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Project Title: Improving Floral Scent Production In Flowers

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Industry Needs And Project Objectives:

Ornamental crops represent a highly important economic commodity with a world market value of over \$30 billion. Unfortunately, many modern floricultural varieties, including cut flowers, foliage and potted plants, have lost their scent during selection and breeding processes. Traditional breeding of ornamental crops has resulted in the production of cultivars with improved vase life, shipping characteristics, and visual esthetic values (i.e., color, shape), however it has sacrificed an other important commercial trait, floral scent. Thus, the manipulation of flower scent would obviously have a great economic impact by increasing crop productivity and the value of ornamentals. Crops with improved scent quality and newly introduced aromas offer possibilities to expand markets and increase grower's income. The objective of this three-year research project is to improve scent quality of cut flowers by manipulation of the output of volatile compounds using recombinant DNA technologies. To achieve this objective, we are investigating the molecular changes that affect the level of scent emission in plants and isolating the BAMT promoter, which can potentially be used to produce transgenic cut flowers with novel scents.

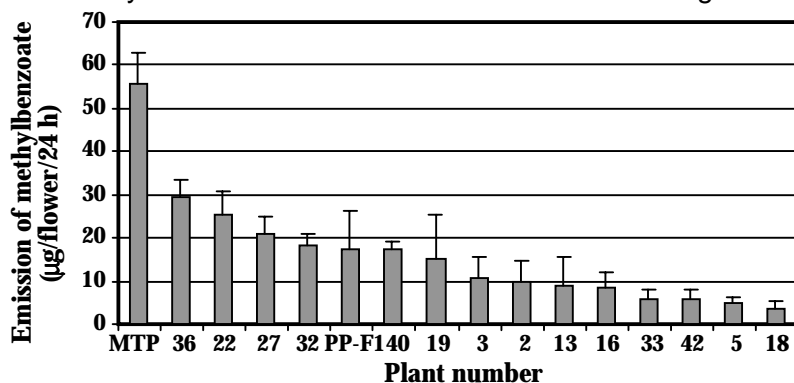
Summary Of Work Completed:

Objective 1. *Determine what is missing in Antirrhinum varieties with low to moderate emission of methylbenzoate.*

We have previously shown that methylbenzoate is produced in upper and lower petal lobes of snapdragon flowers by the action of the biosynthetic enzyme S-adenosyl-L-methionine: benzoic acid carboxyl methyltransferase (BAMT) (Dudareva et al., 2000). During the first year of the project we found that Potomac Pink cultivar emits methylbenzoate at a low level, although it contains a high level of BAMT transcripts. We used RT-PCR and found that Potomac Pink contains two forms of the BAMT gene, one is an active form, which is capable of producing methylbenzoate, and the other is an inactive form in which 10 changes in the amino acid sequence led to the elimination of enzymatic activity. To study the genetic control of methylbenzoate emission in a variety with low methylbenzoate emission, a Potomac Pink plant, which is an F1 hybrid, was self-pollinated, and seeds were grown to generate an F2 population. We now have 14 individual plants from the F2 population. Emission of methylbenzoate was analyzed in all individual plants and is shown on Figure 1. BAMT activity in petal lobes of flowers from individual plants of the F2 population was determined. It was very low when compared with Maryland True Pink, the cultivar with the highest emission of methylbenzoate, and also did not show any correlation with amount of emitted ester. To check whether BAMT expression co-segregates with methylbenzoate emission, the BAMT mRNA expression was analyzed in individual plants. Since the BAMT probe used in RNA blot analysis recognized both types of BAMT (altered-inactive or normal-active), quantitative RT-PCR with type-specific primers was used to determine the contribution of each type of BAMT mRNA in total BAMT expression in the F2 population. We found

that both types of the BAMT gene are expressed in petal tissue at approximately equal levels. We have also analyzed the amount of BAMT protein in individual plants by Western blot analysis using rabbit polyclonal anti-BAMT antibodies. The amount of the BAMT protein was undetectable by Western blot analysis, indicating that the formed protein is probably quickly degraded and that the amount of normal active BAMT, which could be responsible for low activity, is too low for detection.

Figure 1. Methylbenzoate emission of Potomac Pink F2 generation. Potomac Pink F1 and



MTP are included for comparisons. Headspace analysis of emitted volatiles was done on the fifth day after anthesis in controlled growth chamber conditions.

Another possibility is that the low amount of emitted methylbenzoate in Potomac Pink plants could be made by another gene which is, for a example, *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (SAMT). It has been previously shown that *Clarkia breweri* SAMT, although it is highly specific for salicylic acid, does methylate benzoic acid with relatively high efficiency (Ross et al., 1999). To find whether the SAMT gene is involved in methylbenzoate production in Potomac Pink flowers, we have isolated the SAMT gene from snapdragon using a genomic approach. The function of the SAMT gene was biochemically determined using an *Escherichia coli* expression system. Kinetic parameters of SAMT for salicylic acid, benzoic acid and methyl donor SAM were determined using substrate interaction and saturation kinetics. We found that the apparent K_m values for benzoic acid for snapdragon SAMT and BAMT are very close, indicating that SAMT can potentially participate in methylbenzoate biosynthesis in snapdragon flowers.

Objective 2. Characterize temporal and spatial expression of BAMT promoter using a reporter gene.

The focus of this part of the proposal was to determine whether the BAMT promoter is capable of driving transgene expression in a “scent specific” manner (in proper tissue and at the proper stage of development) in other plant species such as petunia and tobacco. Using *Agrobacterium tumefaciens* we have successfully transformed leaf disc explants with a construct containing the putative full-length BAMT promoter region (2kb) translationally fused to the GUS-NOS reporter gene. After shoot and root induction on kanamycin-containing media, plants were transferred to the soil and kept in a greenhouse. For unknown reasons petunia plants died after transferring them to soil. We generated 15 transgenic tobacco plants, but used 10 plants for further analysis.

For functional mapping of *cis*-acting motifs responsible for the temporal and spatial expression of BAMT gene we generated a series of three 5’deletions derived from the full-length BAMT promoter, 1.5, 1.0, and 0.6 kb in size, and translationally fused them to the GUS-NOS reporter gene. These chimeric genes were transformed into *Petunia Mitchell* and *Nicotiana tabacum*. Similar to the construct containing a full-length promoter region, plants were transferred to soil after shoot and root

induction on kanamycin-containing media and kept in a greenhouse. We generated 10 tobacco plants per construct with the exception of the 0.6-kb plants, where only three plants survived the transfer to soilless media. The presence of the promoter-GUS transgene in individual transformed plants was confirmed by PCR using primers designed to recognize the kanamycin resistant gene NPTII that was introduced along with the BAMT:GUS gene. DNA was isolated from each primary transgenic plant and a PCR reaction was performed on each sample. Ten independent transgenic tobacco plants were identified for each of the 2, 1.5, and 1kb BAMT:GUS promoter plants, and all three of the 0.6-kb that survived were transgenic.

Tobacco plants were grown under normal greenhouse conditions until flowering, and flowers were then collected for histochemical analysis of GUS activity. GUS activity was found at a low level in the tobacco corollas and only in the plants containing the full-length (2.0 kb) promoter region. Although GUS activity in the corollas was limited to the outer area and no activity was detected in the tube, which is similar to BAMT expression in snapdragon, these results show that the 2-kb promoter region does not contain sufficient regulatory sequences, which are necessary to reach endogenous levels of BAMT expression. The low level of GUS activity suggests that an enhancer element is missing in the 2-kb BAMT-GUS construct. Additionally, in contrast to BAMT expression in snapdragon, GUS activity was identified in the sepals, ovaries, and receptacles of the 2.0-kb tobacco plants indicating that a suppressor of spatial promoter activity is also missing in the full-length (2.0 kb) promoter region. Transgenic tobacco plants containing the 1.5-kb and the 1.0-kb promoter region of BAMT both showed GUS activity in the ovaries and receptacles whereas plant with the 0.6-kb construct resulted in no GUS activity. Taken together, obtained results show that a 2-kb BAMT promoter region is necessary to direct the transcription of transgene in petal tissue. Additionally, more than one *cis*-element seems to be responsible for the transcriptional regulation of BAMT.

Objectives For The Coming Year:

Recent isolation and characterization of the first several genes encoding scent biosynthetic enzymes opens the door for metabolic manipulation of floral scent to improve its quality. In the last two years isolated scent genes have already been used for the metabolic engineering of plant volatile composition (Lewinsohn et al., 2001; Lucker et al., 2001), however no significant increase in the amount of volatile compounds has been reported (Vainstein et al., 2001). These results clearly show that in addition to the availability of cloned genes encoding enzymes involved in the biosynthesis of floral volatiles, an understanding of the biochemical, molecular and other events controlling the production and emission of volatiles from plant tissues is absolutely required for the bioengineering of floral scent. These results also show the importance of the proposed research. Our objectives for the coming year are to:

1. Complete the investigation of the molecular changes that have occurred in *Antirrhinum* varieties with low to moderate emission of methylbenzoate.

Previously we have shown that BAMT protein is enzymatically active as a homodimer (Murfit et al., 2000). Since Western blot analysis did not detect the BAMT protein in crude extracts from 14 Potomac Pink individual plants from the F2 population despite the high level of BAMT mRNA expression, it is possible that altered-inactive BAMT interacts with normal- active BAMT forming an inactive protein, which is probably degraded quickly. To check this hypothesis, we will coexpress normal and altered BAMT proteins in *E. coli* and check BAMT activity of the resulting recombinant protein. Co-transformation with two plasmids (normal BAMT in pET 11a and mutant BAMT in pET 28a) will be performed in a single transformation event. Positive transformants will be screened for multiple resistances with kanamycin (pET 28a) and ampicillin (pET 11a). Crude extracts of sonicated transformed cells after IPTG induction will be tested for BAMT activity and *E. coli* cells transformed with normal BAMT in pET 11a alone will be used as the control.

Since we have proven that SAMT can potentially participate in methylbenzoate emission in snapdragon flowers, we will analyze expression of the SAMT gene in upper and lower petal lobes of 14 individual plants from the F2 population by RNA gel blot analysis and its possible co-segregation with methylbenzoate emission. Total RNA will be isolated from 4-day-old flowers, one day before the maximum of methylbenzoate emission. If SAMT transcripts are undetectable in floral tissues by RNA-blot hybridization, we will use RT-PCR in the presence of SAMT-specific primers to detect the low levels of SAMT gene expression. Co-segregation of SAMT expression with emission of methylbenzoate will indicate that this gene is responsible for variations in methylbenzoate emission in Potomac Pink plants.

2. Complete characterization of BAMT promoter in transgenic tobacco plants using a reporter gene.

Each primary transformant will be self-pollinated to generate a T1 population to determine the inheritance of the transgene and also for further investigation of the BAMT promoter. This part of work is now in progress. To examine at which stage of flower development the appearance of BAMT expression is initiated and reaches the maximum, GUS activity will be measured in extracts from petals at different stages of flower development.

The discrepancy between the BAMT-driven GUS expression in tobacco transformants and BAMT expression in snapdragon could be due to the absence of specific regulatory proteins required for the expression of genes involved in scent production because *Nicotiana tabacum* used for transformation does not emit methylbenzoate. To check this hypothesis transgenic plants containing the full-length BAMT promoter region –GUS construct from T1 population will be crossed with *Nicotiana suaveolens*, which has been shown to emit methylbenzoate (Loughrin et al., 1991; Kolosova et al., 2001), and GUS expression will be analyzed in the progeny.

We will also check whether expression of the BAMT-GUS construct could be induced by benzoic acid. In these experiments cut flowers will be placed in solution containing different concentrations of benzoic acid in addition to sucrose and GUS activity will be analyzed in floral tissue. These results could be very important in the cut flower industry for increasing scent production by the induction of scent genes.

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