CITRUS ADVANCED TECHNOLOGY PROGRAM

QUARTERLY & FINAL PROGRESS REPORT FORM: Control of Citrus Greening, Canker & Emerging Diseases of Citrus

▶ SELECT PERIOD September 2020 ◉ *Quarterly Report* ○ *Final*

Proposal Title

Investigating the role of transgenic rootstock-mediated protection of non-transgenic scion

Today's Date: 10/12/2020

Sponsoring Organization (drop-down): Citrus Research and Development Foundation

Category (drop down): Plant Improvement

ABSTRACT (750-word Executive Summary-public report-do not disclose proprietary information or intellectual property)

Goals of this project:

1. Evaluate existing transgenic Carrizo and Swingle AtNPR1 overexpressing rootstocks in the laboratory and greenhouse.
2. Conduct a replicated field trial with the best transgenic rootstocks budded with non-transgenic `Valencia' and test for GMO gene products in the fruit or juice.
3. Produce additional transgenic rootstock lines and stack other gene(s) responsible for SAR using mature transformation.
4. Evaluate transgene segregation analyses of the rootstock progeny and large-scale propagation of select lines.

Cumulative (December 1, 2018 to September 30, 2020) progress report:

Objective 1: Evaluate existing transgenic Carrizo and Swingle AtNPR1 overexpressing rootstocks in the laboratory and greenhouse.

Results:

Production of budded plants for the study: We generated a population of AtNPR1 overexpressing transgenic lines prior to the start of this project. Transgenic lines were generated using the Carrizo citrange and Swingle citrumelo cultivars.

Molecular analyses: Two populations of transgenic AtNPR1 expressing citrus have been used in this study. The first is a population of Juvenile tissue derived lines (2300-NPR1-x) and the other a population of mature tissue derived (Ax and ZMx) lines.

Transgenic lines have been evaluated during this period for AtNPR1 expression using qPCR (Table 1).

Table 1: Mean AtNPR1 Ct values from the different mother trees. PCR was performed for 40 cycles. Experiment was repeated thrice.

All the lines were tested for the PR1 and PR2 expression. These PR (Pathogenesis related) genes are markers for the Systemic acquired resistance (SAR) pathway and their overexpression indicates that our NPR1 gene is active. Selected lines with high PR1 and PR2 expression are being used for the greenhouse assays to understand if the transgenic rootstock can confer resistance to the non-transgenic scion. Ct values of the HLB infected budsticks range from 22.5 to 25.1. The first set of data was collected in May 2020. The second set of data was collected in September 2020 (Table 2)

Subsequently we analyzed protein expression in selected A and ZM series mature tissue derived

PI First Name Manjul

PI Last Name Dutt

Email manjul@ufl.edu

Phone 863-956-8679

Organization CREC, UF-IFAS

Sponsor Project Number 18-007

Project Duration (years 3 *Year of Project* 2

% Completion of Objectives 0.00%

RMC 18-007: Investigating the role of transgenic rootstock-mediated protection of non-transgenic scion

P.I. Manjul Dutt, CREC, UF

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Transgenic Line	NPR1 Ct values (±SE)	
	Leaves	Root
2300-NPR1-6	21.40±0.69	23.79±0.49
2300-NPR1-4	22.70±0.42	29.86±0.78
2300-NPR1-7	24.99±0.79	24.22±0.60
2300-NPR1-3	25.17±0.33	22.57±0.22
2300-NPR1-21	25.51±0.99	31.13±0.76
2300-NPR1-1	25.69±0.57	32.08±0.36
2300-NPR1-16	25.80±0.62	32.28±0.84
2300-NPR1-9	26.21±0.75	22.92±0.30

2300-NPR1-12	26.34±0.04	26.12±0.55
2300-NPR1-5	26.41±0.74	22.64±0.30
2300 NPR1-29	26.55±0.43	27.95±0.48
2300-NPR1-20	26.84±0.44	27.81±0.34
2300-NPR1-2	26.91±0.26	24.73±0.57
2300-NPR1-8	27.15±0.79	26.02±0.13
2300-NPR1-15	27.96±0.53	26.22±0.76
2300-NPR1-13	28.11±0.31	28.46±0.83
2300-NPR1-10	28.21±0.99	24.05±0.74
2300-NPR1-24	28.57±0.03	Undetermined
2300-NPR1-19	28.60±0.65	25.96±0.95
2300-NPR1-17	29.04±0.18	34.80±0.35
2300-NPR1-27	29.28±0.93	33.65±0.41
2300-NPR1-25	29.55±0.41	28.00±0.37
2300-NPR1-26	29.83±0.46	20.90±0.93
2300-NPR1-23	30.00±0.76	24.77±0.37
2300-NPR1-14	30.04±0.99	34.60±0.62
2300 NPR1-30	31.40±0.85	37.09±0.41
2300-NPR1-28	31.58±0.44	21.32±0.71
2300-NPR1-22	32.28±0.55	Undetermined
2300-NPR1-11	32.53±0.23	23.20±0.37
2300-NPR1-18	32.70±0.34	Undetermined
A10	25.14±0.62	35.96±0.50
A17	22.36±0.95	36.85±0.03
A22	23.77±0.29	21.72±0.71
A26	23.49±0.99	33.46±0.71
A39	25.01±0.19	35.47±0.43
A42	25.15±0.09	27.65±0.71
A47	23.74±0.86	22.59±0.14
A48	26.63±0.70	21.16±0.16
A49	28.87±0.91	26.23±0.06
A52	29.04±0.93	32.25±0.63
A58	26.07±0.26	Undetermined
A190	25.05±0.71	37.06±0.55
ZM6	27.00±0.48	30.13±0.63
ZM26	26.63±0.93	24.45±0.78
ZM30	24.16±0.92	21.77±0.84
HLB Resistant Sweet Orange control	25.81±0.85	
Wild Type Control	Undermined	Undermined

All the lines were tested for the PR1 and PR2 expression. These PR (Pathogenesis related) genes are markers for the Systemic acquired resistance (SAR) pathway and their overexpression indicates that our NPR1 gene is active. Selected lines with high PR1 and PR2 expression are being used for the greenhouse assays to understand if

the transgenic rootstock can confer resistance to the non-transgenic scion. Ct values of the HLB infected budsticks range from 22.5 to 25.1. The first set of data was collected in May 2020. The second set of data was collected in September 2020 (Table 2)

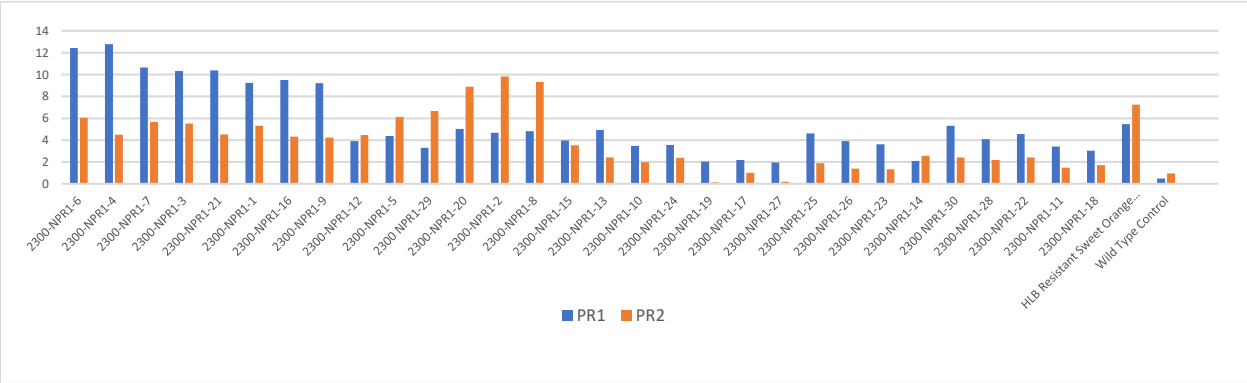

Figure 1: Initial PR1 and PR2 gene expression profile in the transgenic rootstocks.

Subsequently we analyzed protein expression in selected A and ZM series mature tissue derived transgenic lines using western blot with AtNPR1 specific antibody. All transgenic lines tested positive when leaf samples were analyzed (Figure 2). When roots were evaluated, A22, A47, A48, ZM26 and ZM30 had the best AtNPR1 protein expression. (Figure 3).

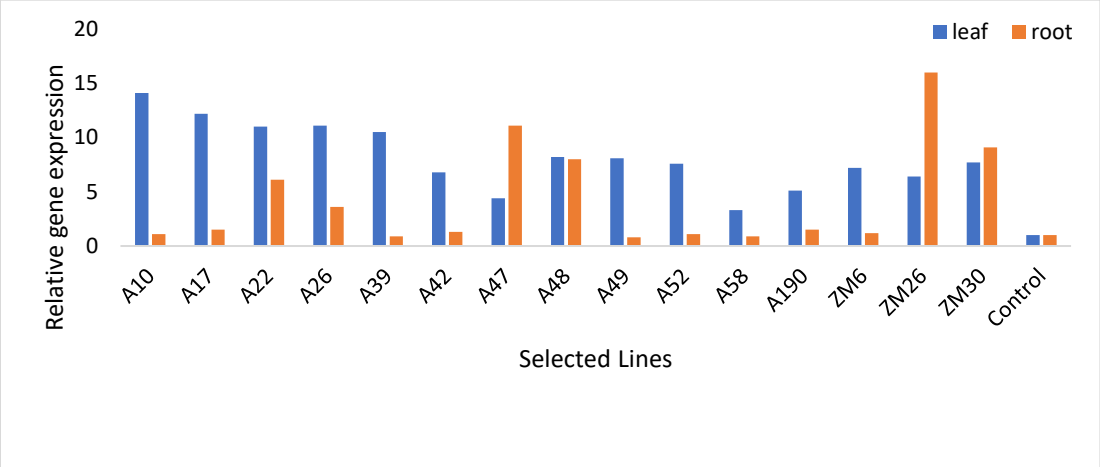

Figure 2: qPR-PCR of the transgenic leaves and roots

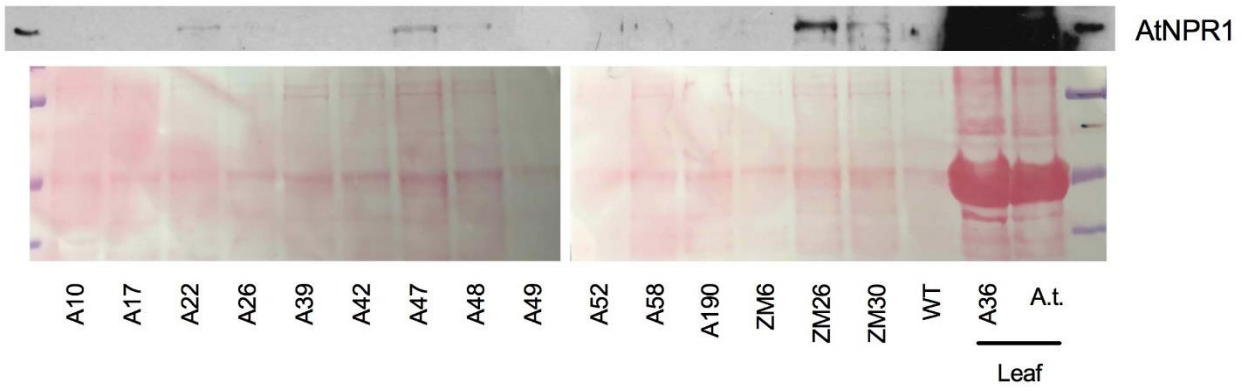

Figure 3: Western blotting of root samples with AtNPR1 specific antibody.

In the first round of clonal rootstock propagation, we focused on the juvenile tissue derived transgenic lines, which had been produced before the start of the current project and were thus bigger in size, compared to the A and ZM series of mature tissue derived transgenic lines. Cuttings from selected lines with low Ct values were propagated under mist. It has been observed that some transgenic lines do not perform well after propagation. They either do not root or the rate of growth is very slow. This is often related to higher trans-protein production in these lines. These lines were discarded and removed from the study. In the second round of propagation, we have focused on propagating cuttings from the A and ZM series of mature transgenic lines.

Figure 4: Propagated transgenic lines in the greenhouse

After 6 months of growth in greenhouse, the rooted cuttings were budded with Valencia 14-19 clone from DPI.

Greenhouse study

The experimental design is a randomized block design setup with at least 6 clones per transgenic line. 8 selected transgenic lines with initial low Ct values are being evaluated. We are testing transgenic lines for the following parameters

1. HLB Ct values – Twice a year, once in the summer and again in the fall.
2. PR1 and PR2 gene expression at a 3 monthly interval

Table 2: Mean CLas Ct values from the transgenic lines evaluated in this study

Transgenic Line	Summer Leaf Ct values	Fall Leaf Ct values
2300-NPR1-1	26.43±1.70	26.24±1.22
2300-NPR1-3	29.02±0.94	30.83±0.31
2300-NPR1-7	29.61±1.49	29.44±1.21
2300-NPR1-9	30.95±1.35	32.74±2.33
2300-NPR1-13	29.08±0.87	27.72±0.51
2300-NPR1-16	25.07±2.63	24.90±0.15
2300-NPR1-21	24.17±1.37	22.86±1.18
2300-NPR1-25	26.85±1.07	31.26±0.10
Non transgenic control	27.76±1.15	25.76±1.26

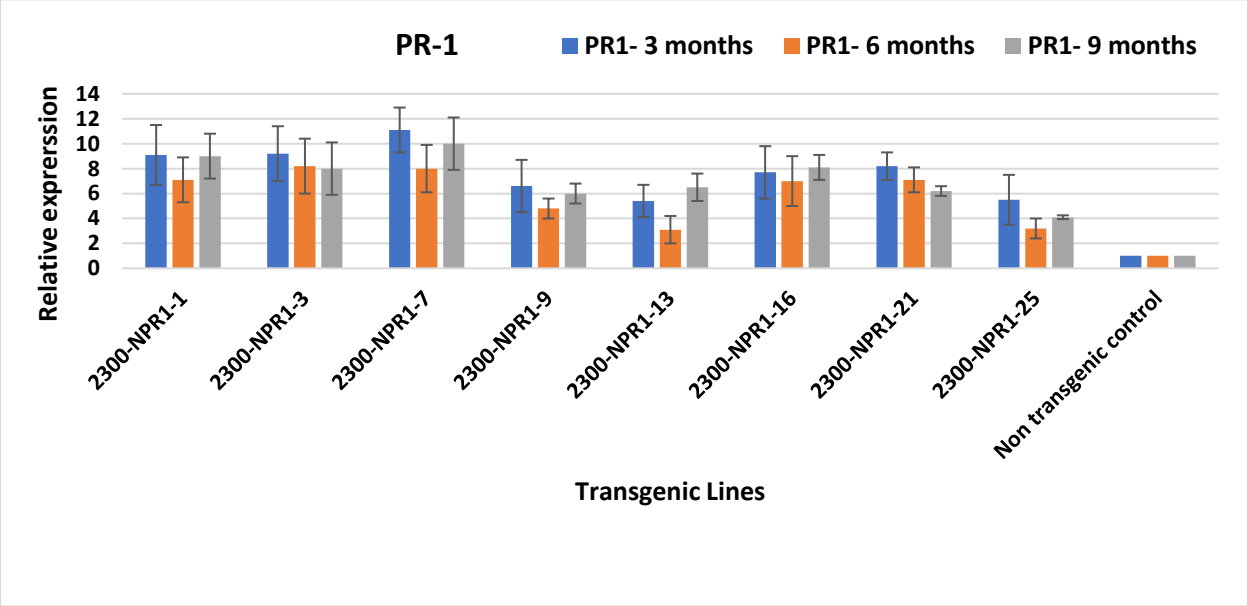

Figure 5: PR-1 gene expression in the transgenic lines in the greenhouse

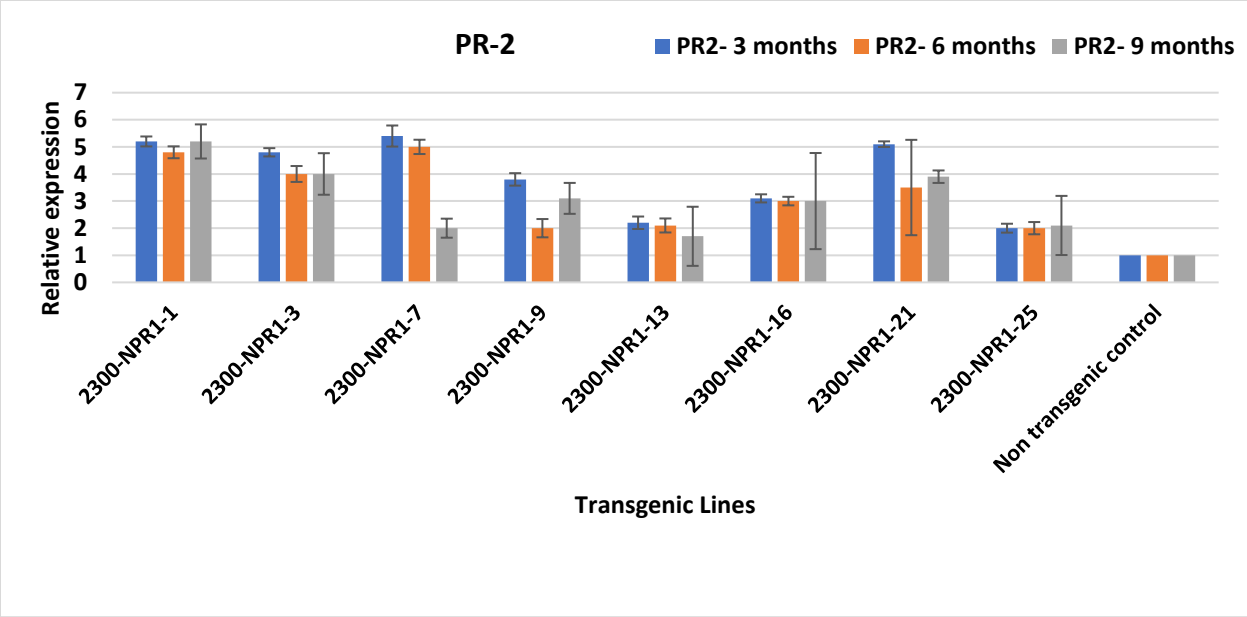

Figure 6: PR-2 gene expression in the transgenic lines in the greenhouse

Discussion:

A greenhouse study is in progress with selected transgenic lines budded with HLB infected sweet orange budsticks. The objective of this study is to understand whether the transgenic AtNPR1 rootstock can protect the aboveground scion against HLB. Three metrics are being evaluated: 1) HLB (Clas) titer, 2) PR-1 gene expression and 3) PR-2 gene expression. The CLas measurements were conducted twice. The first sampling was done in late May 2020 and the second sampling was done in late September 2020. Leaf sampling should have been preferably done in March - April, but due to COVID related and other restrictions had to be delayed. According to our results, all transgenic lines were HLB positive at the time of the initial sampling in late May 2020 (Summer Leaf Ct values - Table 2). When leaves were again sampled in September 2020 (Fall Leaf Ct values - Table 2), Ct values were either statistically similar or had increased in most transgenic rootstock lines. Ct values in the control lines significantly decreased during this period. Thus, this preliminary data provides an indication that transgenic rootstocks could potentially protect the non-transgenic scion from HLB. Although the trees still have HLB, the Ct values have not decreased in the same manner as we see in the controls. This can only be because of the effect of the transgenic rootstock on the scion. Line 2300-NPR1-25 had very high Ct values in the September evaluation, changing from 26.8 to 31.2 within 3 months. We will pay special attention to this line following our next sampling in December – January to confirm the validity of the fall results.

PR-1 and PR-2 gene expression were stable in the budded scions, when compared to the HLB infected non-transgenic control. Samples were collected in March, June and September 2020. We saw decreased gene expression at the 6 month sampling in June

and it may be due to a seasonal fluctuation. Even with the fluctuations, there was consistent upregulation of both genes in our trees. At this point, we cannot correlate enhanced gene expression to the HLB Ct results.

Future directions:

Leaves will be sampled at a 6 monthly interval till the end of the grant cycle and pertinent results will be provided in the quarterly report. An in-depth RNA expression study (RNAseq) on selected lines and control using an illumina HiSeq platform will be conducted to understand differential gene expression in the non-transgenic scion. This data will be essential in getting a detailed understanding of the genetic regulation of the SAR pathways and can provide conclusive evidence on the SAR activity. Additionally, we plan to study the impact of CLas infection on non-transgenic Valencia leaf metabolites using gas chromatography mass spectrometry by a process developed by Nabil Killiny. Expression analysis for genes involved in jasmonic acid (JA), salicylic acid (SA), and proline-glutamine pathways will also be conducted to correlate results observed in the RNAseq and GCMS data.

Objective 2: Conduct a replicated field trial with the best transgenic rootstocks budded with non-transgenic 'Valencia' and test for GMO gene products in the fruit or juice.

Transgenic lines were planted in the field under USDA permit in March 2020. USDA permit allows us to harvest and evaluate seed and fruits from the transgenic lines as necessary for the success of this project. We obtained 250 control trees (non-transgenic Valencia budded onto non-transgenic Kuharske) from Brite leaf nursery to plant as border row trees as stipulated in the transgenic permit in addition to the transgenic rootstocks inside the trial. Another 20% control non transgenic trees were planted within the trial.

Figure 7: Photograph of the field site

Future directions:

COVID related travel restrictions had resulted in a unique management situation for the transgenic field trees soon after trees were planted in March 2020. We are however getting back on track for management of this site. More trees will be planted in Spring 2021, including most of the rootstock seed source lines that have good transgenic expression. These will be planted for fulfilling objective 4. Field trees are managed according to set USDA protocols and leaves will be sampled in March 2021 for year 1 data collection. Subsequently leaves will be sampled at a 6-monthly interval (every spring and fall) and samples will be sent to Southern Gardens diagnostic lab for HLB analysis. RNA from fruit samples will be isolated and tested for presence of AtNPR1 using qPCR.

Objective 3: Produce additional transgenic rootstock lines and stack other gene(s) responsible for SAR using mature transformation.

The mature citrus facility (MCF) is a dedicated facility for mature citrus transformation and we have utilized their services for this project. The MCF received four vectors, each containing two stacked, disease resistance genes conferring Systemic Acquired Resistance (SAR). The four vectors are GNS (*gus*-*NPR*1,*SABP2*), GNO (*gus*-*NPR*1,*OBF*5), GNA (*gus*-*NPR*1,*AZL*1) and GND (*gus*, *NPR*1, *DIR*1). Table 3 lists the transgenic lines produced by the mature transformation facility and available for this project. Trees will be evaluated by qPCR for gene expression and the best lines will be clonally propagated for evaluation in 2021.

Table 3: Transgenic rootstock events produced with different vectors.

Cultivar	Vector	Number Surviving Micrografts
Kuharske	GNS	10
Kuharske	GNO	20
Kuharske	GND	10
Kuharske	GNA	24
US942	GNS	14
UFR17	GNS	1
Total	-	79

Objective 4: Evaluate transgene segregation analyses of the rootstock progeny and large-scale propagation of select lines.

Most of the transgenic rootstocks that show enhanced PR-1 and PR-2 expression have been propagated by budding on US802 rootstock. Trees are to be planted in the field in spring 2021. Several mature tissue derived transgenic lines have already flowered in the greenhouse and fruit harvested from 3 lines. Seedlings were planted from each line. Gus staining and AtNPR1 gene expression of the lines have indicated genetic stability in the progeny. A uniform population has now been generated for budding with non-transgenic scions. Since preliminary results indicate normal seed germination and growth, we are hopeful that overexpression of the AtNPR1 transgene in rootstocks will not have any detrimental effect on the subsequent generations.

Selected transgenic lines are also being mass propagated using in vitro tissue culture to generate a large population of transgenic lines for field testing in two additional USDA approved field sites.

Conclusions:

According to the time frame proposed in our project proposal, we are on track with this project. Year one was spent establishing the framework for this study, getting the plants ready, doing the background molecular analyses and infecting plants with HLB.

We were also able to obtain an USDA permit for field trials and have planted a population of trees in the field.

In year 2, we demonstrate that CLas titers do not significantly fluctuate following infection of the non-transgenic scions budded onto the transgenic rootstocks. Results obtained are only for 12 months after budding and further evaluation will conclusively demonstrate the validity of this hypothesis.

Transgenic rootstock lines with the stacked SAR inducing genes have been produced by the mature citrus facility. Also all existing transgenic lines that have been observed to have enhanced PR-1 and PR-2 gene expression have been propagated.

Seedlings from the first batch of transgenic lines have been phenotypically normal and genotypically stable. Thus, from preliminary indications, it seems the AtNPR1 transgene does not have any deleterious effect on the seedling progeny.