REPORT

Project Title: Screening Lettuce CRISPR Lines for Resistance against Human Pathogens

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Abstract:
It is now widely accepted that the human pathogens *Escherichia coli* O157:H7 and *Salmonella enterica* can colonize edible leaves and survive throughout the production chain causing serious disease outbreaks. We and others have demonstrated that the plant genetic can contribute to this bacterial colonization of leaves, in which some lettuce cultivars are more prone to colonization than others. We have identified a family of nine genes, named *LsJAZ*, that are expected to be associated with the plant susceptibility to microbial colonization. Using the non-GMO genome editing technology (CRISPR/Cas9), we started to develop lettuce lines that do not express each gene. During this reporting period, we have created 28 lines derived from the cultivar Salinas, each of which carrying an individual null mutation in one of four *LsJAZ* genes (*Lsat_1_v5_gn_8_45621*, *Lsat_1_v5_gn_4_91521*, *Lsat_1_v5_gn_5_137041*, and *Lsat_1_v5_gn_8_85820*). These mutations did not seem to cause any undesirable morphological defects in lettuce and the plant lines look healthy under optimal growth conditions. We were also able to phenotype a plant line, temporarily named A3-4, that does not express the gene *Lsat_1_v5_gn_8_45621*. Results confirmed that the population of *E. coli* O157:H7 and *S. enterica* ser. Typhimurium significantly declines in leaves of A3-4 when compared to the wildtype Salinas. Furthermore, A3-4 is more resistant to *Trichoplusia ni* feeding and *Botrytis cinerea* infection.

The results from this research had the following impacts: **(a)** we validated that at least one of the *LsJAZ* genes is a susceptibility factor in lettuce, **(b)** we are providing support to the development of lettuce germplasm with enhanced resistance against the plant colonization by a broad range of pathogens.

Objective:
To phenotype lettuce CRISPR mutants for resistance against human and plant pathogens.

Procedures:

***1. Identification of the LsJAZ genes*:** Sequences of the TIFY motif and Jas motif of AtJAZ4 were obtained from Garrido-Bigotes et al. (2019) and used as query in a TBLASTN search (E-value threshold ≤ 100, BLOSUM62, Word size = 3) against the lettuce genome (version 8) on CoGeBlast (https://genomevolution.org/coge/CoGeBlast.pl). The full protein sequences of all candidates from both search results were analyzed manually to ensure the candidate met the criteria of having both a TIFY motif and Jas motif. Additional searches on NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and CoGeBlast with all Arabidopsis JAZ protein Jas motifs did not identify any additional candidate lettuce orthologs.

LsJAZ candidates were compared to *Arabidopsis thaliana* (At), *Fragaria ves*ca (Fv) (Garrido-Bigotes et al., 2018), *Solanum lycopersicum* (Sl) (Chini et al., 2017) and *Vitis vinifera* (Vv) (Zhang et al., 2012) by phylogenetic analysis of full JAZ protein sequences. The sequences were aligned by the ClustalW function of the MEGA software (Version 10.2.4) and phylogenetic trees were constructed with the aligned sequences using a Maximum Likelihood algorithm and Jones, Taylor, and Thornton (JTT) matrix-based model with a bootstrap consensus inferred from 100 replicates (Jones et al., 1992).

The conserved motifs of the putative LsJAZ proteins were determined by examining aligned sequences and comparing them to known Arabidopsis motifs (Garrido-Bigotes et al., 2019). The motifs were further confirmed by analyzing important residues within each motif as previously determined in Garrido-Bigotes et al. (2019). These residues include the TIFYXG sequence in the TIFY motif, as well as the degron sequence, Jas sequence and nuclear localization signal (NLS) of the Jas motif. The consensus sequences of the lettuce TIFY motif and Jas motif were determined by obtaining the sequences of these motifs from all the LsJAZ genes and inputting them into WebLogo (https://weblogo.berkeley.edu/logo.cgi).

***2. LsJAZ expression analysis by RT-qPCR*:** we used reverse transcriptase-quantitative polymerase chain reaction to assess the expression of all nine putative *JAZ* genes of lettuce cultivar Salinas following standard procedure. As a negative control, we also included three genes that encode TIFY proteins. Briefly, tissues from six-day old seedlings grown of MS medium plates with or without 10 uM jasmonic acid (JA) were harvested, frozen in liquid nitrogen, and stored at -80 °C for RNA extraction. RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) was used for RNA extraction and quantified using Qubit® RNA BR (Broad-Range) Assay Kit (Thermo Invitrogen, Waltham, MA). To assess gene expression, total RNA was synthesized into cDNA using the Takara Prime Script RNA kit (with gDNA eraser) (Clontech, Mountain View, CA, USA). RT-qPCR reaction (10 µl) was performed with 5 µl of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 5 ng of cDNA template, and 300 nM of reverse and forward gene-specific primers. Reactions were carried out in an CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA), using the following cycling parameter: 1 cycle 95°C for 5 min and 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Gene expression levels were normalized based on the expression of the housekeeping gene *LsatAPT1* (Moura Borowski et al., 2014) and relative gene expression was calculated with the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Three biological replicates with two technical replicates were included and the experiment was repeated twice.

***3. Plant material*:** Using a standard pipeline of the non-GMO CRISPR/Cas9 technology explained in the last reporting period, we have created now plant lines carrying a single mutation that disrupts the coding sequence of four of the *LsJAZ* genes. Multiple independent mutant lines for each gene were developed for a robust genetic analysis that can be validated with strong statistical support of the data.

***4. Plant growth conditions*:** Seeds of lettuce (*Lactuca sativa* L.) cultivar Salinas were germinated on water-soaked paper placed in square Petri dishes and maintained at 20°C for 2 days. Germinated lettuce seeds were transferred to the 3 ½" pots (Kord Products, Toronto, Canada) containing a soil mix (Sun Gro® Sunshine® #1 Grower Mix with RESiLIENCE™, Agawam, MA, USA). Lettuce plants were grown at 19 ± 1°C, 75 ± 4% relative air humidity, and photosynthetically active light intensity of 240 ± 10 µmol/m^2/s with a 12-h photoperiod. One liter of deionized water was added to the tray two to three times per week depending on the developmental stage of the plants. At 10 days post germination, lettuce plants were fertilized with 0.05 g/plant of fertilizer (Multi-Purpose 19-11-21; Peters®Excel, OH, USA) dissolved in the deionized water. Four weeks old plants were used for the experimentation with pathogens.

***5. Inoculation assays with human pathogenic bacteria*:** The non-typhoid *Salmonella enterica* subspecies *enterica* serovar Typhimurium strain 14028s (Porwollik et al. 2014) and the enterohemorrhagic *Escherichia coli* serotype O157:H7 strain 86-24 (Sperandio et al. 2001) were grown in Low Salt Luria-Bertani medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.0) at 28°C. Medium supplemented with 50 µg/ml of streptomycin was used to grow O157:H7. When the culture reached OD_{600} of 0.7–0.9, bacterial cells were harvest by centrifuging for 20 min at 2800 rpm. The aerial parts of the plant were inoculated with a final concentration of 1×10^6 CFU/ml (OD_{600} = 0.002), supplemented with 0.01% Silwet L-77 and 1mM $MgCl_2$, by dipping into the inoculum and applying vacuum. This procedure ensures uniform inoculation of the leaf surface and leaf interior. Phenotypic evaluation was performed by enumerating the total bacterial population in leaves at the day of inoculation (Day 0) and 14 days after inoculation using a standard serial-dilution plating technique (Jacob and Melotto, 2020).

***6. Trichoplusia ni non-choice feeding assay*:** *T. ni* eggs (Benzon Research) were incubated on wax paper for 6 days at 22 ºC in petri dishes containing a 1 cm^2 piece of paper moistened with sterile distilled water. Immediately after the emergence of second instar larvae, the larvae were placed on four-week-old plants using a small paintbrush. Each plant was wrapped in a perforated floral sleeve that was tightly sealed above the plant to prevent migration of the larvae. Plants were photographed and larval weight was recorded ten days after feeding.

***7. Botrytis cinerea pathogenesis assay*:** Conidia from sporulating cultures grown on 1% potato dextrose agar (BioPlus) were used to collect fungal spores. Immediately before inoculation, spore suspensions were spun down and resuspended in sterile 50% grape juice. The adaxial side of detached leaves were inoculated with 10 ul of this suspension containing 5 x 10^3 spores or with a mock, spore-free solution. Leaves were kept in covered plates lined with wet filter paper and a loose sheet of wax paper. Samples were incubated at room temperature for three days, at which points they were photographed, and the lesion size was measured using ImageJ.

***8. Experimental design and statistical analysis*:** biological replicates for all experiments represent four separate plants (n=4) for each sample point per genotype. Data points were averaged, the standard error of the mean was calculated, and the statistical difference between the means (Salinas versus CRISPR line) was compared using a two-tailed Student's *t*-test. Experiments were performed at least twice to confirm the results independently.

Results and Discussion:

Recently, a new version of the lettuce genome became available and upon searching for the *LsJAZ* genes, we identified additional related sequences. Our extensive sequence analysis yielded nine putative *LsJAZ* gene sequences based on the predicted domain structure of the encoded protein (**Figure 1A**). All JAZ proteins must contain both a TIFY and a Jas motif. These highly conserved domains (**Figure 1B**) are very important for the function of the proteins and how they regulate the different plant phenotypes. The phylogenetic analysis placed the lettuce proteins in four out of the five groups of JAZ proteins, where group II has no lettuce representatives (**Figure 1A**).

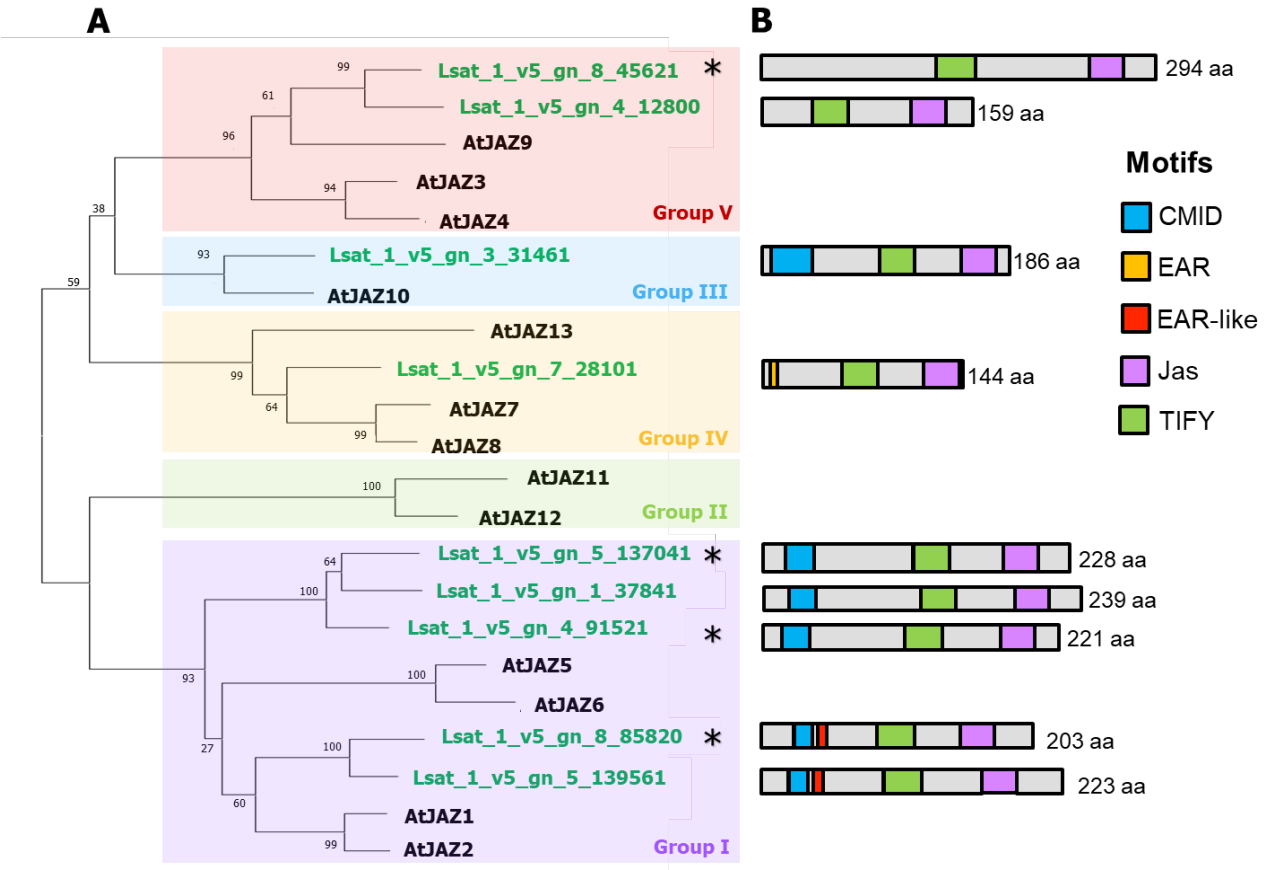

Figure 1. The current version of the lettuce genome revealed nine LsJAZ proteins. (A) Phylogenetic tree depicting the relationship of the full-length JAZ proteins of lettuce (Lsat numbers) with those of the model plant Arabidopsis (At numbers). Asterisks indicate the four proteins for which we have developed null mutants. (B) The predicted domain structure of the nine JAZ proteins of lettuce. The numbers on the right indicate the number of amino acids (aa) for each protein and the colored blocks indicate the relative position of the conserved motifs CMID, EAR, EAR-like, Jas, and TIFY.

There are multiple examples in many plants where *JAZ* genes are induced by the plant hormone jasmonic acid (JA) or its precursors and derivatives. This phenomenon is part of the JA signaling pathway that might protect the plant against biotic stresses (reviewed by Zhang et al., 2017). Thus, we determined whether these *LsJAZ* genes are indeed constitutively expressed or induced in response to JA using RT-qPCR analysis. Our results showed that all of them are expressed at a detectable, yet variable, level in lettuce seedlings (**Figure 2**). Furthermore, JA application significantly induced all nine genes, but not the three control genes that encode proteins containing the TIFY motif but are not considered JAZ proteins due to the lack of the Jas motif (**Figure 2**).

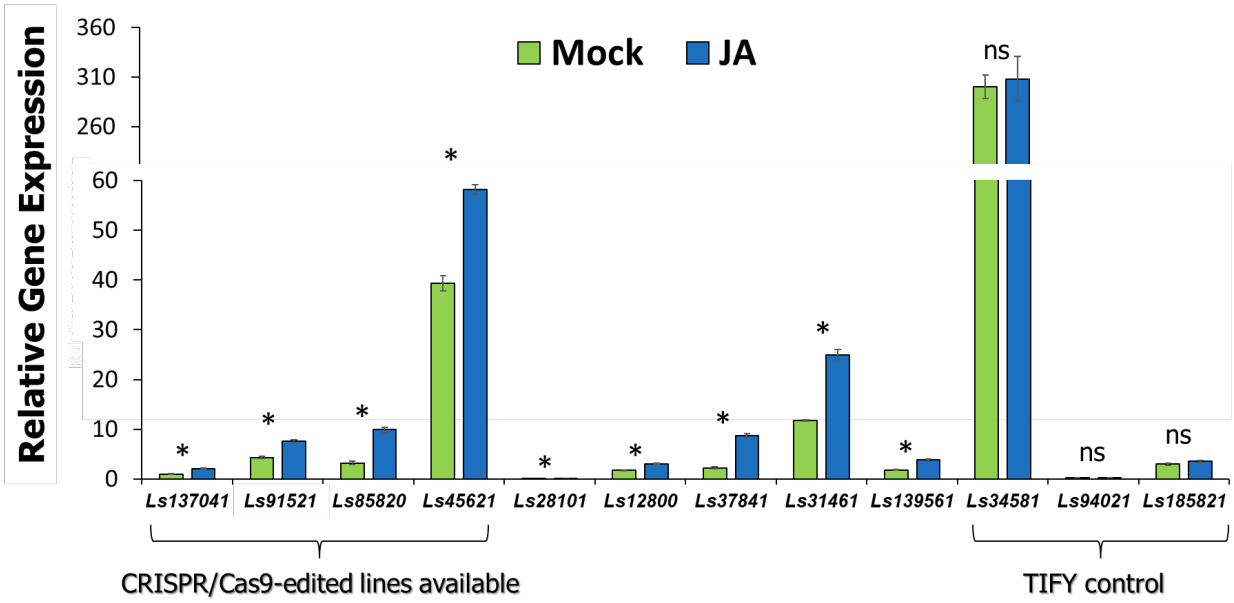

Figure 2. Relative gene expression of nine *LsJAZ* genes in six-day old seedling. Three genes that encode TIFY proteins were included as controls. Statistically significant difference between the means (mock versus JA treatment) was detected with Student's t-test at the threshold of $p<0.05$ indicated by the asterisk on top of the bar. Different letters above the bars indicate statistically significant differences among the means. ns = not significant.

Thus far, we were able to create mutants for the following genes: *Lsat_1_v5_gn_5_137041*, *Lsat_1_v5_gn_4_91521*, *Lsat_1_v5_gn_8_85820*, and *Lsat_1_v5_gn_8_45621* and advance them to the T_2 generation. Genotyping of heterozygous T_2 generation of CRISPR/Cas9 edited plants allowed the concomitant selection of the wildtype siblings of homozygous knockout lines. In total, we have advanced 28 mutants and 14 wildtype lines to produce T_2 seeds. Due to the extensive time and space required for this activity, only one T_2 line, named A3-4, could be selfed to the T_3 generation for microbial testing. The status of these lines is described in **Table 1**.

Table 1. The current number of mutant lines and corresponding wildtype lines selected for each indicated gene. The current generation and whether the lines have been phenotyped for pathogen growth are also listed.

Target Gene	# Confirmed knockout mutants	# Wildtype siblings	Generation	Phenotyped
Lsat_1_v5_gn_8_45621	4	3	T_3	Yes
Lsat_1_v5_gn_4_91521	5	5	T_2	No
Lsat_1_v5_gn_5_137041	6	6	T_2	No
Lsat_1_v5_gn_8_85820	13	0	T_2	No

Next, T_3 plants of the homozygous line A3-4 and its wildtype sibling in the Salinas genetic background (**Figure 3**) were tested for several biotic stresses. Overall, we observed that A3-4 is significantly more resistant to these stresses than the wildtype (**Figure 4**). For instance, the populations of *E. coli* O157:H7 and *S. enterica* Typhimurium were approximately 0.5 Log smaller in A3-4 over the period of 14 days post-inoculation (**Figure 4AB**). Although this reduction is less than optimum, it indicates that other JAZ proteins may also contribute to lettuce susceptibility to the colonization of these human pathogens. To test for the possibility of redundancy among the nine members of the LsJAZ protein family, higher order mutants need to be created to reach the desired reduction in pathogen load on leaves.

Interestingly, A3-4 also showed significant resistance to *B. cinerea* infection and *T. ni* feeding (**Figure 4CD**). These are a generalist fungal pathogen and a pest that attack a wide range of crops. These findings highlight that LsJAZ plays a role in the plant's basal resistance to a broad range of biotic stress. In the future, we will test these plants with other pathogens and pests in the field and greenhouse conditions to validate these results in economically relevant conditions.

Figure 3. Representative photographs of the A3-4 and the wildtype (WT) lines used to run the experiments under controlled environment.

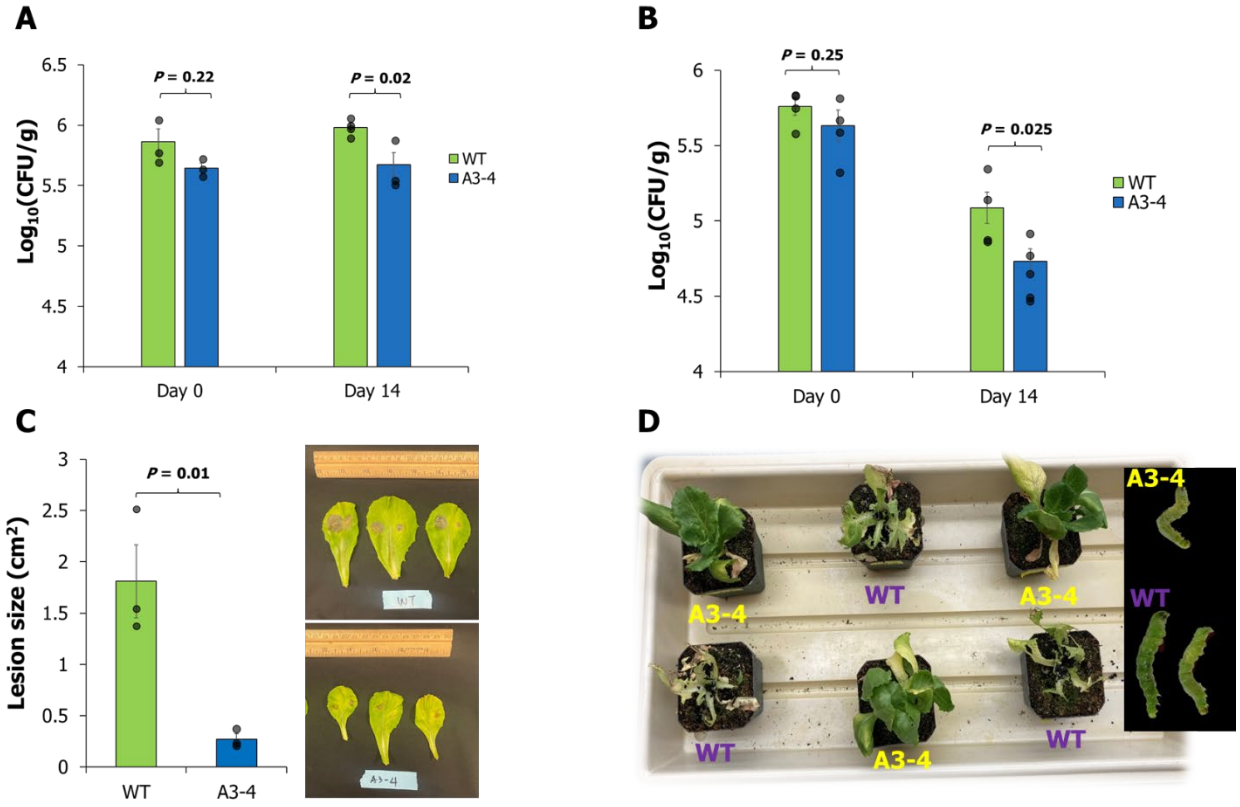

Figure 4. Phenotypes of the A3-4 line under biotic stresses compared to that of the wildtype (WT). (**A**) Persistence of *E. coli* 157:H7. (**B**) Persistence of *S. enterica* Typhimurium. (**C**) *Botrytis cinerea* infection. (**D**) *Trichoplusia ni* feeding showing that A3-4 plants are not as damaged as the WT and the size of the larvae feeding on them is small. Please see methods for details on the assays.

Conclusion:

A new version of the lettuce genome shows nine genes that encode for LsJAZ proteins. We followed a standard CRISPR/Cas9 pipeline to individually knockout each *JAZ* gene. The pipeline is lengthy due to the complex nature of the procedures, the requirement of multiple controls for robust genetic analyses, and the need to advance plant lines to at least three generations before phenotypic analyses can be performed. Nonetheless, we have already completed this work for four *LsJAZ* genes, while others are moving along the pipeline. One line with a null mutation in the *Lsat_1_v5_gn_8_45621* gene showed significant basal resistance to all pathogens tested. We will continue characterizing all genes to achieve desired levels of resistance without compromising the quality of the lettuce crop.