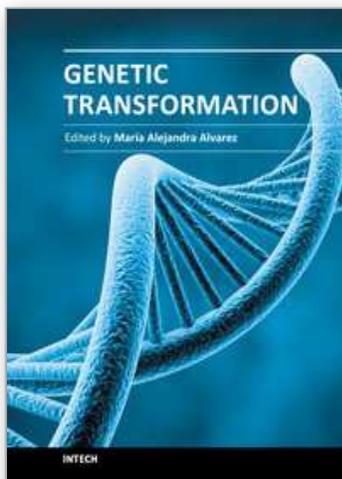
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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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