Interaction of SU91 and SAP6 Marker Linked QTLs on Disease Resistance

Authors/Affiliations

John Raggente

With the Advice and Supervision of Professor Peter Karl Pauls

With the direction and RIL population provided by PhD. Candidate Maryam Vazin

Summary

It has been proven that SU91 and SAP6 are dominant QTLs that both individually provide a major contribution to the resistance of Common Bacterial Blight (CBB). It is assumed that there will be an additive effect of disease resistance since both QTL provide their individual resistance, however it has been seen in literature that the recessive su91 allele interacting with other CBB resistance QTLs can lower the overall effect of CBB resistance in the plant. Through genotyping 70 RILS + 2 parents for SU91 and SAP6, creating marker groups for the possible genotypes, and completing a phenotypic screen, the interaction between these two QTLs affect on disease resistance will be brought to light. Research into this topic is a necessity since CBB can be devastating to pulse crop growers lowering crop yields up to 40% while also laying dormant in the soil for next years crop.

Keywords

Common Bacterial Blight, Disease Resistance, Molecular Markers, Recombinant Inbred Line, Polymerase Chain Reaction, Gel Electrophoresis, Quantitative Trait Loci, Xanthomonas fuscans, Bacteria Culturing

Introduction

Ex-Rico, also known as ICA Bunsi, is a Navy bean cultivar that was introduced into Canada by the International Center for Tropical Agriculture (CIAT) and used for breeding programs because of its tolerance to white mold (*Sclerotinia sclerotiorum*)[1,2] . In a 1970s breeding program for a black bean population (Rico), large variation in the population created a white bean (Ex-Rico).[3]Ex-Rico has two possible pedigrees: Ex-Rico*6/Ex-Rico/PI 326418, and Magdalena/Japon 3 the latter of which being for ICA-Bunsi which is Ex-Rico's alternative name. Ex-Rico also contains the resistance I gene for bean

common mosaic virus (BCMV) and is naturally resistant to ozone pollution making it an excellent candidate for parentage and for pyramiding genes for resistance to many different diseases. [2] It also has does well in its agronomic performance by having acceptable and comparable yields, cooking quality, and time to maturity, however excelling in the presence of white mold in relation to the other cultivars Fleetwood, Kentwood, and Seafarer.[1]

Apex coming from the pedigree Centralia/NY5268//HR67/AC Cruiser, is a navy bean that was designed to have higher CBB resistance, and better agronomic traits including a more upright growth morphology that is better suited for commercial harvesting from combines. [3] The resistance of Apex comes from the presence of two marker-linked QTL (SU91 and SAP6), with SU91 coming from HR67/AC Cruiser and SAP6 coming from Centralia/NY5268 germplasm line HR67. [3] After a two-year multi-location test set under the guidelines of the Ontario Pulse Crop Committee, Apex yielded on average 2%-8% higher than other check cultivars (OAC Rex, OAC Thunder, AC Compass, Nautica, and Vista), had larger seed weight, and other agronomic traits being comparable to check cultivars.[3] Apex has CBB resistance, resistance to BCMV, and some races of anthracnose.[3] Apex is a very good candidate for parentage and a source for CBB resistance. The objective of this research is to genotype CBB resistant markers in a RIL population derived from the progeny of the cross Ex-Rico 23 and Apex, group RIL lines based on marker genotypes and screen for CBB resistance on chromosome 8 (SU91) and 10 (SAP6) and compare disease scores of RILS to see if a significant difference exists between groups.

RESULTS

Parental Genotypes

A codominant SU91-CG11 marker was used for the SU91 allele. To see if the PCR and Gel electrophoresis procedure was correct a trial run was performed using parental lines as the positive

control and water as the negative control. The water was added to the PCR reaction as a substitution for DNA in case the primers anneal to each other. Figure 1.A is the gel representing the trial run split into SU91-CG11 products and SAP6 products. Ex-Rico has a smaller band ~425 bp representing the susceptible SU91 allele, and Apex has a larger band ~464 bp representing the resistant SU91 allele.[4] The arrangement in the gel was Ex-Rico – Apex – Water – Ex-Rico – Apex – Water for both SU91-CG11 allele and SAP6 allele. The same bands appeared for both replicates for the SU91 marker, and none appeared for water showing that the forward and reverse SU91-CG11 primers don't anneal.

From Figure 1.A, Ex-Rico and Apex showed ~ 820 bp fragments representing the dominant allele for SAP6, with no bands for the water amplification meaning that the forward and reverse SAP6 primers don't anneal. The arrangement was also Ex-Rico – Apex – Water – Ex-Rico – Apex – Water, with the same bands appearing for the replicates.

Genotyping RILS for SU91 using SU91-CG11, and NPP

It was harder to diagnose SU91 genotypes for the RILS. Looking at Figure 1.B, only 5 RILS have the same resistant band that Apex has (~464bp) which are RILS 9,22,32,40, and 61. However, it is very difficult to discern since there's only 39 bp separating these two bands. To make sure these are the resistant RILS a replicate was done (Figure 1.C) with the identical results occurring.

To confirm that these RILS are the CBB resistant, the codominant Niemann Pick marker (NPP) was used which amplifies the same region as the SU91-CG11 marker.[5] In Figure 1.C, Ex-Rico and Apex were run first with a space separating them and the five resistant RILS 9,22,32,40, and 61. Apex had a band ~956 bp which is the resistant allele for CBB and Ex-Rico showed a band of ~535 bp which is the susceptible allele for CBB.[5] These results are consistent with Figure 1.A, showing that both markers are amplifying the correct regions and these bands have the correct length. Of the resistant RILS, 9 and 32 had bands at ~956 bp and ~535 bp showing heterozygosity, and RILS 22, 40, and 61 have ~956 bp bands which is the

resistant allele. There was only enough primer to go up to RIL 46, however all the RILS up to 46 showed bands ~535 bp meaning they all had the susceptible allele which agrees with the SU91-CG11 marker diagnosis.

Genotyping for SAP6

SAP6 undoubtedly had more ambiguous results then the SU91 genotyping. Figure 1.D.1 contains the first gel done for the SAP6 marker showing strong bands, light bands, and no bands as possibilities for the RILS. This gel was replicated again in Figure 1.D.2 coming back identical as the first gel (Figure 1.D.1). In Figure 1.E light band (white) and no band (red) RILS were chosen and run one well apart from each with strong band (blue) RILS at the ends as a positive control. A few RILS (18, 47, and 72) had no amplification but others (17, 21, and 46) had light bands. This light band group became a new marker category, now making 6 possible marker groups: SU91/SAP6, SU91/SaP6, SU91/sap6, su91/SAP6, su91/SaP6, su91/sap6. Figure 1.F describes the genotypes for the RIL population + 2 parental lines with five RILS SU91/SAP6, 37 RILS su91/SAP6, 25 RILS su91/SaP6, and 5 RILS su91/sap6.

Phenotyping

After each marker category was created five individuals were chosen from each group. For groups larger then five individuals, random sampling was performed. The SU91/SAP6 group contained Apex and RILs 9, 22, 40, 61, the su91/SAP6 group contained Ex-Rico and RILs 4, 65, 60, 30, the su91/SaP6 group contained RILs 17, 19, 21, 46, and 48, and the su91/sap6 group contained RILs 18, 20, 34, 72, and 45.

ANOVA was done for each disease score evaluation with 6 total ANOVAs being performed and is shown on Table 1. There is no p-value exceeding 0.05 which is the threshold for a significant difference between disease scores. Table 2. Refers to the averages of the disease scores for each group, which is 3.43 for group 1 (SU91/SAP6), 3.12 for group 2 (su91/sap6), 3.10 for group 3 (su91/SaP6), and 3.73 for

group 4 (su91/sap6). These results were also visualized as a boxplot in Figure 2.A showing the averages for each RIL in each group plotted (5 points per boxplot).

Discussion

The SU91-CG11 marker was not the best marker choice for the SU91 allele since the 39bp difference in fragment lengths isn't enough to discern different band lengths. However, to ensure that this difference existed the NPP marker was used as confirmation. For the NPP marker, Apex had the 956 bp fragment (resistance) and Ex-Rico had the 535 bp fragment (susceptible) which agrees with the SU91-CG11 marker.[5] Regarding Figure 1.C, RILS 9 and 32 are heterozygous for the NPP marker, meaning they have both resistant and susceptible SU91 alleles, which is strange seeing as these lines should be homozygous. These RILS were created from the cross of Apex and Ex-Rico, with the F1 being self pollinated, and the subsequent seeds from the F2 generation (largest variation) were inbred using single seed descent until there's homozygosity.[7] Its possible the heterozygosity in RILS 9 and 32, are due to chance or possible cross contamination. There's also heavy favouring for the susceptible 425 bp allele for the SU91-CG11 marker and the analogous 535 bp allele for the NPP marker, which may be dominance for that allele. No CBB screening took place in the creation of these RILS so there was no form of phenotypic selection influencing the genotypic frequency.

For SAP6, the light bands appearing means that something was being amplified in the PCR. It's not the primers annealing together which was reinforced by the negative water controls in Figure 1.A, and it's not spillover since the light band groups had separated one well apart from each other and with light bands still appearing in Figure 1.E. This insinuates that there is another region of genome being amplified by the SAP6 marker with a similar enough DNA sequence that the SAP6 primers can anneal to. It is still unclear what this region is because it's present in some RILS, but not in others seeing as there's certain RILS that had no amplification. Out of the 6 possible genotypes: SU91/SAP6, SU91/SaP6,

SU91/sap6, su91/SAP6, su91/ SaP6, su91/sap6, only four were identified leaving out individuals that had the SU91/SaP6 and SU91/sap6 genotypes. This is largely due to the uneven frequency and heavy favouring for the su91 allele, creating bias in choosing the marker groups since some categories are underrepresented.

There is no significant difference observed between marker groups when looking at their CBB disease scoring. As seen in Table 1, the p-values were all under <0.0001 level of significance and observed in Table 2, the average disease scores for each group range from 3-4 showing no significant level of variation. Averages for groups 1,2,3 and 4, were 3.43, 3.12, 3.10, and 3.73 respectively, showing some level of difference with the su91/sap6 group being the most susceptible, and the su91/SaP6 group being the most resistant, which is also visualized in Figure 2.A. SU91 and SAP6 are disease resistant alleles so it's understandable how complete absence of these alleles would lead to a higher level of susceptibility, however the presence of these alleles don't seem to have increased disease resistance dramatically. The Xanthomonas fuscans variant may be the cause since it's a relatively more aggressive strain.[8] This strain may have been too aggressive and infected everything regardless of genotype leaving little room to see variation. If the same experiment were to be conducted it may be worth it to use both an aggressive and relatively less aggressive strain to see how disease resistance works on both scales. It would also be beneficial to introduce other CBB resistant QTLs in a new RIL population to understand how pyramiding these resistance genes effect CBB resistance for future steps.

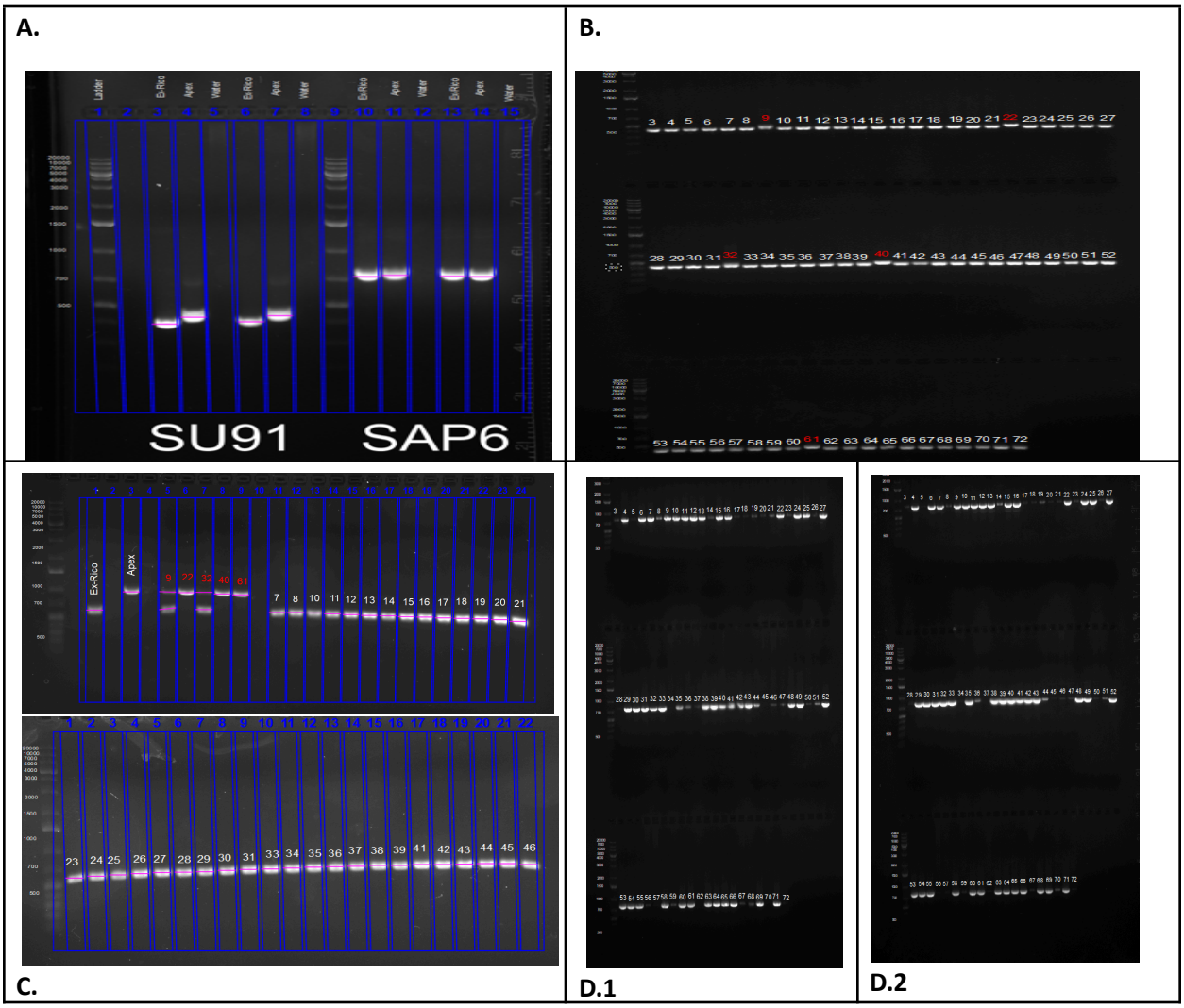
Acknowledgments

I'd like to thank Maryam Vazin for providing the Apex/Ex-Rico RILs created through Apex/Ex-Rico crosses that were advanced through single seed descent at the University of Guelph. These RILS were part of a larger 600 bean population conducted through half-sibling breeding scheme for research

involving anthracnose. This work was supported by funding from Peter K. Pauls research. I'd also like to thank Yarmilla Reinprecht, Lyndsay Schram, Mylene Corzo Lopez, and Sue Couling for direction and advice in during this project.

Figures

Figure 1. PCR and Gels used to genotype 70 RILS + 2 parental lines, for SU91 and SAP6



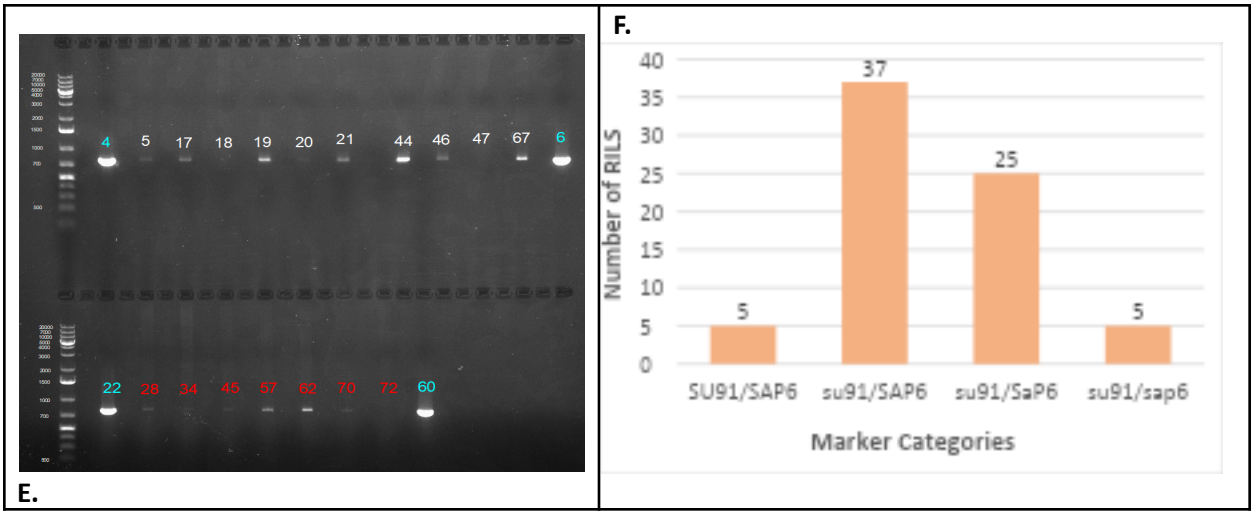


Figure 1.

A) Gel electrophoresis for the SU91 PCR and the SAP6 PCR. Lane 1 contains a 1kb+ DNA ladder while lane 3-5 contain the products from Ex-Rico, Apex, and water SU91 PCR. Lane 8 contains a 1kb+ DNA ladder while lane 10-12 contain the products from Ex-Rico, Apex, and water SAP6 PCR.

B) The Gel Electrophoresis of the PCR amplified SU91-CG11 marker. RILS were put through PCR using the codominant marker SU91-CG11 amplifying a 464 bp fragment (resistant) and a 425 bp fragment (susceptible). The first lane of each section contains a 1kb+ ladder while the rest of the lanes contain RILS. All the fragments amplified as expected, but with little separation since fragments are so close together. However, 5 RILS (colored in red font) are observed to have the resistant SU91-CG11 allele.

C) The Gel Electrophoresis of the PCR amplified NPP (Niemann-Pick) marker. RILS were put through PCR using the codominant marker NPP amplifying a 956bp fragment (resistant) and a 535 bp fragment (susceptible). The first lane of each section contains a 1kb+ ladder while the rest of the lanes contain RILS. All the fragments amplified as expected with a much noticeable band separation due to the larger difference in band size. The red font RILS are thought to have the resistant allele and have separated one well apart from the other samples to avoid spillover.

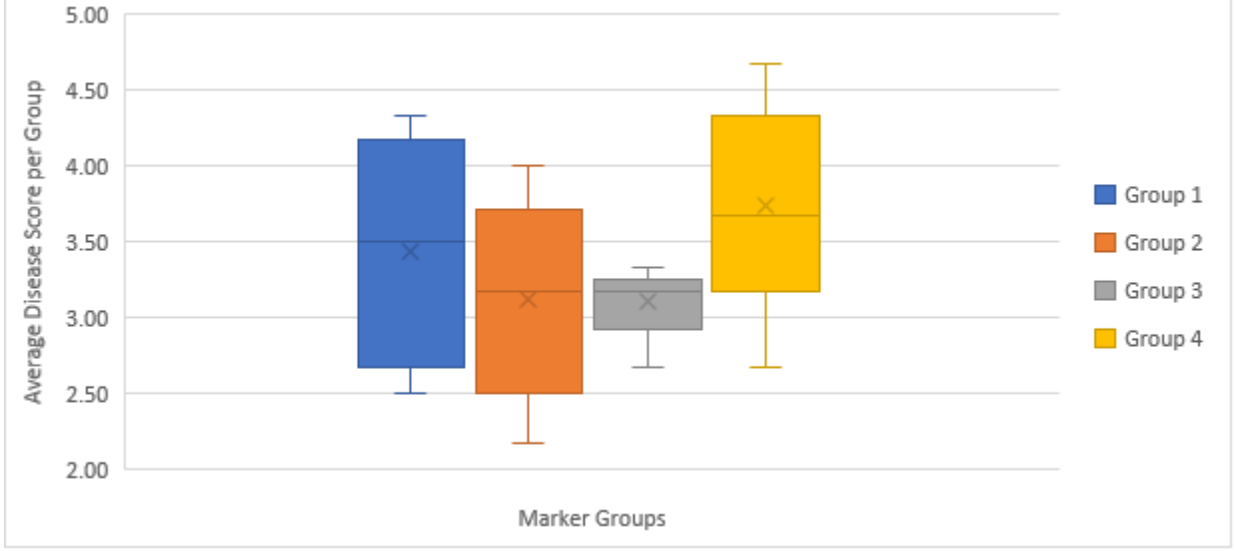
D) The Gel Electrophoresis of the PCR amplified SAP6 marker. RILS were put through PCR using the dominant SAP6 marker amplifying a 820bp fragment (resistant) and no fragment (susceptible). The first lane of each section contains a 1kb+ ladder while the rest of the lanes contain RILS. There were fragments amplified and not amplified, however there was also an in between light banded group. Gels 1 and 2 had two different PCR amplifications and were spaced two days apart however showed identical results.

E) The Gel Electrophoresis of the PCR amplified SAP6 marker. RILS were put through PCR using the dominant SAP6 marker amplifying an 820bp fragment (resistant) and no fragment (susceptible). The first lane of each section contains a 1kb+ ladder while the rest of the lanes contain RILS. The chosen RILS were ones that showed light banded fragments (upper section) and RILS that showed no fragments (bottom section) from Figure 1.D1/D2. The light banded

RILS have white font lettering with RILS 4 and 6 as a positive control. The no band RILS have red font lettering and with RILS 22 and 60 as a positive control.

F) The genotypes regarding the SU91 and SAP6 loci for the 70 RILS + 2 parental lines under study. 4 RILS and Apex have the genotype SU91/SAP6, 37 RILS have the genotype su91/SAP6, 25 have the genotype su91/SaP6, and 4 RILS and Ex-Rico have the genotype su91/sap6.

Figure 2. Boxplots for the disease score averages for each marker group



gggA

Figure 2.

A) Boxplots of the averages of disease scores for each marker groups. Replicate 1,2, and 3 were averaged for each of the five RILS in each group and boxplots were made.

Tables

ANOVA Results for Disease Score Assessments

Disease Score assessment	Mean	Standard Deviation	Variance	P-value
Replicate 1, Evaluation 1	3.5	1.27733275	1.63157895	<0001
Replicate 1, Evaluation 2	4.675	0.51999494	0.27039474	<0001
Replicate 2, Evaluation 1	1.9	1.02083557	1.04210526	<0001

Replicate 2, Evaluation 2	3.95	1.09904265	1.20789474	<0001
Replicate 3, Evaluation 1	2.200000	1.28145	1.64211	<0001
Replicate 3, Evaluation 2	3.85	1.26802789	1.60789474	<0001

[1]

Average Disease Scores for Each Assessment

Line	Group	Rep1Eval1	Rep1Eval2	Rep2Eval1	Rep2Eval2	Rep3Eval1	Rep3Eval2	Averages	Average Per Group
2	1	3	4	2	5	2	5	3.50	3.43
9	1	4	4	2	2	1	2	2.50	
22	1	2	4	2	3	2	4	2.83	
40	1	5	5	3	4	3	4	4.00	
61	1	4	5	3	5	4	5	4.33	
1	2	2	4	1	4	1	5	2.83	3.12
4	2	5	5	2	5	2	5	4.00	
65	2	2	3.5	2	5	3	5	3.42	
60	2	1	5	1	5	2	5	3.17	
30	2	2	5	1	2	1	2	2.17	
17	3	5	5	2	3	2	3	3.33	3.10
19	3	5	5	1	2	1	2	2.67	
21	3	4	5	1	4	1	4	3.17	
46	3	4	5	1	3	3	3	3.17	
48	3	3	5	1	4	1	5	3.17	
18	4	3	4	1	4	2	2	2.67	3.73

[1b] Table 1. ANOVA for each disease score assessment

20	4	3	5	2	5	2	5	3.67	
34	4	5	5	3	5	1	5	4.00	
72	4	3	5	5	5	5	5	4.67	
45	4	5	5	2	4	2	4	3.67	

[2]

STAR Methods

Reagent/Chemical/Abbreviation	**Source/Name**
70 RIL population + 2 parental lines (Ex-Rico/Apex)	Maryam Vazin
Reisolated Xanthomonas fuscans cells	Mylene Corzo Lopez
SU91-CG11 & SAP6 Primers	Agriculture and Food Laboratory, Laboratory Service Division University of Guelph
PCR Reaction kit	Agriculture and Food Laboratory, Laboratory Service Division University of Guelph
DAI	days after infection
CBB	Common Bacterial Blight
Xap	Xanthomonas
RIL	Recombinant Inbred Line

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be filled by the lead contact, John Raggente (jraggent@uoguelph.ca).

DNA Source

Stock DNA for the 70 RILS + 2 parental lines was sampled from previously extracted DNA from the larger PhD project conducted by Maryum Vazin.

Dilutions

[2C] Table 2. Average disease scores broken down for the four marker group. Rep1Eval1 = Replication 1, Evaluation 1
Evaluation 1 = 7DAI
Evaluation 2 = 14DAI
Group 1 = ResistantResistant (SU91/SAP6), Group 2 = SusceptibleResistant (su91/SAP6), Group 3 = SusceptibleLight Band = su91/SaP6, Group 4 = SusceptibleSusceptible = su91/sap6

Stock DNA concentration was calculated using Qubit according to the manufacturer's instructions and then diluted to 12.5 ug/mL. [6] Primers were diluted to 5uM and used in the PCR mix.[6]

Polymerase Chain Reaction (PCR)

Primers of interest will include SU91-CG11, NPP, and SAP6 which will be used in PCR and subsequent gel electrophoresis. SAP6 forward primer is 5'-GTCACGTCTCCTTAATAGTA-3, with reverse primer 5'-GTCACGTCTCAATAGGCAAA-3'.[6] SU91-CG11 forward primer is 5'-GGCGACGGCTTCTTTGAC- 3', and reverse 5'-TCCAAAGACCAAAGGGTGAG-3'. [9] NPP forward primer is 5'- GCTTCTGTTGGTAGTTTGCAT-3', and reverse is 5'-TAGGAATCTCGTGGA AGAGC-3'.[5] The codominant markers SU91-CG11 and NPP are used because they will identify between heterozygosity and homozygosity whereas a dominant marker wont show if the gene is homozygous or heterozygous, or if the PCR worked.

PCR mix (green) 10uL, F primer 1uL, R primer 1uL, H_2O 6uL, DNA 2uL, were used for each of the 72 samples. SAP6 PCR consisted of 94°C for 3 minutes, then 35 cycles of **[**94°C for 10 seconds, 55°C for 42 seconds, and 72°C for 120 seconds**]**.[10] On the last cycle the 72°C extension goes on for 5 minutes, and afterwards the product is kept at 4°C indefinitely [4].[10] SU91-CG11 PCR consisted of 94°C for 3 minutes, then 35 cycles of **[**94°C for 10 seconds, 60°C for 45 seconds, and 72°C for 120 seconds**]**.[11] On the last cycle the 72°C extension goes on for 5 minutes, and afterwards the product is kept at 4°C indefinitely. [11]

NPP PCR consisted of 94°C for 3 minutes, then 35 cycles of **[**94°C for 30 seconds, 65°C for 45 seconds, and 72°C for 60 seconds].[5] On the last cycle the 72°C extension goes on for 10 minutes, with the product kept at 4°C indefinitely.[5]

Gel Electrophoresis

Agarose was weighed in a analytical scale to make a 1% gel and subsequently added to the relative proportion of TBE buffer.[11] The solution was microwaved for three consecutive 1 minute intervals with

swirling in between to dissolve the agarose crystals.[11] Once the mixture was clear it was left to cool following the addition of DNA dye in a ratio of 0.0833 uL DNA dye/mL TBE.[11] It was then poured into the casting and after solidifying, was put into a gel electrophoresis chamber filled with TBE buffer.[11] Each well was filled with 10 uL of PCR product and ran at 100V for ~1-2 hours.[11]

Plant Material

18 RILS + 2 parental lines were germinated in a growth room at 70% relative humidity, 28°C during the day, and 21°C during night, but were subsequently moved to a growth chamber after two weeks. The conviron growth chamber was at 21°C, 80% relative humidity, and 3.0 RFU (Relative Fluorescent Units). The plants were grown in 3x6 black plastic inserts containing 1:1 BM6 All-Purpose Soil to Promix. An additional 20 pots using the exact same lines as the control group were planted so each replicate will utilize 40 plants total. The soil will be wetted and mixed, the seeds will be planted and hand watered once every two days with each plant receiving approximately 50-60 mL of water. Once a week, plants will be watered with a 20-20-20 synthetic fertilizer at 5 g/L of water.

Bacteria Culture

Bacteria was grown in LB broth consisting of 10g Bacto-tryptone, 5g yeast-extract, 10g NaCl, and 800 mL H_2O.[12] pH was adjusted to 7.5 using 5 M [NaOH], and afterwards distillated H_2O was added to make the final volume 1000mL with subsequent autoclaving.[12] Isolated Bacillus-9812 (Xanthomonas fuscans) bacteria was put into the sterilized LB broth and was shaken for 24-48 hours along with a 20 mL negative control (no bacteria).[12] After ~36 hours/visible turbidity, 20 mL of the bacteria broth was added to 150 mL of 0.85% NaCl solution and homogenized and tested for optical density (OD) using a spectrophotometer.[12] OD at 600 nm was tested with an acceptable OD between 0.3 – 0.5.[12]

Inoculation

Plants stayed in growth chamber until reaching trifoliate stage (~2 weeks) and were subsequently put in a growth room under a misting tent for 48 hours prior to inoculation.[12] This room was at 26°C during day and 22°C at night, with sprinkling occurring once every 5 minutes. After 48 hours, plants were inoculated simultaneously and uniformly using the multiple needle method.[13] One trifoliate leaf from of each plant was punctured, and two small culture-soaked sponges were used to squeeze the leaf in between forcing the inoculum through.[14] Each of the inoculations will be labelled and a negative control group will be used to ensure that infections aren't occurring just from the puncture wound. These controls will be punctured and set aside for growth and should show no sign of CBB infection which will be measured by lesion area on infected leaves. After inoculations plants were set outside mist for ~30 minutes for bacterium to dry before going back in the mist for 48 hours.[12] After misting for 48 hours plants were put out of mist in the growth room for assessment. Disease scoring was done using the following criteria, 0 = no visible symptoms, 1 = 1% -10% severity, 2 = 11% - 30% severity, 3 = 31% - 50% severity, 4 = 51% - 80% severity, 5 > 80% severity. Plants with reactions that are <=2 will be classified as resistant, plants that are 3 will be classified as intermediate resistant, and plants >=4 will be seen as susceptible. Two subsequent assessments of symptoms were made 7DAI and 14DAI to measure the disease progression and resistant/susceptibility.[13]

Image 2. located in the supplemental information section, is an example of the multiple needle technique where the yellow circles show the puncture wound made by the needles and having (a) as a control vs (b) which is then inoculated with *Xap.* [13] One sponge will be on the bottom of the leaf and the needle will puncture the leaf going through and hitting the sponge.[15] After all the punctures are made, the second sponge will be placed on top of the leaf and are squeezed together to force infection through needle wounds.[15]

Data Analysis and Graphing

Data collection was conducted through Excel, and data analysis was performed using SAS on Demand for Academics. Gels were imaged using the ImageLab software, and graphs were created using Excel.

Supplemental information

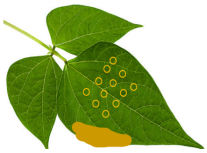
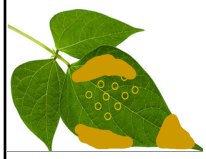
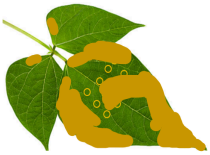
				
Inoculated leaf with a disease score of 1 with 1%-10% severity.	Inoculated leaf with a disease score of 2 with 11%-30$ severity.	Inoculated leaf with a disease score of 3 with 31% -50% severity.	Inoculated leaf with a disease score of 4 with 51%-80% severity.	Inoculated leaf with a disease score of 5 with >80% severity.

Image 1.

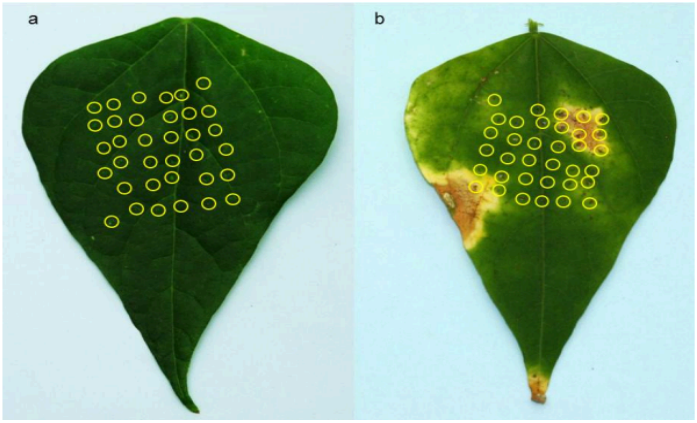
	Represents the CBB symptoms on *P. vulgaris* leaves after (a) mock inoculation, and (b) *Xap* inoculation.[13] The yellow circles represent the puncture wounds which afterwards had *Xap* applied to them. [13]

Image 2.

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