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Citrus x sinensis transgenic lines tolerant to citrus greening disease

Dossier in support of our request for a Regulatory Status Review as described in 7 CFR § 340.4

Submitted to:
Biotechnology Regulatory Services
Animal and Plant Health Inspection Service, USDA
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Summary

Citrus greening disease, also known as Huanglongbing or HLB, was first observed on Florida citrus in 2005. Within a few years, it spread to every citrus growing region of the state. By 2022, statewide citrus yields have declined from 242 million boxes in 2005 to an expected 16 million boxes in 2023 (1), a nearly 95% decline. The disease is caused by an insect-vectored, phloem-limited, unculturable alphaproteobacterium, *Candidatus* Liberibacter asiaticus, also referred to as CLas. Despite enormous efforts by scientists around the world, there are no solutions to the disease on the horizon other than the development and propagation of resistant or tolerant citrus lines. There are tolerant lines of citrus available, such as lime or lemon, but none of these lines do produce commercial oranges. Using those lines to develop tolerant lines by crossing with commercial citrus is underway but is far from a commercially acceptable orange.

Beginning in 2008, Professors Zhonglin Mou and Bill Dawson of the University of Florida collaborated on the construction and efficacy testing of many citrus lines transformed with the *Arabidopsis thaliana NPR1* (*AtNPR1*) plant defense gene by *Agrobacterium*-mediated transformation. Over years of testing in the greenhouse and the field, the number of efficacious lines has been narrowed to six which have the highest level of tolerance to disease over four years in the field, have high levels of the AtNPR1 protein in leaves, and are producing fruit. The genomes of all six lines are now being sequenced to identify the insertion sites of the transfer DNA (T-DNA) from the pBI1.4T-*AtNPR1* vector. These insertion sites will be used to define each line.

All six lines are tolerant to HLB in the field with few, if any, symptoms. However, they are not resistant as all lines are infected by CLas. The primary objective is sufficient tolerance to allow reasonable yields with high fruit quality.

Abbreviations

AtNPRI	*NPR1* gene from *Arabidopsis thaliana*
AtNPRI	NPR1 protein from *Arabidopsis thaliana*
pBI1.4T	plant binary vector used for transformation
pBI1.4T-*AtNPR1*	*AtNPR1* cloned between CaMV 35S promoter and NOS terminator
BLAST	basic local alignment search tool
CaMV 35S	constitutive promoter that drives expression of the transgene
T-DNA	transfer DNA
CLas	*Candidatus* Liberibacter asiaticus, causal agent of citrus greening disease
CREC	University of Florida's Citrus Research and Education Center
HLB	Huanglongbing, Chinese name for citrus greening disease
IFAS	University of Florida's Institute of Food and Agricultural Sciences
MCS	University of Florida's Microbiology and Cell Science Department
NGS	next generation sequencing
NOS	nopaline synthase terminator
NPRI	*NONEXPRESSOR OF PATHOGENESIS-RELATED GENE1*
NPRI	NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEIN1
nptII	*E. coli* neomycin phosphotransferase II - antibiotic resistance marker
NOS promoter	nopaline synthase promoter from *Agrobacterium tumefaciens*
NOS terminator	nopaline synthase terminator from *Agrobacterium tumefaciens*
ONT	Oxford Nanopore sequencing technology
PR	*pathogenesis-related* genes in plants
qPCR	quantitative polymerase chain reaction
RSR	Regulatory Status Review
SA	salicylic acid
SAR	systemic acquired resistance
UF	University of Florida

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1. General Information

1.1 Applicant Details

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1.2 Confidential Information

There is no confidential business information in this dossier.

1.3 Brief Identification of the Modified Plant

Designations: 13-3, 13-29, 24-25, 26-36, 35-30
Plant Species: Citrus x sinensis cv. Hamlin
Phenotype: Tolerance to citrus greening disease under field conditions.
Introduced genes: *AtNPR1*, *nptII*

1.4 Purpose of the Application

This dossier is a request for a Regulatory Status Review (RSR) as described in 7 CFR § 340.4.

1.5 Development of HLB Tolerant Citrus Lines

The construction of the vector used for citrus transformation, pBI1.4T-*AtNPR1*, is described in Zhang et al. (2). The transformation of the Hamlin citrus lines with pBI1.4T-*AtNPR1* was done as described by Orbović and Grosser (3). The only selectable marker in the transgenic plants is the *neomycin phosphotransferase II* (*nptII*) gene from *E. coli*. This gene is on the International Service for the Acquisition of Agri-biotech Applications (ISAAA) database of genetic modifications approved for commercial use (4). The T-DNA insert also includes the *Arabidopsis thaliana NPR1* (*AtNPR1*) gene that codes for a protein that regulates host defense responses in *A thaliana*.

2. Comparator plant

OECD International standards for citrus fruits were published in 2010 (5). The genus *Citrus* is in the Rutaceae family and includes many citrus crops including sweet orange (*Citrus* × *sinensis*), grapefruit (*Citrus* × *paradisi*), kumquat (*Citrus japonica*), key lime (*Citrus* × *aurantiifolia*), lemon (*Citrus* × *limon*), and others. *Poncirus* (trifoliate orange) is a closely related genus. *Citrus* x *sinensis* (also referred to as *Citrus sinensis*) is a hybrid between pomelo (*Citrus maxima*) and mandarin (*Citrus reticulata*). The common name is sweet orange. The diploid genome of *Citrus* x *sinensis* has 18 chromosomes and a genome size of 367 Mb.

3. Genotype of the Modified Plant

Each of the six transgenic lines was transformed with the 15,127 bp pBI1.4T-*AtNPR1* vector (the 6,527 bp T-DNA region sequence is shown in A4 below) using *Agrobacterium* transformation using the methods described previously (2,3). The T-DNA insertion sequence contains two genes, *nptII* and *AtNPR1*. They are constitutively expressed from a NOS promoter and a 35S CaMV promoter, respectively. The gene and protein sequences of both genes are shown in Appendices A and B. The *nptII* gene confers selection of transformants using kanamycin.

NPR1 is a receptor of salicylic acid (SA) and the key transcription coactivator of systemic acquired resistance (SAR). Overexpression of the *AtNPR1* gene in tomato conferred significantly enhanced resistance to bacterial wilt, Fusarium wilt, gray leaf spot, and bacterial spot (6). Importantly, overexpression of *AtNPR1* did not affect tomato overall morphology and horticultural traits in multiple generations (6).

In 1979, SA injection into leaves was shown to reduce tobacco mosaic virus-induced lesions by at least 90% in three tobacco cultivars (7). Since that time, extensive studies have been carried out on this iterating small molecule (8). SA is now recognized as a crucial plant hormone for the induction of plant defenses and the reduction in the disease in plants upon infection (9). Following a period of controversy, the NPR1 protein is now accepted as the receptor for SA that begins the cascade of NPR1's roles in plant defense (10,11). NPR3 and NPR4 are also SA receptors and play a role in ubiquitin-driven NPR1 degradation and suppression of defense gene transcription (10,12). Hence, the ability of SA to bind to all three proteins at different SA concentrations regulates the strength of plant defense responses (10).

Development of New Disease-Resistant Crop Lines Based on the SA Signaling Pathway
Transgenic approaches:

Overexpression of *NPR1* has been shown to be effective in many crop plants for improving disease resistance to bacterial and fungal pathogens (13). The most common means of overexpression is done by transformation of the host using a constitutively expressed *NPR1* gene from *A. thaliana* (13). Other investigators have done this in several crops including (but not limited to): rice (14), citrus (2,15,16), oil seed (17), cotton (18), strawberry (19), apple (20,21), potato (22), and tomato (6).

4. The Intended Trait and Mechanism of Action

4.1 Intended Trait

The intended trait is citrus tolerance to citrus greening disease. Although the *AtNPR1*-transformed citrus plants will still be infected by the disease-causing *Candidatus* Liberibacter asiaticus (CLas), the symptoms will be mild enough to allow production of an economically viable crop. Leaves will have far less mottling. Fruit drop will be significantly curtailed. Tree canopy will be broad and healthy. The roots will remain healthy compared to non-transformed plants.

4.2 Intended Phenotype

The intended phenotype will be citrus plants healthy enough to produce an economically viable crop under conditions of high disease pressure from psyllid-vectored CLas.

Genetically engineered (GE) crops with increased plant defense responses have been under development since 1991 (23-27). NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1) is a key regulator in plant defense pathways but is expressed at a low basal level (28, 29). Shortly after its cloning, there were many reports of overexpression of the *NPR1* gene in

multiple plant species resulting in improved plant disease resistance (6,30-32). In these cases, the *NPR1* gene is typically driven by the 35S cauliflower mosaic virus (CMV) promoter. The resulting cassette is then cloned into an *Agrobacterium* binary Ti-plasmid containing the transfer DNA (T-DNA) for transformation into the host. This was done in tomato with the transgenic plants having strong resistance to several bacterial and fungal pathogens (6).

Improved plant disease resistance through *NPR1*-overexpression has now been accomplished in many hosts including apple, canola, carrot, citrus, cotton, crabapple, mustard, peanut, potato, rice, soybean, tomato, and wheat (13). However, none of these have been commercialized. Part of the reluctance to commercialize is based on unfounded public perceptions of GE-derived products and part on the concerns of unintended non-target effects of T-DNA transformation.

4.3 Mechanism of Action

NPRI protein is a positive regulator of SAR in plants. When induced by the action of NPR1, SAR is both broad-spectrum against pathogens and long-lasting.

The *NPR1* gene expression is typically induced by SA, the hormonal trigger of SAR. SA-induction of *NPR1* is often enough to induce the cascade of events caused by NPR1 interaction with transcription factors that bind the promoters of *PR* (*pathogenesis-related*) genes. This, in turn, upregulates *PR* genes whose products confer resistance to pathogen infection (13). Under low disease pressure, SAR works well, but under high disease pressure (such as exists with citrus greening disease in Florida) infection pressure is so high that the normal levels of NPRI production are insufficient to cope with multitude of infection events across time. Hence, many investigators have studied whether constitutive expression of *NPR1* could confer sufficient disease protection when needed most (2,6,10,23-30). Improved plant disease resistance through *NPR1*-overexpression has now been accomplished in many hosts including apple, canola, carrot, citrus, cotton, crabapple, mustard, peanut, potato, rice, soybean, tomato, and wheat (see Table 1 in reference 13).

4.4 *nptII*, selectable marker used in transformation: trait, phenotype, and mechanism of action.

The *nptII* gene is from *E. coli* and codes for neomycin phosphotransferase II. - antibiotic resistance marker NPTII: trait, phenotype, and mechanism of action. This protein inactivates aminoglycoside antibiotics, such as neomycin and kanamcyin, by phosphorylating an hydroxyl group on the aminoglycoside. The *nptII* gene provides kanamycin tolerance in transformed tissue allowing for growth on media containing kanamycin. Hence, kanamycin selects against untransformed tissue.

5. Concluding Comments.

The AtNPR1 protein is a plant defense regulatory protein and doesn't directly kill plant pathogens. The AtNPR1 protein is not a toxin and is digested into nutritious amino acids in the stomach. AtNPR1 acts through transcription factors to enhance plant defenses. Those transcription factors have no homologs in humans.

Based on allergen assessments using weballergen (http://weballergen.bii.a-star.edu.sg/) and allermatch (http://www.allermatch.org/), we have no evidence that the AtNPR1 protein is allergenic.

Duncan citrus is self-pollinated. Bee pollination is rarely used in commercial citrus production. Citrus is also not propagated from seed but from grafting.

As AtNPR1 protein does not kill the pathogen, there is likely little or no selection pressure for resistance. The *AtNPR1* transformed lines reported are still infected by CLas but show greatly reduced symptoms. Hence, these lines are tolerant, not resistant.

Citrus plants are only considered weeds where groves are abandoned. Citrus is rarely, if ever, a weed in commercial agriculture.

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Appendix A Gene Sequences

A.1 *nptII*

ATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGAC
TGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGT
TCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTG
GCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGC
TGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCAT
CATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAA
ACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGA
GCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGATGATC
TCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCAT
CGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGA
AGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCG
CATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA

A.2 *AtNPR1*

ATCGGAACCTGTTGATGGACACCACCATTGATGGATTCGCCGATTCTTATGAAATCAGCAGCACTAGTTT
CGTCGCTACCGATAACACCGACTCCTCTATTGTTTATCTGGCCGCCGAACAAGTACTCACCGGACCTGAT
GTATCTGCTCTGCAATTGCTCTCCAACAGCTTCGAATCCGTCTTTGACTCGCCGGATGATTTCTACAGCG
ACGCTAAGCTTGTTCTCTCCGACGGCCGGGAAGTTTCTTTCCACCGGTGCGTTTTGTCAGCGAGAAGCT
CTTTCTTCAAGAGCGCTTTAGCCGCCGCTAAGAAGGAGAAAGACTCCAACAACACCGCCGCCGTGAAG
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AACGTCTTTTTCCAACGGAAGCACAAGCTGCAATGGAGATCGCCGAAATGAAGGGAACATGTGAGTTC
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TTGGCGTCATATAGTTTCGCTCTTCGTTTTGCATCCTGTGTATTATTGCTGCAGGTGTGCTTCAAACAAAT
GTTGTAACAATTTGAACCAATGGTATACAGATTTGTAA

A.3 *Citrus sinensis NPR1*

ATGGATAATAGAAATGGGTTCTCGGATTCAAACGAGATCAGTAACAACAGCCGCACCAGCTGTGTAGC
TGCAGCAGCAAATACTGAGTCTTTCTATTCATCAGAACCTGTAAACTCTGACATCACAGCTCTTCGAAT
TCTCTCAAAGACCCTCGAAACAATCTTTGAATCTCAGGATTTTGACTACTTTACAGATGCCAAGATCGT
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TGTATTTGCTGGGACTGGAAAACAGAGAGGACCCAAGTTTGAGCTCAAGGAGTTAGTGAGGGATTATG
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GGTGGAGGTTCTTTATGTGTCTTTTGCTTTTCAGGTTCCAGAGTTGGTGGCTCTTTATCAGAGGCACCTC
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GATGTTGAATTAGTCAGAATGCTATTGAAAGAGGCTCATACTAATCTAGATGACGCACATGCACTTCA
CTATGCCGTGGCATATTGTGATGCAAAGACCACAACTGAGCTTCTTGATCTTGGACTTGCTGATGTCAA
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TCAAAGCGGCTCACTAAGGCTGCAGATTACTATATTCCCACTGAGGAAGGAAAAACAACCCCAAAAG
ATCGGTTATGCATAGAGATATTGGAACAAGCTGAAAGAAGAGATCCCTTGCTCAGAGAAGCTTCTCAT
TCTTTTGCTATGGCTGGTGATGATCTTCGGATGAAACTGCTGTACCTTGAAAACAGAGTTGGACTGGCT
AAACTTCTGTTTCCTATGGAAGCAAAAGTCATAATGGATATTGTCCATCTTGATGGCACTTTGGAGTTT
GCATTAGATGGCATCAAAACTAAGAAAATGGCCGGTGCCCAGAGGACAACTGTGGACTTGAATGAAG
CACCTTTCAAAATGCAAGAGGAGCATCTAAACAGAATGAAAGCACTCTGTAGAACTGTGGAGCTGGG
GAAACGTTTTTTCCCTCGTTGTTCAGAAGTACTTAACAAGATAATGGATGCCGATGACTTAAATCAGCT
AGCATGTCCGGGGAACGATACTCCAGAAGAGCGACTCCTGAAACGAATAAGGTACATGGAACTTCAA
GAAGTTGTAAGTAAGGCGTTTAATGAGGATAAAGAAGAGTTTGATAGGTCTGCTATATCATCTTCTTC
ATCATCAAAATCAGTTGTGAGGCCTCGTGGGGGTAAAAGAACTCACTGA

A.4 Annotated sequence of the T-DNA region of the pBI1.4T-*AtNPR1* construct

>T-DNA_ right border (RB) of the T-DNA from *Agrobacterium tumefaciens* - initiates the transfer of the T-DNA, bases 1-25
GTTTAAACTGAAGGCGGGAAACGAC

>synthetic - intervening sequence, bases 26-30
AATCT

>nopaline synthase (NOS) promoter from *Agrobacterium tumefaciens* - activates transcription of the *nptII* gene, bases 31-349
GATCATGAGCGGAGAATTAAGGGAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTAC
GTTTGGAACTGACAGAACCGCAACGTTGAAGGAGCCACTCAGCCGCGGGTTTCTGGAGTTTAATGAGC
TAAGCACATACGTCAGAAACCATTATTGCGCGTTCAAAAGTCGCCTAAGGTCACTATCAGCTAGCAAATA
TTTCTTGTCAAAAATGCTCCACTGACGTTCCATAAATTCCCCTCGGTATCCAATTAGAGTCTCATATTCAC
TCTCAATCCAAATAATCTGCACCGGATCTGGATCGTTTCGC

>*nptII* gene, bases 350-1144
ATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGAC
TGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGT
TCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTG
GCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGC
TGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCAT
CATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAA
ACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGA
GCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGATGATC
TCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCAT
CGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGA
AGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCG
CATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA

>synthetic - intervening sequence bases 1145-2485
GCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCC
ACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAG
CGCGGGGATCTCATGCTGGAGTTCTTCGCCCACGGGATCTCTGCGGAACAGGCGGTCGAAGGTGCCGAT
ATCATTACGACAGCAACGGCCGACAAGCACAACGCCACGATCCTGAGCGACAATATGATCGGGCCCGG
CGTCCACATCAACGGCGTCGGCGGCGACTGCCCAGGCAAGACCGAGATGCACCGCGATATCTTGCTGC
GTTCGGATATTTTCGTGGAGTTCCCGCCACAGACCCGGATGATCCCCGATCGTTCAAACATTTGGCAATA
AAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAA
GCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATT
ATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCA
TCTATGTTACTAGATCGGGCCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTCT
GAGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGAGGCGGTTCCGGTGGTG
GCTCTGGTTCCGGTGATTTTGATTATGAAAAGATGGCAAACGCTAATAAGGGGGCTATGACCGAAAATG
CCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTACGGTGCTG
CTATCGATGGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATTTTGCTGGC
TCTAATTCCCAAATGGCTCAAGTCGGTGACGGTGATAATTCACCTTTAATGAATAATTTCCGTCAATATTT
ACCTTCCCTCCCTCAATCGGTTGAATGTCGCCCTTTTGTCTTTGGCCCAATACGCAAACCGCCTCTCCCC
GCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCG
CAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGT
ATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAG
CTTGCATGCCTGCAGGTCCCC

>35S promoter from Cauliflower mosaic caulimovirus - activates transcription of the *AtNPR1* gene, bases 2486-3503
AGATTAGCCTTTTCAATTTCAGAAAGAATGCTAACCCACAGATGGTTAGAGAGGCTTACGCAGCAGGTC
TCATCAAGACGATCTACCCGAGCAATAATCTCCAGGAAATCAAATACCTTCCCAAGAAGGTTAAAGATG
CAGTCAAAAGATTCAGGACTAACTGCATCAAGAACACAGAGAAAGATATATTTCTCAAGATCAGAAGTA
CTATTCCAGTATGGACGATTCAAGGCTTGCTTCACAAACCAAGGCAAGTAATAGAGATTGGAGTCTCTA
AAAAGGTAGTTCCCACTGAATCAAAGGCCATGGAGTCAAAGATTCAAATAGAGGACCTAACAGAACTC
GCCGTAAAGACTGGCGAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAAATCTTCGTC
AACATGGTGGAGCACGACACACTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAG
GGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGT
CACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAG
GCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGT
GGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAG
GGATGACGCACAATAGGAACAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTG
TCTAGCCCCTTCCTCTATATAAGGAAGTTAACTTCATTTGGAGAGGACACGGGGGACTCTAGATAATACG
ACTCACTATAGGGAGAGTTTAAACGTCGACGAATTCGGCACGAGTCGATCTTTAACCAAATCCAGTTGA
TAAGGTCTCTTCGTTGATTAGCAGAGATCTCTTTAATTTGTGAATTTCAATTC

> *AtNPR1* full-length gene from *Arabidopsis thaliana* – encodes for a transcription coactivator that activates defense gene transcription by interacting with transcription factors such as TGA factors (**this is the mechanism**) **Trait: disease tolerance, phenotype: grow and produce normally in the presence of the bacterial pathogen *C*Las**, bases 3504-5487
ATCGGAACCTGTTGATGGACACCACCATTGATGGATTCGCCGATTCTTATGAAATCAGCAGCACTAGTTT
CGTCGCTACCGATAACACCGACTCCTCTATTGTTTATCTGGCCGCCGAACAAGTACTCACCGGACCTGAT
GTATCTGCTCTGCAATTGCTCTCCAACAGCTTCGAATCCGTCTTTGACTCGCCGGATGATTTCTACAGCG
ACGCTAAGCTTGTTCTCTCCGACGGCCGGGAAGTTTCTTTCCACCGGTGCGTTTTGTCAGCGAGAAGCT
CTTTCTTCAAGAGCGCTTTAGCCGCCGCTAAGAAGGAGAAAGACTCCAACAACACCGCCGCCGTGAAG
CTCGAGCTTAAGGAGATTGCCAAGGATTACGAAGTCGGTTTCGATTCGGTTGTGACTGTTTTGGCTTATG
TTTACAGCAGCAGAGTGAGACCGCCGCCTAAAGGAGTTTCTGAATGCGCAGACGAGAATTGCTGCCAC
GTGGCTTGCCGGCCGGCGGTGGATTTCATGTTGGAGGTTCTCTATTTGGCTTTCATCTTCAAGATCCCTG
AATTAATTACTCTCTATCAGAGGCACTTATTGGACGTTGTAGACAAAGTTGTTATAGAGGACACATTGGT
TATACTCAAGCTTGCTAATATATGTGGTAAAGCTTGTATGAAGCTATTGGATAGATGTAAAGAGATTATTGT
CAAGTCTAATGTAGATATGGTTAGTCTTGAAAAGTCATTGCCGGAAGAGCTTGTTAAAGAGATAATTGAT
AGACGTAAAGAGCTTGGTTTGGAGGTACCTAAAGTAAAGAAACATGTCTCGAATGTACATAAGGCACTT
GACTCGGATGATATTGAGTTAGTCAAGTTGCTTTTGAAAGAGGATCACACCAATCTAGATGATGCGTGTG

CTCTTCATTTCGCTGTTGCATATTGCAATGTGAAGACCGCAACAGATCTTTTAAAACTTGATCTTGCCGAT
GTCAACCATAGGAATCCGAGGGGATATACGGTGCTTCATGTTGCTGCGATGCGGAAGGAGCCACAATTG
ATACTATCTCTATTGGAAAAAGGTGCAAGTGCATCAGAAGCAACTTTGGAAGGTAGAACCGCACTCATG
ATCGCAAAACAAGCCACTATGGCGGTTGAATGTAATAATATCCCGGAGCAATGCAAGCATTCTCTCAAAG
GCCGACTATGTGTAGAAATACTAGAGCAAGAAGACAAACGAGAACAAATTCCTAGAGATGTTCCTCCCT
CTTTTGCAGTGGCGGCCGATGAATTGAAGATGACGCTGCTCGATCTTGAAAATAGAGTTGCACTTGCTC
AACGTCTTTTTCCAACGGAAGCACAAGCTGCAATGGAGATCGCCGAAATGAAGGGAACATGTGAGTTC
ATAGTGACTAGCCTCGAGCCTGACCGTCTCACTGGTACGAAGAGAACATCACCGGGTGTAAAGATAGCA
CCTTTCAGAATCCTAGAAGAGCATCAAAGTAGACTAAAAGCGCTTTCTAAAACCGTGGAACTCGGGAA
ACGATTCTTCCCGCGCTGTTCGGCAGTGCTCGACCAGATTATGAACTGTGAGGACTTGACTCAACTGGC
TTGCGGAGAAGACGACACTGCTGAGAAACGACTACAAAAGAAGCAAAGGTACATGGAAATACAAGAG
ACACTAAAGAAGGCCTTTAGTGAGGACAATTTGGAATTAGGAAATTCGTCCCTGACAGATTCGACTTCT
TCCACATCGAAATCAACCGGTGGAAAGAGGTCTAACCGTAAACTCTCTCATCGTCGTCGGTGAGACTCT
TGCCTCTTAGTGTAATTTTTGCTGTACCATATAATTCTGTTTTCATGATGACTGTAACTGTTTATGTCTATCG
TTGGCGTCATATAGTTTCGCTCTTCGTTTTGCATCCTGTGTATTATTGCTGCAGGTGTGCTTCAAACAAAT
GTTGTAACAATTTGAACCAATGGTATACAGATTTGTAA

> NOS terminator from *Agrobacterium tumefaciens* – terminates transcription of the *AtNPR1* gene, bases 5488-5785
TATATATTTATGTACATCAACAATAAAAAAAAAAAAAAAGGGCGGCCGCCACCGCGGTGGAGCTCCAGC
TTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGT
TTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCAT
GTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATAC
ATTTAATACGCGATAG

>synthetic - intervening sequence, bases 5786-6502
AAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGA
ATTGGCCCAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTA
ATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTT
CCCAACAGTTGCGCAGCCTGAATGGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCG
CCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCT
CGACCCCAAAAAACTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCG
CCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCC
TATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGAACCACCATCAAACAGGATTTTCG
CCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATC
AGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCCAGTACATTAAAAACGTCCGCAATGTGTTA
TTAAGTTGTCTAAGCGTCAATTT

> left border (LB) of the T-DNA from *Agrobacterium tumefaciens* - terminates the transfer of the T-DNA, bases 6503-6527
GTTTACACCACAATATATCCTGCCA

Appendix B. Protein sequences

B.1 nptII

MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQDEAARLSW
LATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADAMRRLHTLDPATCPFDHQAK
HRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARMPDGDDLVVTHGDACLPNIMVENGRFSGFID
CGRLGVADRYQDIALATRDIAEELGGEWADRFLVLYGIAAPDSQRIAFYRLLDEFF

B.2 AtNPR1

MDTTIDGFADSYEISSTSFVATDNTDSSIVYLAAEQVLTGPDVSALQLLSNSFESVFDSPDDFYSDAKLVLSD
GREVSFHRCVLSARSSFFKSALAAAKKEKDSNNTAAVKLELKEIAKDYEVGFDSVVTVLAYVYSSRVRPPP

KGVSECADENCCHVACRPAVDFMLEVLYLAFIFKIPELITLYQRHLLDVVDKVVIEDTLVILKLANICGKAC
MKLLDRCKEIIVKSNVDMVSLEKSLPEELVKEIIDRRKELGLEVPKVKKHVSNVHKALDSDDIELVKLLLKE
DHTNLDDACALHFAVAYCNVKTATDLLKLDLADVNHRNPRGYTVLHVAAMRKEPQLILSLLEKGASASE
ATLEGRTALMIAKQATMAVECNNIPEQCKHSLKGRLCVEILEQEDKREQIPRDVPPSFAVAADELKMTLLD
LENRVALAQRLFPTEAQAAMEIAEMKGTCEFIVTSLEPDRLTGTKRTSPGVKIAPFRILEEHQSRLKALSKTV
ELGKRFFPRCSAVLDQIMNCEDLTQLACGEDDTAEKRLQKKQRYMEIQETLKKAFSEDNLELGNSSLTDST
SSTSKSTGGKRSNRKLSHRRR

B.3 *Citrus sinensis* NPR1

MDNRNGFSDSNEISNNSRTSCVAAAANTESFYSSEPVNSDITALRILSKTLETIFESQDFDYFTDAKIVLSTGR
EVPVHRCILSSRSGFFKNVFAGTGKQRGPKFELKELVRDYEVGFDPLVAVLAYLYCGKVRPFPIGVCVCVD
DDACSHVACRPAVDFMVEVLYVSFAFQVPELVALYQRHLLDILDKVVADDILVVLSVAHMCGKACEKLL
ERCIEITVKSDIDIVTLDKTLPQHIVKQIIDLRVELSLHRSESCGFPDKHTKRIHRALDSDDVELVRMLLKEAH
TNLDDAHALHYAVAYCDAKTTTELLDLGLADVNHRNSRGYTVLHVAAMRKEPKIIVSLLTKGARPSDLTL
DGRKALQISKRLTKAADYYIPTEEGKTTPKDRLCIEILEQAERRDPLLREASHSFAMAGDDLRMKLLYLENR
VGLAKLLFPMEAKVIMDIVHLDGTLEFALDGIKTKKMAGAQRTTVDLNEAPFKMQEEHLNRMKALCRTV
ELGKRFFPRCSEVLNKIMDADDLNQLACPGNDTPEERLLKRIRYMELQEVVSKAFNEDKEEFDRSAISSSSS
SKSVVRPRGGKRTH

Appendix C. Protein Phylogenetic Tree

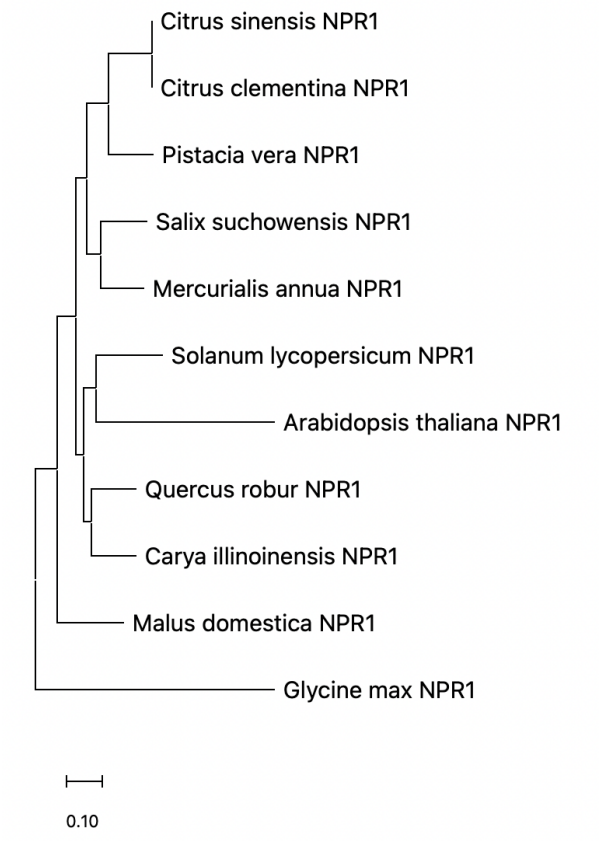

Figure A1. A phylogenetic tree of the NPR1 proteins in several plant species.

Appendix D. Insertion Sites of Transgenes

Citrus DNA was isolated from the leaves of the transgenic lines below. For each citrus line, the DNA was sequenced using the Oxford Nanopore GridION device using 10.4.1 flow cells. Summary statistics are below in Table C1. The vector insert is inserted into precisely the same location of the heme oxygenase gene in the 26-36 and 35-30 Hamlin lines. Hence, these two lines are likely clonal. Both lines will continue to be studies as they serve as useful controls for each other.

Table A1. Summary of genome sequencing assemblies for the citrus genomes. Either one or two flow cells were used for each line. Those with AB are assemblies from two flow cells while A and B individually are based on one flow cell. The number of assembled bases and contigs resulting from the assemblies are listed. The fold-coverage is based on a genome size of 367 Mb for *Citrus sinensis*. BUSCO scores above 95% are considered very good assemblies for eukaryotic organisms with reference genomes (31).

Transgenic line/cultivar	assembly	assembled Gb	fold-coverage	no. contigs	% BUSCO	N50 (kb)
13-3 Hamlin	A	26.79	73.0	21,390	97.9	45
13-29 Hamlin	B	26.47	72.1	22,568	97.6	49
24-25 Hamlin	AB	33.23	90.5	11,505	97.7	103
26-36 Hamlin	AB	32.19	87.7	14,421	98.0	82
35-30 Hamlin	C	29.36	19.0	22,588	97.9	48

Table A2. Insertion sites of the AtNPR1 vector in each transgenic line.

Line/cultivar	contig	5'-end GeneID	3'end GeneID	Chromosome no.*
13-3 Hamlin	41423	LOC102610913	LOC102610603	6
13-29 Hamlin	5513	LOC107174913	LOC102631049	3 or 1
24-25 Hamlin	7819	LOC102624668	LOC18039990	5
26-36 Hamlin	24827	LOC102628729	LOC102628729	1
35-30 Hamlin	9083	LOC102628729	LOC102628729	1

*As no chromosome scaffolds are based on Hamlin citrus, the chromosomal locations of the vector inserts in those line cannot yet be definitively determined.

Table A3. Functions of proteins identified on either side of the pBI1.4T-*AtNPR1* sequence in each *AtNPR1* transgenic line.

GeneID	Line/Cultivar	Function
LOC102610913	13-3 Hamlin	zinc transporter 5
LOC102610603	13-3 Hamlin	transcription factor bHLH75
LOC107174913	13-29 Hamlin	uncharacterized protein
LOC102631049	13-29 Hamlin	12-oxophytodienoate reductase 3
LOC102624668	24-25 Hamlin	sterol 3-beta-glucosyltransferase UGT80B1-like
LOC102628729	35-30 Hamlin	heme oxygenase 1 chloroplastic
LOC102628729	26-36 Hamlin	heme oxygenase 1 chloroplastic
LOC18039990	24-25 Hamlin	protein ROS1

Appendix E. TGA transcription factor protein sequences that interact with NPR1. There are no homologs in humans.

Arabidopsis thaliana

TGA2 (At5g06950.1)
MADTSPRTDVSTDDDTDHPDLGSEGALVNTAASDSSDRSKGKMDQKTLRRLAQNREAARKSRLRKKAYV
QQLENSRLKLTQLEQELQRARQQGVFISGTGDQAHSTGGNGALAFDAEHSRWLEEKNKQMNELRSALNA
HAGDSELRIIVDGVMAHYEELFRIKSNAAKNDVFHLLSGMWKTPAERCFLWLGGFRSSELLKLLANQLEPM
TERQLMGINNLQQTSQQAEDALSQGMESLQQSLADTLSSGTLGSSSSGNVASYMGQMAMAMGKLGTLEG
FIRQADNLRLQTLQQMIRVLTTRQSARALLAIHDYFSRLRALSSLWLARPRE

TGA3 (At1g22070.1)
MEMMSSSSSTTQVVSFRDMGMYEPFQQLSGWESPFKSDINNITSNQNNNQSSSTTLEVDARPEADDNNRV
NYTSVYNNSLEAEPSSNNDQDEDRINDKMKRRLAQNREAARKSRLRKKAHVQQLEESRLKLSQLEQELVR
ARQQGLCVRNSSDTSYLGPAGNMNSGIAAFEMEYTHWLEEQNRRVSEIRTALQAHIGDIELKMLVDSCLNH
YANLFRMKADAAKADVFFLMSGMWRTSTERFFQWIGGFRPSELLNVVMPYVEPLTDQQLLEVRNLQQSSQ
QAEEALSQGLDKLQQGLVESIAIQIKVVESVNHGAPMASAMENLQALESFVNQADHLRQQTLQQMSKILTT
RQAARGLLALGEYFHRLRALSSLWAARPREHT

TGA5 (At5g06960.1)
MGDTSPRTSVSTDGDTDHNNLMFDEGHLGIGASDSSDRSKSKMDQKTLRRLAQNREAARKSRLRKKAYV
QQLENSRLKLTQLEQELQRARQQGVFISSSGDQAHSTAGDGAMAFDVEYRRWQEDKNRQMKELSSAIDSH
ATDSELRIIVDGVIAHYEELYRIKGNAAKSDVFHLLSGMWKTPAERCFLWLGGFRSSELLKLIASQLEPLTEQ
QSLDINNLQQSSQQAEDALSQGMDNLQQSLADTLSSGTLGSSSSGNVASYMGQMAMAMGKLGTLEGFIRQ
ADNLRLQTYQQMVRLLTTRQSARALLAVHNYTLRLRALSSLWLARPRE

TGA6 (At3g12250.1)
MADTSSRTDVSTDGDTDHRDLGFYYLYNVTPGRLVPESLGKTWGILPSDRGHMHAAASDSSDRSKDKLDQ
KTLRRLAQNREAARKSRLRKKAYVQQLENSRLKLTQLEQELQRARQQGVFISSSGDQAHSTGGNGALAFD
AEHSRWLEEKNRQMNELRSALNAHAGDTELRIIVDGVMAHYEELFRIKSNAAKNDVFHLLSGMWKTPAER
CFLWLGGFRSSELLKLLANQLEPMTERQVMGINSLQQTSQQAEDALSQGMESLQQSLADTLSSGTLGSSSS
DNVASYMGQMAMAMGQLGTLEGFIRQADNLRLQTLQQMLRVLTTRQSARALLAIHDYSSRLRALSSLWL
ARPRE

Citrus sinensis

TGA2 (orange1.1g045786m, 82% identical and 86% similar to the *Arabidopsis* homolog)
MADASPRTDISTDADTDEKNQRFDRGQSTAVVASDSSDRSKDKLDQKTLRRLAQNREAARKSRLRKKAY
VQQLESSRLKLTQLEQELQRARQQGIFISSSGDQAHSMSGNGAMAFDVEYARWLEEQNKQINELRSAVNS
HASDTELRMVVDGIMAHYDEIFRLKANAAKADVFHLLSGMWKTPAERCFMWLGGFRSSELLKLLVNQLE
PLTEQQLVGIGNLQQSSQQAEDALSQGMEALQQSLAETLSSGSLGSSGSGNVANYMGQMAMAMGKLGTL
EGFIRQADNLRQQTLQQMHRILTTRQSARALLAIHDYFSRLRALSSLWMARPRE

TGA3 (orange1.1g036441m, 62% identical and 67% similar to the *Arabidopsis* homolog)
MSSPSSQLATLRRMSIYEPFHQISMWGDTFHGDASPNTGSSTIVQVDTRLDNQTEYLSHNSVEPSRSDQEAN
KPSEKTQRRLAQNREAARKSRLRKKAYVQQLESSRLKLAQLEQELDRARRQGIYTGSTSDGSHFGLSGNIN
PGITAFEMEYSHWVEEQNRQIYELRNALQKHITDIELRILVENGLNHYNNLFRMKADAAKADVLSLISGMW
RTSTERFFQWIGGFRPSELLNILMPQLEPLTEQQLIDVCNLRQSSQQAEDALQQGIDKLQQSLVQIITEEQLSS
GIYQSQMVAAAEKLEALESFVNQADHLRQQTMQQMYRVLTTRQAARALLALGEYFHRLRALSSLWAAQ
HLE

TGA5 (orange1.1g045786m, 79% identical and 85% similar to the *Arabidopsis* homolog)
MADASPRTDISTDADTDEKNQRFDRGQSTAVVASDSSDRSKDKLDQKTLRRLAQNREAARKSRLRKKAYV
QQLESSRLKLTQLEQELQRARQQGIFISSSGDQAHSMSGNGAMAFDVEYARWLEEQNKQINELRSAVNSHA
SDTELRMVVDGIMAHYDEIFRLKANAAKADVFHLLSGMWKTPAERCFMWLGGFRSSELLKLLVNQLEPLT
EQQLVGIGNLQQSSQQAEDALSQGMEALQQSLAETLSSGSLGSSGSGNVANYMGQMAMAMGKLGTLEGFI
RQADNLRQQTLQQMHRILTTRQSARALLAIHDYFSRLRALSSLWMARPRE

TGA6 (orange1.1g045786m, 78% identical and 82% similar to the *Arabidopsis* homolog)
MERGQLTAVAASDSSDKSKEKSGDQKTLRRLAQNREAARKSRLRKKAYVQQLESSRLKLTQLEQELQRAR
QQGIFISSSGDQSHSMSGNGAAAFDVEYSRWLEEHNRHIVELRAAVNSHAGDTELRTIVDNVTSHFDEIFRL
KGIASKADVFHILSGMWKTPAERCFMWIGGFRSSELLKLLVNQLEPLTEQQLVGIYNLQQSSQQAEDALSQG
MDALQQSLAETLANGSPSPSGTSGNVANYMGQMAMAMGKLGTLEGFLRQADNLRQQTLQQMHRILTTRQ
SARALLAINDYFSRLRALSSLWLARPRE