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Genetic Engineering of Volatile Chemicals in Ornamental Plants to Repel Insects

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Abstract

Four years ago, we started an effort in functional genomics and DNA sequencing that has led to the development of one of the largest collections of genes expressed in ornamental plants (petunia) in the world. After conducting focused microarray experiments, we have isolated several genes that we have identified to have potential utility for use in genetically engineering plants with altered floral fragrance, and a variety of other potentially valuable characteristics. We have also isolated several genes that appear to be expressed in specific floral tissues (ie. petals), and many of these contain DNA sequence that shares little or no similarity to other genes of known function in any other plant. We are currently engineering many of these genes to either over-express and/or knock out their expression in transgenic plants in an effort to uncover their biological function. Results have been promising to date, leading to the isolation of genes involved in the synthesis of several interesting biochemicals: **methylbenzoate**, a chemical giving flowers a sweet fragrance; **isoeugenol** - clove oil – a chemical giving flowers a spicy fragrance; **benzylbenzoate**, used commercially as an insecticide, miticide, and as an anti-fungal treatment; **2-phenylethanol** (rose oil) and **beta-ionone**, the two major constituents of rose fragrance. As a result of these efforts, we are producing transgenic plants with altered fragrances that are perceived differently by humans, as measured by sensory panelist analysis of engineered flowers. After learning more about how insects and pathogens interact with these volatile biochemicals, we feel that there is good potential for engineering production of volatile chemicals in plants at high enough levels to control some of these pests. Currently we are producing a number of transgenic plants for use in testing whether insect and pathogen activity can be affected through the engineering of volatile biochemicals. We have solidified collaborations with entomologists and pathologists in the US and Europe to begin conducting experiments focused on the analysis of insect and disease pests exposed to transgenic petunia plants with altered volatile synthesis. We think this work will have significant impact on the industry because it will produce the genetic tools and information required to produce transgenic plants with desirable fragrances, and plants that are tolerant to insect or disease pests.

Objectives and Methods

1. Prove the function of floral volatile genes by altering their expression in transgenic petunias.

We utilized microarray analysis to screen through 3400 unique petunia flower genes, and isolated those that were highly expressed in floral tissue compared to other parts of the plant. When comparing the DNA sequences of these genes to known sequences in public databases, many genes appeared to be involved in volatile chemical synthesis:

- Cleavage carotenoid dioxygenase (*PhCCD1*) – now confirmed role in beta-ionone synthesis
- BA:SA Carboxyl methyltransferase (*PhBSMT1&2*) – now confirmed role in methylbenzoate synthesis
- Tyrosine Decarboxylase (*PhTD1*) – potential role in phenylethanol (rose oil) synthesis
- Isoeugenol Synthesis Transferase (*PhIST1*) – potential role in isoeugenol (clove oil) synthesis
- Benzylalcohol benzyltransferase (*PhBEBT1*) – potential role in benzylbenzoate synthesis
- *there are several uncharacterized genes showing no matches to any known sequences

In order to determine the function of the three selected genes, transgenic knockout (using an RNAi approach) and over-expression plants for each gene are being constructed, and are being transformed into petunia using *Agrobacterium tumefaciens*. The plants are being screened to test whether they have altered synthesis of each particular volatile chemical.

2. Screen transgenic plants with altered volatile synthesis for their affect on humans, insects and pathogens.

The above experiments focus on the function of our selected genes. However, we also are interested in identifying the physiological significance of the selected genes, not only for use in the genetic engineering of floral aroma, but also in interactions with insects and pathogens. With this in mind we have devised three experiments to test the physiological significance of our selected genes on human, insect, and fungal/bacterial interactions.

Human Sensory Panels

To test whether knocking out or over-expressing our selected genes results in an alteration in the overall floral aroma that can be perceived by humans, we will setup a “triangle test” where two controls and one transgenic flower, or two transgenic and one control flower are placed in separate jars and closed for 15 minutes. The jars are then given to human panelists who are instructed to identify the jar that smells different from the others. Once we identify the components detected by humans, and which ones they prefer, we will use conventional breeding methods to combine these compounds in different combinations to make different fragrances. Once these combinations are made, flowers will be given to consumers for preference testing. These experiments will be conducted in the UF Food Science and Human Nutrition sensory labs with Dr. Charlie Sims.

Insect Pest Experiments

These experiments will test whether the knockout or over-expression of our selected genes and their subsequent volatiles will increase or decrease the attractiveness of the flowers to insect pests. This year, we started experiments focused on trying to understand how insects interact with plants that have been engineered for altered volatile synthesis. To address insect pollinators, we received vital information from Dr. Rob Raguso’s lab at the University of South Carolina and Dr. Cris Kuhlemeier's lab at the University of Bern (Switzerland) - both are well known for their experiments focused on how pollinators interact with different floral volatile compounds. To determine the potential for engineering volatile synthesis in order to repel insects, we have started conducting insect assays with cooperators at the University of Amsterdam in Dr. Michel Haring and Dr. Robert Schuurink's labs. In particular, we have been trying to work out assays to investigate the activity of whiteflies, aphids and spider mites on our plants.

Antibacterial/Antifungal Experiments

Expression results on the gene *PhBEBT1* have shown that after pollination, levels in the petal decrease while levels increase in the ovary, where seed formation takes place. While this was unexpected, it led to the hypothesis that benzylbenzoate might be a defense compound expressed in tissues critical to the function of the flower like the petals (when attracting pollinators) and the ovary (during seed development). In order to test this, we have isolated several model fungal and bacterial pathogens that will infect petunia flowers. We have grown them on sterile Petri dishes containing physiological levels (as determined by GC-MS) of the volatiles retained internally in floral tissue. Plates in which bacterial or fungal growth is inhibited as compared to untreated controls could represent volatiles with a defense role during the life of the flower. Volatiles that are shown to have defense properties will then be further tested using gene knockout and over-expression plants. The petals of the flowers from these plants are treated with either bacterial or fungal colonies, and the amount of time the flowers resist infection will be measured as compared to the wild-type control. These tests will help us determine whether the increase (over-expression) or decrease (RNAi) in the expression of the selected gene will lead to an alteration in susceptibility as compared to the control flowers.

Results (by objective)

1. Prove the function of floral volatile genes by altering their expression in transgenic petunias.

Currently transgenic gene knockout plants have already been successfully isolated for *PhBSMT1&2, PhBEBT1, PhIST1,* and over expression lines have been produced for *PhBSMT1&2, PhBEBT1, and PhCCD1.* We are currently producing the remaining transgenic plants to provide both knockout and over expression lines for all five genes for maximum ability to make all combinations of floral scent via traditional breeding. Emission from primary transgenic plants is being screened using flame-ionization gas chromatography to look for alterations in floral volatile production. Plants with positive phenotypes are then self-pollinated, and T1 plants grown. Lines showing 3:1 segregation will again be self-pollinated, progeny grown, and lines screened via PCR and gas chromatography in order to identify homozygous lines that can then be studied more extensively in a wide range of environments.

2. Screen transgenic plants with altered volatile synthesis for their affect on humans, insects and pathogens.

Human Experiments
Solid results in this area have been obtained through the assistance of the Food Science and Human Nutrition Department at UF in Gainesville. Trangenic plants with a 99% reduction in sweet-smelling methyl benzoate were correctly detected by 4 out of five human olfactory panel participants. This experiment documented the first time a plant had been engineered that could be detected as smelling different by humans. Preliminary studies with plants that do not produce benzyl benzoate have revealed that even a >95% reduction in emission of this chemical cannot be perceived by humans. Panels to determine the contribution of isoeugenol (clove oil) and phenylethanol (rose oil) to overall fragrance are to be conducted in late Fall 2005 or early Winter 2006. Once we are finished engineering plants to overproduce these individual chemicals, we hope to significantly increase desirable smelling volatiles to see if we can make a flower that humans think smells better than those produced in nature.

Insect Experiments
From several research collaborators, we have learned how to correctly fly hawk moth pollinators (*Manduca sexta*) around our transgenic plants in order to evaluate how they respond to plants with altered volatile synthesis. We are currently scheduled to controlled flight experiments in the greenhouse and large outdoor mesh houses this fall with several lines of plants with altered fragrance. By exposing pollinators to plants with single volatile components knocked out, and measuring their visitation rates to the flowers with a video camera we should be able to get a better understanding of the volatile compounds that are most important in influencing pollinators. We also have an outdoor transgenic field trial planned for next spring to determine how natural insects interact with these plants in a landscape environment – from these experiments we hope to be able to determine whether plants with altered volatile synthesis influence the activities of both pest and beneficial insects.

Unfortunately, we have been unsuccessful in developing assays to determine whether spider mites and aphids are affected differently by our transgenic plants because the glandular trichomes of petunia are an effective 'sticky trap' for these insects and it hinders their movement on the plants. Preliminary efforts focused to determine if mites and aphids were more or less attracted to different petunia genotypes quickly revealed some difficulties that we need to work out before we can get accurate data on these insects. We are currently focusing on developing whitefly assays in Gainesville with Dr. Heather MacAuslane in the Entomology Department.. Our first experiments this Fall/Spring will involve growing a commercial style crop of all of transgenic plants with altered volatile synthesis in randomized complete blocks in a greenhouse. At the stage where seedlings are potted up, we will seed the greenhouse with whiteflies reared on petunias and also stop our normal chemical control of insects. We will then monitor insect reproduction on the plants by counting adults and eggs. We will also monitor the health of the plant by taking various classical physiological data.

Pathogen Experiments
In the past year, we have made significant progress in developing screens for various fungal and bacterial pathogens on petunia flower tissues. In particular, we have very

recently developed an effective screen for susceptibility of flower petal tissue to *Botrytis cinerea* – the most problematic fungal pathogen for petunia in Florida. Preliminary results have shown that flowers with decreased production of sweet-smelling methylbenzoate are no more or less susceptible to *Botrytis* than normal flowers. This is not surprising since this chemical is thought to be involved in pollinator attraction. In upcoming months, we hope to utilize this screen to determine whether flowers engineered to be deficient in production of benzyl benzoate have less fungal tolerance than normal flowers. These plants are currently growing in the greenhouse in preparation for these studies. Since benzyl benzoate is a chemical commonly used as a commercial antimicrobial component, we believe that these plants will be more susceptible to *Botrytis*. New plants engineered to over-produce this compound in flowers are in the pipeline in our lab, and may prove to have increased fungal tolerance. These results should come in mid-2006.

Conclusions and Recommendations

This project will produce an unprecedented number of approaches for experiments focused on determining the function and regulation of virtually any gene expressed in a petunia plant. By determining the function of interesting genes expressed in petunia flowers throughout all stages of floral development, we can now get a better understanding of their role in important physiological processes in floriculture crops. This knowledge will also help us to determine the potential for manipulation of particular genes for commercial utility through the production of the next generation of transgenic plants. As a result of this project, we will continually develop new technologies that will be useful in developing important research collaborations that are vital to supplying the floriculture industry with new innovations. It is our ultimate goal to take promising new technologies discovered during this research into other floriculture crops in the not too distant future, and make flowers smell better, last longer on retail shelves, and have fewer insect and disease pests.

This work has potential use for helping to develop insect and disease management strategies that have not been possible to date in agriculture. By producing transgenic plants with altered levels of volatile chemicals already produced naturally in most plant species, it should be possible to use less synthetic chemicals to control pests. Beyond that, this research will lead to a further understanding of how naturally produced chemicals affect insect and disease pests of floriculture crops. With this information, it may be possible to develop natural chemical strategies that could be used in making new products for use in pest control of floriculture crops. To benefit consumers, this research will lead to the production of petunia plants with altered fragrance that can be detected by humans, and technologies produced as a result of this research will have broader consumer appeal due to enhanced fragrance characteristics.