Item No. 1 of 1

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TISSUE-SPECIFIC ENGINEERING OF TERPENES WITH ACTIVITY AGAINST APHIDS IN CULTIVATED TOMATO

NON-TECHNICAL SUMMARY: Aphids, a group of small pest insects,pose a serious problem in horticultural crop production including tomato, since damage is caused not only by their direct feeding on plants, but even more so by the transmission of viruses for which aphids serve as vectors. This results in reduced crop yield and quality, and often plant death even at low levels of aphid infestation. Current control strategies utilizing synthetic insecticides are increasingly inefficient due to emerging resistances and avoidance behavior of aphids, and are also problematic due to adverse effects on the environment and concerns of consumers. Although significant efforts have been made to generate tomato plants resistant to aphids and viruses, little success has been reported. Tomato is a major vegetable grown throughout the U.S. with a total revenuefrom tomato production in the year 2016of >$2 billion. Although aphidsarea key pest in both field and greenhouse production of tomato, there is no cure for tomato plants infected with viruses transmitted by aphids. Therefore, development of tomato lines resistant to aphids as a preventative control measure is urgently needed.Terpenes, a highly volatile class of compoundsnaturally synthesized by plants, are known to efficiently contribute to the defense of plants by repelling or intoxicatingpest insects. While in wild plants these defensive traits are constantly under positive selection pressure to increase survival, it appears that they have been at least partially lost in crop plants since breeding has favored other agronomic traits. A number of wild tomato species, such as Solanum habrochaites, were found to produce a diverse set of volatile terpenes which are not present in cultivated tomato (Solanum lycoperiscum). Thus wild tomatovarieties can be a good source for defensive volatile terpene traits that act repellent and toxic against pests and could be introduced into cultivated tomato. In the first part of this project we will therefore analyse a collection of wild tomato varieties to identify those producing terpenesthat showrepellent activity against aphids as well as effects on the feeding behavior and survival of aphids. In the second part of the project key genes involved in the biosynthesis of the identifiedhighly effective terpenes will be isolated from respective wild tomato varieties. Subsequently these genes will be used to engineer the production of terpenes with activity against aphids into cultivated tomato plants. We will use regulatory elementsto achieve terpene formation in three specific plant tissues that align with essential stages in the aphid feeding behavior - orientation/landing on host plant, probing of plant tissues, and feeding on plant vascular tissue. The obtained new engineered tomato lines will initially be analyzed to verify the formation of the desired terpene products in the targeted plant tissue

qualitatively and quantitatively. In the third part of this project the newtomato lines will be used to evaluate how the terpene formation engineered into the different plant tissues affects the development, reproduction and feeding behavior of aphids.The long-termgoal of this project is a novel biologically-based, innovative and environmentally-sound pest management strategy that utilizes tissue-specific engineering of terpenes in tomato as a control measure against aphids.This project now providesthe opportunity to obtain base-line data for seeking further research opportunities to investigate the modes of action of the terpenes produced in the engineered tomato plants against aphids. In the future the plant lines generated in this project will also provide the opportunity to test this management strategy for other pests.

OBJECTIVES: The majorgoal of this proposalis to establish a system toward the development of novel aphid control strategies via metabolic engineering of plants. Wild tomato species, such as Solanum habrochaites, were found to produce a diverse set of terpenes, a class of plant metabolitesthat can act repellent and/or toxic against pests. Thus wild tomato accessions can be a good source for defensive traits that can be introduced into cultivated tomato (Solanum lycoperiscum). Our central hypothesis for this proposal is that engineering of highly effective terpenes from wild tomato accessions into specific tissues of cultivated tomato will allow to hinder aphid feeding. Three specific objectives will be addressed in this proposal:Objective 1: Assess if wild tomato accessions with different terpene profils affect the host plant preference and feeding behavior of aphids.Objective 2: Determine how expression of respective terpene biosynthetic genes under the control of different tissue-specific promoters changes the terpene profile in resulting engineered cultivated tomato lines.Objective 3: Determine how engineering of repellent and/or toxic terpenes in three tissues (trichomes, epidermis and phloem) of cultivated tomatochanges the overall performance and feeding behavior of aphids.

APPROACH: To achieve the three specific objectives proposed in this project we will utilized the following methods:Objective 1:For the analysis of the repellent activity of wild tomato accessions against aphids we will use the potato aphid (Macrosiphum euphorbiae). We have already established a collection of 17 wild tomato (Solanum habrochaites) accessions representing 5 subgroups that accumulate distinct terpenes and several cultivated tomato (S. lycopersicum) accessions for comparison in our greenhouse. Our preliminary studies including non-choice tests as well as feeding assays with tomato leaf extracts have indicated that terpenes found in two of these subgroups have an effect on the overall performance and the feeding behavior of the aphids. We will now perform choice tests with a Y-tube olfactometer to determine the respeonse of aphids to volatile terpenes emitted from the wild tomato accessions. Leaves or leaf extracts from a S. habrochaites accession will be placed in one of two chambers connected to the Y-tube arms, while the second chamber will be left empty or filled with S. lycopersicum leaves or extracts. Aphid repellency to an accession will be tested for statistical differences to determine if distribution of choices deviate from random. This assay will further verify if some ofthe S. habrochaites accessions produce terpenes with repellent activity against aphids that are then candidates for metabolic engineering in cultivated tomato.Objective 2:Design of multicistronic expression constructs and transformation of cultivated tomato: In the next step we will engineer the production of 1) β-caryophyllene/α-humulene and 2) santalene/endo-α-bergamotene that already showed activity against aphids in our preliminary studies into cultivated tomato. To achieve terpene formation in tissues that align with essential stages in aphid feeding behavior - host orientation/landing, probing, and phloem feeding - we will use specific promoters to control transgene expression. We have selected the tomato SlTPS9-, the Arabidopsis CER5-, and the Arabidopsis AtSUC2-promoter based on their ability to direct expression in type VI glandular trichomes, epidermal cells, and phloem companion cells, respectively. Under contol of these promoters we will co-express a given terpene synthase (TPS) and a respective prenyl transferase (PTS) providing the required prenyl diphosphate substrate which will result in high level production of desired terpenes. In addition to PTSs and TPSs we will express green fluorescent protein (GFP) under the same promoters, which will serve as visible marker highlighting target tissues and allow to track aphid feeding. To achieve expression of all three genes (PTS, TPS, GFP) in parallel under control of the same promoter, we will utilize a technique for multicistronic gene expression based on the use of viral 2A sequences that

result in ribosomal skipping. For the design of expression constructs promoters will be amplified by PCR from genomic DNA, coding regions of TPSs and PTSs will be amplified by PCR from leaf or trichome cDNA from our available tomato accessions with primers designed on published gene sequences, and then cloned into the binary vectors. Sequences encoding viral 2A peptides will be added to primers so that in the final multicistronic construct all genes will be fused in frame by 2A sequences. All binary vector constructs will be introduced into Agrobacterium tumefaciens. S. lycopersicum line MP-1 will be transformed by Agrobacterium mediated leaf disc transformation. Since stable transformation may take several months, we will use all constructs for transient transformation by Agrobacterium infiltration of leaves. This will allow us to verify function of constructs and do first analysis on infiltrated leaves prior to obtaining stable lines.Genetic and biochemical analysis of engineered tomato lines: By stable transformation we expect to obtain ~20 lines per construct which will be tested for presence of transgenes by PCR on genomic DNA. To screen for terpene production, ground leaves will be extracted with solvent (MTBE) and extracts analyzed by GC-MS. Based on this initial screen 2-3 lines per construct with sufficient terpene formation will be selected. Expression of all transgenes will be confirmed by quantitative RT-PCR. Emitted volatiles will be collected from leaves by closed-loop stripping. Internal terpene pools will be extracted either by dipping leaves in MTBE or by extracting ground tissue in MTBE. Collected volatiles and MTBE extracts will be analyzed by GC-MS to verify qualitative and quantitative composition of terpene profiles. To confirm tissue-specificity transgene expression and terpene production in the targeted tissues will be analyzed (glandular trichomes, epidermis, phloem companion cells). We will verify GFP expression in different lines by fluorescence microscopy. In addition, we will isolate fractions enriched in particular tissues and use these for analysis of gene expression and terpene formation. Trichomes will be isolated by gently scraping tomato tissue frozen in liquidnitrogen with a chilled spatula. Epidermis and vascular tissue will be prepared by mild digestion of leaf discs with cellulose and macerozyme.Objective 3:The obtained tomato lines with engineered terpenes will then be used to assess the effects of terpene production in the three target tissues on the performance of aphids. Towards this goal we will perform non-choice assays by enclosing newly emerged nymphs in clip-cages on the leaf surface of these engineeredtomato lines and checking the status of introduced aphids and numbers of offspring daily. Likewise choice tests with a Y-tube olfactometer will be conducted with the transgenic tomato lines and MP1 control, similar to the choice tests described in Objective 1. For each of thedifferent constructstwo selected transgenic lines will be utilized. The results of these experiments will verify if terpenes produced in engineered tomato lines relative to control affect the development, reproduction, and feeding behavior of aphids. We will also confirm if aphids are acquiring cell/phloem content by analyzing their ingestion of GFP with fluorescence microscopy.

PROGRESS: 2019/05 TO 2020/05
Target Audience:The outcomes of this project during the current reporting period are primarily relevant and of interest to audiences in the research communities of the plant sciences and entomology. The results and knowledge obtained during the current reporting period of this project have been communicated to this audience via an oral presentationat an international scientific conference. A secondplannedpresentation at a national conference had to be canceled due to the Covid-19 related travel ban. In addition, in the current reporting period results of the project have been summarized in two manuscripts that have been submitted for review and have been accepted for publication with minor revisions. However, this project and its results are also relevant and of interest to students in the WVU School of Agriculture and Food. Thus aspects of this project were integrated in the curriculum of the PLSC206 'Principles of Plant Science'class and results of the project were also presented in the AGBI199 'Orientation to Biochemistry' class. Changes/Problems:Due to the Covid-19 related lock down of the WVU campus and the resulting major delay and set back of the project, we have requested and were recently granted a no-cost extension by one year for this project. What opportunities for training and professional development has the project provided?In the current reporting period the project provided training and professional development opportunities for one PhD graduate student and one undergraduate student: Fumin Wang, the graduate student funded through this project, performed all experiments during the current reporting period and received training in entomology, chemical ecology, plant biochemistry and molecular biology under the guidance of the PI

and Co-PI. The graduate student also had the opportunity to contribute significantly to the preparation, submission and current revision of two manuscripts summarizing some of the results of this project. Meleana Santivasci, an undergraduate student majoring in horticulture, assisted in the analysis of some of the tomato lines utilized in the project and received training in basic laboratory skills and in the analysis of terpenes under the guidance of the PI and the graduate student. How have the results been disseminated to communities of interest?In the current reporting period the results of the project have been disseminated to the research community through an oral presentation by the PI at the 14th International Meeting on the Biosynthesis, Function and Synthetic Biology of Isoprenoids, TERPNET 2019, Halle/S. Germany: "Glandular trichome-derived mono- and sesquiterpenes of cultivated and wild tomato accessions have different effects on aphid performance and feeding behavior". The results obtained from our analysis of cultivated and wild tomato accessions, and how their different terpene profiles affect the host plant preference and feeding behavior of aphidshave been summarized during the current reporting period in two manuscripts which have been submitted for review and publication to two scientific journals. Both manuscripts have been reviewed favorably and only minor revisions have been requested by the journal editors prior to publication. The graduate student as well as the PI and CoPI are currently working on the revision of both of these manuscripts: 1) Fumin Wang, Yong-Lak Park and Michael Gutensohn (2020) Glandular trichome-derived sesquiterpenes of wild tomato accessions (Solanum habrochaites) affect aphid performance and feeding behavior. Phytochemistry (under revision) 2) Fumin Wang, Yong-Lak Park and Michael Gutensohn (2020) Glandular trichome-derived mono- and sesquiterpenes of tomato have contrasting roles in the interaction with the potato aphid Macrosiphum euphorbiae. Journal of Chemical Ecology (under revision) What do you plan to do during the next reporting period to accomplish the goals? Considering the recentCovid-19 related campus lock-down we envision the following tentative timetable for the completion of the outstanding portions of the project: With research activities partially resumingon the WVU campus (since earlyJune 2020) we anticipate that we will need approximately six month for the stable transformation of cultivated tomato with the available multicistronic expression constructs, as well as the subsequent genetic and biochemical characterization of the resulting transgenic tomato lines (Objective 2B). Unfortunately, the test of the expression constructs via transient expression in tomato leaves will have to be repeated since the ongoing experiments have been lost due to the Covid-19 related campus lock-down. In general, we will take a stacked approach which will allow us to already characterize one set of transgenic lines while working on the plant transformation with the next expression constructs in parallel. As soon as confirmed transgenic tomato lines will be available, we will utilize these lines for non-choice and choice assays with aphids (Objective 3). We anticipate that we will need additional four to six month for the analysis of the aphid performance on the engineered tomato lines.

IMPACT: 2019/05 TO 2020/05
What was accomplished under these goals? While overall three specific objectives will be addressed in this proposal, we have achieved significant progress on Objectives 1 and 2 during the current reporting period: Objective 1: In the previous reporting period we had analyzed a collection of different tomato accessions (S. habrochaites & S. lycoperiscum) representing 6 different terpene chemotypes for their effect on performance and behavior of potato aphids. Non-choice assays indicated that two groups of tomato accessions producing santalene/bergamotene and caryophyllene/humulene, respectively, had significant effects on the longevity and reproduction of aphids. When terpenes were extracted from tomato accessions and used in aphid feeding assays on artificial diet, extracts from the santalene/bergamotene and caryophyllene/humulene producing accessions significantly affected aphid feeding behavior and survival. Moreover, when these tomato accessions and respective terpene extracts were used for olfactometer choice assays santalene/bergamotene and caryophyllene/humulene had repellent activity towards aphids. To further verify that the observed effects on the performance and behavior of aphids is indeed due to respective terpenes additional control experiments were performed. Commercially available pure caryophyllene and humulene were used in feeding and choice assays and confirmed their effects on aphids. Since santalene and bergamotene are not available as pure compounds we instead used an available tomato introgression line producing these terpenes in non-choice, feeding and choice assays to confirm their effect on

aphids.In the current reporting period these results were summarized in a manuscript (see below) which has been submitted to the journal Phytochemistry and has received favorable reviews (minor revisions). Initiated by some results of this previous analysis we have performed additional experiments in the current reporting period utilizing trichome mutants of cultivated tomato (S. lycoperiscum) that are differently affected in mono- and sesquiterpene production. Non-choice and olfactometer choice assays were performed with these trichome mutants to study effects of these terpenes on performance and choice behavior of aphids. While caryophyllene and humulene contributed to host plant resistance against aphids (confirming our previous results obtained with S. habrochaites), glandular trichome derived monoterpenes in cultivated tomato appear to be exploited as cue for host plant orientation by aphids. These results now provide a good baseline knowledge prior to introduction of new terpene traits into cultivated tomato by metabolic engineering. These results were also summarized in the current reporting period in a manuscript (see below) which has been submitted to the Journal of Chemical Ecology and has received favorable reviews (minor revisions). Objective 2: The goal of this objective is to determine how the expression of respective terpene biosynthetic genes from S. habrochaites under the control of different tissue-specific promoters changes the terpene profile in resulting engineered cultivated tomato lines. In the current reporting period we have continued and completed our work on the design, construction, and cloning of the respective expression constructs and vectors required for the transformation of cultivated tomato that we had already started in the previous reporting period. In order to engineer the formation of these two groups of terpenes in three different tissues of cultivated tomato (glandular trichomes, epidermis, phloem companion cells) we have amplified the promoter regions of three genes (tomato SlTPS9, Arabidopsis AtCER5, Arabidopsis AtSUC2) by PCR and have cloned these into the multiple cloning site of the pMCS:GW vector. Constructs consisting of the open reading frames of the respective farnesyl diphosphate synthases and terpene synthases involved in the biosynthesis of these terpenes as well as enhanced green fluorescent protein linked in frame by viral 2A sequences were designed and then ordered from a commercial provider for gene synthesis. Unfortunately, we had to wait much longer (months instead of weeks) until these synthesized gene constructs were finally delivered to us which resulted in a significant delay of the project. In addition, the subsequent recombination of these large gene constructs under the control of the above promoters into the pMCS:GW vector by Gateway cloning turned out to be by far less efficient than expected which resulted in a further delay. We have completed the cloning of all six final vector constructs (two different gene constructs each under the control of three tissue specific promoter) and have subsequently introduced these vectors into Agrobacterium tumefaciens. We have recently performed a number of transient transformation experiments by Agrobacterium infiltration into tomato leaves with some of these vector constructs to verify their function prior to stable transformation. However, these experiments had to be abandoned due to the Covid-19 related lock-down of the WVU campus before the analysis of transgene expression, terpene formation and GFP localization could be performed and/or completed. Due to the transient nature of these transformation events these experiments are now lost and will have to be repeated. While the engineered tomato lines have not yet been available in the current reporting period, we have used an introgression line producing santalene/bergamotene in glandular trichomes for a first set of analyses. This introgression line was previously obtained by crossing a santalene/bergamotene producing S. habrochaites accession with a cultivated tomato accession and a series of subsequent backcrosses into the cultivated tomato background. We used this introgression line for the same type of non-choice and choice assays (Objective 3) as performed with the respective S. habrochaites accessions. The results indicated that formation of santalene/bergamotene in the background of cultivated tomato indeed has an effect on the performance and behavior of aphids. These results were included in the manuscript (see below) that had been submitted to the journal Phytochemistry and that we are currently revising. Since a respective introgression line is not available for caryophyllene/humulene accessions, in the current reporting period we have also crossed a caryophyllene/humulene producing S. habrochaites accession with a cultivated tomato accession. The F1 plants of this cross were tested for their terpene production and subsequently used for additional backcrosses into the cultivated tomato background.

PUBLICATIONS (not previously reported): 2019/05 TO 2020/05

1. Type: Conference Papers and Presentations Status: Published Year Published: 2019 Citation: Michael Gutensohn*, Fumin Wang and Yong-Lak Park "Glandular trichome-derived mono- and sesquiterpenes of cultivated and wild tomato accessions have different effects on aphid performance and feeding behavior" Oral Presentation, Session: Terpenoids in chemical ecology TERPNET 2019, 14th International Meeting on the Biosynthesis, Function and Synthetic Biology of Isoprenoids
2. Type: Journal Articles Status: Accepted Year Published: 2020 Citation: Fumin Wang, Yong-Lak Park and Michael Gutensohn "Glandular trichome-derived sesquiterpenes of wild tomato accessions (Solanum habrochaites) affect aphid performance and feeding behavior" Phytochemistry (accepted with minor revisions)
3. Type: Journal Articles Status: Accepted Year Published: 2020 Citation: Fumin Wang, Yong-Lak Park and Michael Gutensohn "Glandular trichome-derived mono- and sesquiterpenes of tomato have contrasting roles in the interaction with the potato aphid Macrosiphum euphorbiae" Journal of Chemical Ecology (accepted with minor revisions)