TOWARDS THE DEVELOPMENT OF BROAD-SPECTRUM DISEASE RESISTANCE IN CITRUS

A Dissertation

by

MADHURA BABU KUNTA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Horticulture

TOWARDS THE DEVELOPMENT OF BROAD-SPECTRUM DISEASE RESISTANCE IN CITRUS

A Dissertation

by

MADHURA BABU KUNTA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Co-Chairs of Committee, Mani Skaria
Bhimanagouda S. Patil
Committee Members, Eliezer S. Louzada
John V. da Graca
T. Erik Mirkov
Head of Department, Tim Davis

May 2009

Major Subject: Horticulture

ABSTRACT

Towards the Development of Broad-Spectrum Disease Resistance in Citrus.

(May 2009)

Madhura Babu Kunta, B.S., Andhra Pradesh Agricultural University;

Dip., University of Hyderabad;

M.S., Texas A&M University-Kingsville

Co-Chairs of Advisory Committee: Dr. Mani Skaria
Dr. Bhimanagouda S. Patil

Transgenic plants expressing Cyclic nucleotide-gated channel (*CNGC*) and B-cell lymphoma 2 (*bcl-2*) transgenes have been shown to be resistant to fungal and viral pathogens. A PCR amplification product comprising *CNGCcit* open reading frame (ORF) with *Xba* I and *Eco* RI ends was generated, inserted into pRTL22 plasmid, transformed into *E. coli*, and sequenced. The 3.2 kb *Hind* III fragment of pRTL22/*CNGCcit* containing the CaMV 35S promoter with dual enhancer, *CNGCcit*, and CaMV 35S terminator was inserted into the *Hind* III site of pBin 34SGUS to generate pBin35SCNGCcit construct, and transformed into *E. coli*. The cDNA clone of bcl-2 in the pPTN binary vector was digested with *Hind* III to release a fragment consisting of CaMV 35S promoter, *bcl-2*, and CaMV 35S terminator and inserted into the *Hind* III site of pBin 34SGUS to generate pBin35SBcl-2 construct. These constructs along with pPTN334Bcl-2 were used in *Agrobacterium tumefaciens*-mediated transformation of sour orange rootstock, 'Rio Red', 'Ruby Red' and 'Duncan' grapefruit cultivars. The presence and expression of *CNGCcit* and *bcl-2* in the transgenic plants was

verified by β-glucuronidase histochemical assay, *npt*II enzyme-linked immunosorbent assay, polymerase chain reaction, Southern blot, northern blot, relative quantitative reverse transcription PCR , and quantitative real-time PCR. In this study, two transgenic 'Ruby Red' plants were successfully produced with an incorporated *CNGCcit* gene that gave a positive result with all the analyses. None of the putative transgenic plants was transgenic for *bcl-2* gene based on Southern and northern analyses. Detached leaf assays of the transgenic 'Ruby Red' plants showed an enhanced resistance to *Xanthomonas axonopodis* pv. *citri*, *Alternaria alternata*, and *Phytophthora nicotianae*. Also, the leaves did not show any sensitive response to tentoxin. The citrus cytosolic ascorbate peroxidase (*cAPXcit*) genomic clone was isolated and characterized. Expression of *cAPXcit* in 'Duncan', non-transgenic control 'Ruby Red', and 'Rio Red' was suppressed due to *A. alternata* and *P. nicotianae* inoculations. However, an increased expression of *cAPXcit* was observed in the inoculated transgenic 'Ruby Red' leaves.

ACKNOWLEDGEMENTS

I would like to thank my committee co-chairs, Dr. Skaria and Dr. Patil, and my committee members, Dr. Louzada, Dr. Mirkov, and Dr. da Graca, for their guidance and support. Each member of the committee contributed in different ways for the successful completion of this research project. I appreciate the help of Dr. Bock and Dr. Parker with *Xanthomonas axonopodis* pv. *citri* inoculation studies to evaluate the transgenic plants for resistance.

Thanks also go to all my friends, colleagues, and the department faculty and staff for their support.

I would like to take this opportunity to thank my wife, Madhavi, daughters, Niharika and Ramya, for their encouragement and support. My daughters were the driving force that kept me energized to successfully complete this research.

Finally, I am grateful to the almighty GOD for giving me the life, health, and opportunity to continue my education.

TABLE OF CONTENTS

Page

ABSTRACT iii

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

LIST OF FIGURES ix

LIST OF TABLES xv

INTRODUCTION 1

LITERATURE REVIEW 10

Hypersensitive response 11
Cytosolic calcium 12
CNGC 13
B-cell lymphoma-2 (BCL-2) 15
Agrobacterium-mediated citrus transformation 17

MATERIALS AND METHODS 22

Expression analysis in *E. coli* 22
Plant material 22
Bacterial strains and plasmid vector constructs 23
Transformation and plant regeneration 25
Recovery and selection of putatively transformed shoots. 26
Grafting of the putatively transgenic shoots 26
GUS and *npt*II activity assays 27
Histochemical GUS assay 27
*Npt*II ELISA assay 28
Kanamycin Infiltration 29
Nucleic acid extraction 29
Genomic DNA Extraction 29
Total RNA Isolation 30
Reverse transcription-polymerase chain reaction (RT-PCR) 31
First-strand complementary DNA (cDNA) synthesis 31
PCR 31

Page

Cloning, plasmid DNA extraction, and sequencing 32
PCR to detect *npt*II, *uid*A, and *CNGCcit* 33
Southern analysis 34
Northern analysis 35
Relative quantitative RT-PCR (RQ RT-PCR) 36
Quantitative real-time RT-PCR (Q RT-PCR) 36
Plant inoculation and disease resistance assays. 38
Inoculation of the leaves with *Xac* 38
Fungal Isolates 39
Inoculations with *P. nicotianae* 40
Inoculations with *A. alternata* pv. *citri* 40
Tentoxin assay 41
Isolation of *APXcit* genomic clone 41
Analysis of *cAPXcit* expresion changes due to pathogen inoculations 42

RESULTS 43

Expression analysis in *Escherichia Coli* 43
Plasmid constructs 44
Agrobacterium-mediated transformation and plant regeneration 48
Selection of putative transgenic shoots 56
Grafting 57
GUS and *npt*II-based selection 61
Kanamycin infiltration 62
Molecular analyses 65
PCR 65
Southern blot 66
Northern blot 68
RQ RT-PCR 68
Q RT-PCR 70
Phenotype of the transgenic plants 72
Inoculations with pathogens 73
Xanthomonas axonopodis inoculations 73
P. nicotianae inoculations 76
A. alternata inoculations and tentoxin assay 78
cAPXcit genome organization 83
cAPXcit expression changes due to pathogen inoculations 85

DISCUSSION 90

SUMMARY AND CONCLUSIONS 100

LITERATURE CITED 102

Page

APPENDIX A 117

APPENDIX B 118

APPENDIX C 119

APPENDIX D 120

VITA 121

LIST OF FIGURES

FIGURE Page

1. Expression analysis of *CNGCcit* in *E. coli*. BL21 (DE3) (pET101/D-TOPO) after IPTG induction lanes 1, 2, and 3 = induced after 3 h, 4 h, and 5 h, lane U = un-induced after 5h. The 110 kDa recombinant protein for CNGCcit is shown on left 43

2. Generation of *CNGCcit* ORF using RT-PCR. M= Molecular marker (1 kb DNA ladder, Fermentas), lanes 1-6 = *CNGCcit* ORF (2.1 kb) with the *Xba*I and *Eco*RI ends produced by RT-PCR of total RNA extracted from cold (O^{o}C) acclimated trifoliate orange leaves using the CNGCtrans-F and CNGCtrans-R primers.. 45

3. Restriction digestion of pBin34GUS and pRTL22/CNGCcit with *Hin*dIII. M = Molecular marker (1 kb DNA ladder, Fermentas), lanes 1-4 = pBin34GUS plasmid cut with *Hin*dIII, lanes 6 and 8 = un-cut, lanes 5 and 7 = pRTL22/*CNGCcit* cut with *Hin*dIII 45

4. Restriction digestion of pPTN334Bcl2 plasmid DNA with *Hin*dIII. Lane M = Molecular marker, lane 1 = *Hin*dIII digestion of the pPTN334Bcl2 plasmid DNA to release a 2.17 kb fragment which consists of E35S promoter, TEV leader, *Bcl*-2 gene, and 35S terminator 46

5. The schematic representation of the binary vectors used in the citrus transformation. pBin35SCNGCcit (A), pBin35SBcl2 (B), and pPTN334Bcl2 (C), LB = Left T-DNA border, Pnos = nopaline synthase promoter *npt*II = *neomycin phosphotransferase* II gene, 3'nos = NOS terminator, P35S = CaMV 35S promoter, pd35S = double CaMV 35S promoter, P34S = Figwort mosaic virus 34S promoter, 3'35S = CaMV 35S terminator, PE35S = Tobacco etch virus 35S promoter, *uidAi* = intron inserted *uidA* gene, H = *Hin*dIII, X = *Xba*I, *CNGCcit* = Citrus cyclic nucleotide-gated ion channel, *Bcl-2* = Human B-cell Lymphoma-2 gene, RB = Right T-DNA border (Figures not drawn to scale) 47

FIGURE Page

6. *Hind*III digestion of *A. tumefaciens* plasmid DNA incorporated with pBin35SCNGCcit and PCR amplification of *CNGCcit* from *A. tumefaciens* plasmid DNA. M = Molecular marker (1 kb DNA ladder, Fermentas). (**A,** lanes 1-2) and (B, lanes 1-4) = pBin35SBcl2 and pBin35SCNGCcit digested with *Hind*III, (C, lanes 1-6) = *CNGCcit* gene amplified from the plasmid DNA of *A. tumefaciens* harboring pBin35SCNGCcit using CNGCtrans-F and CNGCtrans-R primers............ 48

7. Citrus seedlings grown on half strength MS medium supplemented with Gamborg's vitamins 49

8. Sour orange internodal stem segments used in transformation. Thorns and leaves were removed from 180 d-365 d-old sour orange seedlings (A) and intermodal stem segments (INS) were used in co-culture with *A. tumefaciens* (B) 49

9. 'Ruby Red' grapefruit epicotyl segments placed horizontally on the co-culture medium... 50

10. Transient GUS staining of the epicotyl end segments after 3 day co-cultivation with *A. tumefaciens* C58C1 carrying binary vector pBin35SCNGCcit. 'Ruby Red' grapefruit (A), 'Duncan' grapefruit (B), 'Rio Red' grapefruit (C), and internodal stem segments of sour orange rootstock (D) 51

11. 'Ruby Red' epicotyl segments placed horizontally on the DBA3 selective medium after co-culture (A), showing callus proliferation at the cut ends (B-C) 52

12. Shoots regenerated from 'Ruby Red', 'Duncan', and sour orange epicotyl segments on DBA3 selective medium (A), a close-up of 'Duncan' (B) 54

13. Sour orange epicotyl segments with *A. tumefaciens* over-growth (A) fungal contaminations (B) 55

14. Sour orange regenerated shoots (A) from the epicotyl segment were cut transversely (B) and placed on BG medium (C) 55

15. 'Ruby Red' grapefruit regenerated shoots placed on the BG medium (A) elongated shoots (B-C) 56

FIGURE Page

16. 'Ruby Red' grapefruit non-transgenic escape shoots showing gradual bleaching due to kanamycin selection using liquid overlay.......................... 57

17. 'Ruby Red' (A) and 'Duncan' (B) grapefruit green shoots that were selected for use in shoot grafting.. 57

18. 'Ruby Red' shoot grafted onto sour orange and the graft union held by plastic tube. (A) 'Duncan' shoot grafted onto C-22 and covered by a plastic pouch to maintain high humidity (B) 58

19. Shoot grafting of putative transgenic shoots. 'Rio Red' on sour orange (A), sour orange on C-22 (B), 'Ruby Red' on sour orange (C), 'Duncan'on sour orange (D) 59

20. Shoot grafting of 'Ruby Red' epicotyl segments with regenerated shoots grafted onto sour orange (A), several successful grafts of 'Ruby Red' on sour orange (B), graft union failure of 'Ruby Red' grafted onto C-22 (C) 60

21. T-grafting of the regenerated 'Ruby Red' epicotyl segments. Grafting onto sour orange (A), bud forcing by breaking the sour orange stem (B), graft failure (C) 60

22. Putative transformed citrus plants growing in the greenhouse...................... 61

23. The putative transgenic plants were screened for *npt*II through ELISA assay. Microtiter plate with positive reactions showing yellow color (7E) for transgenic 'Ruby Red', 7E = transgenic 'Ruby Red' grapefruit, 1A-6E, 1F-6H, 7A-7D = putative transgenic 'Ruby Red' grapefruit, 7F and 7G = water and PBST buffer negative control, 7H = positive control. The absorbance readings at 450 nm are shown in the following Table 3 63

24. Non-transgenic 'Ruby Red' leaf showing no blue stain (A), transgenic 'Ruby Red'leaves showing blue stain (B).. 64

25. Kanamycin infiltration assay. The leaves infiltrated with 10 µL of100 mg mL^{-1} kanamycin showing yellowing (chlorosis) of the non-transgenic sour orange (A) and 'Ruby Red' grapefruit (B). Transgenic 'Ruby Red' leaves (C) did not show chlorosis... 65

FIGURE Page

26. PCR analysis of the DNA from the transgenic 'Ruby Red' plants. Lane M = Molecular marker (1 kb DNA ladder, Fermentas), lanes 1-2 *CNGCcit* (2.1 kb), 3-4 = *npt*II gene fragments of sizes 350 bp and 700 bp, lane 5-6 = *uid*A gene fragment of 450 bp and 880 bp, and lane 7 = non-transformed control. 66

27. *uid* A and *Bcl-2* fragments used in Southern and northern analyses. Lane M = Molecular marker (1 kb DNA ladder, Fermentas), the *uid* A probe DNA (2 kb) generated by restriction digestion *uid* A plasmid with *Sac*I and *Bam*HI (A lanes 1-2), the *Bcl-2* probe DNA (791 bp) was produced by the restriction digestion of pPTN334Bcl2 plasmid DNA with *Xba*I and *Nco*I (A lane3-4), gel-purified *uid*A and *Bcl-2* probes (B lanes 1-2). 67

28. Southern blot analysis of the citrus plants transformed with *CNGCcit* gene. The DNA was digested with *Hin*dIII and hybridized with *uidA* probe. Lanes 1 and 11 = transgenic 'Ruby Red', lanes 2-9 = putative transgenic 'Ruby Red', lane 10 = non-transgenic negative control. Molecular weights are indicated on the left. 68

29. RQRT-PCR analysis of the relative *CNGCcit* gene (2.1 kb) expression in the transgenic 'Ruby Red' plants compared to non-transgenic control plants. Lane M= Molecular marker (1 kb DNA ladder), lanes 1, 3, and 5 = non-transgenic control, lanes 2 and 4 = transgenic 'Ruby Red' plants.......... 69

30. Q RT-PCR performed on the first-strand cDNA from 'Ruby Red' grapefruit plants. Graphs showing the Ct values for *CNGCcit* (A), graphs for COX (B), 5, 7, and 9 = transgenic 'Ruby Red', 4, 6, 8, 10 = non-transgenic, NTC = non-template control (water). The results are shown in the following table 71

31. A comparison of similar aged non-transgenic control 'Ruby Red' and transgenic 'Ruby Red' leaf and whole plant non-transgenic control 'Ruby Red' leaf (A top) and transgenic 'Ruby Red' leaf (A bottom), transgenic 'Ruby Red' plant (B left) and non-transgenic control 'Ruby Red' plant (B right) 73

32. 'Duncan' grapefruit leaves inoculated with *Xac* and photographed after 3 d of inoculation. Intact leaves (Ft. Pierce, FL) showing typical citrus canker lesions (A), detached leaves (Fort Pierce, FL) (B), and detached leaves (Weslaco, TX) (C).......... 75

FIGURE Page

33. 'Rio Red' leaves inoculated with *Xac* showing senescence with no clear citrus canker symptom development. 76

34. Non-transgenic control 'Ruby Red' (A) and transgenic 'Ruby Red' (B) inoculated with *Xac*. The control 'Ruby Red' showed clear citrus canker lesion symptoms while the transgenic 'Ruby Red' did not show any symptoms. 76

35. *P. nicotianae* morphology under a standard light microscope. Sporangium releasing the motile zoospores (left) well branched coenocytic mycelia bearing sporangia filled with the zoospores (right). 77

36. Confirmation of the *P. nicotianae* by PCR with the gene specific primers Pn5B and Pn6 M = Molecular marker (100 bpDNA ladder, Fermentas), lane 1 and 2 = 120 bp amplification product from *P. nicotianae* fungal mycelium. 78

37. *P. nicotianae* inoculation of detached leaves 'Duncan' (A), 'Rio Red' (B), non-transgenic control 'Ruby Red' (C), and transgenic 'Ruby Red' (D). The pictures were taken after 4 d of inoculation.......................... 78

38. Diseased Pummelo (*C. grandis*) leaves showing the typical necrotic lesions associated with the *A. alternata* infection. 80

39. Actively growing *A. alternata* culture on potato dextrose agar (PDA) medium. 80

40. Microscopic picture of *A. alternata* conidiophore and multi-cellular, ellipsoidal, and pale brown conidiospore (A), close-up of a conidiospore (B). 81

41. The detached leaves were inoculated with *A. alternata* and photographed after 4d of inoculation 'Duncan' (A), 'Rio Red' (B), non-transgenic control 'Ruby Red' (C), and transgenic 'Ruby Red' (D). 81

42. Tentoxin sensitivity assay on different grapefruit cultivars. Detached leaves of 'Duncan' (A), 'Rio Red' (B), non-transgenic control 'Ruby Red' (C), and transgenic 'Ruby Red' (D). The pictures were taken after 4 d of infiltration 82

FIGURE Page

43. Negative controls for the pathogen inoculation experiments where the leaves were infiltrated with sterile water scratched leaves (A) and punctured leaves (B)... 83

44. Schematic representation of *cAPXcit* showing exons and introns. [exon symbol] = exon, [intron symbol] = intron, 3' UTR = 3' un-translated region, 5' UTR = 5' un-translated region, bp = base pair.. 84

45. The *cAPXcit* expression was examined by RQ RT-PCR. 'Duncan', Ruby Red' and transgenic 'Ruby Red' leaves were infiltrated with *A. alternata* and sampled after 3 d of inoculation. M= Molecular marker (1 kb DNA ladder, Fermentas), lanes 1 = non-inoculated 'Duncan', 3 = non-inoculated non-transgenic control 'Ruby Red', 5 = non-inoculated transgenic 'Ruby Red', 2 = inoculated 'Duncan', 4 = inoculated non-transgenic control 'Ruby Red', 6 and 7 = inoculated transgenic Ruby Red'. The densities of the amplification products are shown in the following table. 86

46. The *cAPXcit* expression was examined by RQ RT-PCR. 'Duncan', 'Rio Red', mutated 'Rio Red', non transgenic control 'Ruby Red', and transgenic 'Ruby Red' leaves were infiltrated with *P. nicotianae* and sampled after 3 d of inoculation. M= Molecular marker (1 kb DNA ladder, Fermentas), lanes 1 = non-inoculated 'Duncan', 3 = non-inoculated non-transgenic control 'Ruby Red', 5 = non-inoculated 'Rio Red', 7 = non- inoculated mutated 'Rio Red', 9 = non-inoculated transgenic 'Ruby Red', 2 = inoculated 'Duncan', 4 = inoculated non-transgenic control 'Ruby Red', 6 = inoculated 'Rio Red', 8 = inoculated mutated 'Rio Red', and 10 and 11 = inoculated transgenic 'Ruby Red'.The densities of the amplification products are shown in the following table.. 88

LIST OF TABLES

TABLE Page

1. List of the primers used in this study 33
2. Summary of the number explants used and shoot regeneration in the *Agrobacterium*-mediated transformation of citrus 53
3. Microtiter plate absorbance readings at 450nm wavelength using Bio Rad plate reader 64
4. The RQRT-PCR amplification product densities for transgenic 'Ruby Red' plants compared to non-transgenic control plants 69
5. Results table showing Ct values for Q RT-PCR performed on the first-strand cDNA from transgenic and non-transgenic 'Ruby Red' plants.......... 72
6. Summary of susceptibility of different grapefruit cultivars to *A. alternata* and sensitivity to tentoxin.. 79
7. Splice sites and percentage of A + T of introns in *cAPXcit* gene................. 85
8. RQRT-PCR amplification product densities for *cAPXcit* expression due to *A. alternata* infection in non-transgenic 'Duncan' and 'Ruby Red' compared to transgenic 'Ruby Red' plants 87
9. RQRT-PCR amplification product densities for *cAPXcit* expression due to *P. nicotianae* infection in non-transgenic 'Duncan', 'Rio Red', and 'Ruby Red' compared to transgenic 'Ruby Red' plants......................... 89

INTRODUCTION

Citrus is believed to have originated in south-east Asia. Commercial citrus species belong to the order Geraniales, sub-family Aurantioidaea, and family Rutaceae. The commercially important citrus cultivars including oranges, grapefruit, limes, and lemons were believed to be originated from nucellar seedlings or bud sports. It is one of the oldest known fruit crops under cultivation of over 4000 years and it has been an important fruit crop in the world with an estimated 100 million ton production and grown in an area of 7.6 million hectares (FAO, 2006). Commercial citrus production is located in both sub-tropical and tropical weather conditions predominantly spread between 40° N and 40° S latitudes. The areas under citrus cultivation are very widespread with sweet oranges grown in 114 countries, lemons and limes in 94, and grapefruit in 74 (FAO, 2003). The important citrus producing countries include Brazil, USA, Mexico, Argentina, Spain, Italy, Turkey, China, Japan, and India (Talon and Gmitter Jr, 2008).

Citrus is one of the most important fruit crops in USA, with four states including Florida, California, Arizona, and Texas being the predominant citrus producing states. In 2003-2004, the total area under citrus production in US was approximately 404,685 ha including Florida 275,186, California 101,171, Arizona 10,926, and Texas 10,926 (NASS, USDA, 2004). The overall value of citrus at the national level was over $8 billion in 1996 (Tsai et al., 1999) and $10 billion in 2007 (NASS, USDA, 2008).

This dissertation follows the style of the Journal of the American Society for Horticultural Science.

The citrus industry annually contributes approximately $1.4 billion, $893 million, $46 million, and $29 million to Florida, California, Arizona, and Texas economies, respectively. The added values such as juice production and export are not included in the above production values. In Texas, the commercial citrus production was limited to about 27000 acres in 2005 and located totally in the Lower Rio Grande Valley with a total value of $200 million and with a crop value to the growers $50 million (LRGV) (Sauls, 2008).

The citrus production is severely limited by several abiotic and biotic stress factors. Among all, the devasting exotic diseases which are rapidly spreading pose a great threat for the survival of the citrus industry. Citrus is susceptible to various nematode, fungus, bacteria, phytoplasma, virus and viroid pathogens. Some of the economically important diseases include slow decline caused by citrus nematode, *Tylenchulus semipenetrans*, spreading decline caused by *Radopholus similis*, root rot and gummosis caused by *Phytophthora* spp., fruit rot caused by *Alternaria alternata*, citrus greening or huanglongbing (HLB) caused by *Candidatus* Liberibacter spp., citrus canker (CC) caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*), citrus variegated chlorosis (CVC) caused by *Xylella fastidiosa*, stubborn disease caused by *Spiroplasma citri*, and quick decline, yellowing, and stem pitting caused by C*itrus tristeza virus* (CTV).

Nematodes are transparent, microscopic worm-like organisms that infest several plant species. Although there are more than 40 nematodes reported in citrus worldwide, a single nematode, *Tylenchulus semipenetrans*, is the most important nematode that causes significant economic losses in US. It was estimated that the infested citrus areas range approximately 90% in Texas and Arizona.

Around 60 species of *Phytophthora* have been reported that cause economic losses which accounts for billions of dollars worldwide (Erwin and Ribeiro, 1996). It poses a serious threat to the citrus industry in Florida and the management cost for the *Diaprepes-Phytophthora* complex was estimated to be as high as $500 to 600 per hectare (Muraro, 2000). Stem end rot and black rot are the post-harvest disease problem in the fruits caused by *A. alternata*. This fungus also causes brown spot lesions surrounded by a yellow halo in fruits and leaves.

Citrus greening, also called huanglongbing (HLB) or yellow dragon disease is a disastrous disease, posing a serious threat to Florida's $9 billion citrus industry. It is considered to be the most dangerous citrus pathogen worldwide (Halbert and Manjunath, 2004). The disease is vectored by the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama and African citrus psyllid, *Trioza erytreae*. Moreover, the disease is spread by only ACP in US. The presence of ACP was reported in Alabama, Georgia, Florida, California, Mississippi, and South Carolina. Also, ACP is established with high populations in Florida and Texas states (Halbert, 1998; French et al., 2001). In the US, the disease was first reported in the year 2005 in Florida and later, in 2008 in Louisiana. All the citrus species and the citrus relatives are susceptible to the disease. At present, the whole US citrus industry is prone to the serious threat from the HLB disease.

Although several bacteria strains were implicated with canker in citrus (Alvarez et al., 1991; Civerolo and Fan, 1982), the Asiatic citrus canker (ACC) caused by *Xac* is the most dangerous disease. The bacteria enter the plant through the natural openings such as wounds and stomata (Brunings and Gabriel, 2003) and form canker lesions.

Xylella fastidiosa, a Gram negative, xylem-limited bacterium which was well described as the causative organism of Pierce's disease of grapevine (Hopkins, 1989). This bacterium also causes *CVC* in citrus, a major disease in Brazilian citriculture with direct losses amounting to one billion US dollars (Qin et al., 2001). CVC has been reported in Brazil (Chagas et al., 1992), Argentina (Contreras et al., 1992), and Costa Rica (Aguilar et al., 2005). The disease is transmitted by several sharpshooter species (Marucci et al., 2008).

There are more than 30 viruses or virus-like citrus diseases known in the world. Among them, CTV is one of the most economically important virus pathogen of citrus (Bar-Joseph et al, 1989). The virus is efficiently vectored by the brown citrus aphid, *Toxoptera citricida*. CTV was attributed to a world wide loss of around 50 million trees in 1989 (Bar-Joseph et al., 1981).

Citrus psorosis disease caused by *Citrus psorosis virus*, and bud union crease caused by Citrus tatter leaf virus are economically important viral diseases in Texas (Timmer, 1974; Herron and Skaria, 2000). Citrus stubborn disease is an economically important disease in young plants of California, Arizona, and North Africa (Bové and Garnier, 2000). Citrus viroids cause two economically important diseases, exocortis and cachexia (xyloporosis) (Roistacher., 1991) and were also reported from Texas (Oslon, 1952; Kunta et al., 2007a).

One of the most important objectives of the citrus breeding is to produce disease resistant plants, but, several limiting factors including pollen or ovule sterility, apomixis, heterozygosity, and long juvenile periods impede the citrus improvement programs (Roose and Close, 2008). However, small size genome (Arugumanathan and

Earle, 1991), considerable natural diversity, ease of propagation through grafting, and well established genetic transformation procedures makes citrus an excellent model plant for genomic studies (Roose and Close, 2008). Sexual compatibility of citrus with trifoliate orange (*Poncirus trifoliata*) and kumquat (*Fortunella spp.*) offers several advantages in improvement programs. Trifoliate orange is being used as source of genes for cold, CTV, and burrowing nematode resistance. Kumquat and Calamondin (*C. mitis*) may provide genes for CC resistance.

Agrobacterium-mediated citrus transformation was used in the efforts aimed at disease resistance such as CTV resistance (Ananthakrishnan et al., 2007), citrus canker (Gonzalez-Ramos et al., 2005; Mirkov, personal communication), *Phytophthora* (Azevedo et al., 2006), and citrus blight (Kayim et al., 2004). Some of the recent advancements in improving the transformation efficiency may provide new opportunities for the extensive efforts in studying a variety of genes for disease resistance.

Since the first transgenic tobacco plant was produced (Horsch et al., 1985), the area under cultivation with transgenic plants has drastically increased in recent years with an estimated market value of $4500 million US dollars (Cesar and Burgos, 2005). However, there is the problem of consumer acceptance of transgenic or genetically modified (GM) plants. Moreover, in case of the perennial trees, many ecologists were concerned for the possibility of transgene escape to their wild relatives either by sexual or non-sexual means.

Furthermore, it is a very difficult task to predict the long-term evolutionary and ecological impact of the transgenic perennial trees (Johnson and Kirby, 2001). Also,

the pleiotropism due to the expression of the transgene and mutated non-target genes pose a problem as it takes several years before the detrimental effects become apparent (Irwin and Jones, 2006).

Use of the antibiotic resistance marker genes such as neomycin phosphotransferase (*npt*II) that confers resistance to kanamycin and its analogues is a common practice in the selection of the transformants. A herbicide tolerance gene such as *bar* (bialaphos resistance) is also used in the transformation of several plants. These genes may pose human health problems such as allergies and transgene escape may confer unexpected tolerance of the insects and microbial pathogens to the antibiotics.

Tissue specific expression of the transgene, sterility, and plastid transformation to avoid the transgene escape with pollen are some of the suggested strategies to deal with the problems of transgene escape in the perennial trees. Use of safer marker genes such as Green fluorescent protein (*gfp*), β-glucuronidase (*GUS*), phosphomannose isomerase (*PMI*), and Betain aldehyde dehydrogenase (*BADH*) from spinach (Daniell et al., 2001) can avoid the problems associated with the antibiotic selection marker genes. Moreover, several methods of antibiotic marker gene removal from the plants such as co-transformation, use of transposases, and intra-chromosomal recombination have also been reported. Recently, cisgenic or intragenic plants which are genetically engineered with the genes from the plant's own genome instead of other non-related organisms is gaining popularity (Schouten and Jacobsen, 2008). These plants may overcome the consumer perception problems associated with the acceptance of the GM plants.

Calcium (Ca^{2+})-signaling is a widely accepted mechanism of signal transduction during biotic or abiotic stresses. In the normal plant cell, the cytosolic calcium levels are maintained at a very low concentration. However, perception of any stress causes a huge influx of the Ca^{2+} across the Ca^{2+}-permeable channels on the plasma membrane leading to an elevated cytosolic calcium levels. Furthermore, Ca^{2+} acts as a secondary signaling molecule in the activation of the stress response Ca^{2+}-dependent and calmodulin (CaM)-dependent protein kinases (Case et al., 2007). An elevated cytosolic calcium levels also proved to trigger defense response genes such as phytoalexins and phenylalanine ammonia lyase (PAL) (Vögeli et al., 1992).

Plant cyclic nucleotide gated ion channels (CNGCs) are non-selective ion channels that are permeable to cations including Ca, Na, and K. They are localized on the plasma membrane and endomembranes, playing a very important role in the ion homeostasis of the plant cells by influx of the cations into the cell. The CNGCs are shaker-type voltage-gated channels with a six-transmembrane domain, a pore loop, and a CN binding site at the C-terminus with an overlapping CaM binding domain. The binding of CN activates the CNGCs and the binding of the CaM deactivates the function of CNGCs. In *Arabidopsis thaliana*, cyclic nucleotide gated-ion channels (CNGCs) play a crucial role in Ca^{2+}-signaling (Talke et al., 2003).

Plants do not have a defined immunity system such as in animals. However, the hypersensitive response (HR) involving localized cell death and thereby limiting the further spread of the pathogen, serves as an alternative tool for this purpose. Using the *A. thaliana* defense-no-death (*dnd1*) mutants which lack *AtCNGC2*, nitric oxide (NO) was shown to be a critical signaling molecule in downstream of elevated cytosolic

calcium levels in the plant innate immunity response to pathogens (Ali et al., 2007). It was also reported that plants lacking the functional *AtCNGC2* could not show any HR response due to *Pseudomonas syringae* infection. This study clearly showed that the CNGCs play a direct role in the HR and the disease resistance.

Ca^{2+}-dependent as well as Ca^{2+}-independent accumulation of the reactive oxygen species (ROS) was previously reported due to pathogen invasion. ROS and NO are the key role players in the signal transduction and further regulation of the programmed cell death (PCD). The initiation of PCD due to the ROS and NO also involve the mitogen-activated protein kinase (MAPK) cascades. ROS accumulation is also correlated to several abiotic stresses such as heat, drought, salinity, and cold. The plant defense responses involve an overlapping of several cellular processes. At the cellular level, Ca^{2+} and ROS are shown to be the key role players of the signaling function in response to both abiotic and biotic stresses. Plant defense is a very complex mechanism and a cross talk between signaling components such as ROS and Ca^{2+} offers a challenging area of research to produce more disease resistant plants.

Despite several attempts made in improving citrus for disease tolerance, there is a necessity for the programs aimed at broad-spectrum disease resistance. This is because introduction of exotic citrus diseases via to natural means, insects, and human activity is a continuous problem. Although broad-spectrum disease resistance is a long-term goal which may involve an integrated effort of many scientists from different fields of plant science and several years of laborious research, we initiated a step towards this goal.

The objectives of this research include production of the transgenic citrus plants for *CNGCcit* and *Blc-2* genes, evaluation of any conferred broad-spectrum disease resistance, and elucidate changes in the *cAPXcit* mRNA transcript levels due to the infection of necrotrophic fungi. In this study, we could successfully produce transgenic 'Ruby Red' grapefruit plants with a gene encoding CNGC in citrus (*CNGCcit*). This is the first report of transformation of citrus with a *CNGC* gene. The present work also investigated whether *CNGCcit* gene conferred any disease resistance to *Xac*, *P. nicotianae*, and *A. alternata*. Furthermore, the citrus cytosolic ascorbate peroxidase (*cAPXcit*) mRNA level changes due to *P. nicotianae* and *A. alternata* infection was studied through relative quantitative reverse transcription- polymerase chain reaction (RQ RT-PCR).

LITERATURE REVIEW

Plants are consistently confronted with a wide variety of pathogens. The capability of the plant to recognize a pathogen and an early initiation of stress response mechanism determines the disease resistance. The elicitors released by a pathogen activate host receptors which trigger early defense responses through several signal transduction pathways (Dixon et al., 1994).

There are two types of elicitors, non-race-specific (NRE) and race-specific (RSE). NRE can be of biotic or abiotic origin, such as fungal and bacterial cell wall fragments released during infection process, hydrolytic enzymes of plant or pathogen origin, glycoproteins, and polyunsaturated fatty acids, heavy metals and ultra-violet (UV) light. RSE are peptides encoded by avirulence (*Avr*) genes in the pathogen, which are believed to bind to receptors in the plants, encoded by resistance (*R*) genes (De Wit, 1998). This will trigger several signal transduction pathways leading to a massive shift in gene transcription and plant cell metabolism resulting in a defense response that helps to minimize disease (Dangl, 1995).

Thus, host-pathogen specific disease resistance is determined by the interaction of products of *Avr* genes and elicitors of the pathogen and *R* genes of the host (Keen, 1990; De Wit, 1992).

HYPERSENSITIVE RESPONSE

Hypersensitive response (HR) is a type of widely used mechanism by plants to prevent the further spread of a pathogen after the pathogen attack (Heath, 2000). It is an *R* gene-dependent early disease resistance response, involving rapid cell death and localized cell necrosis within the infection site (Goodman and Novacky, 1996). The *R* genes detect the pathogen, activating the ion fluxes and the production of H_2O_2 which bring a change in the membrane potential and ion permeability of the plasma membrane. It was shown that these processes are mediated by regulation of plasma membrane-bound enzymes, including changes in activities of H^+-ATPase (Vera-Estrella et al., 1994; Xing et al., 1996), activation of membrane-bound ion channels (Gelli et al., 1997; Zimmermann et al., 1997), and the induction of NADPH oxidase (Desikan et al., 1996; Xing et al., 1996).

In phase one of the HR, the *R* genes trigger an increase in extra cellular pH and K^+ (Orlandi et al. 1992), while eliciting an influx of calcium (Ca^{+2}) and hydrogen ions (H^+) into the cell. The efflux of K+ and influx of Ca^{+2} and H^+ ion are dependent and trigger the HR.

In phase two, the cells undergoing the HR rapidly produce reactive oxygen species (ROS), including super oxide anions, hydrogen peroxide, and hydroxyl radicals (Baker et al. 1993). This oxidative burst may affect the host plant cell as well as the pathogen and thereby limiting the spread of the disease (Mehdy, 1994).

In a classical gene-for-gene (*avrRpm1/RPM1*) study in *Arabidopsis* inoculated with *Pseudomonas syringae* pv. tomato, it was shown that increased cytosolic calcium precedes oxidative burst during the HR response (Grant et al., 2000).

CYTOSOLIC CALCIUM

Several molecules such as Ca^{2+} (Talke et al., 2003; Guo et al., 2003), cyclic nucleotide monophosphates (Moutinho et al., 2001), inositol triphosphates (Carland and Nelson, 2004), hydrogen peroxide (Apel and Hirt, 2004), and nitric oxide (Wilson et al., 2008) are shown to act as messengers, playing a significant role in plant cell signal transduction.

In the plant cell, Ca^{2+}-signaling occurs in three important steps, cytosolic calcium levels increase due to various signals perceived by Ca^{2+}–permiable channels (Leng et al., 2002), Ca^{2+}-ATPases (Sanders et al., 2002), and H^{+}/ Ca^{2+} antiporters (Hirschi et al., 1996). Sensing the Ca^{2+} signature by Ca^{2+} binding proteins such as calmodulin (CaM) (Snedden and Fromm, 1998) and calcineurin B-like proteins (CBL) (Kudla et al., 1999), which undergo conformational changes to further regulate the target protein kinases (Sanders et al., 2002) and gene expression (Hua et al., 2003; Hu et al., 2004).

Moreover, some functionally distinct proteins from CaM that regulate Ca^{2+}-mediated cellular responses were reported (Snedden and Fromm, 1998). In *Arabidopsis*, at least 6 *CBL* genes, involved in abiotic stress responses, were reported (Kudla et al., 1999).

Recent studies in plants provided evidence for elevated levels of cytosolic calcium due to pathogen attack with an associated activation of many defense responses and the onset of HR (Tavernier et al., 1995; Xu and Heath, 1998; Blume et al., 2000; Grant et al., 2000), where free cytosolic Ca^{2+} may serve as a secondary signaling messenger. Thus, calcium homeostasis plays a vital role in plant responses to

pathogens. Lecourieux et al. (2002) showed that cryptogein receptor-mediated increase in cytosolic Ca^{2+} in *N. plumbaginifolia* lead to influx of extracellular calcium which initiated successive HR and SAR.

Specific receptor-mediated activation of a Ca^{2+}permeable ion channels by elicitors shows an increased cytosolic Ca^{2+} levels can initiate various defense responses, including the production of ROS, phenolics and phytoalexins, callose and lignin depositions, and induction of pathogenesis-related (PR) proteins (Gelli et al., 1997; Zimmermann et al., 1997). Cordeiro et al. (1998) showed that centrin (a Ca^{2+} binding protein) is involved in *Arabidopsis* plant defense to bacterial inoculation.

CNGC

Cyclic nucleotide-gated ion channels (CNGCs) are well characterized in animals as important signal transducers, especially in visual and olfactory systems but until recently, less so in plants. There are about 20 known genes in *Arabidopsis* that encode CNGCs with an overall genome sequence similarity of 55-83% (Maser et al., 2001).

Plant CNGCs play a significant role in the permeabilization of both monovalent (K^+, Na^+, and Cs^+) and divalent (Ca^{2+} , Ni^{2+}, and Pb^{2+}) cations (Kohler et al., 1999; Leng et al., 1999; Talke et al., 2003; Leng et al., 2002). CNGC2 was shown to function as an ion channel, mediating Ca^{2+} and K^+ influxes without Na^+ influx (Leng et al., 1999, 2002). In 2003, it was demonstrated that CNGC2 is critical for plant development by using null mutants of CNGC2, which were negatively affected in growth, programmed cell death, and adaptation to external stimuli (Chan et al., 2003). It was also shown that a mutation in CNGC2 [defense no death (DND1)] resulted in

almost loss of the HR (Yu et al., 1998). The *dnd1* mutants showed gene for gene resistance without the HR.

Broad-spectrum disease resistance and loss of HR in *Arabidopsis* was attributed to AtCNGC2 by studying *dnd1* mutants (Clough et al., 2000). Elevated salicylic acid levels were observed in the resistance caused by dnd1 mutation. *Arabidopsis* DND2 mutant plants showed enhanced broad-spectrum disease resistance against *P. syringae* with elevated levels of salicylic acid and PR gene expression (Jurkowski et al., 2004). *HLM1* gene encoding CNGC4 was shown to be an essential signaling component in the HR and broad-spectrum disease resistance in *Arabidopsis* (Balague et al., 2003).

Expression of *HLM* 1 in *Xenopus* oocytes showed that CNGC4 is activated by 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP) and is permeable to both Na+ and K^+. In *Arabidopsis thaliana*, it was shown that a novel chimeric gene, *A. thaliana* CNGC (ATCNGC) 11/12 activates multiple disease resistance responses upon the inoculation with *Hyaloperonospora parasitica* (Yoshioka et al., 2006). Although the disease resistance mechanism was unclear, ion fluxes were associated with early events in defense signaling. It was also demonstrated by several studies that phenotypes with a mutation in CNGC resulted in loss of HR cell death without loss of disease resistance (Jurkowski et al., 2004; Chan et al., 2003; Clough et al., 2000; Jirage et al., 1999).

Using *Arabidopsis* knock-out mutants of gene encoding *CNGC2*, Chen et al. (2007) showed that *CNGC2* could be a crucial element in adaptation of the plants to abiotic stresses. They also speculated that *CNGC2* is primarily involved in the signal transduction and ion homeostasis and may not be involved in calcium uptake from the

growth medium. The functional analysis of plant CNGC was conducted by heterologous expression of AtCNGC1 and AtCNGC1M2 (CaM binding domain (CNBD) deleted) in yeast (Ali et al., 2006). Moreover, it was speculated that endogenous yeast CaM and cyclic nucleotides in the cytosol may affect the activity of the plant CNGCs in yeast. Also, in a different study, the physical association of CaM with CNGC was shown to depend on cytosolic Ca^{2+} levels (Hua et al., 2003). However, the function of the CNGCs in plants was shown to be regulated by secondary messenger molecules such as cytosolic cAMP, CaM, and Ca^{2+}.

The sub-cellular localization of HvCBT1 and NtCBP4 showed that both of them are localized in the plasma membrane (Arazi et al., 1999). In a recent study, by heterologous expression of AtCNGC3 in yeast, AtCNGC3 was shown to play a critical role in ion transport during germination (Gobert et al., 2006).

B-CELL LYMPHOMA-2 (BCL-2)

There are about 15 *Bcl-2* family members were identified in the mammals (Jin et al., 2008). Bcl-2 is an anti-apoptotic protein which plays a crucial role in the mitochondrial outer membrane permeabilization (Adams and Cory, 2007).

Transgenic tobacco (*Nicotiana tabacum*) plants expressing anti-apoptotic genes, human (*Homo sapiens*) Bcl-2 and Bcl-xl, nematode (*Caenorhabditis elegans*) Ced-9, or baculovirus Op-IAP, showed heritable resistance to several necrotrophic fungi (*Scerotinia sclerotiorum*, *Botrytis cinerea*, and *Cercospora nicotianae*) and a necrogenic virus, *Tomato spotted wilt virus* (TSWV) (Dickman et al., 2001). Xu et al. (2004) reported that transgenic tomato (*Solanum lycopersicum*) plants expressing

Bcl-xl and Ced-9 genes were tolerant to *Cucumber mosaic virus* (CMV)-induced necrosis. The plants displayed a significant delay in the chilling-induced leaf senescence. Moreover, accumulation of anthocyanins was observed in the leaves which may protect the plants from oxidative stress and thereby enhancing the cell survival.

It was also reported that overproduction of Bcl-xl and Ced-9 in tobacco plants (cultivar 'Samsun NN') conferred resistance to paraquat and UV-B irradiation (Mitsuhara et al., 1999). Moreover, the plants showed suppressed HR upon inoculation with *Tobacco mosaic virus* (TMV). These results also proved that Bcl-xl and Ced-9 can inhibit plant cell death. Tobacco suspension cells over-expressing Bcl-xl and Ced-9 showed resistance to salt (0.2M NaCl), cold (10°C), and wounding (Qiao et al., 2002). Improved function of the organellae including mitochondria, vacuoles, and chloroplasts, enhanced ATP generation, and suppressed reactive oxygen species (ROS) generation were the possible reasons attributed to the resistance mechanism.

In a recent study, it was reported that in human leukemia U937 cells over-expressing Bcl-2 gene, naringenin-induced apoptosis was inhibited by suppressing the caspase-9 and caspase-3 activation (Jin et al., 2008). In this study, the regulatory role of Bcl-2, downstream mitochondria, and the restoration of the susceptibility of U937/Bcl-2 cells to apoptosis upon co-administration of the inhibitors HA14-1 and naringenin was successfully demonstrated.

AGROBACTERIUM-MEDIATED CITRUS TRANSFORMATION

Agrobacterium-mediated citrus transformation has been widely reported in several important cultivars and rootstocks since the first citrus genetic transformation (Kobayashi and Uchimiya, 1989). Grapefruit (*C* x *paradisi*), an economically important citrus crop plant, was transformed and successfully regenerated from epicotyl segments (Luth and Moore, 1999). In this study, rooting as an alternative to the micro-grafting was proposed, however, rooting grapefruit plants was a very laborious and slow process.

Nucellar seedlings of four grapefruit cultivars, ‘Duncan’, ‘Flame’, ‘Marsh’, and ‘Ruby Red’ were transformed with CTV-derived candidate resistance genes, ‘392’ and ‘p23 hairpin’ genes (Ananthakrishnan et al., 2007). This was the first report of ‘Flame’ and ‘Marsh’ grapefruit transformation. However, complete resistance was not achieved; about 5.2% of the plants transformed with ‘392’ showed some degree of reduction in CTV replication in the protoplasts.

Recently, ‘Ruby Red’ grapefruit transformed with genes encoding protein for potential resistance to CC was reported (Gonzalez et al., 2005). In an attempt to produce transgenic resistance to CTV, ‘Duncan’ grapefruit plants were transformed with several constructs derived from the CTV genome (Febres et al., 2008). In this study, it was reported that only one line, transformed with the 3' end of CTV was resistant, six lines were partially resistant, and 27 were susceptible. Post-transcriptional gene silencing (PTGS) was suggested as the mechanism of resistance in the resistant line. ‘Duncan’ grapefruit plants transformed with several CTV genes, including capsid

protein gene, p20 gene, and 3' end of the genomic RNA and evaluated for CTV resistance (Febres et al., 2003).

‘Rio Red’ was transformed with untranslatable coat protein gene (*uncp*) from CTV strain SY568 and agglutinin gene (*gna*) gene from *Galanthus nivalis* (Yang et al., 2000). A successful protocol to produce transgenic ‘Washington’ navel orange [*C. sinensis* (L.) Osbeck] was reported (Bond and Roose, 1998). They also reported that *Agrobacterium* strain C58C gave higher transformation efficiency compared to EHA101-5 and LBA4404 and using 21-d-old epicotyl stem segments gives high transformation efficiency compared to 35-or 56-d-old epicotyl segments.

Transgenic ‘Mexican’ lime containing *CTV-CP* was reported using *Agrobacterium* strain EHA 105 carrying the binary plasmid pBI121/*CVT-CP* (Dominguez et al., 2000). A high number of escapes (92.1%) were reported in the transformation of sweet orange (Peña et al., 1995). The transgenic plants were successfully regenerated by shoot grafting onto 5-month old Rough lemon seedlings.

Two lines of transgenic Rangapur lime (*C. limonia* Osbeck) containing bacterio-opsin (*bO*) were produced and preliminary screening for *P. nicotianae* resistance showed that one of the two lines showed greater tolerance with significantly smaller lesions compared to the control (Azevedo et al., 2006). A citrus blight-associated gene, p12, was incorporated into carrizo citrange and evaluations for disease resistance are under progress (Kayim et al., 2004).

Yu et al. (2002) reported that inclusion of 2,4-dichlorophenoxyacetic acid (2,4-D) in the explant pre-treatment medium and co-culture medium increased *Agrobacterium*-mediated transformation efficiency. Earlier, Cervera et al. (1998)

reported that callus development by exposing the explants to 28 d on selection medium and a cocultivation of 3 d on the medium supplemented with auxins improved the transformation efficiency of citrange.

Genetic transformation of 'Nagpur' mandarin was reported using Agrobacterium strain EHA105 (Khawale et al., 2006). They also showed that the regeneration frequency on MS medium supplemented with auxins, 8.87 mM BAP, 2.32 mM kinetin, and 5.37 mM NAA was higher compared to the control medium without the auxins. *Agrobacterium* strain EHA-105 was used in the transformation of 'Valencia' and 'Natal' sweet oranges and 'Rangpur' lime (*C. limonia*) epicotyl segments (Almeida et al., 2003). Use of $1mg.L^{-1}$ BA for bud induction and $1mg.L^{-1}$ IBA for rooting gave the best results in 'Natal', 'Valencia', and 'Hamlin' sweet oranges and use of 0.5-2.5 $mg.L^{-1}$ BA followed by $1mg.L^{-1}$ IBA gave good number of transformants for 'Rangpur' lime (Almeida et al., 2002).

By adding BAP and NAA in the regeneration and selective media and using 4 month-old explants, highly efficient genetic transformation of sour orange with CTV coat protein (*CTV-CP*) genes was achieved (Ghorbel et al., 2000). Transformation of sour orange (*C. aurantium*) and key lime [*C. aurantiifolia* (Christm.) Swing.] with CTV coat protein gene using *Agrobacterium* strains EHA 101 and A518 was reported (Gutierrez et al., 1997).

It was reported that more than 30% of the transgenic Mexican lime plants regenerated under non selective conditions showed silenced genes (Dominguez et al., 2002). This was the first study ever reported on quantification of gene silencing after transformation. Dominguez et al. (2004) also reported that at least 25% of the plants

had integrated transgenes that were considered escapes based on *npt*II and *uid*A selectable markers. However, Peña et al. (1995) reported that prolonged periods of kanamycin selection improved the recovery of the transformed shoots with a reduced number of escape shoots, however, favored the regeneration of the chimeric shoots.

To improve the selection of the transgenic shoots and elimination of the escapes, use of antibiotics alternate to kanamycin were tried. However, an unsuccessful attempt of using geneticin as an alternative to kanamycin in the production of 'Mexican' lime was reported by Peña et al. (1997). PMI/mannose based selection showed high transformation rates of 30% in Carrizo citrange and 13% in 'Pineapple' sweet orange (Alida et al., 2008).

Prolonged juvenility is a problem in the reproductive development of citrus. To overcome this problem, carrizo citrange was transformed with *Arabidopsis LEAFY* or *APETALA1* genes (Peña et al., 2001). The transgenic plants showed flowering as early as in one year with out any developmental abnormality. Incorporation of additional copies of *vir*G from pTiBo542 into *Agrobacterium* strains EHA105 and C58 dramatically increased the transformation ability (Ghorbel et al., 2001). Moreover, internodal segments from the greenhouse grown 'Pera', 'Valencia', 'Natal', and 'Hamlin' sweet orange plants were used as explants in the transformation and recovery of the transgenic plants (Almeida et al., 2003). This study could provide an opportunity to release new improved varieties in short time as the regenerated plants would lack juvenile characteristics. Furthermore, a transformation efficiency of 8.6% was reported in Swingle citrumelo using thin epicotyl sections (Molinari et al., 2004).

Oliveira et al. (2008) showed that 2 s sonication-assisted *Agrobacterium*-mediated transformation combined with vacuum infiltration for 10 min increases the transformation efficiency in 'Pineapple' sweet orange and 'Swingle' citrumelo [*C.* x *paradisi* Macf. X *P. trifoliata* (L.) Raf.]. Recently, an efficient transformation procedure for the transformation of trifoliate orange [*P. trifoliata* (L.)] via indirect organogenesis was reported (Zou et al., 2008). Supplementation of the co-cultivation medium with 8.86 µM 6-benzylaminopurine (BA) and 1.43 µM indole-3-acetic acid (IAA) to induce callus formation and a 7 d co-cultivation drastically improved the transformation efficiency with a reduction in the non-transgenic escapes. The rooting frequency reached to 96% by multiplication of the transgenic shoots on shoot induction medium supplemented with 1.11 µM BA and 5.71 µM IAA.

Recently, eight new pGREEN-derived green fluorescent protein (GFP) binary vectors were developed for citrus transformation and were used in the transformation of the 'Duncan' grapefruit transformation (Chen et al., 2007). This may provide an opportunity for high transformation efficiency coupled with early *in vivo* screening of the trasnsformants. Costa et al. (2002) reported an improved protocol for 'Duncan' grapefruit transformation by evaluating six different factors that influence the transformation efficiency, and concluded that pre-culturing the explants and the composition of the cocultivation medium are the major influencing factors. In this study, transgenic grapefruit plants containing the carotenoid biosynthetic genes phytoene synthase, phytoene desaturase, and lycopene- β-cyclase were successfully produced.

MATERIALS AND METHODS

EXPRESSION ANALYSIS IN *E. COLI*

The *CNGCcit* ORF was amplified using *Pfx* DNA polymerase (Invitrogen). The amplified fragment was cloned into the protein expression vector pET101/D-TOPO (Invitrogen) and transformed into BL21 Star (DE3) *E. coli* cells. The cells were cultured at 37°C overnight in 10mL LB broth supplemented with 100µg.mL^{-1} ampicillin. The bacteria culture was split into equal parts (5 mL), and one of them was used for IPTG induction.

To the induced culture, IPTG was added to a final concentration of 1mM and samples were collected at 0 h, 1 h, 2 h, 3 h, 4 h, and 5 h of incubation at 37°C with shaking. At each set time, an aliquot of 500µL was removed from each culture, centrifuged at 14,000 rpm for 30 s and the supernatant aspirated.

The cell pellets were suspended in tris-glycine SDS buffer (Invitrogen), the proteins were separated by Novex tris-glycine polyacrylamide gel (Invitrogen), and stained with comassie blue. The gel was observed for a band of increasing intensity in the size range of the expected recombinant protein.

PLANT MATERIAL

About 0.5-1.0 cm epicotyl segments from in vitro grown 21-28 d-old 'Ruby Red' grapefruit (*C.* x *paradisi* Macf.), 'Duncan', and 'Rio Red' grapefruit and sour orange rootstock (*C. aurantium* L.) seedlings grown *in vitro* were used as a source of tissue for *Agrobacterium*-mediated transformation. Under aseptic conditions, the seeds

were extracted from mature fruit of these plants and dried at room temperature for about 24 h. The seeds were surface sterilized as described by Yang et al. (2000) and either used immediately or stored at 4°C. Briefly, the seeds were immersed in a beaker with 10% Clorox and 0.1% Tween 20 with constant stirring for 2 h and rinsed with sterile water for four times. Under sterile conditions, the seed integuments were removed and the embryos were placed on a medium consisting of half strength Murashige and Skoog basal medium (Murashige and Skoog, 1962) supplemented with the Gamborg's vitamins (Appendix A) (Gamborg et al., 1968) (Sigma-Aldrich, St. Louis, MO), and 0.4% Phytagel (Sigma-Aldrich) in majenta GA-7 vessels (Sigma) and kept in dark at 27 ^{o}C.

Three weeks after germination, the etiolated seedlings were placed in cool fluorescent light for 5 d and epicotyl segments of 0.5-1.0 cm were collected for the transformation experiments. In the initial experiments, the embryos were also germinated on RMA medium (Appendix B) with charcoal and 0.8% agar. In a separate experiment, 180-360 d-old greenhouse-grown sour orange rootstock seedlings were taken, the thorns and leaves were removed from the stem pieces, surface sterilized as described above, and intermodal segments (INS) of 0.5-1.0 cm were cut from the stem pieces and used in the transformation.

BACTERIAL STRAINS AND PLASMID VECTOR CONSTRUCTS

A. tumefaciens EHA 105 (Hood et al., 1993) and C58C1 (pGV3850) (Zambryski et al., 1983) carrying binary plasmids of either pBin35SCNGCcit, pPTN334Bcl2, and pBin35SBcl2 were used in the genetic transformation of 'Ruby Red', 'Duncan', and

'Rio Red' grapefruits and sour orange rootstock. *CNGCcit* ORF with *Xba* I and *Eco* RI ends was produced and inserted into pRTL22 plasmid, and the construct was transformed into *E. coli* TOP 10 cells (Invitrogen, Carlsbad, CA). The plasmid DNA was extracted using Qiaprep Miniprep kit (Qiagen, Valencia, CA) and digested with *Hin*d III to release a 3.2 kb fragment of pRTL22/*CNGCcit*. This fragment consisting of the CaMV35S promoter with a dual enhancer, *CNGCcit*, and CaMV35S terminator was inserted into the *Hin*d III site of pBin34SGUS (Yang et al., 2000) to generate pBin35SCNGCcit.

Another plasmid vector construct, pBin35SBcl2, was generated by *Hin*d III digestion of the pPTN334Bcl2 (provided by Martin B. Dickman, Institute for plant genomics and biotechnology, Texas A&M University, College Station, TX) DNA to release a 2171 bp fragment consisting of E35S promoter, TEV leader, *Bcl-2* ORF, and 35S terminator. This fragment was inserted into the *Hin*d III site of pBin34SGUS to generate pBin35SBcl2 construct, and transformed into *E. coli* March-T1 cells (Invitrogen).

The three plasmid vectors including pBin35SCNGCcit, pPTN334Bcl2, and pBin35SBcl2 were introduced into the *A. tumefaciens* strains EHA 105 and C58C1 by electroporation using Gene Pulser II (Bio Rad, Hercules, CA). The electroporation conditions were voltage of 2.5kV voltage, field strength of 12.5 $kV.cm^{-1}$, capacitance of 25 µF, resistance of 200 Ω, and 4-5 s time constant. The plasmid DNA was extracted from the transformed *A. tumefaciens* cells (Qiagen) and the incorporation of the transgene was verified through PCR.

TRANSFORMATION AND PLANT REGENERATION

The *Agrobacterium* cells harboring pBin35SCNGCcit and pBin35SBcl2 constructs were cultured for either overnight or 1 day in 100 mL Luria broth (LB) (Sambrook et al., 1989) containing 50 $mg.L^{-1}$ kanamycin and 20 µM acetosyringone at 28°C. The bacterial cells containing pPTN334Bcl2 construct were also grown under similar conditions except that the LB medium was supplemented with 100 $mg.L^{-1}$ streptomycin and 100 $mg.L^{-1}$ spectinomycin. The bacterial cells were pelleted by centrifugation at 5000 rpm for 5 min at 4°C, washed with 40mL DBA3 medium (Deng et al., 1992) (Appendix C) containing 200 µM acetosyringone, and resuspended in DBA3 medium with 200 µM acetosyringone to a final OD of 1 at 600nm.

The fresh cut 0.5-1cm epicotyl segments were added to the bacterial cells and incubated for 20 min with gentle shaking. Then, the epicotyl segments were blotted on sterile Kimwipes (Kimberly-Clark, Roswell, GA), placed horizontally on the co-culture medium (DBA3 medium containing 100 µM acetosyringone) and incubated 3 days at 22°C in the dark or low light conditions. A transient assay of β-glucuronidase (GUS) expression was performed on thin transverse sections of the epicotyl segment ends. After 3 d of co-culture, the epicotyl segments were gently rinsed twice in the sterile water for no more than 5 min each wash and transferred to DBA3 medium containing 400$mg.L^{-1}$carbenicillin, 100 $mg.L^{-1}$ cefatoxime, and 100 $mg.L^{-1}$ kanamycin, kept 15 d in dark, and later transferred to light. The epicotyl segments were transferred to the fresh DBA3 medium every 21 d.

RECOVERY AND SELECTION OF PUTATIVELY TRANSFORMED SHOOTS

The epicotyl segments with the regenerated shoots were cut transversely close to the base of the shoots and placed vertically in semi-solid BG shoot elongation medium (Appendix D) containing 200 $mg.L^{-1}$ carbenicillin, 100 $mg.L^{-1}$ cefatoxime, and 75 $mg.L^{-1}$ kanamycin and grown for 21 d under cool fluorescent light at 26^oC. An optimized procedure to reduce the number of the non-transformed escape shoots (Yang et al., 2000) was also adopted.

In this procedure, the liquid BG medium containing 200 $mg.L^{-1}$ carbenicillin, 100 $mg.L^{-1}$ cefatoxime, and 75 $mg.L^{-1}$ kanamycin was added to the surface of the semi-solid BG medium such that the basal ends of the shoots were submerged in the liquid medium. The explants were subsequently transferred in every 14 d interval to the fresh BG medium with the same antibiotics but, with kanamycin concentration reduced to 50 $mg.L^{-1}$.

GRAFTING OF THE PUTATIVELY TRANSGENIC SHOOTS

The shoots with green leaves were selected and micro-grafted onto the 180 d-old sour orange rootstock seedlings. The sour orange seedlings were decapitated, a vertical incision was made into which the putatively transgenic shoot, cut into V-shape, was inserted. The graft union was held in place with a malleable plastic tube to facilitate good contact of scion and rootstock and covered with a small transparent plastic bag. Three or four grafted seedlings were placed in a container and covered with a transparent plastic bag to keep high relative humidity (RH) and kept in fluorescent light

for 28 d. After 28 d, seedlings with successful grafts developed new leaves. The small plastic bags and tubes were removed and the seedlings were moved to the greenhouse for another 14 d for acclimation before transferring to larger pots containing commercial soil mix.

In a separate experiment, the epicotyl segments with green shoots and leaves were directly grafted onto either 180 d-old sour orange rootstock or C-22, a hybrid rootstock of Sunki mandarin (*C. reticulata* L.) and Swingle trifoliate orange (*Poncirus trifoliata*). Moreover, 10 epicotyl segments with putatively transgenic shoots were cut longitudinally and T-grafted onto sour orange rootstock for a comparison of grafting success.

GUS AND *NPT* II ACTIVITY ASSAYS

Histochemical GUS Assay. The epicotyl end segments from the 3 d co-culture and leaves from the putatively transformed shoots were tested for GUS expression through histochemical GUS assay (Jefferson et al., 1987) using the *β-Glucuronidase* (GUS) reporter staining kit (Sigma). The staining solution was prepared by mixing 2.5 mL reagent A [200mM sodium phosphate (pH 7.0), with 4mM EDTA], 10 µL reagent B (100mM potassium ferricyanide), 10 µL reagent C (100mM potassium ferrocyanide), 5.5 mL deionized water, 2.0 mL methanol, and 20 µL 5-Bromo-4-Chloro-3-Indolyl- β-D-Glucuronide (X-GlucA).

The plant tissue was immersed in the staining solution in a 1.5mL microcentrifuge tube or in 96-well microtiter plate. The tube or the plate was placed in a vaccum dessicator and vacuum was applied for 3 min to facilitate the uptake of the

staining solution, covered with a lid, and incubated overnight at 37°C. The chlorophyll was removed from the tissue with 70% ethanol and observed under stereoscope for the blue color. The tissue was kept in ethanol for long term storage.

***NPT*II ELISA Assay.** The leaves from the putatively transgenic plants were assayed for neomycin phosphotransferase II (*NPT* II) activity through enzyme-linked immunosorbent assay (ELISA) using the Pathoscreen *NPT* II kit (Agdia, Elkhart, IN) according to the manufacturer's instructions. Leaf tissue was ground with 5 mL 1X PEB1 extraction buffer (1:5) using pulverizer (Kinetic Labs, Visalia, CA).

100 micro-liter of the prepared sample was dispensed into a 96-well microtiter plate. An aliquot of 100 µL each of the known positive control, buffer, and deionized water were also added to the wells. The plate was incubated for 2 h at room temperature (RT) in a humid chamber. The enzyme conjugate diluent was prepared by mixing one part of MRS-2 component to four parts 1X PBST buffer. Then, the enzyme conjugate was prepared by mixing enzyme conjugate A and B and enzyme conjugate diluent in a ratio of 1:100.

After completion of the first incubation was complete, the plate was washed four times with overflowing 1X PBST, quickly emptied, and the wells dried by holding the plate upside down and tapping firmly on paper towel. An aliquot of 100 µL of the prepared enzyme conjugate was added to each well and incubated for 2 h at RT. Then, 100 µL TMB substrate solution was added per well, and the plate was incubated for 15 min in a humid chamber. To stop the reaction, 50 µL of sulfuric acid was added. After 5 min the substrate color change from blue to yellow was observed visually and also

measured on a plate reader (Bio Rad, Hercules, CA) at 450 nm. The wells with yellow color were designated as positive and no significant color change as negative.

Kanamycin Infiltration. In order to screen the transformed plants for *Bcl-2* transgene, about 1mL of 100mg/mL sterile kanamycin was infiltrated into the leaves in at least four spots, using syringe and needle. After 10-15 days of infiltration, the leaves were observed for conspicuous chlorotic spots. The plants that showed the chlorotic spots were considered as non-transgenic and vice versa.

NUCLEIC ACID EXTRACTION

Genomic DNA Extraction. The genomic DNA was isolated from the leaves of 'Ruby Red' grapefruit and 'Duncan' grapefruit using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

The leaf tissue was pulverized in the presence of liquid nitrogen, with a mortar and pestle. To the powdered tissue, 400 µL buffer AP1 and 4 µL RNase A (100mg/mL) were added and vortexed vigorously. The mixture was incubated for 10 min at 65°C with intermittent mixing. Then, 130 µL of buffer AP2 was added to the lysate, mixed, and incubated on ice for 5 min. The lysate was added to a QIAshredder Mini spin column, centrifuged for 2 min at 14,000 rpm, the flow-through was transferred to a new 1.5 mL tube without disturbing the cell-debris pellet. To this lysate, 675 µL of AP3/E buffer was added, mixed, transferred by pipette to DNeasy Mini spin column, and centrifuged for 1 min at 10,000 rpm. Further washing was performed twice by adding 500 µL buffer AW and spinning at 14,000 rpm to dry the

membrane. The genomic DNA was eluted into a 1.5 mL microcentrifuge tube using 100 µL of AE buffer. The quality of the DNA extracted was verified by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Total RNA Isolation. Total RNA was isolated from the leaves of 'Ruby Red' grapefruit, 'Duncan' grapefruit, trifoliate orange, and sour orange rootstock using the RNeasy Mini Kit (Qiagen).

To 100 mg of the ground leaf tissue, 450 µL of buffer RLT was added and vortexed vigorously. The lysate was collected in a new tube after removal of the cell-debris using QIAshredder spin column. To the cleared lysate, 0.5 volume of ethanol was added, transferred to RNeasy spin column, centrifuged for 15 s, and the flow through was discarded. After washes with 700 µL buffer RW1 and 500 µL RPE, total RNA was eluted into a clean microcentrifuge tube with 40 µL RNase-free water. The RNA was treated with DNase1, RNase-free (Ambion, Austin, TX) and was incubated at 37°C for 30 min to remove trace to moderate amount of contaminating DNA from the RNA.

The RNA was quantified by spectrophotometer (Amersham Biosciences, Piscataway, NJ) and the quality and integrity of RNA was checked by running 1 µg of each RNA sample on 1.0% native agarose gel and staining with ethidium bromide.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

First-Strand Complementary DNA (cDNA) Synthesis. First-strand cDNA was synthesized from 3 µg of total RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's recommendations.

In a 0.5 mL tube, a mixture of 3 µg RNA, 10mM dNTP mix, and either oligo dT (0.5 µg) or random hexamers (50 ng) were mixed and incubated at 65°C for 5 min, then placed on ice for 2 min. A 9 µL reaction mixture constituting 2 µL 10X RT buffer [200mM Tris-HCl (pH 8.4), 500 mM KCl], 4 µL of 25mM $MgCl_2$, 2 µL of 0.1M DTT, and 1 µL of RNaseOUT Recombinant RNase Inhibitor (40 $U.\mu L^{-1}$) was added to the tubes and incubated at 42°C for 2 min. Finally, 1 µL (50 U) of Superscript II RT was added to each tube, mixed, and incubated at 42°C for 50 min. Later, the reaction was terminated by heating the reactions at 70°C for 15 min. The cDNA prepared was either immediately used in PCR or stored at -20°C until further use.

PCR. The first-strand cDNA obtained was used as a template in the PCR. The platinum *Pfx* DNA polymerase (Invitrogen) was used in the amplification of the *CNGCcit* ORF and all other PCR reactions were carried out using Platinum *Taq* DNA polymerase (Invitrogen).

The PCR reaction with the platinum *Pfx* DNA polymerase was carried out in a 50 µL reaction volume containing 5 µL 10X *Pfx* amplification buffer, 1 µL of 50mM $MgSO_4$, 1.5 µL of 10mM dNTP mix, 1.5 µL of 10 µM sense primer (Table 1), 1.5 µL of 10 µM antisense primer (Table 1), 0.5 µL of Platinum *Pfx* DNA Polymerase

(2.5 U.µL^{-1}), 2 µL of cDNA, and 37 µL of nuclease-free water. The PCR reaction with the platinum *Taq* DNA polymerase was carried out in a 50 µL reaction volume containing 5 µL of 10X PCR buffer minus Mg [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 1.5 µL of 50 mM $MgCl_2$, 1 µL of 10mM dNTP mix, 1 µL of 10 µM sense primer, 1 µL of 10 µM antisense primer, 0.4 µL of Platinum *Taq* DNA Polymerase (5 U.µL^{-1}), 2 µL of cDNA, and 38.1 µL of nuclease-free water.

The PCR conditions were optimized at 94°C for 2 min followed by 35 cycles of 94°C for 30 s for denaturation, 52°C for 30 s for annealing (the annealing temperature was changed for different PCR reactions based on primer's melting temperature), 72°C for 2 min for polymerization, and 72°C for 10 min for final extension. The PCR amplicon was subjected to electrophoresis on a 1% agarose gel and stained with ethidium bromide for visualization under UV light.

CLONING, PLASMID DNA EXTRACTION, AND SEQUENCING

In all the experiments, the DNA was purified from the agarose gel by using QIAquick Gel Extraction Kit (Qiagen). The gel-purified PCR products were cloned into either pCR4-TOPO vector (Invitrogen) for sticky-ended products or Zero Blunt II TOPO vector (Invitrogen) for blunt-ended products, transformed into TOP 10 or Max Efficiency DH 5α *E. coli* competent cells (Invitrogen), and sequenced (IOWA state DNA sequencing Facility, Ames, IA).

The plasmid DNA was extracted from 5 mL *E. coli* or 10 mL *A. tumefaciens* overnight cultures using QIAprep Miniprep kit (Qiagen). The bacterial cells were pelleted by centrifugation, resuspended in 250 µL of the buffer P1, 250 µL of buffer P2

was added, and mixed. To the mixture, 350 µL of buffer N3 was added, inverted 4-6 times to avoid localized precipitation, centrifuged at 13,000 rpm for 10 min, and the supernatant was added to the QIAprep spin column. Further washes were performed with 500 µL of buffer PB and 750 µL of buffer PE, and the DNA was eluted with 50 µL buffer EB.

PCR TO DETECT *NPT* II, *uid*A, AND *CNGCcit*

Standard PCR technique was employed to detect the presence of the *npt*II, *uid*A, and *CNGCcit* sequence in the putatively transgenic leaves. The gene specific primer sequences used in the PCR reactions are listed in the Table 1.

Table 1. List of the primers used in this study.

Name	Sequence	Purpose
NPTII-F1	5'-TCACTGAAGCGGCGGGAAGGGACT-3'	Amplification of NPT II gene
NPTII-R1	5'-CATCGCCATGGCTCACGACGA-3'	
NPTII-F2	5'-TCGGCTATGACTGGGCACAACAGA-3'	
NPTII-R2	5'-AAGAAGGCGATAGAAGGCGATGCG-3'	
GUS-F1	5'-GGTGGGAAAGCGCGTTACAAG-3'	Amplification of *uid*A (GUS) gene
GUS-R1	5'-TGGATCCCGGCATAGTTAAA-3'	
GUS-F2	5'-TGTAGAAACCCCAACCCGTGA-3'	
GUS-R2	5'-GCGGATTCACCACTTGCAAAG-3'	
cAPX-F	5'-CACCATGGGAAAGTGTTATC-3'	*cAPXcit* gene amplification
cAPX-R	5'-TTACTCAGCATCCGCAAATCCC-3'	
Pn5B	5'-GAACAATGCAACTTATTGGACGTTT-3'	*P. nicotianae* identification
Pn6	5'-AACCGAAGCTGCCACCCTAC-3'	

SOUTHERN ANALYSIS

Genomic DNA was isolated from the putatively transgenic 'Ruby Red' and 'Duncan' grapefruit leaves as described above (Qiagen). For Southern analysis, 20 µg of the DNA was digested with *Hin*d III restriction enzyme and separated on a 0.8% agarose gel. The gel was stained with ethidium bromide and pictures taken (Biospectrum 500 imaging system, Upland, CA) with a ruler on the side. The gel was washed with 0.25 M HCl for 10 min with gentle shaking and with 0.4M NaOH for 5 min. In the subsequent steps, the DNA was transferred overnight to a positively charged nylon membrane (Amersham Hybond XL, GE Healthcare Bio-Sciences, Piscataway, NJ) by downward alkali transfer method and fixed by using a commercial crosslinker (UVP, Upland, CA).

The *uid* A probe DNA was generated by restriction digestion *uid* A plasmid with *Sac*I and *Bam*HI. The *Bcl-2* probe DNA (791 bp) was produced by the restriction digestion of pPTN334Bcl2 plasmid DNA with *Xba*I and *Nco*I. The restriction digestion products were separated on a 0.8% agarose gel, gel purified using QIAquick Gel Extraction Kit (Qiagen), labeled with alkaline phosphatase, and further detection was performed by chemiluminescence detection with CDP-Star (Amersham Gene Images AlkPhos Direct Labelling and Detection System, GE Healthcare, Piscataway, NJ). Briefly, the DNA to be labeled was mixed with 20 µL cross-linker solution, denatured by heating for 5 min in a vigorously boiling water bath. The DNA was immediately cooled on ice for 5 min, 10 µL of reaction buffer was added, mixed, and 2 µL of labeling reagent was added, and incubated for 30 min at 37°C.

The blot was transferred to the hybridization buffer and pre-hybridization was performed for 15 min at 58°C in a hybridization oven (Boekel Scientific, Feasterville, PA) with shaking. The labeled probe (10 $ng.mL^{-1}$ of buffer) was added to the hybridization buffer and hybridization was carried out overnight at 58°C. The post hybridization stringency washes were performed twice with the primary wash buffer for 10 min at 58°C and twice with the secondary wash buffer at for 5 min at RT. Further steps included pipetting CDP-Star detection reagent (40 $\mu L.cm^{-2}$) onto the blot, exposing to the hyperfilmECL (GE Healthcare), and developing.

NORTHERN ANALYSIS

The total RNA was extracted from the putatively transgenic plants as described and RNA was denatured by mixing with equal volume of Glyoxal Load Dye (Ambion) and incubating for 30 min at 50°C. The denatured RNA samples were loaded into the wells of an agarose gel made with 1 g of agarose, 90 mL of water, and 10 mL of 10X Gel Prep/Gel Running buffer (Northern Max-Gly kit) and electrophoresis was performed at 5 $V.cm^{-1}$.

The RNA was transferred from the agarose gel to a positively charged nylon membrane, Amersham Hybond XL (GE Healthcare Bio-Sciences), by downward alkali transfer method (Chomczynski, 1992) for 3 h using RNA transfer buffer (0.01 N NaOH, 3 M NaCl) and fixed by using a commercial crosslinker (UVP, Upland, CA). The same procedure that was used for Southern analysis was followed for hybridization with the *Bcl-2* probe labeled with alkaline phosphatase, stringency washes, exposure to the film, and development of the film.

RELATIVE QUANTITATIVE RT-PCR (RQ RT-PCR)

RQ RT-PCR was performed for the quantitative analysis of the *CNGCcit* gene expression in the transgenic 'Ruby Red' grapefruit plants. First-strand cDNA was synthesized from 1 µg of total RNA using Superscript II reverse transcriptase and random hexamers (Invitrogen) according to the manufacturer's instructions. The 18S internal control from Quantum RNA 18S Internal Standards (Ambion) was used as an endogenous standard.

The PCR reaction was performed as described above using Platinum *Taq* DNA polymerase (Invitrogen). The PCR reaction mixture of 50 µL containing 5 µL of 10X PCR buffer minus Mg [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 1.5 µL of 50 mM $MgCl_2$, 1 µL of 10mM dNTP mix, 1 µL of 10 µM sense primer, 1 µL of 10 µM antisense primer, 0.4 µL of Platinum *Taq* DNA Polymerase (5 $U.\mu L^{-1}$), 1 µL of cDNA, 1 µL of 18S Primer:Competimer mixture (either 4:6 or 3:7) and 39.3 µL of nuclease-free water. The resultant PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. The PCR amplification product densities were measured using visionworks analysis software (UVP Bioimaging systems, Upland, CA). The relative value was expressed as a ratio of the density of the gene-specific signal and 18S internal control signal and was used in the estimation of relative gene expression.

QUANTITATIVE REAL-TIME RT-PCR (Q RT-PCR)

The relative *CNGCcit* mRNA levels in the transgenic 'Ruby Red' grapefruit plants compared to the non-transgenic control plants was analyzed by the Q RT-PCR.

Based on the *CNGCcit* ORF, two primers and one Taqman probe were designed using OLIGO version 6 (Molecular Biology Insights Inc, Cascade, CO) which amplify a 136 bp amplicon.

The *CNGCcit* probe (CNGCp) was labeled at 5´-terminal nucleotide with 6-carboxy-fluorescein (FAM) reporter dye and the 3´-terminalnucleotide was labeled with Black hole Quencher (BHQ)-1. The internal probe was cytochrome oxidase (COX)p which was previously designed based on the conserved COX gene from citrus (Li et al., 2006). COXp was labeled at the 5´-terminal nucleotide with tetrachloro-6-carboxy-fluorescein (TET) reporter dye and the 3´-terminal nucleotide was labeled with BHQ-2. The cDNA was generated from the total RNA (1µg) of the transgenic 'Ruby Red' grapefruit plants and non-transgenic 'Ruby Red' control plants, 2 µL was used as template in the PCR.

All real–time PCR amplifications were performed using a Smart Cycler II machine (Cepheid, Sunnyvale, CA) and the data was analyzed using the Smart Cycler software version 2. The real-time RT-PCR amplification of 25 µL reaction volume was constituted of 2.5 µL of 10X buffer minus Mg [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 3 µL of 50 mM $MgCl_2$, 0.6 µL of 10mM dNTP mix, 3 µL of CNGC primer mix (2 µM each), 3 µL of 1 µM CNGCp, 3 µL of COX primer mix (2 µM each), 3 µL of 1 µM COXp, 0.2 µL of Platinum *Taq* DNA Polymerase (5 $U.\mu L^{-1}$), 2 µL of cDNA, and 4.7 µL of nuclease-free water. The real-time PCR program was set-up as 95°C for 20 s with optics off followed by 40 cycles of 95°C for 1 s with optics off and 58°C for 40 s with optics on.

PLANT INOCULATION AND DISEASE RESISTANCE ASSAYS

The transgenic ‘Ruby Red’, and the non-transgenic ‘Ruby Red’, ‘Rio Red’, and ‘Duncan’ control plants were evaluated for disease resistance against a bacterium, *X. axonopodis* pv. *citri* (*Xac*) and two necrotrophic fungi: *P. nicotianae* and *Alternaria alternata* pv. *citri*. In a separate experiment, tentoxin (Sigma), a phytotoxic cyclic tetrapeptide from *A. alternata*, which causes chlorosis of the leaves, was also used to evaluate relative phytotoxicity in transgenic and non-transgenic citrus plants.

In all these experiments, detached leaves were used as the detached leaf assays respond similarly to the inoculated whole plants (Dickman et al., 2001), experimentally very convenient, and facilitate transportation of the leaves to other labs for inoculations with exotic disease causing pathogens. All the experiments with the pathogen inoculations were repeated at least three times.

Inoculation of the leaves with *Xac*. The fully expanded young leaves from non-transgenic control ‘Duncan’, ‘Ruby Red’, ‘Rio Red’, and transgenic ‘Ruby Red’ plants were collected from the greenhouse. The leaves were surface sterilized with 70% ethanol for 1 min, rinsed in 4 changes of sterile distilled water, and blotted dry on sterile Kimwipes (Kimberly-Clark). The leaves were placed on either wet filter paper or agar medium.

In the initial inoculation experiments, the leaves were shipped in 50 mL tubes, sterile bags, and majenta GA-7 vessels (Sigma). All the *Xac* inoculations were performed at the United States Agriculture Department-Agricultural Research Station (USDA-ARS), Fort Pierce, FL and the inoculation with *Xac* was in collaboration with

Clive Bock (USDA, ARS, Ft. Pierce, FL) and Paul Parker (USDA-APHIS, Edinburg, TX).

In later studies, the transgenic ‘Ruby Red’ grapefruit leaves along with the non-transgenic control leaves were inoculated with *Xac* and the relative tolerance was observed visually. Inoculation of both the ‘Duncan’ detached leaves and the intact leaves on the whole plants from the greenhouse, Fort Pierce, FL served as a positive control for the experiments.

A predetermined sterile inoculation suspension (IS) of approximately 108 and 103 CFU.ml^{-1} *Xac* or a suspension of ground lesions from leaves was prepared and the suspension was poured into a syringe. Holding a finger behind the upper leaf surface for support, the end of the syringe (no needle attached) was placed on the lower leaf surface and the plunger depressed to dispense 100 µL into the infiltration site. The suspension flows from the syringe orifice into the stomata on the leaf surface and water-soaking of the leaf was evident.

Fungal Isolates. *P. nicotianae* and *A. alternata* were isolated from infected plant material, using standard isolation techniques. Root and soil samples from the infected citrus trees in the South Research Farm (SRF), TAMU-Kingsville Citrus Center, Weslaco, TX were collected and *P. nicotianae* was isolated using grapefruit leaf disc baiting. The leaf discs were transferred to selective agar medium plates with final concentration of antibiotics 10 mg.L^{-1} Pimaricin, 200 mg.L^{-1} Vancomycin, 100 mg.L^{-1} PCNB, and 50 mg.L^{-1} Hymexazole. These plates were incubated at room temperature in the dark for 7 d.

The colonies showing morphological characteristics of *P. nicotianae* were observed under microscope. The fungal DNA was isolated and PCR was performed using the *P. nicotianae* gene specific primers (Table 1). The agar plugs with *P. nicotianae* were transferred to sterile water and zoospores were released by chilling at 4°C for 20 min and placing for 15 min at RT.

A. alternata was isolated from diseased pummelo [*C. maxima* (Merr.)] leaves from the SRF, as described by Logrieco et al. (1990). The tissue from the infected plant material was transferred to water agar with 10 mg.L^{-1} streptomycin, incubated for 5 d at RT, and the fungal mycelium was further transferred to potato dextrose agar (PDA), and incubated for a further 5 d at RT. The fungal spores were observed under microscope to confirm that the fungus was *A. alternata*.

Inoculations with *P. nicotianae*. Fully expanded young leaves from plants were collected and surface sterilized as described above. The leaves were placed with adaxial side down in petri dishes with a wet filter paper. A puncture was made at the inoculation site using a fine syringe needle. An aliquot of 10 µL of the inoculation suspension (IS) with about 400-500 zoospores were dispensed using a micropipette on the abaxial side of the leaf. At least 2 sites on each leaf were inoculated and incubated at RT. The lesion development was observed daily either visually or using a microscope (Olympus, Center Valley, PA).

Inoculations with *A. alternata* pv. *citri*. The detached leaves from both the transgenic and non-transgenic plants were used in the inoculations with *A. alternata*.

The IS was prepared in sterile water with either 14 d-old fungal cultures or crushed lesions from the leaves. The leaves were inoculated on the abaxial side of the leaf with 20 µL IS at least in two different sites and wounded with a fine syringe needle.

The lesion development was observed visually starting from 48 h after incubating the inoculated leaves in a petri dish with a wet filter paper at RT. The leaves were scored for the occurrence of the lesion development.

Tentoxin Assay. Tentoxin (Sigma-Aldrich), a natural cyclic tetrapeptide from *A. alternata*, was used in the phytotoxicity assays on detached leaves from the above mentioned citrus cultivars and transgenic 'Ruby Red'.

The toxin was dissolved in 95% ethanol and diluted with sterile water to 100, 10, 1, and 0.1 $\mu g.mL^{-1}$. Young leaves were placed on a wet filter paper in the petri dishes, gently scratched on the abaxial side with a pipette tip or punctured with a syringe needle and the toxin solution of different concentrations was placed on the wounded sites. The plates were incubated for 3 d in the dark at 27°C and later, development of leaf necrosis was recorded.

ISOLATION OF *APXCIT* GENOMIC CLONE

The full-length citrus cytosolic ascorbate peroxidase (c*APXcit*) cDNA was previously isolated (Kunta et al., 2006). To obtain the genomic clone, PCR was performed on the genomic DNA using primers flanking the *APXcit* ORF (Table 1) and the PCR product was cloned and sequenced. The genomic organization of

APXcit was determined by homologous alignment of c*APXcit* cDNA and its genomic sequence.

ANALYSIS OF *cAPXcit* EXPRESION CHANGES DUE TO PATHOGEN INOCULATIONS

Changes in the expression levels of *cAPXcit* gene in the *P. nicotianae* and *A. alternata* inoculated 'Duncan', 'Rio Red', and 'Ruby Red', and transgenic 'Ruby Red' plants compared to the un-inoculated control plants was examined by RQ RT-PCR. Briefly, the total RNA was isolated from both the control and the inoculated leaves using RNeasy Mini kit (Qiagen); single-strand cDNA was synthesized from 3 µg RNA using the Superscript RT reverse transcriptase enzyme (Invitrogen), and PCR was performed on 1 µL of cDNA using Platinum *taq* DNA polymerase (Invitrogen). The 18S internal standard (Ambion) was used as an endogenous standard.

RESULTS

EXPRESSION ANALYSIS IN *ESCHERICHIA COLI*

To verify if the *CNGCcit* ORF was functional, *CNGCcit* cDNA was cloned into pET101/D-Topo vector and analyzed by PCR. Recombinant plasmid was transformed into the expression host, BL 21 Star (DE3) *E. coli*, and a recombinant protein of approximately 110 kDa was obtained after IPTG induction (Fig. 1). The protein was detected as a single band in the fraction of the recombinant bacterial cells, while it was absent in the non-induced *E.coli* BL 21. At zero induction time, no protein was produced, and from 1h to 5h induction, the protein production was drastically increased (Fig. 1). This confirms that the *CNGCcit* ORF is functional.

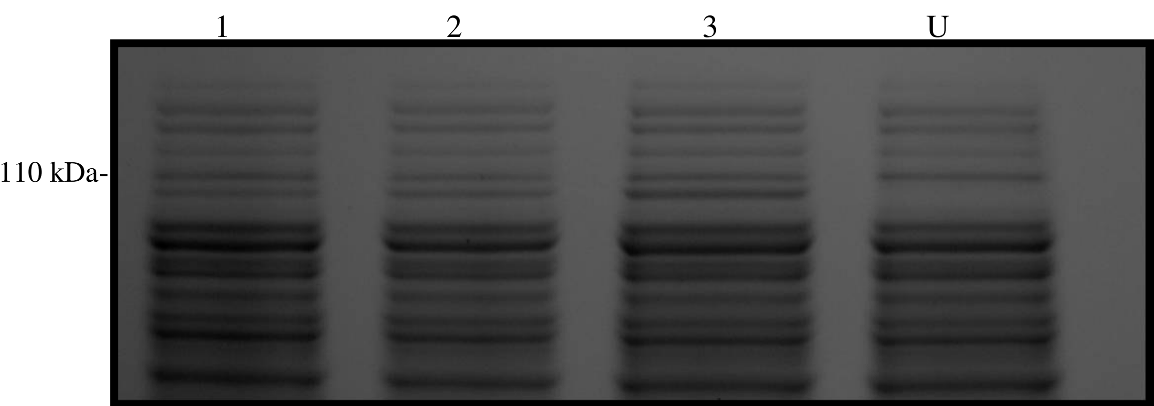


Fig. 1. Expression analysis of *CNGCcit* in *E. coli*. BL21 (DE3) (pET101/D-TOPO) after IPTG induction lanes 1, 2, and 3 = induced after 3 h, 4 h, and 5 h, lane U = un-induced after 5h. The 110 kDa recombinant protein for CNGCcit is shown on left.

PLAMID CONSTRUCTS

RT-PCR to amplify the CNGCcit OFR produced a 2.1 kb amplicon (Fig. 2). The *CNGCcit* gene was cloned into pRTL22 plasmid and the incorporation of the gene was verified by digestion with *Hin*dIII enzyme (Fig. 3). Restriction digestion of pPTN334Bcl2 with HindIII released a 2.17 kb fragment consisting of E35S promoter, TEV leader, Bcl-2 gene, and 35S terminator (Fig. 4).

Three binary vectors including pBin35SCNGCcit (Fig. 5A), pBin35SBcl2 (Fig 5B), and pPTN334Bcl2 (Fig. 5C) were used in the transformation of three grapefruits 'Ruby Red', 'Duncan', and 'Rio Red' and sour orange rootstock.

The pBin35SCNGCcit and pBin35SBcl2 constructs have an intron-inserted *uid*A gene under the control of Figwort mosaic virus (FMV) 34S promoter at the right T-DNA border and *npt*II gene under the control of nopaline synthase (NOS) promoter at the left T-DNA border (Fig. 5A and 5B).

The pPTN334Bcl2 construct has the *Bcl-2* gene under the control of *Tobacco etch virus* 35S promoter and CaMV 35S terminator. This construct also has *npt*II gene under the control of NOS promoter at the left T-DNA border (Fig. 5C).

In pBin35SCNGCcit and pBin35SBcl-2 constructs, *npt*II gene served in selection and *uid*A gene in screening putative transformants. In case of pPTN334Bcl-2, *npt*II gene served for both selection and screening of the putative transformants. The binary vectors were transformed into *A. tumefaciens* EHA 105 and C58C1 strains by electroporation and the transformation was verified by PCR amplification of the genes and *Hin*dIII digestion (Fig. 6).

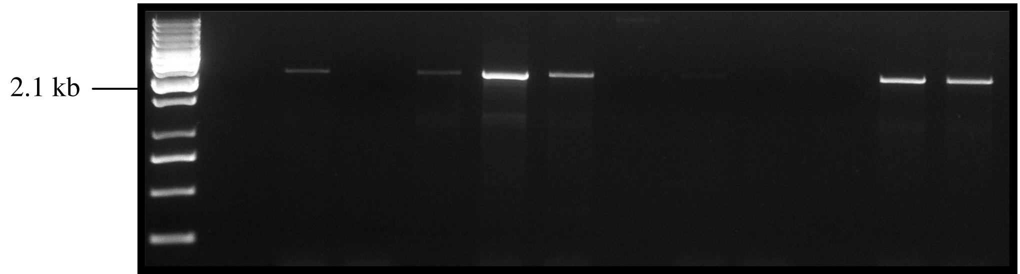


Fig. 2. Generation of *CNGCcit* ORF using RT-PCR. M= Molecular marker (1 kb DNA ladder, Fermentas), lanes 1-6 = *CNGCcit* ORF (2.1 kb) with the *Xba*I and *Eco*RI ends produced by RT-PCR of total RNA extracted from cold (O^{o}C) acclimated trifoliate orange leaves using the CNGCtrans-F and CNGCtrans-R primers.

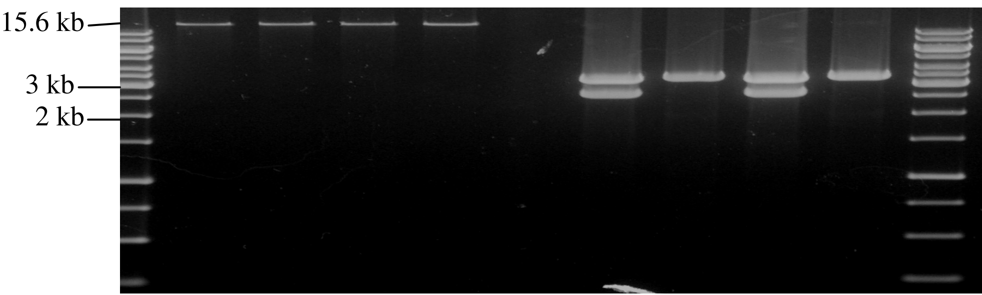


Fig. 3. Restriction digestion of pBin34GUS and pRTL22/CNGCcit with *Hin*dIII. M = Molecular marker (1 kb DNA ladder, Fermentas), lanes 1-4 = pBin34GUS plasmid cut with *Hin*dIII, lanes 6 and 8 = un-cut, lanes 5 and 7 = pRTL22/*CNGCcit* cut with *Hin*dIII.

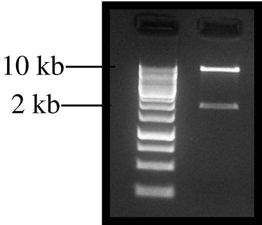


Fig. 4. Restriction digestion of pPTN334Bcl2 plasmid DNA with *Hin*dIII. Lane M = Molecular marker, lane 1 = *Hin*dIII digestion of the pPTN334Bcl2 plasmid DNA to release a 2.17 kb fragment which consists of E35S promoter, TEV leader, *Bcl*-2 gene, and 35S terminator.

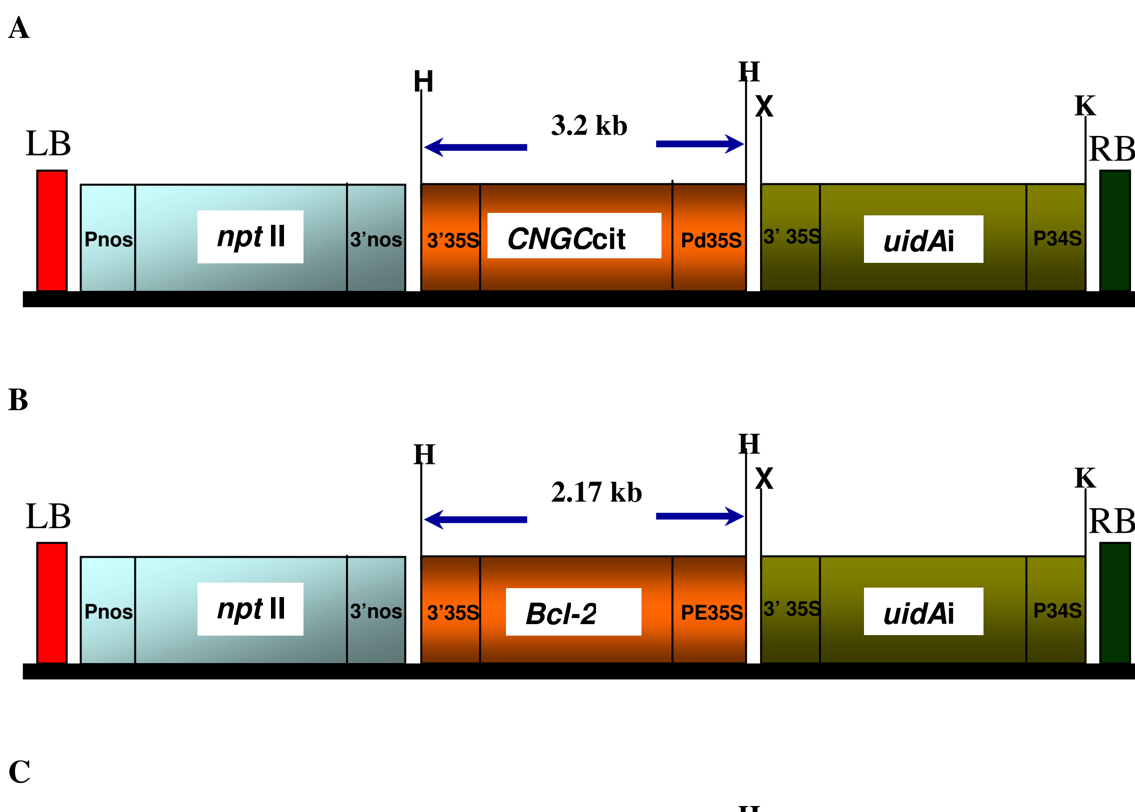


Fig. 5. The schematic representation of the binary vectors used in the citrus transformation. pBin35SCNGCcit (**A**), pBin35SBcl2 (**B**), and pPTN334Bcl2 (**C**), LB = Left T-DNA border, Pnos = nopaline synthase promoter, *npt*II = *neomycin phosphotransferase* II gene, 3'nos = NOS terminator, P35S = CaMV 35S promoter, pd35S = double CaMV 35S promoter, P34S = Figwort mosaic virus 34S promoter, 3'35S = CaMV 35S terminator, PE35S = Tobacco etch virus 35S promoter, *uidAi* = intron inserted *uidA* gene, H = *Hin*dIII, X = *Xba*I, *CNGCcit* = Citrus cyclic nucleotide-gated ion channel, *Bcl-2* = Human B-cell Lymphoma-2 gene, RB = Right T-DNA border (Figures not drawn to scale).

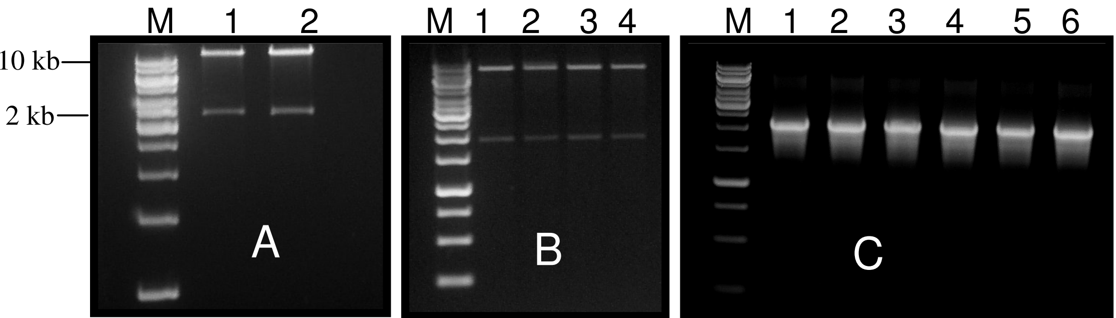


Fig. 6. *Hin*dIII digestion of *A. tumefaciens* plasmid DNA incorporated with pBin35SCNGCcit and PCR amplification of *CNGCcit* from *A. tumefaciens* plasmid DNA. M = Molecular marker (1 kb DNA ladder, Fermentas). (**A,** lanes 1-2) and (**B,** lanes 1-4) = pBin35SBcl2 and pBin35SCNGCcit digested with *Hin*dIII, (**C,** lanes 1-6) = *CNGCcit* gene amplified from the plasmid DNA of *A. tumefaciens* harboring pBin35SCNGCcit using CNGCtrans-F and CNGCtrans-R primers.

AGROBACTERIUM-MEDIATED TRANSFORMATION AND PLANT REGENERATION

A. tumafaciens EHA 105 and C58C1 strains harboring the above described binary vectors were used to transform 'Ruby Red', 'Duncan', and 'Rio Red' grapefruit fresh cut 0.5-1.0 cm epicotyl segments from 21-28 d-old seedlings (Fig. 7) and sour orange INS 180 d- 365 d old plants (Fig. 8).

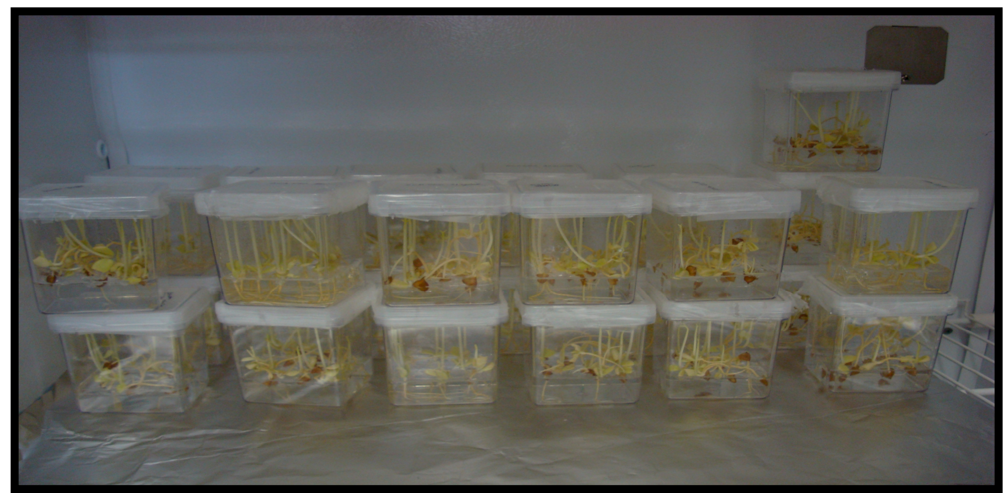


Fig. 7. Citrus seedlings grown on half strength MS medium supplemented with the Gamborg's vitamins.

A **B**

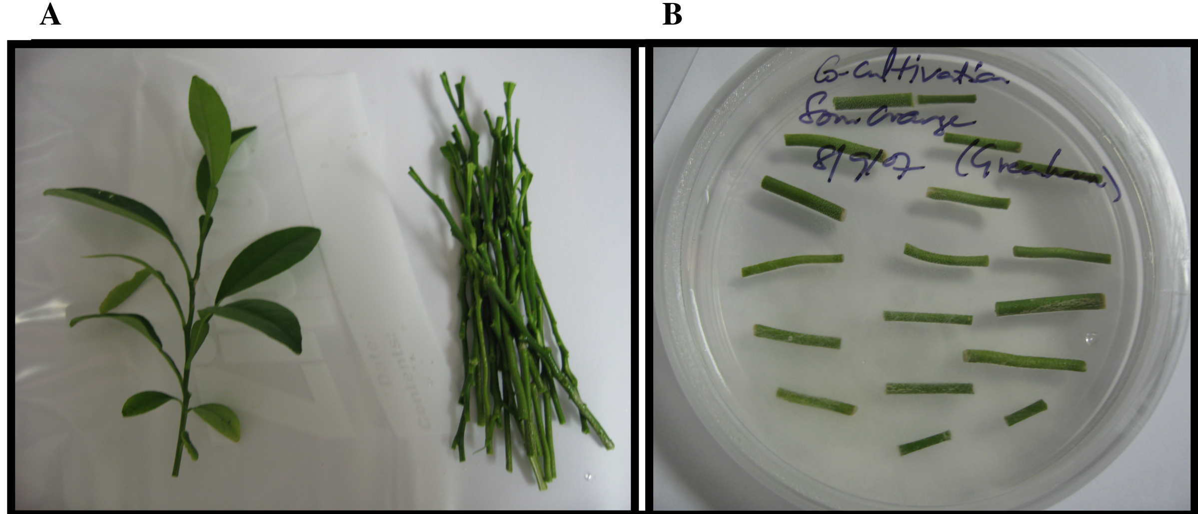


Fig. 8. Sour orange internodal stem segments used in transformation. Thorns and leaves were removed from 180 d-365 d-old sour orange seedlings (**A**) and intermodal stem segments (INS) were used in co-culture with *A. tumefaciens* (**B**).

The 'Duncan', 'Rio Red', 'Ruby Red', and sour orange epicotyl segments and sour orange INS were transformed with *A. tumefaciens* EHA105 and co-cultivated on DBA3 medium supplemented with 100µM acetosyringone. In the initial experiments, embryos were also germinated on RMA medium with charcoal and 0.8% agar (Appendix B). Both media worked very well in supporting the germination of the citrus embryos. After 20 min incubation with the bacterial cells, the epicotyl segments and sour orange INS were placed horizontally on the co-culture medium (Fig. 9) and incubated for 3 days at 22°C in the dark.

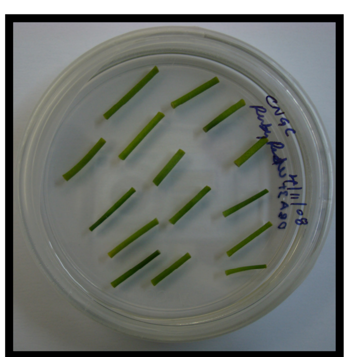


Fig. 9. 'Ruby Red' grapefruit epicotyl segments placed horizontally on the co-culture medium.

Transient histochemical GUS assays were performed on the thin sections of the epicotyl end segments after co-culture to evaluate the transformation efficiency of the *A. tumefaciens* strains EHA105 and C58C1. An evaluation of 25 'Ruby Red' grapefruit epicotyl segments showed consistent intense blue staining in 22 when transformed with C58C1 strain compared to 13 with EHA105 strain. Further transformation experiments were conducted only with the *A. tumefaciens* strain C58C1.

Based on the transient GUS staining, it was believed that 'Duncan' and 'Ruby Red' grapefruit were easier to transform than sour orange rootstock and 'Rio Red' grapefruit (Fig. 10). It was also observed that the blue staining was predominantly confined to the cambial cells (Fig. 10).

A **B** **C** **D**

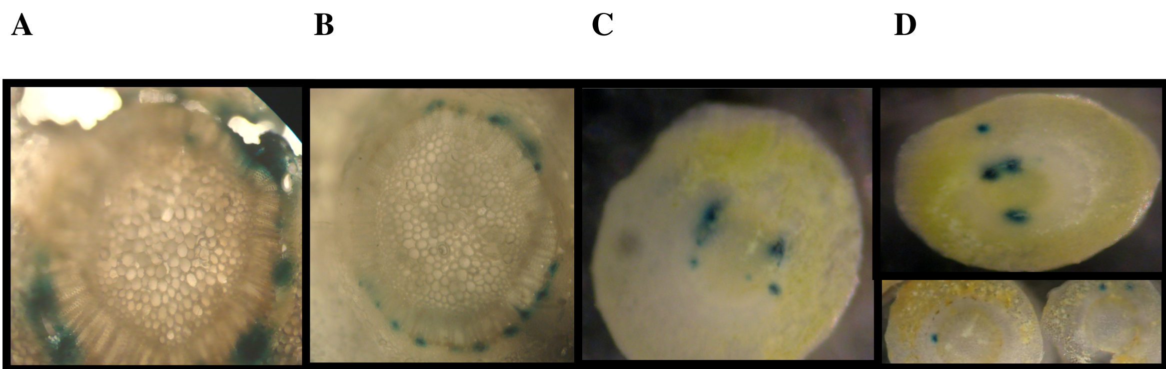


Fig. 10. Transient GUS staining of the epicotyl end segments after 3 day co-cultivation with *A. tumefaciens* C58C1 carrying binary vector pBin35SCNGCcit. 'Ruby Red' grapefruit (**A**), 'Duncan' grapefruit (**B**), 'Rio Red' grapefruit (**C**), and internodal stem segments of sour orange rootstock (**D**).

After co-cultivation, the epicotyl segments were transferred to DBA3 medium containing 400mg L^{-1}carbenicillin, 100 mg L^{-1} cefatoxime, and 100 mg L^{-1} kanamycin and kept 10 days in dark for callus formation (Fig. 11) and later transferred to light. It was previously reported that callus formation may facilitate regeneration of higher number of transgenic shoots and suppress non-transgenic escape shoots (Cervera et al., 1998).

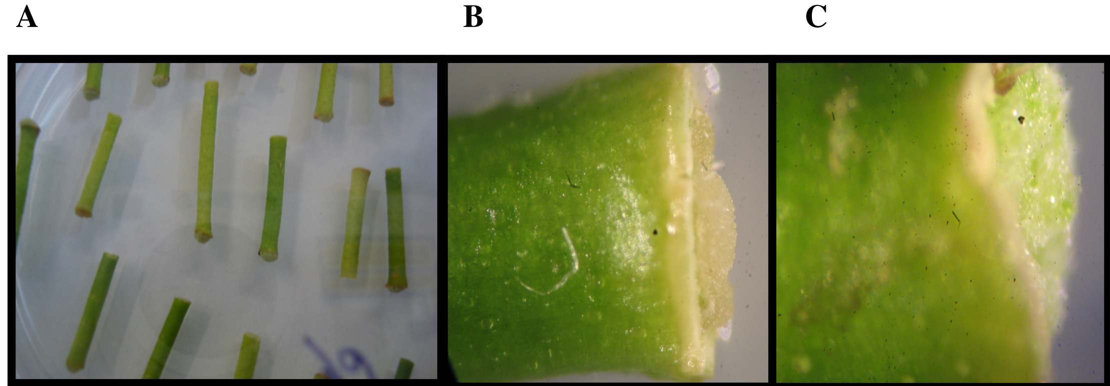


Fig. 11. 'Ruby Red' epicotyl segments placed horizontally on the DBA3 selective medium after co-culture (**A**), showing callus proliferation at the cut ends (**B-C**).

Explants were sub-cultured in fresh DBA3 medium supplemented with antibiotics. Green shoots regenerated from callused area approximately 15 d after culturing. Higher numbers of shoots were regenerated from 'Ruby Red' and 'Duncan' grapefruit epicotyl segments compared to 'Rio Red' grapefruit and sour orange rootstock (Table 2) (Fig. 12). The regeneration efficiency of the epicotyl segments on the DBA3 medium was improved by the addition of benzylaminopurine (BAP), a cytokinin, at 3 $m.gL^{-1}$ and 2,4-dichlorophenoxyacetic acid (2,4-D), an auxin, at $10\mu g.L^{-1}$.

In the initial experiments, we lost several regenerated shoots due to over-growth of *A. tumefaciens* (Fig. 13A). After the co-culture, the epicotyl segments were rinsed twice with sterile water for no more than 5 min each time (Yang et al., 2000) and then transferred to DBA3 selective medium to eliminate *A. tumefaciens* over-growth problems. Initially, we also encountered fungal contamination (Fig. 13B) problems, which were overcome by working under strict sterile techniques.

All epicotyl segments from the mature greenhouse grown sour orange seedlings either died due to fungal and bacterial infections or never regenerated. We re-emphasize that accurate working autoclave and laminar flowhood, and cleaner facility are the prerequisites for the tissue culture success.

Table 2. Summary of the number explants used and shoot regeneration in the *Agrobacterium*-mediated transformation of citrus.

Cultivar	Number of explants	Number of shoots regenerated	Percentage of regeneration
Ruby Red	750	342	45.6
Duncan	815	430	52.7
Rio Red	432	77	17.8
Sour orange	2153	166	7.7

In the initial experiments, regenerated shoots were cut transversely (Fig. 14) and placed vertically on the selective shoot elongation medium (BG medium) supplemented with GA3 (Appendix D). In this procedure, the shoots never showed any elongation and eventually died.

A

B

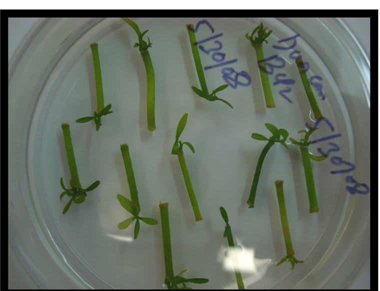


Fig. 12. Shoots regenerated from 'Ruby Red', 'Duncan', and sour orange epicotyl segments on DBA3 selective medium (**A**), a close-up of 'Duncan' (**B**).

Fig. 13. Sour orange epicotyl segments with *A. tumefaciens* over-growth (**A**) fungal contaminations (**B**).

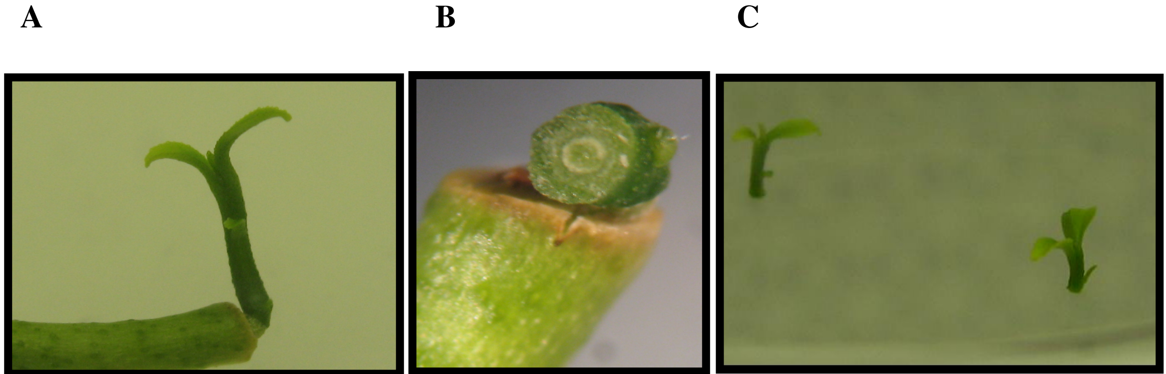


Fig. 14. Sour orange regenerated shoots (**A**) from the epicotyl segment were cut transversely (**B**) and placed on BG medium (**C**).

In all further experiments, the regenerated shoots were cut close to the base of the shoots leaving a small piece of the epicotyl tissue along with the shoot and placed vertically on the BG medium. This method gave promising results and the regenerated shoots started to elongate in 12 days after transfer to the BG medium (Fig. 15).

A **B** **C**

Fig. 15. 'Ruby Red' grapefruit regenerated shoots placed on the BG medium (**A**) elongated shoots (**B-C**).

SELECTION OF PUTATIVE TRANSGENIC SHOOTS

Regeneration and growth of the non-transgenic escape shoots is a major problem in citrus transformation. In the initial experiments, it was found that many sour orange explants were bleached and gradually died because of prolonged exposure to kanamycin at 100mg L^{-1} in the DBA3 regeneration medium and the BG medium. Moreover, the growth of the regenerated shoots and success of grafting of the shoots were severely effected.

A two step protocol using kanamycin at a concentration of 100 mg.L^{-1}in the DBA3 medium and reducing the concentration to 50 mg.L^{-1} in the BG medium was found to be very effective in the recovery of the putative transformants and grafting success.

To further reduce the escape shoots, we adopted the application of a liquid overlay of BG medium containing 75 mg.L^{-1} kanamycin on the semi-solid BG medium. This method was previously reported by several researchers to improve selection in citrus transformation (Yang et al., 2000; Gutierrez et al., 1997). By using a

liquid overlay, the number of escape shoots was dramatically reduced (Fig. 16) and only green shoots (Fig. 17) were further used in the grafting.

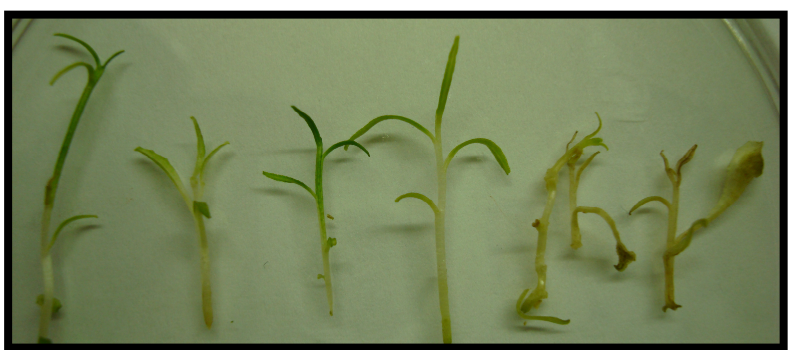


Fig. 16. 'Ruby Red' grapefruit non-transgenic escape shoots showing gradual bleaching due to kanamycin selection using liquid overlay.

A **B**

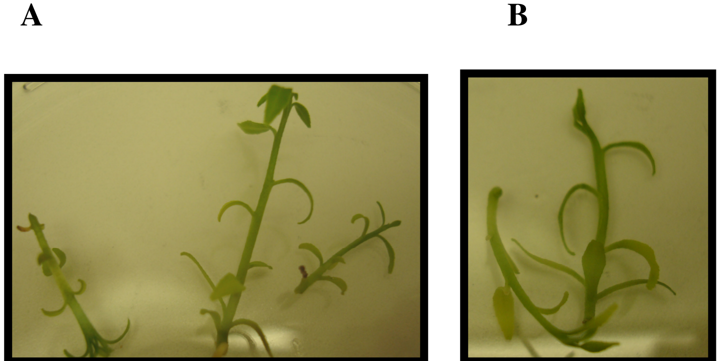


Fig. 17. 'Ruby Red' (**A**) and 'Duncan' (**B**) grapefruit green shoots that were selected for use in shoot grafting.

GRAFTING

A total of 52 'Ruby Red', 46 'Duncan', 7 'Rio Red', and 16 sour orange shoots were shoot grafted onto either sour orange or C-22 seedlings to recover the putative transgenic citrus plants. A survival rate of over 75% was obtained with the grafting.

The graft union between the scion and the rootstock was held by a malleable plastic tube and the high humidity was maintained by keeping a plastic pouch around the graft union (Fig. 18). The graft union was healed in about 15 d and after 30 d; the plants with successful graft union (Fig. 19) were moved to the greenhouse.

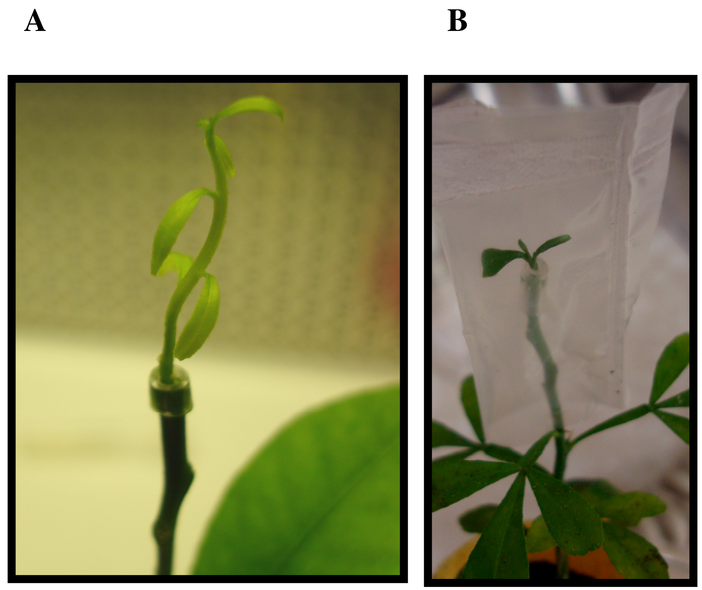


Fig. 18. 'Ruby Red' shoot grafted onto sour orange and the graft union held by plastic tube. (**A**) 'Duncan' shoot grafted onto C-22 and covered by a plastic pouch to maintain high humidity (**B**).

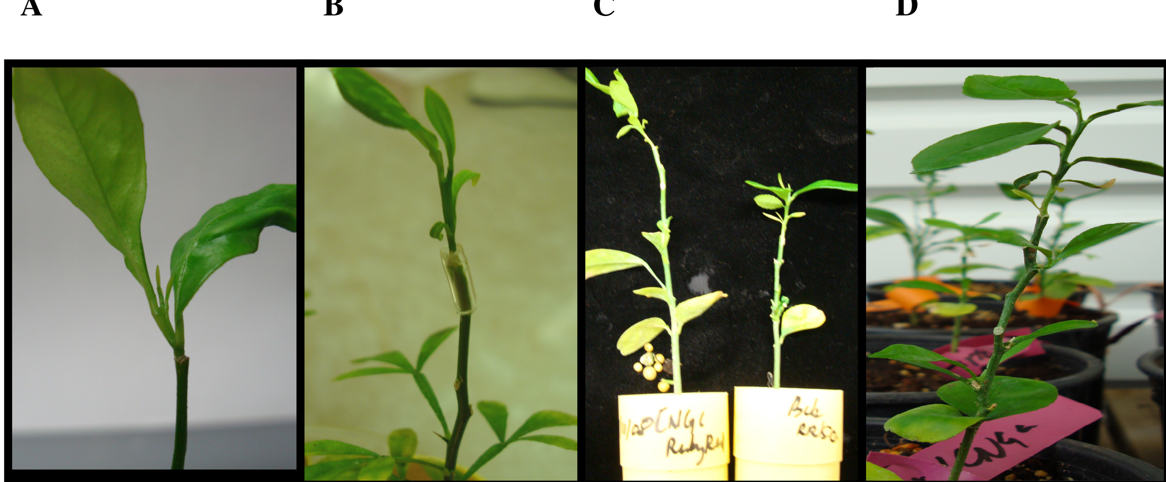


Fig. 19. Shoot grafting of putative transgenic shoots. ‘Rio Red’ on sour orange (**A**), sour orange on C-22 (**B**), ‘Ruby Red’ on sour orange (**C**), ‘Duncan’ on sour orange (**D**).

In a separate experiment, 25 each of ‘Ruby Red’ epicotyl segments with regenerated shoots were grafted onto sour orange and C-22 seedlings. A 100% survival was obtained in the ‘Ruby Red’ grafted onto sour orange and only three successful grafting onto C-22 (Fig. 20). The difference in the grafting success could be attributed to the vigorous nature of the rootstock to support the survival of the scion. The plants grew vigorously after transfer to the greenhouse. However, none of these plants were transgenic based on molecular analyses.

Fig. 20. Shoot grafting of 'Ruby Red' epicotyl segments with regenerated shoots. grafted onto sour orange (**A**), several successful grafts of 'Ruby Red' on sour orange (**B**), graft union failure of 'Ruby Red' grafted onto C-22 (**C**).

T-grafting was performed with 10 regenerated 'Ruby Red' epicotyl segments grafted onto sour orange but, none of them survived (Fig. 21).

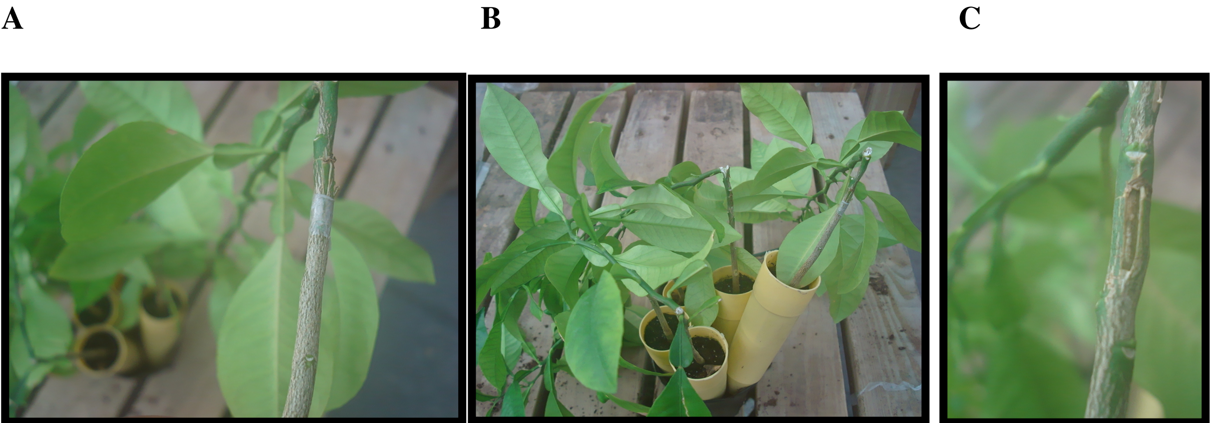


Fig. 21. T-grafting of the regenerated 'Ruby Red' epicotyl segments. Grafting onto sour orange (**A**), bud forcing by breaking the sour orange stem (**B**), graft failure (**C**).

Fig. 22. Putative transformed citrus plants growing in the greenhouse.

GUS AND NPTII-BASED SELECTION

The putative transgenic plants (Fig. 22) transformed with binary vectors pBin35SCNGCcit and pBin35SBcl2 were screened by *npt*II ELISA assay using the Pathoscreen NPTII kit (Fig. 23) and histochemical GUS assays (Fig. 24). In case of the plants transformed with pPTN334Bcl-2, *npt*II Elisa assay served as a screening method for transformants. Out of 53 putative transgenic plants screened by *npt*II ELISA, the absorbance readings (0.154-2.852) for nine putative transgenic 'Ruby Red' plants showed a positive reaction. The absorbance readings for non-transgenic plants never exceeded 0.22, however, in transgenic plants it was 2.6-2.8. The NPTII enzyme

concentration in the transgenic plant leaf extract was estimated to be in the range of 14-18 ng.mL^{-1}.

Histochemical GUS analysis of the putative transgenic plants showed a positive reaction (blue stain) only for two putative transgenic 'Ruby Red' plants. However, Southern analysis confirmed that the two putative transgenic' Ruby Red' plants that gave a positive reaction with both *nptII* ELISA and histochemical GUS are transgenic with the incorporated *CNGCcit* gene.

KANAMYCIN INFILTRATION

Yellowing of the leaves of the non-transgenic plants around the kanamycin unfiltered area was observed 10 days after infiltration (Fig. 25). This method can serve as a crude method of *npt*II screening for kanamycin sensitivity. However, the age of the leaf, mineral deficiencies, and diseases can influence the results.

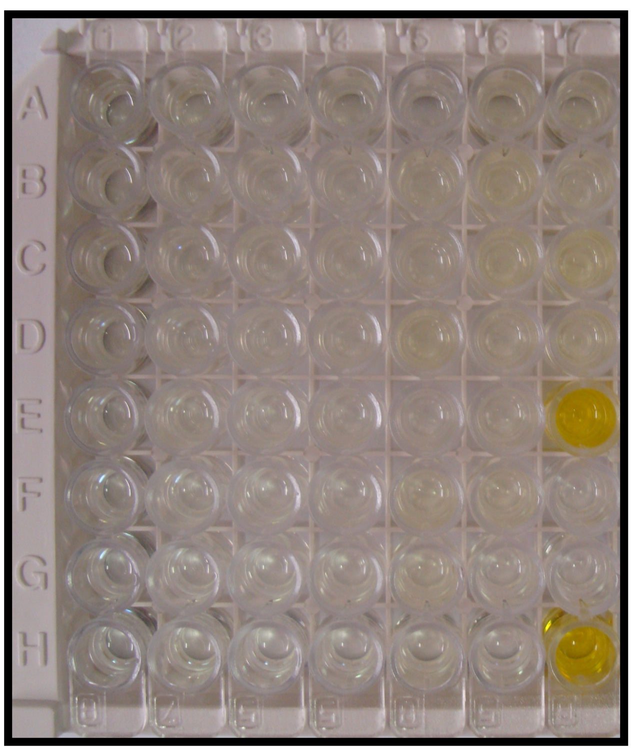


Fig. 23. The putative transgenic plants were screened for *npt*II through ELISA assay. Microtiter plate with positive reactions showing yellow color (**7E**) for transgenic ‘Ruby Red’, 7E = transgenic ‘Ruby Red’ grapefruit, 1A-6E, 1F-6H, 7A-7D = putative transgenic ‘Ruby Red’ grapefruit, 7F and 7G = water and PBST buffer negative control, 7H = positive control. The absorbance readings at 450 nm are shown in the following Table 3.

Table 3. Microtiter plate absorbance readings at 450nm wavelength using Bio Rad plate reader.

	1	2	3	4	5	6	7
A	0.119	0.134	0.109	0.127	0.089	0.154	0.156
B	0.114	0.119	0.113	0.101	0.131	0.181	0.139
C	0.116	0.117	0.112	0.115	0.106	0.168	0.221
D	0.11	0.110	0.106	0.111	0.168	0.132	0.157
E	0.11	0.118	0.102	0.107	0.090	0.112	2.852
F	0.103	0.103	0.100	0.093	0.139	0.144	0.000
G	0.104	0.102	0.097	0.102	0.131	0.102	0.000
H	0.103	0.120	0.098	0.103	0.120	0.103	2.611

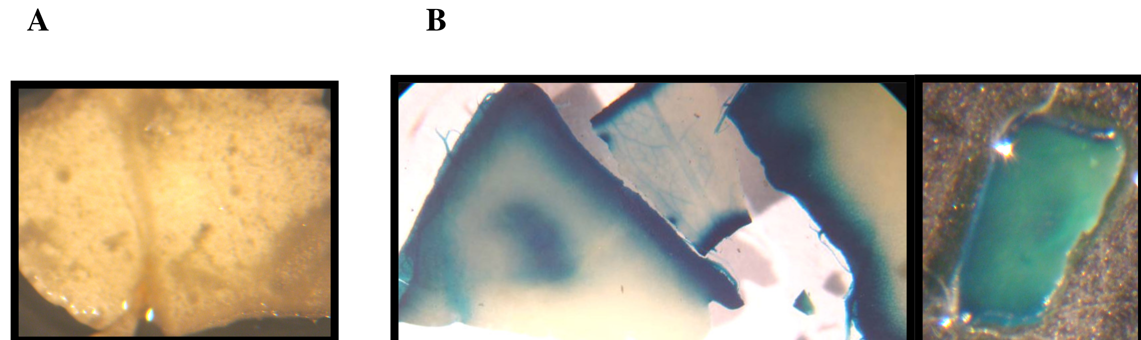


Fig. 24. Non-transgenic 'Ruby Red' leaf showing no blue stain (**A**), transgenic 'Ruby Red' leaves showing blue stain (**B**).

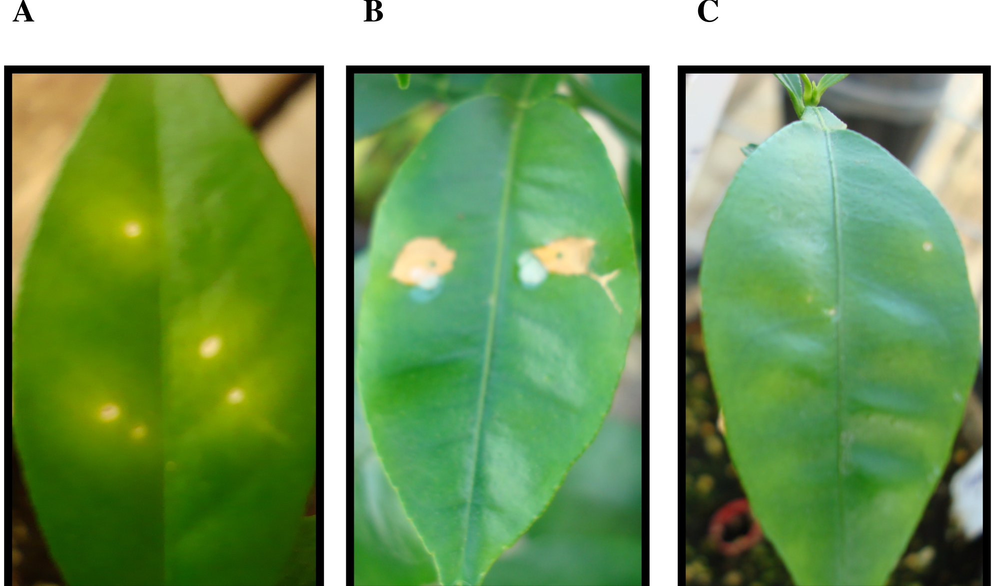


Fig. 25. Kanamycin infiltration assay. The leaves infiltrated with 10 µL of 100 $mg.mL^{-1}$ kanamycin showing yellowing (chlorosis) of the non-transgenic sour orange (**A**) and 'Ruby Red' grapefruit (**B**). Transgenic 'Ruby Red' leaves (**C**) did not show chlorosis.

MOLECULAR ANALYSES

PCR. PCR analysis was performed on the genomic DNA extracted from the transgenic plants. The primers CNGCTrans-F and CNGCTrans-R produced an amplicon of 2.1 kb. Furthermore, NPTII-F1 and NPTII-R1, NPTII-F2 and NPTII-R2 produced PCR fragments of 350 bp and 700 bp, and GUS-F1 and GUS-R1, GUS-F2 and GUS-R2 produced PCR fragments of 450 bp and 880 bp (Fig. 26).

The two 'Ruby Red' *CNGCcit* transgenic plants consistently showed all the amplification products.

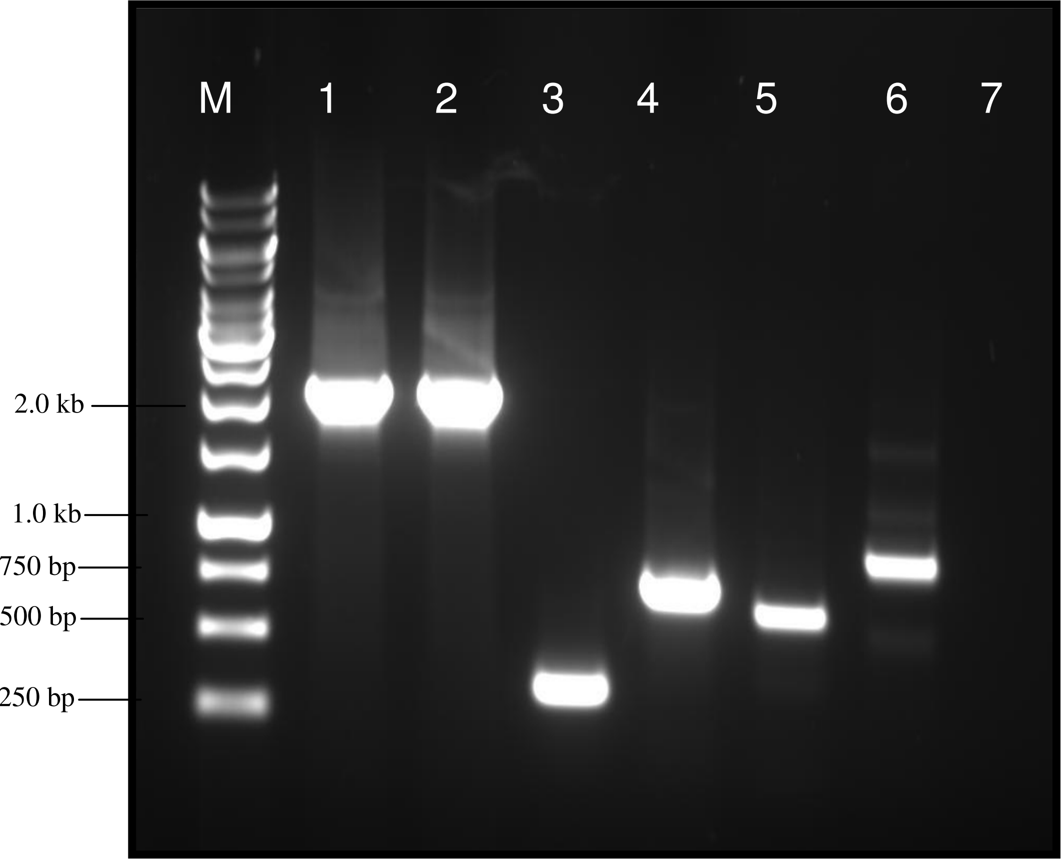


Fig. 26. PCR analysis of the DNA from the transgenic ‘Ruby Red’ plants. Lane M = Molecular marker (1 kb DNA ladder, Fermentas), lanes 1-2 *CNGCcit* (2.1 kb), 3-4 = *npt*II gene fragments of sizes 350 bp and 700 bp, lane 5-6 = *uid*A gene fragment of 450 bp and 880 bp, and lane 7 = non-transformed control.

Southern Blot. Genomic DNA was isolated from 14 of the selected putative transgenic plants for *CNGCcit and Bcl-2* genes. DNA from the non-transgenic ‘Ruby Red’ plants with empty vector served as a negative control. The DNA was digested with *Hin*dIII and analyzed by Southern blot to verify the integration of the transgenes and to determine the copy number.

The *uid*A probe and *Bcl-2* probe (Fig. 27) were used to check the presence of the *uid*A gene and *Bcl-2* gene, respectively. Two *CNGCcit* transgenic ‘Ruby Red’ plants showed positive result, one with single copy insertion and the other with double copy insertion (Fig. 28). These two plants were also GUS and *npt*II positive. The non-transgenic control plants did not show any hybridization signal. These results confirm the stable integration of the *uidA* gene into the genome of the ‘Ruby Red’ grapefruit plants. However, Southern analysis showed no hybridization signal with the *Bcl-2* gene probe indicating that none of the plants incorporated the gene.

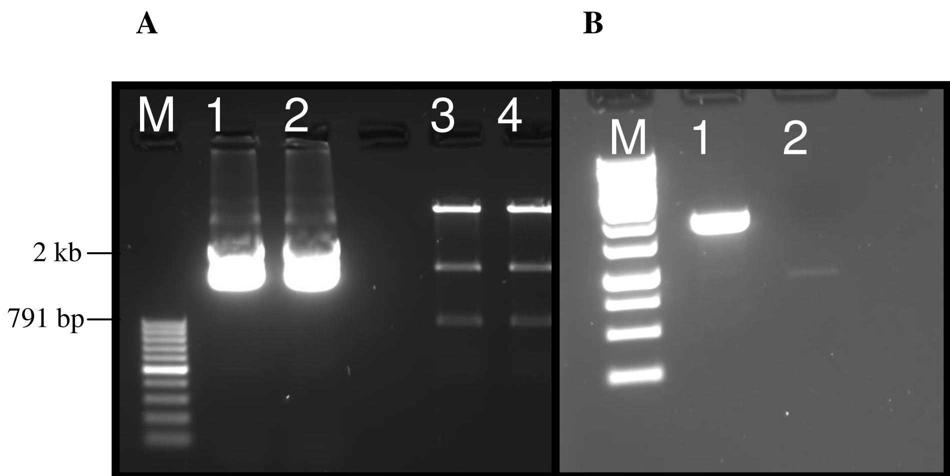


Fig. 27. *uid* A and *Bcl-2* fragments used in Southern and northern analyses Lane M = Molecular marker (1 kb DNA ladder, Fermentas), the *uid* A probe DNA (2 kb) generated by restriction digestion *uid* A plasmid with *Sac*I and *Bam*HI (**A** lanes 1-2), the *Bcl-2* probe DNA (791 bp) was produced by the restriction digestion of pPTN334Bcl2 plasmid DNA with *Xba*I and *Nco*I (**A** lane3-4), gel-purified *uid*A and *Bcl-2* probes (**B** lanes 1-2).

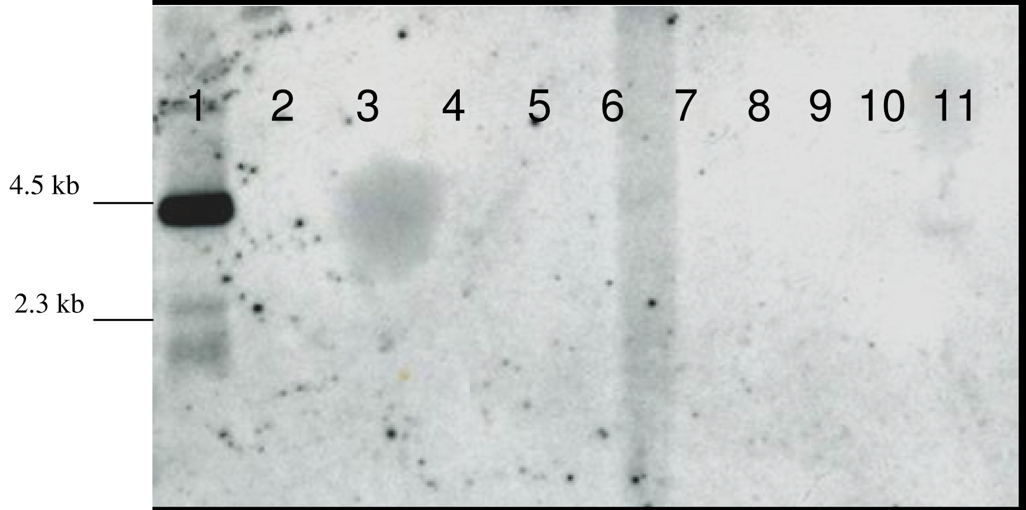


Fig. 28. Southern blot analysis of the citrus plants transformed with *CNGCcit* gene. The DNA was digested with *Hin*dIII and hybridized with *uidA* probe. Lanes 1 and 11 = transgenic 'Ruby Red', lanes 2-9 = putative transgenic 'Ruby Red', lane 10 = non-transgenic negative control. Molecular weights are indicated on the left.

Northern Blot. Northern blot was performed on the total RNA extracted from the putative transgenic plants for *Bcl-2* gene and hybridized with *Bcl-2* probe labeled with alkaline phosphatase. However, no hybridization signal was observed indicating the gene was not expressed in the plants (results not shown).

RQ RT-PCR

The *CNGCcit* gene expression pattern in the transgenic 'Ruby Red' plants compared to the non-transgenic control plants was analyzed by RQ RT-PCR. The 18S internal control was used to ensure equal loading of the RNA. The results show an increased expression of the *CNGCcit* gene in the transgenic 'Ruby Red' plants compared to the non-transgenic control plants (Fig. 29) (Table 4). This relates to the

constitutive high expression of the *CNGCcit* gene under the control of the CaMV35S promoter with dual enhancer in the transgenic plants.

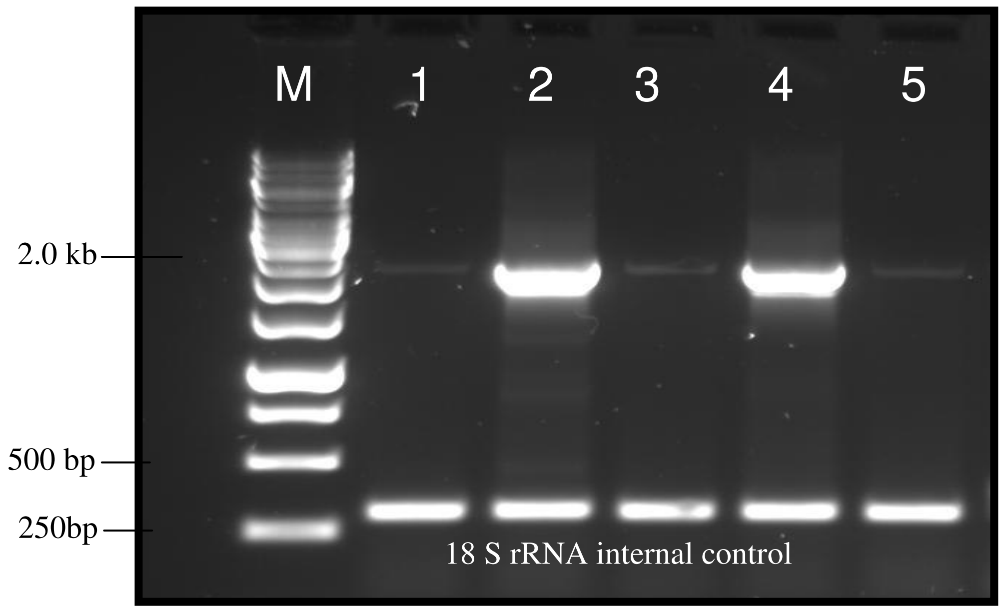


Fig. 29. RQRT-PCR analysis of the relative *CNGCcit* gene (2.1 kb) expression in the transgenic 'Ruby Red' plants compared to non-transgenic control plants Lane M= Molecular marker (1 kb DNA ladder), lanes 1, 3, and 5 = non-transgenic control, lanes 2 and 4 = transgenic 'Ruby Red' plants.

Table 4. The RQRT-PCR amplification product densities for transgenic 'Ruby Red' plants compared to non-transgenic control plants.

Lane	Total Density	Total Background	Total Raw Density	Mean Raw Density	Ratio
1	17656.4135	210201.8133	224695	50.9859	0.10 [a]
2	702474.5724	623245.2775	1309658	199.1572	3.50 [b]
3	21898.9342	161199.5921	182322	56.745[a]	0.10 [a]
4	656875.3279	556489.7297	1204312	186.715[b]	3.06 [b]

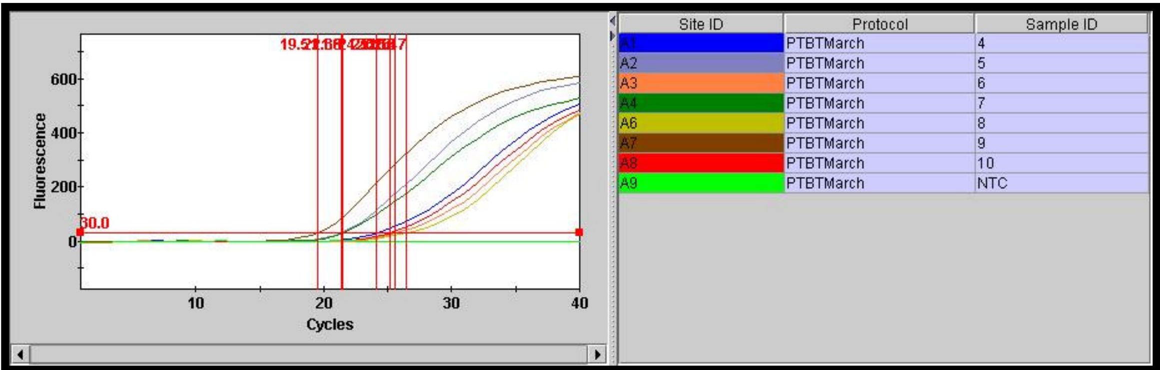
[a]Non-transgenic 'Ruby Red'.
[b]Transgenic 'Ruby Red'.

Q RT-PCR

Q RT-PCR is an extremely sensitive technique to analyze and quantify the mRNA transcripts in the plant cells. Q RT-PCR was performed on the first-strand cDNA made from the total RNA of the *CNGCcit* transgenic 'Ruby Red' plants and the non-transgenic control plants. The primers and Taqman probe labeled with FAM were used to amplify a 136 bp fragment from the *CNGCcit* ORF.

The COX gene served as an internal control and the COX probe was labeled with TET. The fluorescence threshold was kept at 30 and when the fluorescence crossed the threshold, the threshold cycle (Ct), was inversely related to the abundance of the mRNA transcripts. The difference in the Ct values between the transgenic and non-transgenic plants was at least five units confirming the relative abundance of the *CNGCcit* mRNA in the transgenic 'Ruby Red' plants compared to the non-transgenic control plants (Fig. 30) (Table 5).

A



B

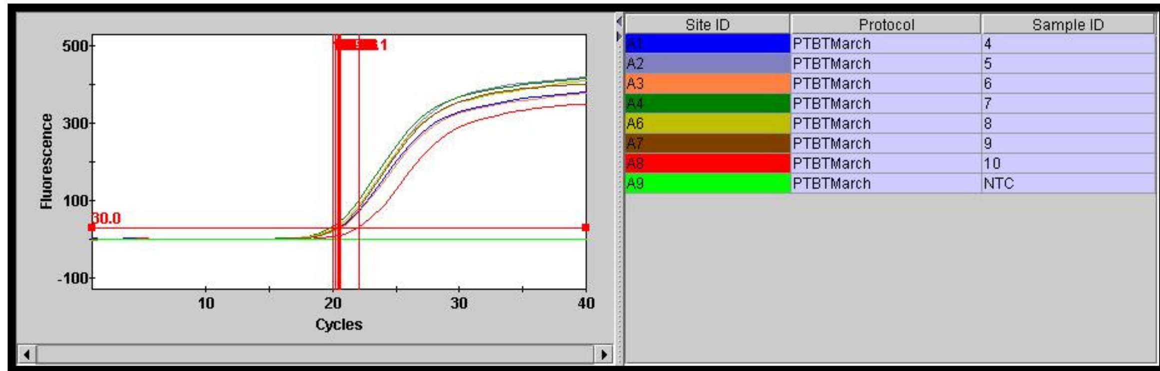


Fig. 30. Q RT-PCR performed on the first-strand cDNA from ‘Ruby Red’ grapefruit plants. Graphs showing the Ct values for *CNGCcit* (**A**), graphs for COX (**B**), 5, 7, and 9 = transgenic ‘Ruby Red’, 4, 6, 8, 10 = non-transgenic, NTC = non-template control (water). The results are shown in the following table.

These results are in agreement with the RQ RT-PCR, which is an end-point analysis. Furthermore, based on the difference in the Ct values (ΔCt), the relative abundance of the genes can be approximately quantified (Livak and Schmittgen, 2001). Target A/Target B = $2^{-\Delta Ct}$

The Ct difference between the non-transgenic control plants (NT) and transgenic plants (T) is equal to 5. This can be expressed as

NT/T = $2^{-\Delta Ct} = 2^{-5} = 1/2^5$ = 1/32. Thus, the *CNGCcit* is 32 times relatively more expressed compared to the non-transgenic control plants.

Table 5. Results table showing Ct values for Q RT-PCR performed on the first-strand cDNA from transgenic and non-transgenic 'Ruby Red' plants.

Site ID	Protocol	Sample ID	Status	FAM Std/Res	FAM Ct	TET Std/Res	TET Ct
A1	CNGC	Non-Trans(4)	OK	POS	24.11	POS	20.54
A2	CNGC	Trans(5)	OK	POS	21.38	POS	20.41
A3	CNGC	Non-Trans(6)	OK	POS	25.55	POS	20.63
A4	CNGC	Trans(7)	OK	POS	21.5	POS	19.97
A6	CNGC	Non-Trans(8)	OK	POS	26.47	POS	20.17
A7	CNGC	Trans(9)	OK	POS	19.51	POS	20.41
A8	CNGC	Non-Trans(10)	OK	POS	25.15	POS	22.1
A9	CNGC	NTC	OK	NEG	0	NEG	0

PHENOTYPE OF THE TRANSGENIC PLANTS

The two 'Ruby Red' plants were consistently confirmed of transgenic carrying and expressing the *CNGCcit* gene by histochemical GUS analysis, *npt*II Elisa, PCR (amplification of the CNGCcit gene, *npt*II gene fragment, and *uid*A gene fragment), Southern blot, RQRT-PCR, and QRT-PCR.

These transgenic ’Ruby Red’ plants over-expressing the *CNGCcit* initially showed slow growth, reduced leaf size, and dwarfism (Fig. 31). However, the plants recovered after 180 d and started growing normally.

A **B**

Fig. 31. A comparison of similar aged non-transgenic control ‘Ruby Red’ and transgenic ‘Ruby Red’ leaf and whole plant non-transgenic control ‘Ruby Red’ leaf (**A** top) and transgenic ‘Ruby Red’ leaf (**A** bottom), transgenic ‘Ruby Red’ plant (**B** left) and non-transgenic control ’Ruby Red’ plant (**B** right).

INOCULATIONS WITH PATHOGENS

***Xanthomonas axonopodis* inoculations.** Intact leaves of ‘Duncan’ and detached leaves of non-transgenic ‘Duncan’, ‘Ruby Red’, ‘Rio Red’, and transgenic ‘Ruby Red’ were inoculated with *Xac*. Both the detached and the intact leaves of greenhouse grown ‘Duncan’ grapefruit from Fort Pierce, FL were inoculated which served as a control for the experiments. All the experiments were repeated at least three times. In the initial inoculations, the leaves were shipped on agar medium in majenta GA-7 vessels

(Sigma). The early symptoms were observed only after 7 d of inoculation. However, the leaves started to show senescence before full symptom development.

In all the further experiments, the leaves were surface sterilized and shipped on a moist filter paper in the petri dishes. In this method, the leaves showed no senescence even for extended periods of 30 d. The mature leaves were found to be more resistant to the infection. Furthermore, fully expanded very young leaves are highly suitable for the *Xac* inoculation studies. Among all the cultivars tested, 'Duncan' was highly susceptible to the *Xac* infection and the typical CC lesions were observed both on the detached and intact leaves within 7-10 days (Fig. 32).

'Rio Red' did not show any symptoms in the initial inoculations. In the later inoculations, the leaves showed lesion development and senescence, however, no clear development of CC symptoms were observed (Fig. 33). Non-transgenic control 'Ruby Red' showed clear symptoms of CC while the transgenic 'Ruby Red' leaves showed no CC symptom development (Fig. 34). However, one leaf showed a little development of lesion without any CC symptoms. This could be a HR reaction of the transgenic leaves to the *Xac* infection.

The repeated inoculation studies proved that the transgenic 'Ruby Red' leaves are resistant to *Xac*, however, more studies on intact leaves on the plant, twigs, and fruit should be conducted before arriving at a definite conclusion that the transgenic 'Ruby Red' is totally resistant.

A **B**

C

Fig. 32. ‘Duncan’ grapefruit leaves inoculated with *Xac* and photographed after 3 d of inoculation. Intact leaves (Ft. Pierce, FL) showing typical citrus canker lesions (**A**), detached leaves (Fort Pierce, FL) (**B**), and detached leaves (Weslaco, TX) (**C**).

Fig. 33. ‘Rio Red’ leaves inoculated with *Xac* showing senescence with no clear citrus canker symptom development.

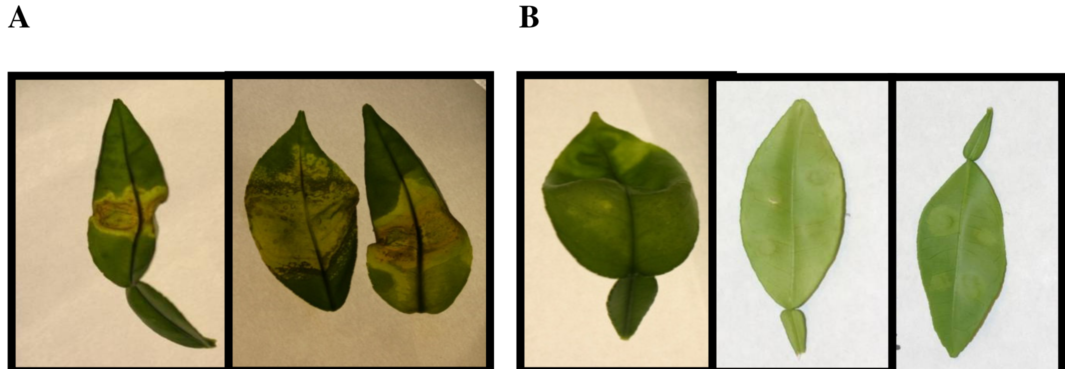


Fig. 34. Non-transgenic control ‘Ruby Red’ (**A**) and transgenic ‘Ruby Red’ (**B**) inoculated with *Xac*. The control ‘Ruby Red’ showed clear citrus canker lesion symptoms while the transgenic ‘Ruby Red’ did not show any symptoms.

P. nicotianae **inoculations.** *P. nicotianae* was isolated from the root and soil sample from the infected grapefruit trees. The fungal colonies showing the typical morphology (Fig. 35) were transferred to sterile water and the zoospores were released (Fig. 35) by chilling treatment. The zoospores were counted using hemacytometer and an aliquot of 10 μL of IS was used to inoculate the leaves.

The fungus was characterized to be *P. nicotianae* based on the PCR amplification of a fragment of the ribosomal DNA (rDNA) as described (Kunta et al., 2007b) (Fig. 36). The leaves were scored for necrotic lesion development daily. 'Duncan' was highly susceptible to *P. nicotianae* infection showing severe necrosis after 1 d of inoculation (Fig. 37). After 4 d of inoculation, 'Ruby Red', and 'Rio Red' also showed severe necrosis (Fig. 37). However, the transgenic 'Ruby Red' did not show any necrotic lesion even after 5 d of inoculation.

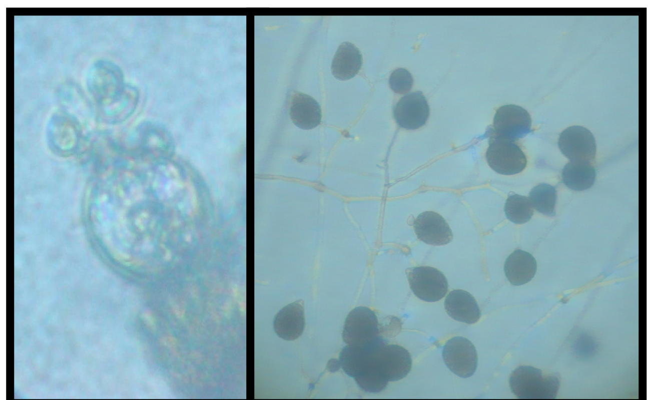


Fig. 35. *P. nicotianae* morphology under a standard light microscope. Sporangium releasing the motile zoospores (left) well branched coenocytic mycelia bearing sporangia filled with the zoospores (right).

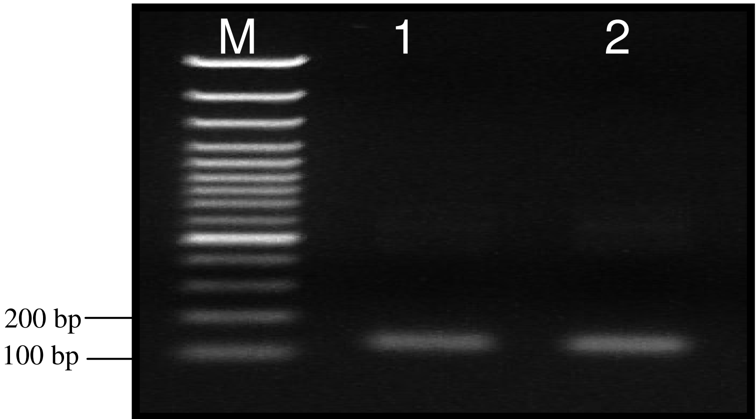


Fig. 36. Confirmation of the *P. nicotianae* by PCR with the gene specific primers Pn5B and Pn6 M = Molecular marker (100 bpDNA ladder, Fermentas), lane 1 and 2 = 120 bp amplification product from *P. nicotianae* fungal mycelium.

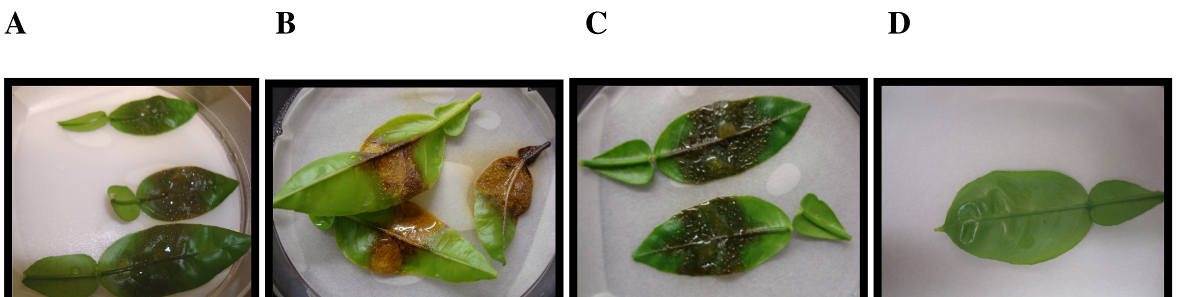


Fig. 37. *P. nicotianae* inoculation of detached leaves 'Duncan' (**A**), 'Rio Red' (**B**), non-transgenic control 'Ruby Red' (**C**), and transgenic 'Ruby Red' (**D**). The pictures were taken after 4 d of inoculation.

***A. alternata* inoculations and tentoxin assay.** *A. alternata* fungus was isolated from the diseased pummelo leaves (Fig. 38), maintained on the PDA plates (Fig. 39), and the morphological observations of mycelia and spore structure was performed to characterize the fungus (Fig. 40).

The leaves from all the cultivars tested were susceptible to fungal inoculation except the transgenic ‘Ruby Red’ (Fig. 41). However, transgenic ‘Ruby Red’ leaves also showed a small hypersensitive response-like leaf lesion. The susceptibility to the fungus and sensitivity to the toxin in different cultivars is listed in Table 6.

Different grapefruit cultivars and transgenic ‘Ruby Red’ plants were examined for susceptibility to *A. alternata* fungus and phytotoxic sensitivity to tentoxin. In the initial experiments, tentoxin was applied to the leaves at different concentrations of 100, 10, 1, and 0.1 $\mu g.mL^{-1}$. The leaves showed sensitive response to the toxin only at the concentration of 100 $\mu g.mL^{-1}$. So, further toxin tests were performed only at this concentration.

Among all the cultivars, ‘Duncan’ was highly susceptible to fungal inoculation and very sensitive to the tentoxin (Fig. 42). In ‘Duncan’, the leaf necrosis due to fungal inoculation and tentoxin application could be readily observed within 24 h of inoculation. However, the transgenic ‘Ruby Red’ showed complete resistance to the tentoxin (Fig. 42). Non-transgenic ‘Ruby Red’ and ‘Rio Red’ showed moderate sensitivity to the toxin.

Table 6. Summary of susceptibility of different grapefruit cultivars to *A. alternata* and sensitivity to tentoxin.

Cultivar	Fungus Inoculation	Tentoxin
Duncan'	S	++
Non-transgenic 'Ruby Red'	S	+
Rio Red'	S	+
Transgenic 'Ruby Red'	R	-

The 20 µL drops of inoculation suspension with *A. alternata* spores and 10 µL of tentoxin (100 µg.mL^{-1}) were applied on the abaxial side of the leaf and leaf necrosis was observed after 3 d. Reactions to tentoxin was scored as fully susceptible (S), resistant (R), severe necrosis (++), moderate or slight necrosis (+), and no necrosis (-).

Fig. 38. Diseased Pummelo (*C. grandis*) leaves showing the typical necrotic lesions associated with the *A. alternata* infection.

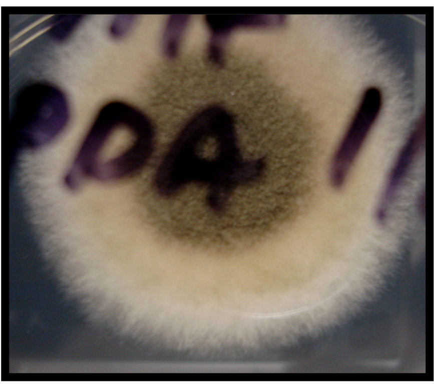


Fig. 39. Actively growing *A. alternata* culture on potato dextrose agar (PDA) medium.

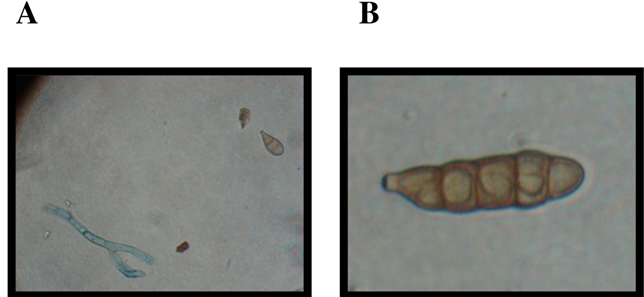


Fig. 40. Microscopic picture of *A. alternata* conidiophore and multi-cellular, ellipsoidal, and pale brown conidiospore (**A**), close-up of a conidiospore (**B**).

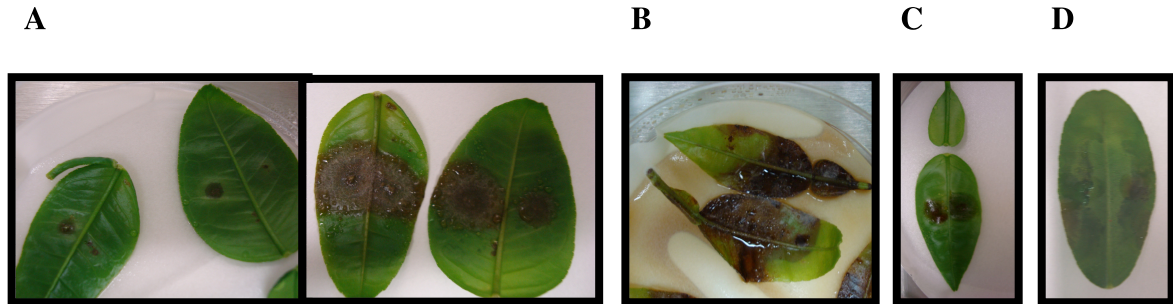


Fig. 41. The detached leaves were inoculated with *A. alternata* and photographed after 4d of inoculation 'Duncan' (**A**), 'Rio Red' (**B**), non-transgenic control 'Ruby Red' (**C**), and transgenic 'Ruby Red' (**D**).

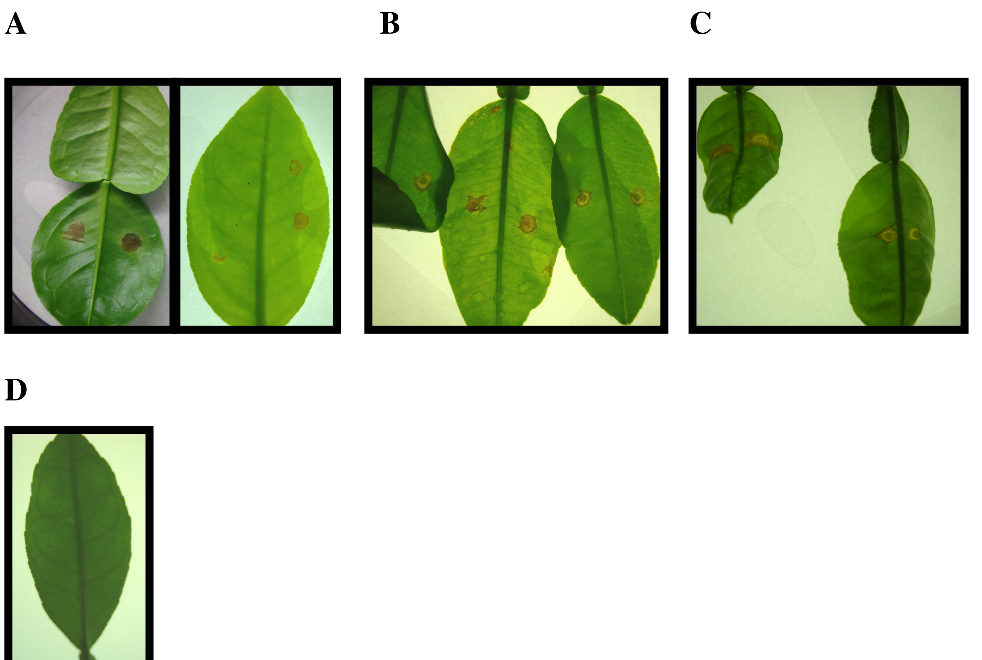


Fig. 42. Tentoxin sensitivity assay on different grapefruit cultivars. Detached leaves of ‘Duncan’ (**A**), ‘Rio Red’ (**B**), non-transgenic control ‘Ruby Red’ (**C**), and transgenic ‘Ruby Red’ (**D**). The pictures were taken after 4 d of infiltration.

In the initial inoculation studies, the leaves were gently scratched with the pipette tip and the IS was applied along with the control experiments with the water. The scratching did not induce any necrotic lesion development when sterile water was used (Fig. 43). However, all the further leaf inoculations were performed by applying the predetermined amount of the IS and puncturing with a fine syringe needle tip. The results were consistent with both methods. However, the point inoculations with minimal leaf damage are more suitable for the disease resistance studies involving more than a week.

A **B**

Fig. 43. Negative controls for the pathogen inoculation experiments where the leaves were infiltrated with sterile water scratched leaves (**A**) and punctured leaves (**B**).

cAPXcit **GENOME ORGANIZATION**

APX is a ubiquitous H2O2 scavenging enzyme in the cell and exists in different isozyme forms in higher plants, algae, and certain cyanobacteria. It utilizes ascorbate as a reducing substrate for detoxification of H2O2 (Asada, 1992) and it is found in chloroplasts, cytosol, microbodies, and mitochondria of higher plants (Asada, 1992; Mittler and Zilinskas, 1992; Thomsen et al., 1992; Patterson and Poulos, 1995; Jimenez et al., 1997).

Cytosolic *APX* (*cAPX*) has been known to be highly responsive under oxidative stress conditions (Shigeoka et al., 2002). Moreover, post-transcriptional suppression of cAPX accelerated pathogen-induced programmed cell death in tobacco probably due to the accumulation of H_2O_2 (Mittler et al., 1998).

The full-length *cAPXcit* cDNA was previously isolated from mature 'Dancy' tangerine (*Citrus reticulata* Blanco) juice vesicles (Kunta et al., 2006). In this study, the experiments were further continued to isolate *cAPXcit* genomic clone.

The isolated *cAPXcit* gene was found to contain nine exons interrupted by eight introns as shown in Fig. 44. The exon size varied from 19bp to 175bp and intron from 90 to 559. The internal seven exons of *APXcit* was exactly the same size and order of the internal exons of *cAPX* of *Oryza sativa* (BAB17666), *A. thaliana* (At3g09640), *Pisum sativum* (M93051), and *Fragaria ananassa* (AF158652, AF158653, and AF158654). This may suggest that *cAPXcit* is localized in the cytosol.

The introns were found to conform to 5' and 3'-splice site consensus sequence (Table 7). Moreover, introns contained a high percentage of A+T (average of 67.5%) compared to exons (58%). Furthermore, the intron-exon splice junctions conform to the consensus sequences GT at the donor site and AG at the acceptor site (Brown, 1986).

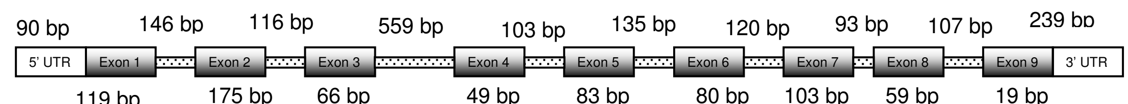


Fig. 44. Schematic representation of *cAPXcit* showing exons and introns. = exon, = intron, 3’ UTR = 3’ un-translated region, 5’ UTR = 5’ un-translated region, bp = base pair.

Table 7. Splice sites and percentage of A + T of introns in *cAPXcit* gene.

Intron	5'–Splice site	3'-Splice site	A+T (%)
1	GGC **GT**AA**G**	TGA**AG** A	71.2
2	CAG **GT**AAT	TGA**AG** T	70.6
3	CCG **GT**GA**G**	TGC**AG** G	67
4	AAG **GT**CA**G**	TGC**AG** G	68.9
5	TTG **GT**CT**G**	TAC**AG** G	62.2
6	TAA **GT**AA**G**	GAT**AG** G	68.3
7	GCA **GT**AA**G**	TTC**AG** G	65.5
8	GGG **GT**TA**G**	TTC**AG** A	65.4
	AG **GT**AA**G**	TGC**AG** G	(Plant consensus sequence)

***cAPXcit* EXPRESSION CHANGES DUE TO PATHOGEN INOCULATIONS**

The expression of *cAPXcit* in ‘Duncan’ and non-transgenic control ‘Ruby Red’ leaves was suppressed in general after 3 d of inoculation with *A. alternata*. Moreover, in ‘Duncan’ it was totally inhibited. However, in case of the transgenic ‘Ruby Red’, an increased expression was detected after 3 d of inoculation (Fig. 45) (Table 8).

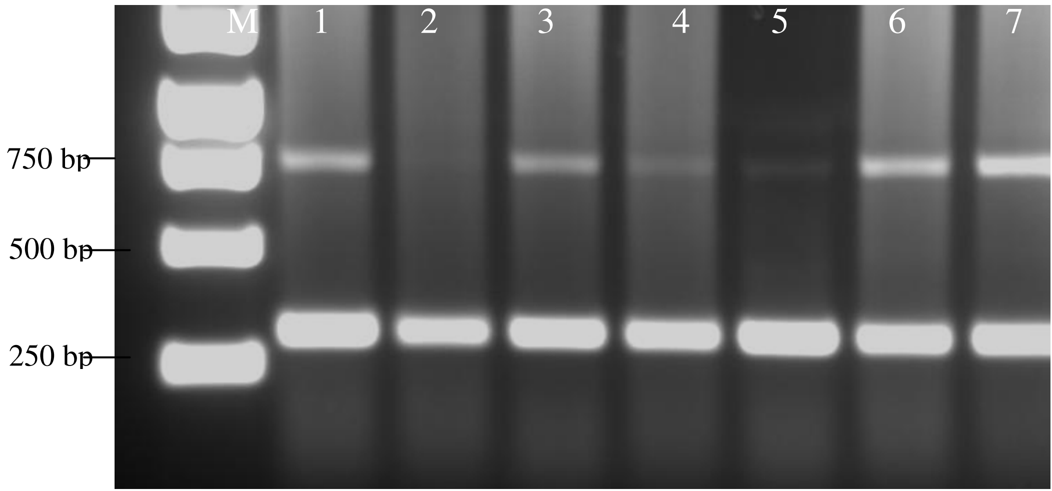


Fig. 45. The *cAPXcit* expression was examined by RQ RT-PCR. 'Duncan', 'Ruby Red', and transgenic 'Ruby Red' leaves were infiltrated with *A. alternata* and sampled after 3 d of inoculation. M= Molecular marker (1 kb DNA ladder, Fermentas), lanes 1 = non-inoculated 'Duncan', 3 = non-inoculated non-transgenic control 'Ruby Red', 5 = non-inoculated transgenic 'Ruby Red', 2 = inoculated 'Duncan', 4 = inoculated non-transgenic control 'Ruby Red', 6 and 7 = inoculated transgenic 'Ruby Red'. The densities of the amplification products are shown in the following table.

Table 8. RQRT-PCR amplification product densities for *cAPXcit* expression due to *A. alternata* infection in non-transgenic 'Duncan' and 'Ruby Red' compared to transgenic 'Ruby Red' plants.

Region	Total Density	Total Background	Total Raw Density	Mean Raw Density	Ratio
1	99902.3213	206489.1892	301202	143.0209	1.00 [a]
2	9684.9194	123510.4839	131268	94.71	0.006 [b]
3	64579.2571	168744.5556	232201	114.3847	0.36 [a]
4	28041.419	165521.1429	192125	92.814	0.17 [b]
5	670.8086	105635.3135	105846	60.4834	0.004 [a]
6	70110.1193	166039.367	234534	109.2889	0.27 [b]
7	99166.8208	191119.8113	285864	136.1257	0.35 [b]

[a]Before inoculation
[b]After inoculation.

The *cAPXcit* gene expression changes in 'Duncan', 'Rio Red', mutated 'Rio Red', non-transgenic control 'Ruby Red', and transgenic 'Ruby Red' due to *P. nicotianae* infection was also studied after 3 d of inoculation (Fig. 46) (Table 9).

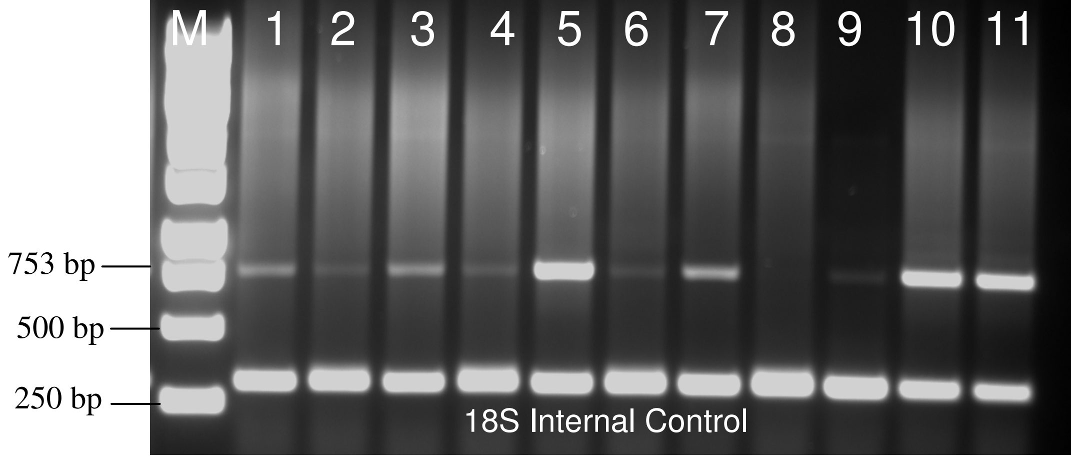


Fig. 46. The *cAPXcit* expression was examined by RQ RT-PCR. 'Duncan', 'Rio Red', mutated 'Rio Red', non transgenic control 'Ruby Red', and transgenic 'Ruby Red' leaves were infiltrated with *P. nicotianae* and sampled after 3 d of inoculation. M= Molecular marker (1 kb DNA ladder, Fermentas), lanes 1 = non-inoculated 'Duncan', 3 = non-inoculated non-transgenic control 'Ruby Red', 5 = non-inoculated 'Rio Red', 7 = non-inoculated mutated 'Rio Red', 9 = non-inoculated transgenic 'Ruby Red', 2 = inoculated 'Duncan', 4 = inoculated non-transgenic control 'Ruby Red', 6 = inoculated 'Rio Red', 8 = inoculated mutated 'Rio Red', and 10 and 11 = inoculated transgenic 'Ruby Red'. The densities of the amplification products are shown in the following table.

Table 9. RQRT-PCR amplification product densities for *cAPXcit* expression due to *P. nicotianae* infection in non-transgenic 'Duncan', 'Rio Red', and 'Ruby Red' compared to transgenic 'Ruby Red' plants.

Region	Total Density	Total Background	Total Raw Density	Mean Raw Density	Ratio
1	63089.9802	312978.0791	366924	143.3297	0.27 [a]
2	11276.4471	187690.0385	191461	114.5102	0.07 [b]
3	73241.0556	287796.4444	348645	135.3436	0.30 [a]
4	29846.1373	243541.2346	271355	121.1406	0.17 [b]
5	189537.4266	305427.2218	482572	200.4869	1.22 [a]
6	25901.4174	229155.4071	252696	103.4791	0.14 [b]
7	91704.173	194746.8679	282820	140.2877	0.47 [a]
8	7237.5333	165489.9556	170985	86.0086	0.04 [b]
9	13222.1176	152081.4706	164689	83.1763	0.06 [a]
10	203962.4298	267148.4386	468972	186.3959	1.08 [b]
11	198486.9718	285611.8644	478057	162.6044	1.25 [b]

[a]Before inoculation.
[b]After inoculation.

In general, a similar trend of suppressed expression of cAPX was observed in all the cultivars inoculated with *A. alternata*. However, only transgenic 'Ruby Red' showed an increased expression of *cAPXcit* due to *P. nicotianae* infection compared to the un-inoculated control.

DISCUSSION

Although several citrus species transformation has been reported, in general, transformation efficiencies are very low (Peña et al., 2004). This recalcitrant nature appears to be genotype dependent. Several researchers reported that sour orange is highly recalcitrant to transformation and the successful production of transgenic sour orange is highly incidental. Moreover, sour orange transformation efficiency was reported to be less than 0.1% (Gutierrez et al., 1997).

The results from the present study show this general phenomenon. When compared to 'Ruby Red', 'Duncan', and 'Rio Red', sour orange showed the least number of shoot regeneration, with a maximum 8%. Moreover, after working with more than 2,000 explants, no transgenic sour orange plant was produced. Furthermore, several researchers observed that highly recalcitrant plant species show either a very low regeneration or no regeneration at all (Peña et al., 2004).

Several strategies including the modification of the cultural medium were proposed to increase the regeneration efficiency in citrus. Additionally, an increased regeneration and transformation frequencies were observed by using auxin rich co-cultivation medium (Ghorbel et al., 2000). In this study, a 3 $mg.L^{-1}$ BAP in our co-cultivation and regeneration medium (DBA3 medium) was used along with a reduced BAP concentration of 0.2 $mg.L^{-1}$ in the shoot elongation medium (BG medium). The DBA3 medium also consisted of a very low concentration 2,4-D at 0.001 $mg.L^{-1}$.

It was previously reported in Petunia leaves that addition of BAP and 2,4-D enhanced the transformation frequency (Villemont et al., 1997). It was speculated that 2,4-D triggered the dedifferentiation of the cells which might promote the competence

of the cells to genetic transformation. This study produced successful shoot regeneration from 'Duncan', 'Ruby Red', 'Rio Red', and sour orange epicotyl segments; however, the regeneration frequencies were different.

In Carrizo citrange, a co-cultivation medium with BAP at 3 $mg.L^{-1}$ and later a reduced concentration at 0.2 $mg.L^{-1}$ in the subsequent culture medium showed faster bud differentiation (Peña et al., 2004). An enhanced cell division and bud differentiation due to the addition of BAP to the culture medium was observed by Garcia-Luis et al. (1999).

The period of the co-cultivation appears to influence the Agrobacterium-mediated transformation in citrus (Almeida et al., 2002). In all the experiments, a co-cultivation of 3 d on DBA3 supplemented with 100 µM acetosyringone was used and incubation of the explants in the dark. Similar conditions were successfully used in the transformation of 'Rio Red' grapefruit cultivar (Yang et al., 2000). Transformed plants could not be regenerated in rice and barley when acetosyringone was not added to the co-culture medium (Rashid et al., 1996; Kumlehn et al., 2006). A co-culture incubation of 20 min was used to inoculate the epicotyl segments.

In a comparative study of co-culture incubation times of 5, 10, 20, and 40 min., it was observed that 20 min. is the best for sweet orange and lime transformation (Almeida et al., 2003). Several factors influencing the success of citrus transformation during co-cultivation were evaluated (Mendes et al., 2002). They concluded that in sweet orange, a co-cultivation period of 3 d and incubation of the explants in dark is the best co-cultivation condition. For trifoliate orange transformation, a 7 d co-

cultivation on a medium supplemented with 8.86 μM BAP and 1.43 μM was used to improve callus formation from epicotyl segments (Zou et al., 2008).

In this study, epicotyl segments from 21-28 d-old seedlings were used for transformation. However, citrus transformation using the juvenile material such as epicotyl segments is a lengthy process. Citrus plants from juvenile tissue may take upto 15 y for blooming. This could delay the ability to evaluate the transgenic plants for various incorporated traits. Hence, transformation of mature tissue is better to avoid juvenile characteristics. Transformation of mature tissue and recovery of transgenic plants was reported in sweet orange (Cervera et al., 1998).

In the initial transformation experiments based on the transient histochemical GUS analysis, it was observed that the transformation efficiency of A. tumefaciens strain C58C1 was superior to that of EHA105. Moreover, all the further transformations were performed using C58C1 strain. In sweet orange, the transformation efficiency of three A. tumefaciens strains, namely LBA4404, EHA101, and C58C1, were compared based on the GUS analysis. They also concluded that the transformation efficiency was higher with C58C1 (45%) compared to EHA101 (29%) and LBA4404 (0%). The present results along with the results from the previous studies conclude that C58C1 may be a better A. tumefaciens strain for citrus transformation.

The transient GUS analysis of the epicotyl segment end pieces after the co-cultivation always showed a high number of positive blue stains. However, it should be noted that not all transformed cells can regenerate and the presence of the A. tumefaciens bacterial cells may contribute to this result. Furthermore; the transient

histochemical GUS assays revealed that the blue staining is mostly confined to the meristematic cells in the cambium. This may suggest that the competent cells for the transformation are localized mostly in the meristematic tissue. Moreover, in Carrizo citrange, it was shown that the callus forms from the cambial cells (Peña et al., 2004). Cambium is a very thin layer of cells which could be one of the reasons for low transformation efficiency as the number of available cells for transformation is limited.

The development of non-transgenic escape shoots is one of the most important limitations to citrus transformation. A selective marker gene, nptII, is widely used in the Agrobacterium-mediated citrus transformation to select the putative transformed shoots and to discard the escape shoots. In our experiments, all three plasmid vectors have nptII gene under the control of NOS promoter to facilitate the screening of the putative transgenic shoots.

A total of 52 'Ruby Red', 46 'Duncan', 7 'Rio Red', and 16 sour orange shoots were selected and shoot grafted onto sour orange rootstock. However, only two 'Ruby Red' plants were confirmed to be transgenic based on several molecular and non-molecular qualitative assays. These results support some earlier observations that kanamycin selection is not an efficient screening method for citrus transformation. Generation of the escape shoots under kanamycin selection was attributed to the persistence of resistant A. tumefaciens, masking the non-transformed cells with the transformed cells, which precludes the exposure to the surrounding kanamycin (Dominguez et al., 2004). Furthermore, several alternative selection strategies using phosphomannose isomerase (PMI) and multi-auto transformation vector (MAT) based selection are shown to be effective for Carrizo citrange and sweet orange. Also, use of

transversely cut thin epicotyl segments and increased kanamycin selection pressure reduced the escape shoot population in ‘Swingle’ citrumelo (Molinari et al., 2004).

The positive aspects of the kanamycin selection include the successful use of kanamycin in the transformation of several citrus genotypes, that allows a stable integration and expression of the marker genes such as GUS, and the transgenic plants produced are non-harmful to humans, other animals, and the environment (European Food Safety Authority, 2007).

Several researchers have reported improved methods to enhance the Agrobacterium-mediated citrus transformation efficiency. Recently, green fluorescent protein (GFP) binary vectors were developed for efficient citrus transformation (Chen et al., 2007). The selection of the transformants using gfp gene expression has an advantage that it is a non-destructive method unlike the GUS selection where the tissue is destroyed during the analysis. However, the persistence of the *A. tumefaciens* bacterial cells on the explants or the transgenic plants may give false-positive results (Dominguez et al., 2004). Recently, the highest transformation efficiency of 8.4% in sweet orange and 11.2% in ‘Swingle’ citrumelo was reported by using a novel sonication-assisted *Agrobacterium*-mediated transformation (Oliveira et al., 2008).

Southern blot analysis of the two putative transgenic ‘Ruby Red’ plants with *uid*A probe confirmed the stable integration of the transgenes into the plant genome. All transgenes including, *CNGCcit*, *uid*A, and *npt*II were successfully amplified using PCR. Screening of the *nptII* gene by ELISA and histochemical GUS assay to screen for the *uid*A gene clearly showed positive results for these two plants. All these tests confirm that all the three transgenes were stably incorporated into the plant’s genome.

However, for the plants transformed with *Bcl-2* gene, no hybridization signal was observed when hybridized with Bcl-2 probe.

In this study, a qRT-PCR based on TaqMan detection and RQ RT-PCR were used to analyze the *CNGCcit* gene expression in the transgenic 'Ruby Red' plants. Both the methods produced similar conclusive results showing constitutive high expression of the gene. Moreover, qRT-PCR is a highly sensitive and accurate method of quantitatively measure subtle differential gene expression. A TaqMan probe labeled with FAM at the 5'-end along with the gene specific primers were used in measuring the differential gene expression in the transgenic plants compared to the non-transgenic control plants. The *COX* gene specific primers and the probe labeled with TET were used as an internal control. The real-time accumulation of the fluorescence due to the separation of FAM and TET upon the amplification of the gene was measured as cycle threshold (cT) values. A cT difference of at least 5 was observed between the transgenic and non-transgenic control plants. QRT-PCR was previously used in the quantitative expression of *CcGA20ox1* (a key enzyme of GA biosynthesis) gene in both sense and anti-sense Carrizo citrange transgenic plants (Fagoaga et al., 2007). Furthermore, qRT-PCR was effectively used in the estimation of the gene copy number encoding lipid transfer protein (*LTP*) in transgenic sweet oranges (Omar et al., 2008).

The detached leaf assays with *Xac*, *P. nicotianae*, and *A. alternata* inoculations revealed that the transgenic 'Ruby Red' plants over-expressing *CNGCcit* were resistant compared to the non-transgenic 'Ruby Red', 'Duncan', and 'Rio Red' plants. Both the transgenic 'Ruby Red' and the above non-transgenic leaves were inoculated with *P. nicotianae* and *A. alternata* for 3 d and the differential expression analysis of *cAPXcit*

gene was performed using RQ RT-PCR. The results showed an enhanced expression of the gene in the inoculated transgenic plants while the non-transgenic plants showed a suppressed expression.

The observed resistance in the transgenic 'Ruby Red' plants could be attributed to *CNGCcit* over-expression which might have resulted in an elevated cytosolic Ca^{2+} levels. The eleveated cytosolic Ca^{2+} acts as a secondary signaling molecule to activate the calcium- and CaM-dependent protein kinases, which further result in the disease response. Furthermore, enhanced *cAPXcit* gene expression in the inoculated transgenic plants might have contributed to the continuous ROS scavenging, thereby, providing an enhanced tolerance to the pathogen.

In plants, ROS generation due to both abiotic and biotic stresses and subsequent oxidative stress is well established. ROS production is also suggested as an early event in the programmed cell death (PCD) or necrosis (Levine et al., 1996) due to biotic stress such as invasion of pathogens. Suppression in *cAPX* gene expression was reported during pathogen-induced PCD was in tobacco (Mittler et al., 1998). In the initial phase of the infection, an elevated ROS may also act as signaling molecules for HR (Mittler et al., 1999). However, over a long period, high accumulation of ROS is also detrimental to the cell due to oxidative stress which leads to the cellular damage and ultimately to PCD. The plant's capability to scavenge these ROS determines the disease tolerance capacity.

The genome organization of APX isoforms localized in the same sub-cellular compartments is highly conserved. The cytosolic and peroxisomal isoforms are composed of nine exons and eight introns, while the chloroplastic contain 11 or

12 exons and 10 or 11 introns. Additionally, the cytosolic isoforms does not contain the intron that separates the exon 2 and 3 in the peroxisomal. Furthermore, the last exon in the cytosolic isoforms is short while the peroxisomal has a large last exon comprising the hydrophobic transmembrane domain which is required for peroxisomal localization (Teixeira et al., 2004). The genomic organization of the *cAPXcit* gene revealed that the seven exons were of the same size and order compared to the *cAPX* genes from other plant species, which may suggest that the *cAPXcit* is localized in the cytosol.

Exotic citrus diseases such as HLB and CC pose a serious threat to the citrus industry. In citrus, both these diseases are caused by gram-negative bacteria, *Ca*. Liberibacter spp. and *Xac*, respectively. *Xac* has been reported in more than 30 citrus producing countries (Gottwald et al., 2002). *Xac* has been reported to cause damage to citrus industry in Asia, Australia, South America, North America including US, and Australia (Gottwald et al., 2002). CC was first found in Florida in 1912 (Stevens, 1915). In spite of several eradication efforts, the recent out-breaks caused damages to millions of dollars. In 2001, the Florida CC eradication program costs exceed $200 millions (Brown, 2001).

Asiatic citrus canker (ACC) is one of the most important citrus diseases worldwide (Schaada et al., 2006). Duan et al. (1999) reported that expression of *pthA* gene which encodes for host plant specific pathogenic protein, PthA is necessary for *Xac* to cause ACC in citrus.

Several approaches including use of transgenic plants, insecticides to control Asian leaf miner, and bacteriophages were reported for ACC disease management.

Recently, grapefruit cultivars including ‘Rio Red’, ‘Ruby Red’, ‘Duncan’, and ‘Hamlin’ sweet orange were transformed with two genes encoding antimicrobial proteins including spinach defensin gene and bovine lysozyme gene (Gonzales et al., 2005). The transgenic plants are under field evaluations for ACC resistance. ‘Pera’ sweet orange plants constitutively expressing antibacterial peptides such as sarcotoxin IA showed significant resistance to ACC (Bespalhok et al., 2005). ‘Early Gold’ sweet orange and ‘Murcott’ tangor plants (Guo and Grosser, 2004) and ‘Hamlin’ sweet orange plants (Omar and Grosser, 2007) expressing *Xa21* gene from rice are under evaluation for ACC resistance.

Using subtractive cDNA libraries, the differentially expressed defense genes and transcription factors in ‘Nagami’ kumquat due to *Xac* inoculation were identified (Khalaf et al., 2007). In this study, involvement of superoxide dismutase enzyme in scavenging harmful ROS was elucidated. ‘Lakeland’ kumquat, an intergeneric hybrid between kumquat and ‘Key’ lime was reported as highly resistant to ACC (Reddy, 1997). Further studies proved that ‘Lakeland’ kumquat could serve as a promising seed parent to breed citrus for ACC resistance (Viloria et al., 2004).

In Swingle citumelo, induced systemic resistance (ISR) due to the application of harpin and Actigard did not reduce the ACC incidence (Graham and Leite, 2004). It was concluded that ISRs with or without copper formulations did not have any effect on reducing ACC occurrence. An attempt of using bacteriopphages with or without copper formulations found to be ineffective in

controlling ACC in grapefruit, however, a significant reduction of ACC incidence was observed in 'Valencia' oranges (Balogh et al., 2008).

In this study, the preliminary results showed that the transgenic 'Ruby Red' grapefruit is resistant to *Xac*. A continuation of the field evaluations of the transgenic plants for resistance to *Xac* is desirable.

HLB is the most destructive citrus disease in the world (da Graca and Korsten, 2004). The disease was first discovered in Florida in 2005 (Halbert, 2005). Since then, the disease caused severe economic losses to US citrus industry. Although the disease is not yet found in Texas citrus, widespread of the insect vector, ACP, pose a serious threat (French et al., 2001).

The efforts to isolate and grow *Ca*. Liberibacter in culture have been unsuccessful so far, which limits the opportunity to further study the biology of the bacterium. Recently, Davis et al. (2008) reported the isolation and culture of the bacterium, however, in co-cultures with the presence of actinobacteria.

The peroxidase activity was higher in the HLB-"tolerant" Tahiti limes compared to susceptible cultivars (van Lelyveld and van Vuuren, 1988). In the present study, *Xac* inoculated transgenic 'Ruby Red' plants showed an increased *cAPXcit* gene expression, however, it was decreased in all non-transgenic control grapefruit plants. The preliminary results showing resistance to *Xac* and an increased *cAPXcit* gene activity upon *Xac* inoculation may suggest that in future, these transgenic plants may prove to be HLB-tolerant. Hence, further studies on the conferred resistance to HLB are recommended.

SUMMARY AND CONCLUSIONS

In this study, two ‘Ruby Red’ transgenic plants expressing *CNGCcit* were successfully produced through *Agrobacterium*-mediated transformation. This is the first report of citrus transformation with a gene encoding CNGC, moreover, this is also the first report of transformation of a perennial tree with a gene encoding CNGC to investigate disease resistance.

The results showed that sour orange is highly recalcitrant to transformation. The research suggests superior transformation capability of *A. tumefaciens* strain C58C1 compared to EHA105. The occurrence of a large number of escape plants due to kanamycin-based selection of the transformed shoots was in agreement with earlier reports. It is suggested that there is a necessity for an alternative and improved selection strategy to avoid escape shoot regeneration.

The preliminary results from the detached leaf inoculation assays showed promising results. The transgenic plants showed an enhanced resistance to necrotrophic fungi, *P. nicotianae* and *A. alternaria*, and an economically important bacterium, *Xac*. The transgenic plant leaves were totally resistant to *P. nicotinae* infection and insensitive to tentoxin. The leaves did not show any signs of necrosis at all. However, before concluding that the transgenic plants are totally resistant to these pathogens, several repeated and long-term studies including detached leaves, intact leaves on the trees, and total plant inoculations are needed.

The transgenic plants inoculated with *P. nicotianae* and *A. alternata* showed an increased expression of *cAPXcit*, an important ROS scavenger even after 3 d of inoculation. However, in the susceptible grapefruit cultivars including ‘Ruby Red’,

'Duncan', and 'Rio Red', there was a suppressed *cAPXcit* expression. Based on these results, it is proposed that a cross-talk between an elevated cytosolic Ca^{2+} and an enhanced ROS scavenging capability due to increased *cAPXcit* expression or other ROS scavenging enzymes play a crucial role in disease tolerance, especially in prolonged exposure to the pathogen.

The objective of this study was to produce citrus with broad-spectrum disease resistance. This is a long-term project which may involve years of integrated efforts of researchers in different fields of plant science. However, the first step towards this goal proved to be promising. Further studies should be continued towards any conferred broad-spectrum resistance CTV and a bacterium, *C.* Liberibacter causing HLB disease in citrus.

In order to further continue the studies, the transgenic plants should be multiplied by grafting onto a vigorous rootstock such as rough lemon. Further investigation of expression changes in PR genes and genes involved in ethylene, ABA, jasmonic acid, and salicylic acid pathways may give more insight into the broad-spectrum disease resistance development.

A. thaliana AtCNGCs are shown to play a major role in resistance to several abiotic stress factors. Recent advancements in plant science unraveled a convergence of signaling pathways showing cross-talk among different abiotic and biotic stress responses. The transgenic plants should be evaluated for any conferred resistance to abiotic stress factors including cold, salinity, and uptake of the metals. The results of this study are the foundation for future citrus with a broad-spectrum disease resistance.

LITERATURE CITED

Adams, J.M. and S. Cory. 2007. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr. Opin. Immunol. 19:488-96.

Aguilar, E., W. Villalobos, L. Moreira, C.M. Rodriguez, E.W. Kitajima, and C. Rivera. 2005. First report of *Xylella fastidiosa* infecting citrus in Costa Rica. Plant Dis. 89:687.

Alida, B., M. Cervera, and L. Peña. 2008. Evaluation of selection strategies alternative to nptII in genetic transformation of citrus. Plant Cell Rep. 27:1005-1015.

Ali, R., R.E. Zielinski, and G.A. Berkowitz. 2006. Expression of plant cyclic nucleotide-gated cation channels in yeast. J. Expt. Bot. 57:125-138.

Ali, R., W. Ma, F. Lemtiri-Chlieh, D. Tsaltas, Q. Leng, S. von Bodman, and G.A. Berkowitz. 2007. Death don't have no mercy and neither does calcium: *Arabidopsis* cyclic nucleotide gated channel2 and innate immunity. The Plant Cell 19:1081-1095.

Almeida, W.A.B., F.A.A. Mourão filho, B.M.J. Mendes, and A.P.M. Rodriguez. 2002. *In vitro* organogenesis optimization and plantlet regeneration in *Citrus sinensis* and *C. limonia*. Sci. Agric. 59:35-40.

Almeida, W.A.B., F.A.A. Mourão filho, B.M.J. Mendes, A. Pavan, and A.P.M. Rodriguez. 2003. Agrobacterium-mediated transformation of *Citrus sinensis and C. limonia* epicotyl segments. Sci. Agric. 60:23-29.

Alvarez, A.M., A.A. Benedict, C.Y. Mizumoto, L.W. Pollard, and E.L. Civerolo. 1991. Analysis of *Xanthomonas campestris* pv. *citri* and *X. c. citrumelo* with monoclonal antibodies. Phytopathology 81:857-865.

Ananthakrishnan, G., V. Orbović, G. Pasquali, M. Ćalović, and J.W. Grosser. 2007. Transfer of *citrus tristeza virus* (CTV)-derived resistance candidate sequences to four grapefruit cultivars through *Agrobacterium*-mediated genetic transformation. *In vitro* Cellular & Develop. Biol. 43:593-601.

Apel, K. and H. Hirt. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55:373-399.

Arazi, T., R. Sunkar, B. Kaplan, and H. Fromm. 1999. A tobacco plasma membrane calmodulin-binding transporter confers Ni^{2+} tolerance and Pb^{2+} hypersensitivity in transgenic plants. Plant J. 20:171-182.

Arugumanathan, K. and E.D. Earle. 1991. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. 9:208-218.

Asada, K. 1992. Ascorbate peroxidase-A hydrogen scavenging enzyme in plants. Physiol. Plant. 85:235-241.

Azevedo, F.A., F.A.A. Mourão Filho, B.M.J. Mendes, W.A.B. Almeida, E.H. Schinor, R. Pio, J.M. Barbosa, S. Guidetti-Gonzalez, H. Carrer, and E. Lam. 2006. Genetic transformation of Rangpur lime (*Citrus limonia* Osbeck) with the bO (bacterio-opsin) gene and its initial evaluation for *Phytophthora nicotianae* resistance. Plant Mol. Biol. Rep. 24:185-196.

Baker, J. C., N. Mock, J. Glazener, and E. Orlandi. 1993. Recognition responses in pathogen/non-host and race/cultivar interactions involving soybean (*Glycine max*) and *Pseudomonas syringae* pathovars. Physiol. Mol. Plant Path. 43:81-94.

Balague, C., B. Lin, C. Alcon, G. Flottes, S. Malmstorm, C. Kohler, G. Neuhaus, G. Pelletier, F. Gaymard, and D. Roby. 2003. HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. The Plant Cell 15:365-379.

Balogh, B., B.I. Canteros, R.E. Stall, and J.B. Jones. 2008. Control of citrus canker and citrus bacterial spot with bacteriophages. Plant Dis. 92: 1048-1052.

Bar-Joseph, M., C.N. Roistacher, S.M. Garnsey, and D.J. Gumpf. 1981. A review on tristeza, an ongoing threat to citriculture. Proc. Intl. Soc. Citricult.1:419-423.

Bar-Joseph, M., R. Marcus, and R.F. Lee. 1989. The continuous challenge of citrus tristeza virus control. Annu. Rev. Phytopathol. 27:291-316.

Bespalhok F.J.C., A.K. Kobayashi, I. Mitsuhara, H.B. Molinari, R.P. Leite, L. Meneguim, Y. Ohashi, S. Natori, and LG.E. Vieira. 2005. Enhanced resistance to citrus canker in transgenic sweet orange plants expressing the antibacterial peptide sarcotoxin IA. Proc. Intl. Citrus Canker and Huanglongbing Res.Workshop. Nov 7-11, Orlando, FL.

Blume, B., T. Nürnberger, N. Nass, and D. Scheel. 2000. Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. The Plant Cell 12:1425-40.

Bond, J.E. and M.L. Roose. 1998. *Agrobacterium*-mediated transformation of the commercially important citrus cultivar Washington navel orange. Plant Cell Rep.18:229-234.

Bové, J.M., and M. Garnier. 2000. Stubborn, p. 48-50. In: Timmer, L. W., S.M. Garnsey, and J.H. Graham (eds.). Compendium of Citrus Diseases. APS Press, St. Paul, MN.

Brown, J.W.S. 1986. A catalogue of splice junction and putative branch point sequences from plant introns. Nucleic Acids Res. 14:9549-9559.

Brown, K. 2001. Florida fights to stop citrus canker. Science 292:2275-2276.

Brunings, A.M. and D. Gabriel. 2003. *Xanthomonas citri*: Breaking the surface. Mol. Plant Pathol. 4:141-157.

Carland, F.M. and T. Nelson. 2004. Cotyledon vascular pattern 2-mediated inositol (1,4,5) triphosphate signal transduction is essential for closed venation patterns of *Arabidopsis* foliar organs. The Plant Cell 16:1263-1275.

Case, R.M., D. Eisner, A. Gurney, O. Jones, S. Muallem, and A. Verkhratsky. 2007. Evolution of calcium homeostasis: From birth of the first cell to an omnipresent signalling system. Cell Calcium 42:345-350.

Cervera, M., J.A. Pina, J. Juárez, L. Navarro, and L. Peña. 1998. *Agrobacterium*-mediated transformation of citrange: Factors affecting transformation and regeneration. Plant Cell Rep. 18:271-278.

César, P. and L. Burgos. 2005. Transformation of fruit trees. Useful breeding tool or continued future prospect? Transgenic Res.14:15-26.

Chagas, C.M., V. Rossetti, and M.J.G. Beretta. 1992. Electron microscopy studies of a xylem-limited bacterium in sweet orange affected with citrus variegated chlorosis disease in Brazil. J. Phytopathol. 134:306-312.

Chan, C.W.M., M. Laura, M. Schorrak, K.S.R. Smith, A.F. Bent, and M.R. Sussman. 2003. A cyclic nucleotide-gated ion channel, CNGC2, is crucial for plant development and adaptation to calcium Stress. Plant Physiol.132:728-731.

Chen, C., Q. Zheng, X. Xu, J.R. Soneji, S. Huang, Y.A. Choi, M.N. Rao, and F.G. Gmitter. 2007. Development of pGreen-derived GFP binary vectors for citrus transformation. HortScience 42:7-10.

Chomczynski, P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Anal. Biochem. 201:134-139.

Civerolo, E.L. and F.F. Fan. 1982. *Xanthomonas campestris* pv. *citri* detection and identification by enzyme-linked immunosorbent assay. Plant Dis. 66:231-236.

Clough, S.J., K.A. Fengler, I.-C. Yu, B. Lippok, R.K. Smith, and A.F. Bent. 2000. The Arabidopsis dnd1 ‘‘defense, no death’’ gene encodes a mutated cyclic nucleotide-gated ion channel. Proc. Natl. Acad. Sci. 97:9323-9328.

Contreras, J.D.V. 1992. Pecosita ou falsa mancha grasienta na Argentina. Laranja Cia 31:4.

Cordeiro, M.C., R. Piqueras, D.E. de Oliveira, and C. Castresana. 1998. Characterization of early induced genes in Arabidopsis thaliana responding to bacterial inoculation: Identification of centrin and of a novel protein with two regions related to kinase domains. FEBS Lett. 434:387-393.

Costa, M., W. Otoni, and G. Moore. 2002. An evaluation of factors affecting the efficiency of *Agrobacterium*-mediated transformation of *Citrus paradisi* (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. Plant Cell Rep. 21:365-373.

da Graça, J.V. and L. Korsten. 2004. Citrus huanglongbing: Review, present status and future strategies. p229-245. In: S.A.M.H. Naqvi (ed.). Diseases of fruits and vegetables, Vol.1, Kluwer, Dordrecht, The Netherlands.

Dangl, J.F. 1995. Piece de Resistance: Novel class of plant disease resistance genes. Cell 80:363-366.

Daniell, H., B. Muthukumar, and S.B. Lee. 2001. Marker-free transgenic plants: Engineering the chloroplast genome without the use of antibiotic selection. Curr. Genet. 39:109-116.

Davis, M.J., S.N. Mondal, H. Chen, M.E. Rogers, and R.H. Brlansky. 2008. Co-cultivation of '*Candidatus* Liberibacter asiaticus' with actinobacteria from citrus with huanglongbing. Plant Dis. 92:1547-1550.

Deng, X.X., J.W. Grosser, and F.G. J. Gmitter. 1992. Intergenic somatic hybrid plants from protoplast fusion of *Fortunelle crassifolia* "Meiwa" with *Citrus sinensis* cultivar "Valencia". Scientiae Horticulturae 49:55-62.

Desikan, R., J.T. Hancock, M.J. Coffey, and S.J. Neill. 1996. Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by a NADPH oxidase-like enzyme. FEBS Lett. 382:213-217.

De Wit, P.J.G.M. 1992. Molecular characterization of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. Annu. Rev. Phytopathol. 30:391-418.

De Wit, P.J.G.M. 1998. Pathogen avirulence and plant resistance: A key role for recognition. Trends Plant Sci. 12:452-458.

Dickman, M.B., Y.K. Park, T. Oltersdorf, W. Li, T. Clemente, and R. French. 2001. Abrogation of disease development in plants expressing animal antiapoptotic genes. Proc. Natl. Acad. Sci. 98:957-6962.

Dixon, R.A., M. J. Harrison, and C. J. Lamb. 1994. Early events in the activation of plant defense responses. Annu. Rev. Phytopathol. 32:479-501.

Dominguez, A., J. Guerri, M. Cambra, L. Navarro, P. Moreno, and L. Peña. 2000. Efficient production of transgenic citrus plants expressing the coat protein gene of citrus tristeza virus. Plant Cell Rep. 19:427-433.

Dominguez, A., C. Fagoaga, L. Navarro, P. Moreno, and L. Peña. 2002. Regeneration of transgenic citrus plants under non-selective conditions results in high-frequency recovery of plants with silenced transgenes. Mol. Genet. Genom. 267:544-556.

Dominguez, A., M. Cervera, R.M. Perez, J. Romero, C. Fagoaga, J. Cubero, M.M. Lopez, J.A. Juarez, L. Navarro, and L. Peña. 2004. Characterisation of regenerants obtained under selective conditions after *Agrobacterium*-mediated transformation of citrus explants reveals production of silenced and chimeric plants at unexpected high frequencies. Mol. Breeding 14:171-183.

Duan, Y.P., A. Castañeda, G. Zhao, G. Erdos, and D.W. Gabriel. 1999. Expression of a single, host-specific, bacterial pathogenicity gene in plant cells elicit division, enlargement, and cell death. Mol. Plant Microbe Int. 12:556-560.

EFSA, European Food Safety Authority. 2007. Statement of the scientific panel on genetically modified organisms on the safe use of the nptII antibiotic resistance marker gene in genetically modified plants. 1 March 2009. <http://www.efsa.europa.eu/en/ science/gmo/statements0/npt2.html>.

Erwin, D.C. and O.K. Ribeiro. 1996. Phytophthora diseases worldwide. APS Press, St. Paul, MN.

Fagoaga, C., F.R. Tadeo, D.J. Iglesias, L. Huerta, I. Liso, A.M. Vidal, M. Talon, L. Navarro, J.L. Garcia-Martinez, and L. Peña. 2007. Engineering of gibberellin levels in citrus by sense and antisense over-expression of a GA 20-oxidase gene modifies plant architecture. J. Expt. Bot. 58:1407-1420.

FAO, Food Agriculture Organization of the United Nations. 2003. 3 Feb. 2009. <http://www.fao.org/>.

FAO, Food Agriculture Organization of the United Nations. 2006. 3 Feb. 2009. <http://www.fao.org/>.

Febres, V.J., R.F. Lee, and G.A. Moore. 2003. Characterization of grapefruit plants (Citrus paradisi Macf.) transformed with citrus tristeza closterovirus genes. Plant Cell Rep. 21:421-8.

Febres, V.J., R.F. Lee, and G.A. Moore. 2008. Transgenic resistance to Citrus tristeza virus in grapefruit. Plant Cell Rep. 27:93-104.

French, J.V., C.J. Kahlke, and J.V. da Graça. 2001. First record of the Asian citrus psylla, *Diaphorina citri* Kuwayama (Homoptera: Psyllidae), in Texas. Subtrop. Plant Sci. 53:14-15.

Gamborg, O. L., R.A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.

García-Luis, A., Y. Bordón, J.M. Moreira-Dias, R.V. Molina, and J.L. Guardiola. 1999. Explant orientation and polarity determine the morphogenic response of epicotyl segments of Troyer citrange. Annals Bot. 84:715-723.

Gelli, A., V.J. Higgins, and E. Blumwald. 1997. Activation of plant plasma membrane Ca^{2+}-permeable channels by race-specific fungal elicitors. Plant Physiol. 155:35-45.

Ghorbel, R., A. Domínguez, L. Navarro, L. Peña L. 2000. High efficiency genetic transformation of sour orange (*Citrus aurantium*) and production of transgenic trees containing the coat protein gene of citrus tristeza virus. Tree Physiol. 20:1183-1189.

Ghorbel, R., S. LaMalfa, M.M. López, A. Petit, L. Navarro, and L. Peña. 2001. Additional copies of virG from pTiBo542 provide super-transformation ability to *Agrobacterium tumefaciens* in citrus. Physiol. Mol. Plant Pathol. 58: 103-110.

Gobert, A., G. Park, A. Amtmann, D. Sanders, and F.J. Maathuis. 2006. *Arabidopsis thaliana* cyclic nucleotide gated channel 3 forms a non-selective ion transporter involved in germination and cation transport. J. Expt. Bot. 57:791-800.

Gonzalez-Ramos, J., J. Graham, and T.E. Mirkov. 2005. Transformation of citrus cultivars with genes encoding potential resistance to citrus canker (*Xanthomonas axonopodis* pv. *citri*). Phytopathology 95:S35.

Goodman, R. N. and A. J. Novacky. 1996. The hypersensitive reaction in plants to pathogens: a resistance phenomenon. APS Press, St. Paul, MN.

Gottwald, T.R., J.H. Graham, and T.S. Schubert. 2002. Citrus canker: The pathogen and its impact. Plant Health Progress doi:10.1094/PHP-2002-0812-01-RV. 1 Feb. 2009. <http://www.plantmanagementnet work.org/pub/php/review/citruscanker/>.

Graham, J.H. and R.P. Leite. 2004. Lack of control of citrus canker by induced systemic resistance compounds. Plant Dis. 88:745-750.

Grant, M., I. Brown, S. Adams, M. Knight, A. Ainslie, and J. Mansfield. 2000. The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J. 23: 441-450.

Guo, F. Q., M. Okamoto, N.M. Crawford. 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signalling. Science 302:100-103.

Guo, W.W. and J.W. Grosser. 2004. Transfer of a potential citrus canker resistance gene into citrus protoplasts using gfp as the selectable marker Acta Hort. (ISHS) 632:255-258.

Guo, Y., L. Xiong, C.P. Song, D. Gong, U. Halfter, and J.K. Zhu. 2002. A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signalling in *Arabidopsis*. Develop. Cell 3:233-244.

Gutiérrez-E, M.A., D. Luth and G.A. Moore. 1997. Factors affecting Agrobacterium-mediated transformation in citrus and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of citrus tristeza virus. Plant Cell Rep.16:745-753.

Halbert, S. 1998. Asian citrus psyllids and greening disease of citrus. In: Pest alert: A literature review. Florida Department of Agriculture and Consumer Services, Division of Plant Industry. 3 Feb. 2009. <http://www.doacs.state.fl.us/pi/enpp/ento /dcitri.htm>.

Halbert, S.E. 2005. The discovery of Huanglongbing in Florida. P. H-3. In: Proc. 2nd Intl. Citrus Canker and Huanglongbing Research Workshop. Florida Citrus Mutual. Orlando, FL.

Halbert, S.E. and K.L. Manjunath. 2004. Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: A literature review and assessment of risk in Florida. Florida Entomol. 87:330-353.

Heath, M. C. 2000. Hypersensitive response-related death. Plant Mol. Biol. 44:321-334.

Herron, C.M. and M. Skaria, M. 2000. Further studies on citrus tatter leaf virus in Texas. In: Proc. Conf. IOCV. 14:185-194.

Hirschi, K.D., R. Zhen, K.W.Cunningham, P.A. Rea, and G.R. Fink. 1996. CAX1, a H+ /Ca^{2+} antiporter from Arabidopsis. Proc. Natl. Acad. Sci. 93:8782-8786.

Hood, E. E., S.B. Gelvin, L.S. Melchers, and A. Hoekema. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 2:208-218.

Hopkins, D.L. 1989. *Xylella fastidiosa*: Xylem limited bacterial pathogen of plants. Annu. Rev. Phytopathol. 27:271-290.

Horsch, R. B., J.E. Fry, N.L. Hoffman, D. Eichholtz, S.G. Roger, and R.T. Fraley. 1985. A simple and general method of transferring genes into plants. Science 227:1229-1230.

Hu, X.Y., S.J. Neill, W.M. Cai, and Z.C. Tang. 2004. Induction of defense gene expression by oligogalacturonic acid requires increases in both cytosolic calcium and hydrogen peroxide in *Arabidopsis thaliana*. Cell Res. 14:234-240.

Hua, B.G., R.W. Mercier, Q. Leng, G.A. Berkowitz. 2003. Plants do it differently: A new basis for potassium/sodium selectivity in the pore of an ion channel. Plant Physiol. 132: 1353-1361.

Irwin, R., and P.B.C. Jones. 2006. Landscapes, genomics and transgenic conifers. p245-261. Springer, Dordrecht, The Netherlands.

Jefferson, R. A., T.A. Kavanagh, and M.W. Bevan. 1987. GUS fusion: *β-glucuronidase* is a sensitive and versatile fusion marker in higher plants. EMBO J. 6:3901-3907.

Jimenez, A., J. A. Hernandez, L. A. del-Rio, and F. Sevilla. 1997. Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. Plant Physiol. 114: 275–284.

Jin, C.Y, C. Park, J.H. Lee, K.T. Chung, T.K. Kwon, G.Y. Kim, B.T. Choi, and Y.H. Choi. 2008. Naringenin-induced apoptosis is attenuated by Bcl-2 but restored by the small molecule Bcl-2 inhibitor, HA 14-1, in human leukemia U937 cells. Toxicol. *In vitro*. doi:10.1016/j.tiv.2008.12.005.

Jirage, D., T.L. Tootle, T.L. Reuber, L.N. Frost, B.J. Feys, J.E. Parker, F.M. Ausubel, and J. Glazebrook. 1999. *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. Proc. Natl. Acad. Sci. 96:13583-13588.

Johnson, B. and K. Kirby. 2001. Potential impacts of genetically modified trees on biodiversity of forestry plantations: A global perspective, p. 176-186. In: Strauss, S.H. and H.D. Bradshaw (eds.). Proc. 1st Intl. Symp. Ecol. Soc. Aspects of Transgenic Plantations. College of Forestry, Oregon State Univ., Corvallis, OR.

Jurkowski, G.I., R.K. Smith, I.C. Yu, J.H. Ham, S.B. Sharma, D.F. Klessig, K.A. Fengler, and A.F. Bent. 2004. Arabidopsis DND2, a second cyclic nucleotide-gated ion channel gene for which mutation causes the "defense, no death" phenotype. Mol. Plant Microbe Interact. 17:511-520.

Kayim, M., T. Ceccardit, M.J.G. Berretta, G.A. Barthe, and K.S. Derrick. 2004. Introduction of a citrus blight-associated gene into Carrizo citrange [*Citrus sinensis* (L.) Osbc. x *Poncirus trifoliata* (L.) Raf.] by *Agrobacterium*-mediated transformation. Plant Cell Rep. 23:377-385.

Keen, N.T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annu. Rev. Genet. 24:447-463.

Khalaf, A., G.A. Moore, J. Jeffrey, and F.G. Gmitter. 2007. New insights into the resistance of Nagami kumquat to canker disease. Physiol.Mol. Plant Pathol. 71:240-250.

Khawaler, R.N., K. Singh, G. Garg, V.K. Baranwal, and S.R. Alizadeh. 2006. *Agrobacterium*-mediated genetic transformation of Nagpur mandarin (*Citrus reticulata* Blanco). Current Sci. 91:1700-1705.

Kobayashi, S. and H. Uchimiya. 1989. Expression and integration of a foreign gene in orange (Citrus sinensis Osb.) protoplasts by direct DNA transfer. Jpn. J. Genet. 64:91-97.

Köhler, C., T. Merkle, and G. Neuhaus. 1999. Characterization of a novel gene family of putative cyclic nucleotide- and calmodulin-regulated ion channels in *Arabidopsis thaliana*. Plant J 18: 97-104.

Kudla, J., Q. Xu, K. Harter, W. Gruissem, and S. Luan. 1999. Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. Proc. Natl. Acad. Sci. 96:4718-4723.

Kumlehn, J.L., S.G. Hensel, D. Becker, and H. Lorz. 2006. Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. Plant Biotechnol. J. 4:251-261.

Kunta, M., H.S. del Rio, and E. Louzada. 2006. Isolation and molecular characterization of a putative ascorbate peroxidase from citrus. HortScience 41:1021.

Kunta, M., J.V. da Graça, and M. Skaria. 2007a. Molecular detection and prevalence of citrus viroids in Texas. HortScience 42:600-604.

Kunta, M., M. Hongqin, and M. Skaria. 2007b. Molecular distinction of citrus *Phytophthora* isolates in the Lower Rio Grande Valley of Texas. Subtrop. Plant Sci. 59:1-5.

Lecourieux, D., C. Mazarsb, N. Paulyb, R. Ranjevab, and A. Pugin. 2002. Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. The Plant Cell 14:2627-2641.

Leng Q., R.W. Mercier, W. Yao, and G.A. Berkowitz. 1999. Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. Plant Physiol. 121: 753-761.

Leng, Q., R.W. Mercier, B.G. Hua, H. Fromm, and G.A. Berkowitz. 2002. Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels. Plant Physiol. 128:400-410.

Levine, A., R.I. Pennell, M.E. Alvarez, R. Palmer, and C. Lamb. 1996. Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr. Biol. 6: 427-437.

Li, W., J.S. Hartung, and L. Levy. 2006. Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus huanglongbing. J. Micro. Methods. 66:104-115.

Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Logrieco, A., A. Visconti, and A. Bottalica. 1990. Mandarin fruit rot caused by *Alternaria alternata* and associated mycotoxins. Plant Dis. 74:415-417.

Luth, D. and G. Moore. 1999. Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*-mediated transformation. Plant Cell, Tissue and Organ Cult. 57:219-222.

Marucci, R.C., J.R.S. Lopes, R.R. Cavichioli. 2008. Transmission efficiency of *Xylella fastidiosa* by sharpshooters (Hemiptera: Cicadellidae) in coffee and citrus. J. Econ. Entomol. 101:1114-1121.

Mäser, P., S. Thomine, J.I. Schroeder, J.M. Ward, K. Hirschi, H. Sze, I.N. Talke, A. Amtmann, F.J.M. Maathuis, D. Sanders, J.F. Harper, J. Tchieu, M. Gribskov, M.W. Persans, D.E. Salt, S.A. Kim, and M.L. Guerinot. 2001. Phylogenetic relationships with cation transporter families of *Arabidopsis*. Plant Physiol. 126:1646-1667.

Mehdy, M.C. 1994. Active oxygen species in plant defense against pathogens. Plant Physiol. 105:467-472.

Mendes, B.M.J., R.L. Boscariol, F.A.A. Mourao Filho, and W.A.B. Almeida. 2002. *Agrobacterium*-mediated transformation of citrus Hamlin cultivar (*Citrus sinensis* L. Osbeck) epicotyl segments. Pesquisa Agropecuária Brasileira. 37:955-961, 2002.

Mitsuhara, I., K.A. Malik, M. Miura, and Y. Ohashi. 1999. Animal cell-death suppressors Bcl-XL and Ced-9 inhibit cell death in tobacco plants. Curr. Biol. 9:775-778.

Mittler, R. and B.A. Zilinskas. 1992. Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. J. Biol. Chem. 267: 21802-21807.

Mittler, R., X. Feng, and M. Cohen, M. 1998. Post-transcriptional suppression of cytosolic ascorbate peroxidase expression during pathogen-induced programmed cell death in tobacco. The Plant Cell 10:461-474.

Mittler, R., V. Shulaev, E. Lam, and M. Cohen. 1999. Signals controlling the expression of cytosolic ascorbate peroxidase during pathogen-induced programmed cell death in tobacco. Plant Mol. Biol. 39:1025-1035.

Molinari, H.B.C.., C.J. Marur, and J.C.B. Filho. 2004. Osmotic adjustment in transgenic citrus rootstock Carrizo citrange (*Citrus sinensis* Osb. x *Poncirus trifoliata* L. Raf.) overproducing proline. Plant Sci.6:1375-1381.

Moutinho, A., P.J. Hussey, A.J. Trewavas, and R. Malhó. 2001. cAMP acts as a second messenger in pollen tube growth and reorientation. Proc. Natl. Acad. Sci. 28:10481-10486.

Muraro, R.P. 2000. Cost benefit analysis for controlling Diaprepes, p. 105-110. In: S. H. Futch (ed.). Proc. *Diaprepes* Short Course. Univ. Fla. IFAS Coop. Ext. Serv. Lake Alfred, FL.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

NASS, National Agricultural Statistical Survey-United States Department of Agriculture. 2004. 1 Jan. 2009. <http://usda.mannlib.cornell.edu/reports/nassr /fruit/zcf-bb/>.

NASS, National Agricultural Statistical Survey-United States Department Agriculture. 2008. 1 Jan. 2009. <http://usda.mannlib.cornell.edu/usda/nass/CropValuSu//2000s /2008/CropValuSu-02-14-2008.pdf.>.

Oliveira, M.L., V.J. Febres, M.G. Costa, G.A. Moore, and W.C. Otoni. 2008. High-efficiency *Agrobacterium*-mediated transformation of citrus via sonication and vacuum infiltration. Plant Cell Rep. DOI 10.1007/s00299-008-0646-2.

Olson, E.O. 1952. Investigations of citrus rootstock diseases in Texas. Proc. Rio Grande Valley Hort. Inst. 6:46-50.

Omar, A.A., W.Y. Song, and J.W. Grosser. 2007. Introduction of Xa21, a *Xanthomonas*-resistance gene from rice, into 'Hamlin' sweet orange [*Citrus sinensis* (L.) Osbeck] using protoplast-GFP co-transformation or single plasmid transformation. J. Hort. Sci. Biotechnol. 82:914-923.

Omar, A.A., M.G.H. Dekkers, J.H. Graham, and J.W. Grosser. 2008. Estimation of transgene copy number in transformed citrus plants by quantitative multiplex real-time PCR. Biotechnol. Progress. 24:1241-1248.

Orlandi, E.W., S.W. Hutcheson, and C.J. Baker. 1992. Early physiological responses associated with race-specific recognition in soybean leaf tissue and cell suspensions treated with *Pseudomonas syringae* pv. *Glyn*. Physiol. Mol. Plant Path. 40:173-180.

Patterson, W.R., and T. L. Poulos. 1995. Crystal structure of recombinant pea cytosolic ascorbate peroxidase. Biochemistry 34: 4331-4341.

Peña, L., M. Cervera, J. Juarez, L. Navarro, J.A. Peña, N. Duran-Vila, and L. Navarro. 1995. *Agrobacterium*–mediated transformation of sweet orange and regeneration of transgenic plants. Plant Cell Rep. 14:616-619.

Peña, L., M. Cervera, J. Juarez, L. Navarro, J.A. Pina, and L. Navarro. 1997. Genetic transformation of lime (*Citrus aurantifolia*): factors affecting transformation and regeneration. Plant Cell Rep. 16:731-737.

Peña, L., M. Martín-Trillo, J. Juárez, J.A. Pina, L. Navarro, and J.M. Martínez-Zapater. 2001. Constitutive expression of Arabidopsis LEAFY or APETALA1 genes in citrus reduces their generation time. Nature Biotechnol. 19:263-267.

Peña, L., R.M. Perez, M. Cervera, J.A. Juarez, and L. Navarro. 2004. Early events in *Agrobacterium*-mediated genetic transformation of citrus explants. Ann. Bot. 94:67-74.

Qiao, J., I. Mitsuhara, Y. Yozaki, K. Sakano, Y. Gotoh, M. Miura, and Y. Ohashi. 2002. Enhanced resistance to salt, cold and wound stresses by overproduction of animal cell death suppressors Bcl-xl and Ced-9 in tobacco cells-their possible contribution through improved function of organelle. Plant Cell Physiol. 43:992-1005.

Qin, X., V.S. Miranda, M.A. Machado, E.G.M. Lemos, and J.S. Hartung. 2001. An evaluation of the genetic diversity of *Xylella fastidiosa* isolated from diseased citrus and coffee in São Paulo, Brazil. Phytopathology 91:599-605.

Rashid, H., S. Yokoi, K. Toriyama, and K. Hinata. 1996. Transgenic plant production mediated by *Agrobacterium* in *indica* rice. Plant Cell Rep.15:727-730.

Reddy, M.R.S. 1997. Sources of resistance to bacterial canker in citrus. J. Mycol. Plant Pathol. 27:80-81.

Roistacher, C.N. 1991. Graft-transmissible diseases of citrus. Handbook for detection and diagnosis. FAO, Rome, Italy. 286 p.

Roose, M.L. and T.J. Close. 2008. Genomics of citrus, a major fruit crop of tropical and subtropical regions, p. 187-200. In: Moore, P.H. and R. Ming (eds.). Genomics of tropical crop plants. Springer, Dordrecht, The Netherlands.

Sambrook, J., E.F. Fritsch, and T. Maniatis eds. 1989. Molecular cloning: A laboratory manual, 2 ed.: Cold Spring Harbor Laboratory Press, New York, NY.

Sanders, D., J. Pelloux, C. Brownlee, and J.F. Harper. 2002. Calcium at the crossroads of signaling. The Plant Cell (Suppl.) 14: S401–S417.

Sauls, J.W. 2008. The Texas citrus industry. 1 Jan 2009. <http://aggie-horticulture.tamu.edu /citrus/l2286.htm>.

Schaada, N.W., E. Postnikovaa, G. Lacy, A. Sechlera, I. Agarkovac, P.E. Stromberga, V.K. Stromberg, and A.K. Vidaverc. 2006. Emended classification of xanthomonad pathogens on citrus. Syst. Appl. Microbiol. 29:690-695.

Schouten, H.J. and E. Jacobsen. 2008. Cisgenesis and intragenesis, sisters in innovative plant breeding. Trends Plant Sci. 13:260-261.

Shigoeka, S., T. Ishikawa, M. Tamoi, Y. Miyagawa, T. Takeda, Y. Yabuta, and K. Yoshimura. 2002. Regulation and function of ascorbate peroxidase isoenzymes. J. Exp. Bot. 53: 1305-1319.

Snedden, W.A. and H. Fromm. 1998. Calmodulin, calmodulin–related proteins and plant responses to the environment. Trends Plant Sci. 3:299-304.

Stevens, H.E. 1915. Studies of citrus canker. Fla. Agric. Exp. Stn. Bull. 124:31-53.

Talke, I.N., B. Damien, J. Frans, M. Maathuis, and D. Sanders. 2003. CNGCs: prime targets of plant cyclic nucleotide signaling? Trends Plant Sci. 286-293.

Talon, M. and F.G. Gmitter Jr. 2008. Citrus genomics. Intl. J. Plant Genom. doi:10.1155/2008/528361.

Tavernier, E., D. Wendehenne, J.P. Blein, and A. Pugin. 1995. Involvement of free calcium in action of cryptogein, a proteinaceous elicitor of hypersensitive reaction in tobacco cells. Plant Physiol. 109:1025-1031.

Teixeira, F.K., L. Menezes-Benzvente, R. Margis, and M. Margis-Pinheiro. 2004. Analysis of the molecular evolutionary history of the ascorbate peroxidase gene family: Inferences from the rice genome. J. Mol. Evol. 59:761-770.

Timmer, L.W. 1974. A necrotic strain of citrus ringspot virus and its relationship to citrus psorosis virus. Phytopathology 64:389-394.

Thomsen, B., H. Drumm-Herrel, and H. Mohr. 1992. Control of the appearance of ascorbate peroxidase (EC 1.11.1.11) in mustard seedling cotyledons by phytochrome and photooxidative treatments. Planta 186:600–608.

Tsai, J.H., R.F. Lee, Y.H. Liu, and C.L. Niblett. 1999. IPM World textbook. 5 Jan 2009. <http://ipmworld.umn.edu/chapters/tsaietal.htm>.

Van Lelyveld, L.J. and S.P. van Vuuren. 1988. Peroxidase activity as a marker in greening disease of citrus for assessment of tolerance and susceptibility. J. Phytopathol. 121:357-362.

Vera-Estrella, R., B.J. Barkla, V.J. Higgins, and E. Blumwald. 1994. Plant defense response to fungal pathogens: Activation of host-plasma membrane H^{+}-ATPase by elicitor-induced enzyme dephosphorylation. Plant Physiol. 104:209-215.

Villemont, E., F. Dubois, R.S. Sangwan, G. Vasseur, Y. Bourgeois, B.S. Sangwan-Norreel. 1997. Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: Evidence of an S-phase control mechanism for T-DNA transfer. Planta 201:160–172.

Viloria, Z., D.L. Drouillard, J.H. Graham, and J.W.Grosser. 2004. Screening triploid hybrids of 'Lakeland' limequat for resistance to citrus canker. Plant Dis. 88:1056-1060.

Vögeli, U., R. Vögeli-Lange, and J. Chappell. 1992. Inhibition of phytoalexin biosynthesis in elicitor-treated tobacco cell-suspension cultures by calcium/calmodulin antagonists. Plant Physiol. 100:1369-1376.

Wilson I.D., S.J. Neill, and J.T. Hancock. 2008. Nitric oxide synthesis and signalling in plants. Plant, Cell and Environ. 31: 622-631.

Xing, T., V.J. Higgins, and E. Blumwald. 1996. Regulation of plant defense response to fungal pathogens: Two types of protein kinases in the reversible phosphorylation of the host plasma membrane H^{+}-ATPase. The Plant Cell 8:555-564.

Xu, H. and M.C. Heath. 1998. Role of calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the cowpea rust fungus. The Plant Cell 10:585-597.

Xu, P., S.J. Rogers, J. Marilyn, and M.J. Roossinck. 2004. Expression of antiapoptotic genes bcl-xL and ced-9 in tomato enhances tolerance to viral-induced necrosis and abiotic stress. Proc. Natl. Acad. Sci. 101: 15805-15810.

Yang Z.N., I.L. Ingelbrecht, E. Louzada, M. Skaria, and T.E. Mirkov. 2000. *Agrobacterium*-mediated transformation of the commercially important grapefruit cultivar Rio Red (*Citrus paradisi* Macf.). Plant Cell Rep. 19:1203-1211.

Yoshioka, K., W. Moeder, H.G. Kang, P. Kachroo, K. Masmoudi, G. Berkowitz, and D. F. Klessig. 2006. The chimeric *Arabidopsis* cyclic nucleotide-gated ion channel11/12 activates multiple pathogen resistance response. The Plant Cell 18:747-763.

Yu, C., S. Huang, C. Chen, Z. Deng, P. Ling, F.G. Gmitter Jr. 2002. Factors affecting *Agrobacterium*-mediated transformation and regeneration of sweet orange and citrange. Plant Cell, Tissue and Organ Cult. 71:147-155.

Yu, I.C., J. Parker, and A. F. Bent. 1998. Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis* dnd1 mutant. Proc. Natl. Acad. Sci. 95:7819-7824.

Zambryski, P., H. Joos, C. Genetello, J. Leemans, M. Van Montagu, and J. Schell. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J. 2:2143-2150.

Zimmermann, S., T. Nürnberger, J.M. Frachisse, W. Wirtz, J. Guern, R. Hedrich, and D. Scheel. 1997. Receptor-mediated activation of a plant Ca^{2+}-permeable ion channel involved in pathogen defense. Proc. Natl. Acad. Sci. 94: 2751-2755.

Zou, X., D. Li, X. Luo, K. Luo, and Y. Pei. 2008. An improved procedure for *Agrobacterium*-mediated transformation of trifoliate orange (*Poncirus trifoliata* L. Raf.) via indirect organogenesis. *In vitro* Cellular & Develop. Biol.-Plant. 44:169-177.

APPENDIX A

HALF-STRENGTH MS MEDIUM

Ingradient	For 1 Liter
MS basal medium with Gamborg's vitamins (Sigma)	2.2 g
pH to 5.8 with Potassium hydroxide (KOH)	

APPENDIX B

RMA MEDIUM

Ingredient	For 1 Liter
[1] MT macro nutrient stock	10 mL
[2] MT micro nutrient stock	5 mL
[3] MT vitamin stock	5 mL
[4] MT calcium stock	15 mL
[5] MT Iron stock	5 mL
[6] NAA stock	20 µL
Activated charcoal	0.5 g
Sucrose	25 g
pH to 5.8 with Potassium hydroxide (KOH)	
Agar	8 g

[1] 82.5 g NH_4NO3; 95 g KNO_3; 18.5 g $MgSO_4.7H_2O$; 7.5 g KH_2PO_4; 1 g K_2HPO_4.

[2] 0.62 g H_3BO_3; 1.68 g $MnSO_4$. H_2O; 0.86 g $ZnSO_4$. $7H_2O$; 0.083 g KI; 0.025 g $Na_2MoO_4.2H_2O$; 0.0025 g $CuSO4.5H_2O$; 0.0025 g $CoCl_2.6H_2O$.

[3] 10 g mio-inositol; 1 g thiamine-HCl; 1 g pyridoxine-HCl; 0.5 g nicotinic acid; 0.2 g glycine.

[4] 29.33 g $CaCl_2.2H_2O$

[5] 7.45 g Na_2EDTA; 5.57 g $FeSO_4.7H_20$

[6] NAA 1mg mL^{-1}

APPENDIX C

DBA3 MEDIUM

Ingredient	For 1 Liter
MS basal medium with Gamborg's vitamins	4.3 g
[1] K2HPO4 stock	1 mL
Sucrose	25 g
[2] 2,4-D stock	10 µL
[3] BAP stock	3 mL
pH to 5.8 with Potassium hydroxide (KOH)	
Malt extract	1.5 g
Coconut water	20 mL
Phytagel	3 g

[1] K_2HPO_4 2 g 100 mL^{-1}

[2] 2,4-D 1 mg mL^{-1}

[3] BAP 1 mg mL^{-1}

APPENDIX D

SHOOT ELONGATION MEDIUM (BG)

Ingredient	For 1 Liter
MS basal medium with Gamborg's vitamins	4.3 g
[1] BAP stock	0.2 mL
[2] Gibberellic acid (GA3) stock	0.5 mL
Sucrose	25 g
pH to 5.8 with Potassium hydroxide (KOH)	
Phytagel	2 g

[1] BAP 1 mg mL^{-1}

[2] GA3 1 mg mL^{-1}

VITA

Madhura Babu Kunta received his Bachelor of Science degree from Acharya NG Ranga Agricultural University (formerly, Andhra Pradesh Agricultural University, India) in 1995. In 1998, he received a postgraduate diploma in Planning and Project Management from the University of Hyderabad, India. He worked with United Phosphorus Limited and Pioneer Hi-Bred companies in India (1998-2001). He entered the Plant and Soil Science program at the Texas A&M University-Kingsville in 2001 and received his Master of Science degree in 2003. He worked at Texas A&M University and Children Nutrition Research Center, Baylor College of Medicine, Houston (2004), TAMU-Kingsville (2005-2009). His research interests include: plant diseases, disease resistance, gene expression, transgenic plants, abiotic stress, and signaling pathways in plant response to both abiotic and biotic stresses.

Madhurababu Kunta can be reached at TAMUK-Citrus Center, 312 North International Blvd, Weslaco, TX 78596, email MBKunta@ag.tamu.edu.