דף פותח

דו"ח לתכנית מחקר מספר **20-02-0075**

שנת המחקר:_____3_____ מתוך_____3_____ שנים

שיפור היעילות של מדביר ביולוגי באמצעות הגברת מנגנון השראת העמידות בצמח

מוגש לקרן המדען הראשי במשרד החקלאות

ע"י: דר' מיה בר

פתולוגיה של צמחים וחקר עשבים, מינהל המחקר החקלאי ,בית דגן

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תקציר

פתוגנים פטרייתיים וחיידקיים מייצרים מחלות הרסניות וגורמים לאובדן יבול משמעותי ברחבי העולם. בגלל הדברה כימית הפוגעת בסביבה ובבריאות האדם, אסטרטגיות למניעת מחלות אלטרנטיביות, כולל מיקרו-אורגניזמים המהווים הדברה ביולוגית, מבוקשות יותר ויותר בחקלאות. הדברה ביולוגית של מחלות נוף מהווה גישה חלופית לבקרת מחלות צמחים. מנגנון הפעולה העיקרי של מיקרואורגניזמים בעלי פעילות הדברה ביולוגית, כגון פטריות ממיני טריכודרמה, Trichoderma spp, הוא השראת עמידות בצמח המאחסן. עם זאת, דוגמאות למיקרואורגניזמים יעילים בהדברה ביולוגית במערכות חקלאיות עדיין חסרות, בעיקר בגלל חוסר עקביות ביעילות ובהדירות הביולוגית, מה שמוביל לעיתים קרובות למחלה נרחבת לפני שפעול השראת העמידות הנדרשת במארח.

כחלק מאסטרטגיית ההתיישבות שלהן בצמחים, פטריות ממיני טריכודרמה יכולות להפריש תרכובות ומולקולות שונות אשר יכולות להשרות עמידות בצמח. אחת המולקולות הללו המסונתזות ומופרשות מכמה מינים של טריכודרמה היא אנזים הקסילנאז ממשפחת Xyn11 המכונה EIX , קסילנאז המשרה אתילן. EIX משרה עמידות במיני צמחים וזנים ספציפיים. התגובה ל- EIX בזני טבק ועגבניה נשלטת על ידי לוקוס דומיננטי יחיד, המכונה *LeEix*, המכיל שני קולטנים, *LeEix1* ו- *LeEix2*, שניהם שייכים למשפחה של גליקופרוטאינים המוצגים על פני התא. שני הקולטנים מסוגלים לקשור EIX, עם זאת, בעוד ש- *LeEix2* מתווך בתגובות הגנה בצמחים, *LeEix1* משמש כקולטן decoy ומחליש איתות חיסוני של קולטן ה- *LeEix2* המושרה על ידי EIX.

במחקר זה אנו משתמשים במדביר ביולוגי נחקר ביותר, תבדיד T39 של טריכודרמה הרציאנום, המהווה מערכת מודל לחקר המנגנונים המעורבים בהדברה ביולוגית. T39 מדביר פתוגנים באמצעות מנגנון של השראת עמידות סיסטמית. במחקר זה, השתמשנו בשיטת cripsr / CAS9 על מנת לעשות מוטציה בקולטן *LeEix1*, כדי להגביר את השראת העמידות של מיני טריכודרמה, וכתוצאה מכך, לגרום לשיפור ההדברה הביולוגית באמצעות טריכודרמה.

במהלך המחקר, ייצרנו צמחים מוטנטים ברצפטור *LeEix1*, ווידאנו את המוטציות בצמחים שיוצרו, וכן ווידאנו שאון הצימוח ואיכות העגבניות לא נפגעו כתוצאה מהמוטציה. השתמשנו בשיטות מולקולריות לאפיין את השראת העמידות בצמחי המוטנטים, ובחרנו בקווים בעלי השראת העמידות החזקה ביותר בתגובה לטריכודרמה, כפי שאופיינה בפרמטרים של מערכת החיסון הצמחים וכן באמצעים גנטיים. השתמשנו במספר מיני טריכודרמה על מנת לבחון את עמידות הצמחים המוטנטים למחלות, ומצאנו כי לצמחים המוטנטים עמידות מוגברת למחלות עובש אפור וקשיונה גדולה, הנגרמים על ידי פתוגנים נקרוטרופיים, וכן למחלת קמחון, הנגרמת על ידי גורם מחלה ביוטרופי, לאחר הטיפול במיני טריכודרמה, בהשוואה לצמחי ביקורת. יכולת האכלוס של הצמחים המוטנטים על ידי טריכודרמה נבדקה גם היא.

<u>מעריכים מומלצים לבדיקת הדוח המדעי</u>

1. דר' אופיר בהר, מנהל המחקר החקלאי
2. דר' נועם אלקן, מנהל המחקר החקלאי
3. פרופ' שאול בורדמן, האוניברסיטה העברית בירושלים

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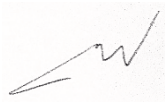
<u>הצהרת החוקר הראשי</u>:

הממצאים בדו"ח זה הינם תוצאות ניסויים.

הניסויים מהווים המלצות לחקלאים: ~~כן~~*/לא (<u>מחק את המיותר</u>)

<u>*במידה וכן, על החוקר להמציא פרטים על הגוף שבאמצעותו מופץ הידע (כמו: שה"ם)</u>

חתימת החוקר  **תאריך 04.04.21**

<u>רשימת פרסומים שנבעו מהמחקר:</u>

1. חסינות קונסטיטוטיבית ככלי לייצור צמחים עמידים למחלות, **מיה בר**, כנס הסטודנטים של המכון להגנת הצומח, מכון וולקני, 2018.

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Introduction

Plant pathogens are the foremost yield limiting factor for many crops in open field and greenhouse cultivation systems [1], and this trend is expected to increase. Fungal and bacterial pathogens are the cause of significant tomato crop losses worldwide, generating devastating diseases, in some cases due to wide host range, relatively limited information on pathogen biology and infection strategies, and their ability to remain quiescent for long periods of time and become virulent upon changing conditions. Pesticidal strategies can lack effectivity and are often a source of pollution and detrimental effects to consumer health [2], with many pesticides becoming increasingly banned worldwide.

Biocontrol of foliar diseases is an alternative, non-toxic means of management of foliar pathogens. A combination of several modes of action, including induced resistance, has been documented as responsible for biocontrol. Induced resistance is recognized as an important mode of action to achieve biocontrol in vegetative tissues [3,4]. Induced systemic resistance (ISR) caused by various micro-organisms can protect plants against soil or foliar pathogens [5].

One of the most studied commercial biocontrol agents (BCAs) is isolate T39 of *Trichoderma harzianum* which can be regarded as a model for commercial biocontrol and the mechanisms involved. T39 has been shown to control foliar pathogens such as *Botrytis cinerea*, *Pseuperonospora cubensis*, *Sclerotinia sclerotiorum* and *Sphaerotheca fusca* (syn. S. fuliginea) under commercial greenhouse conditions [6–8].

Induced systemic resistance can be demonstrated by applying a BCA at a location separated from the plant organ that is challenged by a pathogen. T39 induced plant defence against *B. cinerea* in tomato, lettuce, pepper, bean and tobacco: T39 was applied to the soil (in all crops) or to the lower leaves (in bean) whereas the disease was induced on the upper canopy parts of the plants. Given the spatial separation of the T39 application from the pathogens, this effect could be attributed to the ISR imparted by *T. harzianum* T39 [9,10].

Given the lack of examples of highly successful BCAs in practical disease management, we may conclude that the space for current BCA is unlikely to be in the region where satisfactory and stable disease control could be reached rapidly. The characteristics of BCA are such that slight changes in the external environment could result in drastic changes in the system dynamics and hence biocontrol efficacies. This may explain often observed inconsistencies in biocontrol efficacy in practice since spatio-temporal environmental hetero-geneity is a rule rather than an exception, and can lead to

widespread disease prior to the required colonization or ISR induction by the biocontrol agent. Generating increased receptivity in the host to immunity activation by BCAs could potentially improve BCA effectivity and disease control.

As part of its plant colonization strategy, trichoderma spp. can secrete various compounds and molecules which can effect host priming/ ISR. One of these molecules synthesized by, and secreted from several species of Trichoderma, including *Trichoderma viride* and *Trichoderma reseii*, is a Family 11 xylanase enzyme known as ethylene inducing xylanase, EIX. The Trichoderma fungal protein elicitor EIX, induces ethylene biosynthesis, electrolyte leakage, expression of PR proteins and the hypersensitive response (HR) in specific plant species and/or varieties [11–14]. EIX was shown to specifically bind to the plasma membrane of responsive cultivars of both tomato and tobacco [15]. The response to EIX in tobacco and tomato cultivars is controlled by a single dominant locus, termed LeEix [16]. The LeEix locus contains two receptors, *LeEix1* and *LeEix2*, both belonging to a class of leucine-rich repeat cell-surface glycoproteins. Both receptors are able to bind the EIX elicitor while only the LeEix2 receptor mediates plant defense responses [16,17]. LeEix1 acts as a decoy receptor and attenuates EIX induced signaling of the LeEix2 receptor [18,19].

Considering that EIX acts to increase plant immunity through binding and downstream signaling of LeEIX2, and that LeEIX1 can act to block this immunity promoting downstream signaling, we reasoned that knocking out LeEIX1 might result in stronger immune activation, leading to increased host response to *trichoderma* biocontrol agents and enhancement of disease resistance conferred by *trichoderma*.

The goal of this work was therefore to increase trichoderma response and disease resistance, by knocking out LeEIX1.

Results

Generation of LeEIX1 CRISPR mutants

We generated *LeEIX1* knockouts using CRISPR/CAS9. Designing specifics gRNAs to target only *LeEIX1* and not *LeEIX2*, we obtained >10 mutant tomato lines with different mutations. We analyzed the different lines for zygosity and were able to obtain two homozygous lines, one with a two base deletion (1-b5) and the other with a two base deletion and additional insertions (1-1-4), at a PAM site ~330 nucleotides after the LeEIX1 ATG (Figure 1a). Both mutations are predicted to result in a frame shift causing a stop codon, resulting in a truncated 113 amino acid protein from the N-terminus of the 1031 amino acid full LeEIX1, with leeix1-1-4 additionally having 13 "nonsesnse" amino acids prior to the premature stop (Figure 1b). The truncated proteins formed in the mutants contain only the signal peptide and N-terminal Leucine zipper, and do not have the LRR domains important for ligand recognition and protein-protein interactions, or the transmembranal domain required for PM insertion (Figure 1c). Thus, these truncated proteins formed would likely not be inserted in the membrane, or be able to bind the xylanase ligand or protein interactors- leading to null of LeEIX1 functionality.

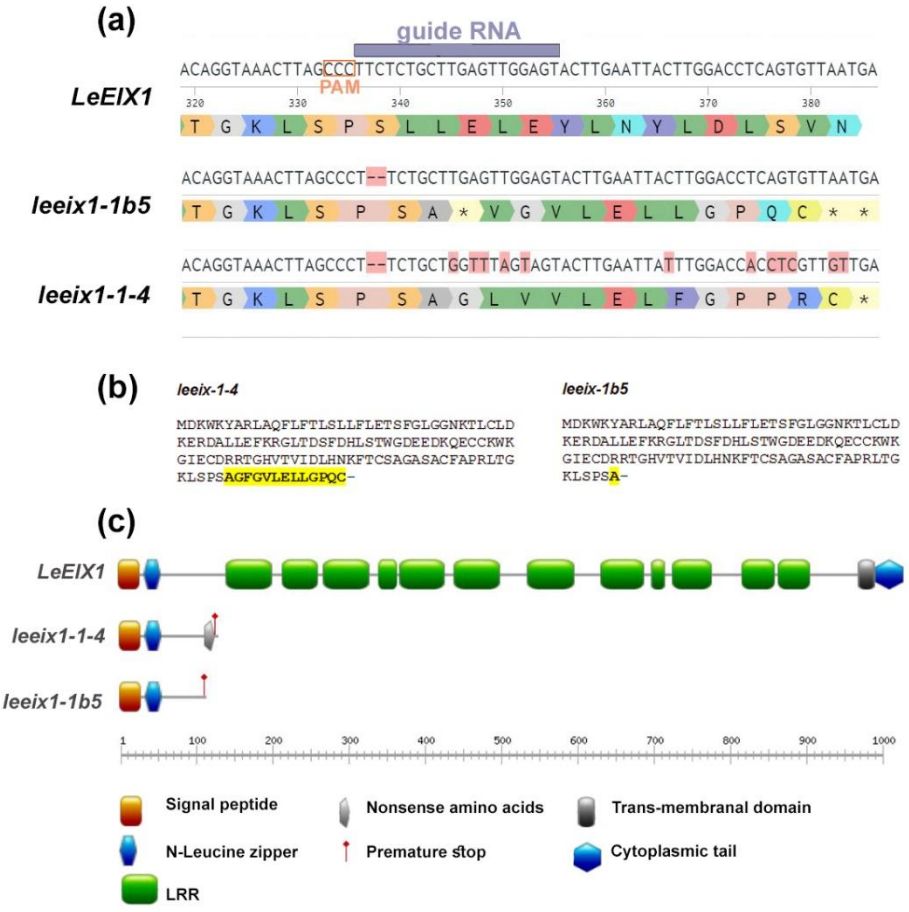

Figure 1: Description of generated *leeix1* lines.

(a) Representation of gRNA/ PAM site and resultant mutations in the LeEIX1 sequence.

(b) Predicted protein sequence of the mutants.

(c) Protein domain analysis of LeEIX1 alongside the mutant truncated proteins.

Verification of agricultural quality of generated mutants

LeEIX1 and *LeEIX2* were originally identified using a screen of the *S. penellii* introgression populations [13,16,20]. Based on our previous knowledge that the introgression line IL-7-5 that lacks ~40 genes on the short arm of chromosome 7, including both LeEIX1 and LeEIX2, has ostensibly normal development, we surmised that the mutated *LeEIX1* lines would likely not have developmental defects. We analyzed their agricultural quality, finding that the *leeix1* plants have similar developmental progression, agricultural quality, yield, and tomato quality, as their wild type M82 background line (Figures 2,3).

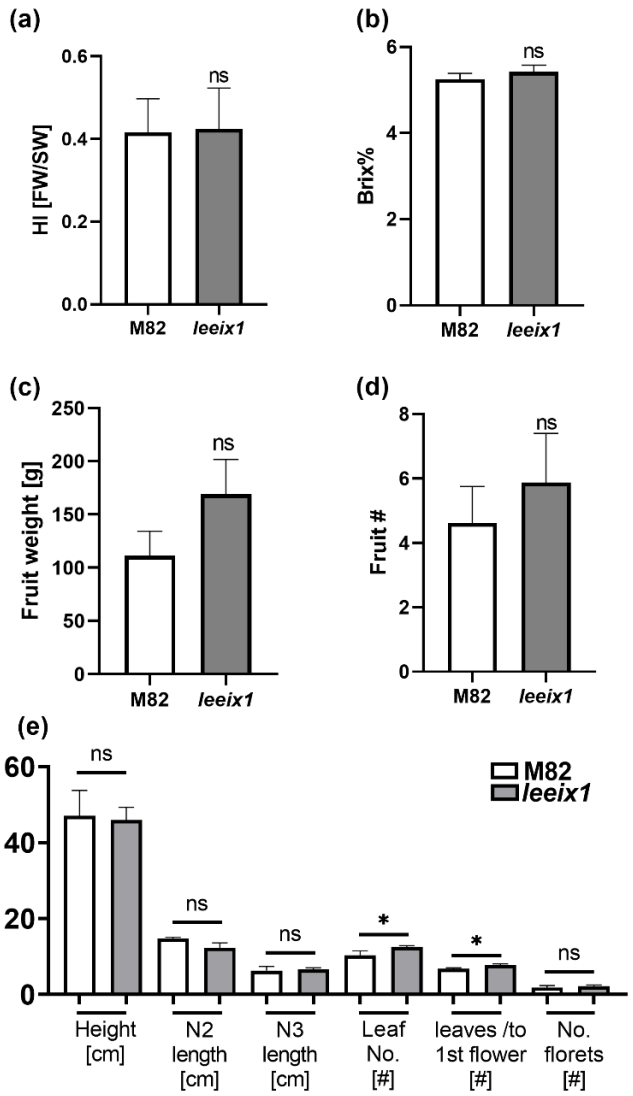

Figure 2: *leeix1* mutants have similar agricultural quality as the background M82 line.

Agricultural and developmental parameters were measured in M82 and *leeix1* plants.

(a) Harvest index (HI) of plants was calculated as the ratio between the total mass of fruit yield and the total biomass.

(b) Total soluble sugars were measured using a refractometer and are expressed as °Brix.

(c) Total fruit weight per plant.

(d) The average total number of tomato fruits produced per plant.

(e) Analysis of growth and development parameters: Height, length of nodes 2 and 3 (N2, N3), Number of leaves, Number of leaves produced in the vegetative stage (before the first flower), and Number of florets. Average ±SEM of at least four independent replicates is shown, N=18. No statistically significant differences were observed among WT and *leeix1* (t-test, welch correction).

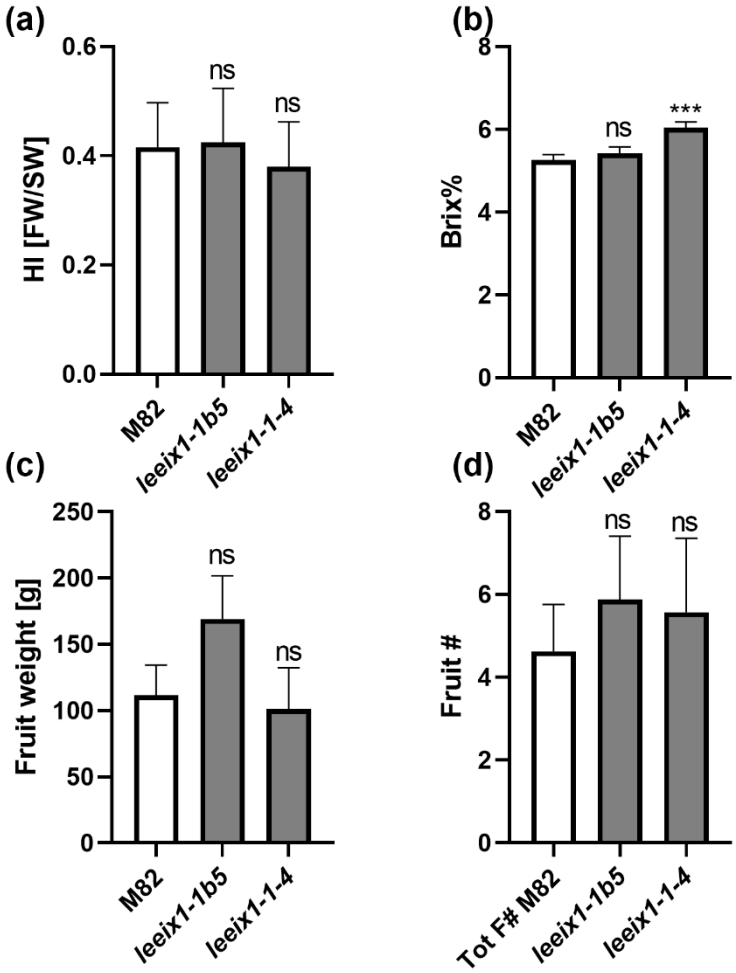

Figure 3: *leeix1* mutants have similar agricultural quality as the background M82 line- separate analysis of two independent *leeix1* mutant lines.

Agricultural and developmental parameters were measured in M82 and *leeix1* plants of lines *1-4* and *1b5*.

(a) Harvest index (HI) of plants was calculated as the ratio between the total mass of fruit yield and the total biomass.

(b) Total soluble sugars were measured using a refractometer and are expressed as °Brix.

(c) Total fruit weight per plant.

(d) The average total number of tomato fruits produced per plant. Average ±SEM of at least three independent replicates is shown, N=8. No statistically significant differences were observed among WT and *leeix1* lines except in the case of Brix, where line 1-4 had increased soluble sugars (t-test, Welch's correction, ***p<0.001).

leeix1 mutants display stronger disease reduction in response to T. harzianum treatment than their WT counterparts

To examine whether *T. harzianum* mediated biocontrol was improved in *leeix1* mutants, we treated WT and *leeix1* plants with *T. harzianum* T39, and infected the plants with the necrotrophic fungi *B. cinerea* or *S. sclerotiorum* 3 days after the first treatment. *leeix1* plants displayed greater reduction in disease levels following T39 treatment than WT plants (Figures 4, 5).

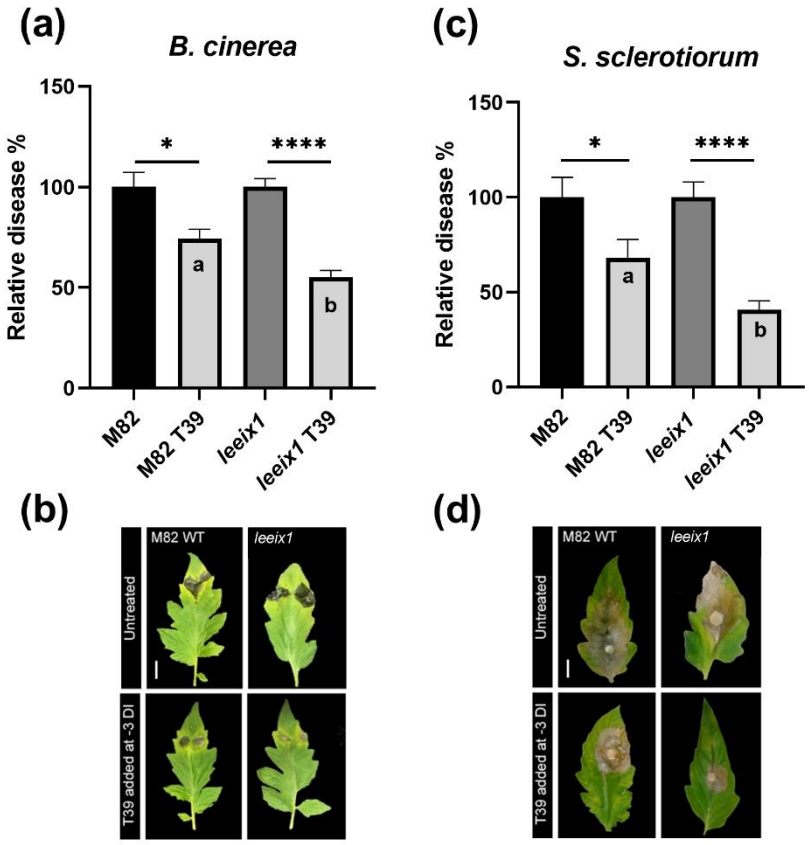

Figure 4: *leeix1* mutants have improved disease resistance in response to *T. harzianum.*

WT M82 and *leeix1* plants were challenged with *B. cinerea* ($10X^6$ spores /mL) (**a-b**) and *S. sclerotiorum* (mycelial plugs from 5 day old plates) (**c-d**).

(**a,c**) Relative disease area was calculated as the lesion area measured 5 days after inoculation in each genotype treated with T39 as compared to mock-treated controls.

(b) Representative images of *B. cinerea* symptomatic leaves.

(d) Representative images of *S. sclerotiorum* symptomatic leaves. (a) Average ±SEM of 6 independent replicates comprising 7 plants each is shown, N=85. (c) Average ±SEM of 3 independent replicates comprising 7 plants each is shown, N=20. In both cases, asterisks represent statistical significance of T39 treatment over control in a one way analysis of variance with a Bonferroni post-hoc test, $p<0.0165$, and letters represent statistical significance between the WT and *leeix1* T39 treated samples in a one way analysis of variance with a Bonferroni post-hoc test, $p<0.03$.

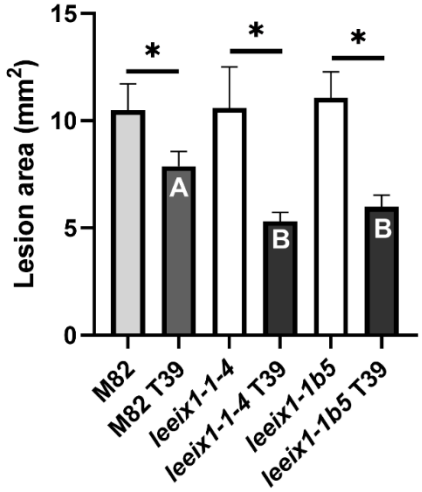

Figure 5: *leeix1* mutants have improved *B. cinerea* disease resistance in response to *T. harzianum*- separate analysis of two independent *leeix1* mutant lines.

WT M82 and *leeix1* plants of lines *1-4* and *1b5* were challenged with *B. cinerea* ($10X^6$ spores /mL). Lesion area was measured 5 days after inoculation in each genotype treated with T39 as compared to mock-treated controls. Average ±SEM of 3 independent replicates is shown, N=7. Asterisks represent statistical significance of T39 treatment over control, and letters represent statistical significance between the WT and *leeix1* T39 treated samples, in unpaired two-tailed ttests ($p<0.05$). No significant difference between the two *leeix1* mutant lines was observed.

Similar results were also observed with the biotrophic fungal pathogen *Oidium neolycopersici* (Figure 6).

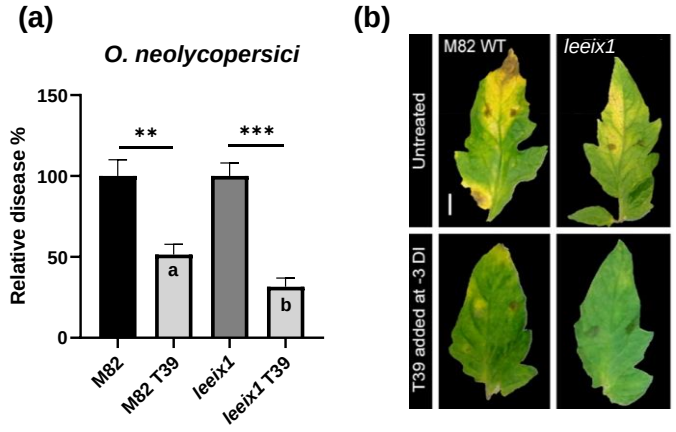

Figure 6: *leeix1* mutants have improved disease resistance to *Oidium neolycopersici* in response to *T. harzianum*.

WT M82 and *leeix1* plants were challenged with *O. neolycopersici* (10^4 spores/ mL).

(a) Relative disease area was calculated as the necrotic area measured 10 days after inoculation in T39 treatments, as compared to mock-treated controls. Average ±SEM of 3 independent replicates comprising 4 plants each is shown, N=12. Asterisks represent statistical significance of T39 treatment over control, and letters represent statistical significance between the WT and *leeix1* T39 treated samples- in a one way analysis of variance with a Bonferroni post-hoc test, $p<0.025$.

(b) Representative images of *O. neolycopersici* symptomatic leaves.

leeix1 mutants have stronger defense response activation in response to Trichoderma harzianum treatment than WT plants

What is the basis for the increased disease resistance we observed in our *leeix1* mutants? To examine whether *leeix1* mutants had stronger immune responses to trichoderma than control plants, we examined defense responses following treatment with the trichoderma derived elicitor Ethylene Inducing Xylanase (EIX), in *leeix1* mutants and control plants. *leeix1* plants displayed greater

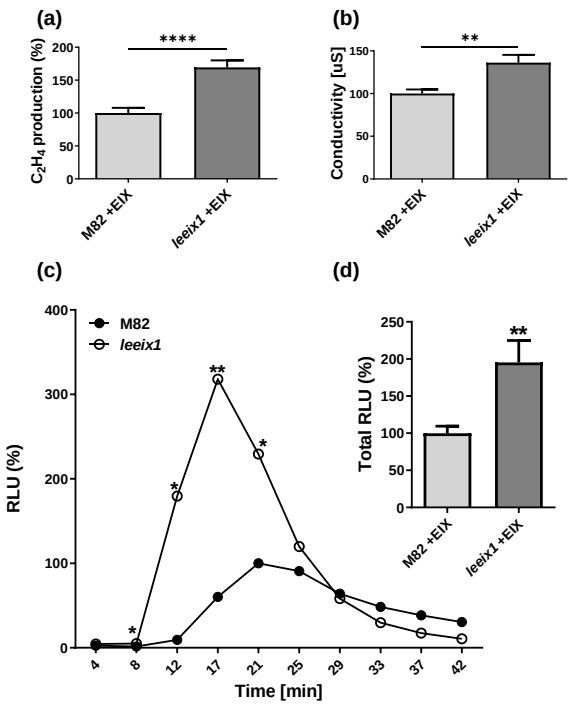

Figure 7: *leeix1* mutants have improved defense responses to the Trichoderma elicitor Xyn11/ EIX.

(a) Ethylene induction after EIX elicitation in M82 and *leeix1* was measured using gas-chromatography. M82 average ethylene production is defined as 100%, average ± SEM of 3 independent experiments comprising 4 plants each is presented, N=12. Asterisks represent statistical significance in t-test with Welch's correction (****, p-value <0.0001).

(b) Conductivity of M82 and *leeix1* samples immersed in water supplemented with EIX for 24 h was measured. Average ± SEM of 3 independent replicates comprising 4 plants each is shown, N=12. Asterisks represent statistical significance in t-test with Welch's correction (**, p-value <0.01).

(c) ROS production of WT M82 and *leeix1* was measured immediately after EIX application every 4 minutes using the HRP-luminol method. Average ± SEM of 3 independent experiments comprising 4 plants each, with 2 technical replicates, is shown, N=24 per time point. Asterisks represent statistical significance in multiple t-tests (one per time point), *p<0.05, **p<0.01.

(d) Shows total ROS production, **p<0.01 in t-test with Welch's correction, N=24.

responses to EIX, generating significantly higher levels of Ethylene (Figure 7a) and ion leakage (Figure 7b), as well as reactive oxygen species (ROS, Figure 7c-d) in response to EIX treatment.

To further characterize the immune response in *leeix1* plants, we examined defense gene expression in response to T39 treatment as well as botrytis infection, in WT and *leeix1* plants. T39 appears to effect similar expression of the genes *PR1a*, *Pti5*, *PI-2* and *beta-glucanase* in WT and *leeix1* plants (Figure 8), while defense gene expression in response to botrytis is reduced in *leeix1* mutants as compared with WT in several cases.

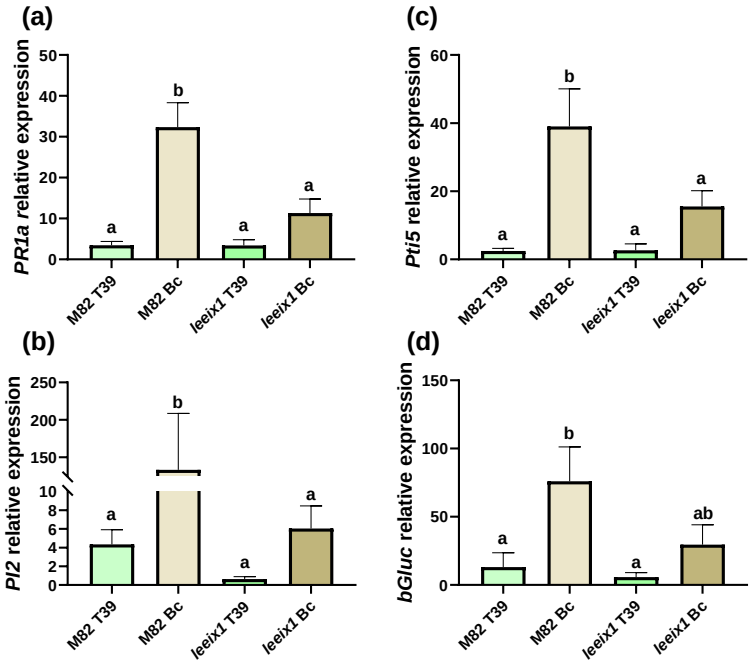

Figure 8: *leeix1* mutants have similar T39 induced gene expression and reduced defense gene induction in response to *B. cinerea*.

Gene expression analysis of pathogen responsive genes was measured by RT-qPCR in M82 and *leeix1* plants 24 h after application of T39 by soil drench, or plant spraying of *B. cinerea* ($10X^6$ spores /mL). Relative expression normalized to control M82. Average ±SEM of three independent experiments is shown.

(a) *Pathogenesis Related Protein 1a* (*PR1a*, Solyc01g106620): letters indicate statistical significance in a one-way anova with a Dunnett post-hoc test, $p<0.034$, N=11.

(b) *Proteinase inhibitor 2* (*PI-2*, Solyc03g020080): letters indicate statistical significance in a one-way anova with a Tukey post-hoc test, $p<0.037$, N=8.

(c) *Pto-interacting 5* (*Pti5*, Solyc02g077370): letters indicate statistical significance in a one-way anova with a Tukey post-hoc test, $p<0.016$, N=7.

(d) *β-1,3-glucanase* (*bGluc*, Solyc01g060020): letters indicate statistical significance in a one-way anova with a Tukey post-hoc test, $p<0.046$, N=7.

Trichoderma harzianum leaf colonization is similar in WT and leeix1 mutants

Biocontrol of microorganisms can be a result of induced systemic resistance, as was reported previously in several cases including for T39 [7,9,21], or, alternatively, can also be the result of microorganism colonization of the plant and direct or chemical effects of said colonizing microorganisms on the attacking pathogens. Additionally, BCA colonization of the plant may be a requirement for the induction of ISR. We examined T39 colonization of tomato leaves, finding no difference in colonization between WT and *leeix1* (Figure 9), leading to the conclusion that colonization is not a requirement for induction of ISR in our system, as was previously reported for several BCAs/ trichoderma strains [7,21,22].

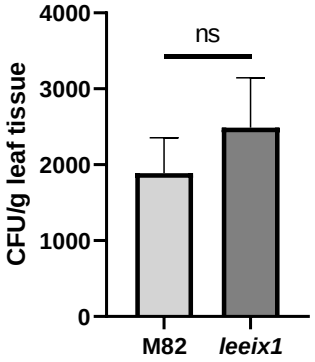

Figure 9: *leeix1* mutants are colonized with T39 to similar levels as M82 WT.

CFU amount of T39 in leaf tissue was measured 5 days post inoculation in 3 independent experiments, graph represents average CFU per leaf ±SEM of three independent experiments, N=12. No significant differences among samples were found (t-test).

Leeix1 responds similarly to Additional Trichoderma strains

To further characterize the improved receptivity to biocontrol we observed in our *leeix1* mutants, we examined two more trichoderma isolates in their ability to both induce resistance and colonize plants. Another isolate of *T. harzianum*, TCIM, was selected, as well as an isolate of a different species of trichoderma, *T. longibrachiatum*, known as Ti166. Both isolates have proven biocontrol activity [23]. Based on our experience with different species of trichoderma, we expected the activity of TCIM and TI66 to likely be lower than that of the outstanding T39 isolate. As expected, all 3 trichoderma isolates showed improved biocontrol activity against *B. cinerea* in *leeix1* as compared with the WT M82 background (Figure 10a), while no differences in colonization were observed (Figure 10b).

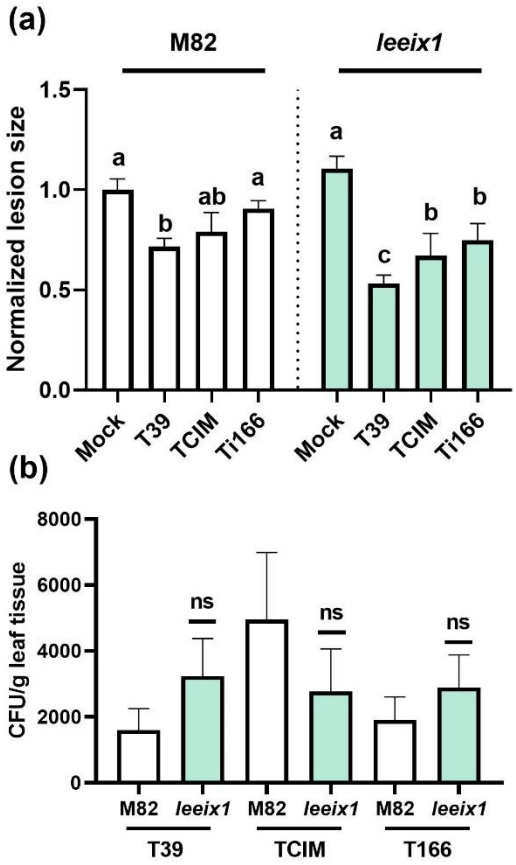

Figure 10: Improved biocontrol and similar colonization of different trichoderma strains in *leeix1* mutants.

(a) Resistance to gray mold after treatment with various trichoderma strains. Plants were treated with trichoderma by irrigation with water containing 10^7 spores, and were inoculated after 3 days with drops (10 µl) of $5x10^5$ botrytis suspension) produced from a ten-day old PDA plate. *leeix1* mutants have a more significant reduction in gray mold disease than the wild-type M82 after treatment with all trichoderma strains. Graph shows the mean and standard error of four experiments, (N = 18). The letters denote statistical significance in a one way ANOVA with a Tukey post hoc test, p <0.0002.

(b) CFU amount of different trichoderma strains in leaf tissue was measured 5 days post inoculation in 3 independent experiments, graph represents average CFU per leaf ±SEM of three independent experiments, N=12. No significant differences among samples were found (t-test).

Discussion

This work was based on the idea that removing a plant receptor that blocks immune responses in response to trichoderma treatment, will increase these immune responses and, thus, result in improved biocontrol. In order to ensure that the mutant lines generated will be agriculturally valuable, we used Crispr/cas9 technology, to preserve the ability of outcrossing the transgene to generate non-GMO mutants. We generated several lines of *leeix1* mutants in this manner. In order to work with fixed lines, following our initial screens on the mutants generated, we selected two homozygous lines with different mutations to continue our work with (Figure 1). Agricultural quality of these lines, which was expected to be similar to WT M82 based on the knowledge available for the introgression line IL7-5 [20] which lacks the *LeEIX1* gene, was found to be unaffected (Figures 2,3), with the exception of an increase in soluble sugars in line 1-1-4. The underlying causes for this increase in soluble sugars are not known, and will be investigated in future work aimed a deciphering the effects of trichoderma on tomato agricultural quality. We subsequently observed that there are no significant differences between the two selected *leeix1* mutant lines (Figures 3,5).

We examined biocontrol responsiveness by assaying disease levels with 3 different pathogens in *leeix1* mutants following trichoderma treatment. **While gray mold, white rot, and powdery mildew disease levels were reduced in the background WT M82 following trichoderma treatment, the reductions were more significant in all cases in the *leeix1* mutants** (Figures 4-6).

Trichoderma biocontrol works primarily by inducing resistance in the host plant [7,24]. Molecular analysis of the underlying cause for increased trichoderma receptivity in *leeix1* mutants revealed that these mutants respond more strongly to the trichoderma elicitor EIX (Figure 7). Gene expression analysis indicated that while defense genes are activated to similar levels in *leeix1* mutants as compared with WT M82 plants following trichoderma treatment, subsequent *B. cinerea* inoculation results in significantly lower induction of defense genes. This indicates that induced resistance in *leeix1* mutants is more effective, as evidenced by defense assays (Figure 7) and reduction in disease levels (Figures 4-6). **This confirms that the mechanism of action for *leeix1* increased receptivity to trichoderma biocontrol is likely more efficient/ stronger induced resistance**.

We also examined the possibility of a colonization based mechanism, finding no evidence of preferential colonization of *leeix1* mutants by trichoderma (Figure 9). Given that we observed an increase in induced resistance in the *leeix1* mutants, which is known not to depend on colonization, this was expected [22].

To examine the breadth of the increased responsiveness of *leeix1* mutants to trichoderma based biocontrol, we examined two additional isolates, TCIM and Ti166, and observed similar results to those obtained with T39. In all three cases, disease reduction was improved in the *leeix1* mutants following trichoderma treatment, and colonization was found unaffected (Figure 10). T39 was more effective in reducing disease than the other two trichoderma isolates we examined. T39 is known to be a potent biocontrol agent [8,9], while the other two isolates we used were less investigated, and never examined in tomato. Thus, it is possible that a host specificity mechanism governs plant receptivity to trichoderma and the resultant induced resistance, or that T39 specifically possess characteristics that render it an improved plant immunity inducer. This could potentially be an interesting question to be investigated in future work.

The proposal work has successfully concluded, and we have obtained tomato lines with an improved response to trichoderma biocontrol which stems from increased induced resistance. We now hope to submit a continuation proposal, which will focus on (1) testing *leeix1* mutant plants, from which the cas9 transgene has been outcrossed, in field trials, to examine if their improved trichoderma response can translate to improved yield in agricultural conditions following trichoderma treatment; and (2) examining whether this improved induced resistance is trichoderma-specific, or can be applied to additional biocontrol agents and immunity inducers.

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