CHEMOTYPIC VARIATION OF CARYOPHYLLENE DERIVATIVES IN COTTON GERMPLASM LINES

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Abstract

² -caryophyllene is a ubiquitous secondary metabolite found in many plant species, including cotton. Several studies have reported that ² -caryophyllene affects the activities of insect pests in various crops. While ² -caryophyllene is present in most cotton species, including commercial upland cultivars, ² -caryophyllene derivatives are not. Interestingly, the diploid cotton species *Gossypium armourianum*, *G. harknessii* and *G. turneri* have been reported to produce three unique ² -caryophyllene derivatives identified as 12-hydroxy-² -caryophyllene, 12-hydroxy-² -caryophyllene acetate, and 12-hydroxy-² -caryophyllene-4,5-oxide acetate. Furthermore, these diploid species have been reported to possess resistance to various biotic and abiotic stresses. Hence, these wild cotton species may be used to produce new cotton hybrids with resistance to multiple pests and adverse environmental conditions. USDA recently developed several tri-species cotton lines having *G. armourianum* or *G.turneri* in their genetic background. The tri-species hybrids are HAA ((*G. hirsutum x G. arboreum*)^2 x *G. armourianum*), HHA ((G. *hirsutum x G. herbaceum*)^2 x *G. armourianum*), and HAT ((*G. hirsutum x G. arboreum*)^2 x *G. turneri*). Backcrosses with *G. hirsutum* have resulted in promising hybrids with good agronomic characteristics. However, the trait for the production of ² -caryophyllene derivatives is not always expressed in subsequent progeny. As such we have developed a chemotypic method using 12-hydroxy-² -caryophyllene and 12-hydroxy-² -caryophyllene acetate as biomarkers to aide in the identification of hybrids containing the genes responsible for their production. Gas chromatography-mass spectrometry (GC-MS) was used for the separation and identification of ² -caryophyllene extracted from terminal leaves of hybrid plants. Initially, the presence of 12-hydroxy-² -caryophyllene acetate in *G. armourianum* was confirmed by using the molecular weight and fragmentation patterns obtained from GC-MS analysis. Tri-species hybrids were developed and grown under both greenhouse and field conditions. The terminal leaves of plants were analyzed for the presence of 12-hydroxy-² -caryophyllene and 12-hydroxy-² -caryophyllene acetate, and plants containing these compounds have been selected for further breeding efforts. Given the ease, sensitivity and reproducibility of this GC-MS analytical procedure, breeding efforts may be expedited to fulfill industry needs for cotton cultivars with multiple pest resistance platforms and appealing agronomic traits.

Introduction

Cotton plants (*Gossypium spp.*) produce secondary metabolites known as terpenoids, such as ² -caryophyllene (Fig. 1), that protect the plants from insect pests. ² -caryophyllene is a component of the volatile profile of many plant species. The role of ² -caryophyllene on the activities of entomophages and herbivores in cotton has been reviewed by Langeheim in 1994. ² -caryophyllene was observed to be an attractant for the adult predatory green lacewings (*Chrysopa carnea*), but toxic to the tobacco budworm. Interestingly, Williams et al. (1997) identified three diploid cotton species, *Gossypium armourianum*, *G. harknessii* and *G. turneri*, that have three unique ² -caryophyllene derivatives. These derivatives were identified as 12-hydroxy-² -caryophyllene, 12-hydroxy-² -caryophyllene acetate, and 12-hydroxy-² -caryophyllene-4,5-oxide acetate (Fig. 2). Due to the specific presence of these compounds in these three wild cotton species, they are ideal biomarkers in breeding efforts in the introgression of various resistance traits found in these three species. For example, diploid cotton species *G. armourianum*, *G. turneri* and *G. herbaceum* have been reported to possess resistance to insect and bacterial diseases, as well as resistance to abiotic stress factors (Gotmare et al. 2000). Currently, USDA has developed various tri-species cotton cultivars with the goal of producing cotton germplasm lines that have resistance to a wide range of pests, while still maintaining good agronomic traits. The tri-species hybrids are HAA ((*G. hirsutum x G. arboreum*)^2 x *G. armourianum*), HHA ((G. *hirsutum x G. herbaceum*)^2 x *G. armourianum*), and HAT ((*G. hirsutum x G. arboreum*)^2 x *G. turneri*). Backcrosses of the tri-species hybrids with *G. hirsutum* have resulted in several promising hybrid lines with good agronomic characteristics.

One drawback of traditional breeding methodologies is that they abide by mendelian genetics, meaning that all the progeny will not necessarily express the desired traits. One way to verify the presence or absence of a desired trait is to use various genetic tools to analyze the expression of a particular set of genes. Tools such PCR and Western blotting are usually employed for this analysis. While extremely effective, they involve tedious sample preparation steps and expensive reagents. Herein, we describe and test a method involving the detection and identification of ² -caryophyllene derivatives using Gas Chromatography-Mass Spectrometry (GC-MS). This method involves extraction of secondary metabolites from cotton terminal leaves and direct injection of the extract for GC-MS analysis.

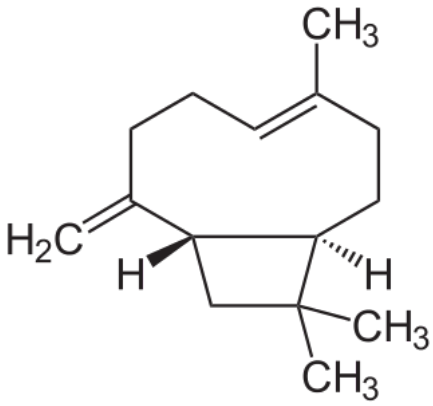

Figure 1. Structure of ² -caryophyllene.

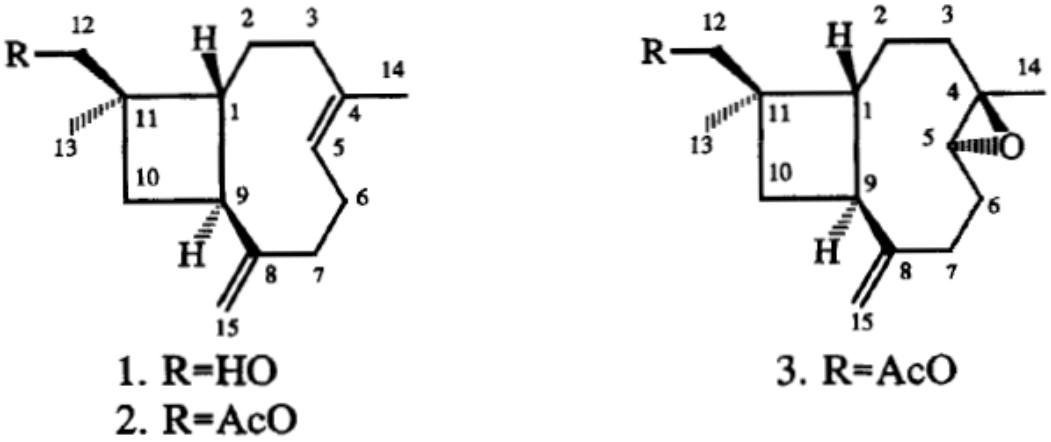

Figure 2. Structures of ² -caryophyllene derivatives found in *Gossypium armourianum*, *G. harknessii* and *G. turneri.*

Materials and Methods

A *G. armourianum* plant was initially analyzed by GC-MS for the presence of 12-hydroxy-² -caryophyllene acetate in terminal leaves and compared to a commercial upland cotton cultivar (Fibermax 966). *G. armourianum* is known to have various caryophyllene derivatives present in terminal leaf tissue, while no such compounds are found in upland cotton. One terminal leaf was excised from each plant and immediately frozen until extraction of metabolites was performed. The extraction of ² -caryophyllene derivatives was carried out by pulverizing the terminal leaf material in a 1.5 ml microcentrifuge tube followed by the addition of 1 ml of dichloromethane. The mixture was then vortexed for 10 seconds and placed in an ultrasonic water bath for 20 min. The samples were then centrifuged for 10 min at 3000 rpm, and the extract was decanted into a separate 1.5 ml tube. The remaining leaf

material was extracted again as previously described. The second extract was pooled with the first extract and concentrated to dryness under room temperature conditions in a fume hood. Once dried the extract was reconstituted in 0.5 ml of dichloromethane and transferred to a GC-MS vial fitted with a volume reducing insert. The extract was then submitted for GC-MS analysis.

GC-MS analysis was carried out using a Shimadzu GCMS-QP2010 Ultra (Shimadzu Scientific Instruments, Columbia, MD) equipped with a Zebron ZB-WAX plus (30 m length × 0.25 mm I.D. × 0.25 µm film thickness; (Phenomenex, Torrence CA, USA). The carrier gas was helium at a flow rate of 1.2 ml min^{-1}. The temperature of the injection port was 220°C and a 1µL sample was injected in a split-less mode. The column temperature program consisted of an initial temperature of 100°C, held for 5 min followed by a 10 °C/min ramp to 220°C and held for 10 min. The program was ended with a 40°C/min ramp to an ending temperature of 250°C. The mass spectrometry conditions were: electron impact ionization (EI); interface temperature of 250 °C; and ion source temperature of 200°C. Identification of cotton volatile compounds was made by comparison of mass spectra of experimental samples to those stored in the National Institute of Standards and Technology (NIST) and Wiley Registry (10th Edition) libraries, in addition to verification of reported molecular mass and fragmentation patterns.

Over 20 tri-species hybrid lines developed at USDA-ARS were screened for the presence of 12-hydroxy-^{2}-caryophyllene and 12-hydroxy-^{2}-caryophyllene acetate. An example of a typical chromatogram of a tri-species hybrid is presented in Figure 3. Several hundred greenhouse grown plants were screened and plants that expressed the two caryophyllene derivatives or only 12-hydroxy-^{2}-caryophyllene acetate were selected for further back crossing experiments. It is important to note that the trait of interest is the expression of 12-hydroxy-^{2}-caryophyllene acetate, but plants having both caryophyllene derivatives were also selected because 12-hydroxy-^{2}-caryophyllene is a precursor molecule of the acetate form. Hence, the presence of both metabolites in the leaf extracts may indicate that the biosynthesis of the acetate from the alcohol was not complete. Selected plants were used for further backcrossing experiments. Backcrossed 2 selfed (BC_2S1) and backcross 3 (BC_3) seeds from selected plants were used for field trials. Seeds were germinated in Jiffy-7 peat pellets and transplanted into the field. About 50 plants from each line were transplanted and assessed for the presence of 12-hydroxy-^{2}-caryophyllene and 12-hydroxy-^{2}-caryophyllene acetate by GC-MS as outlined above.

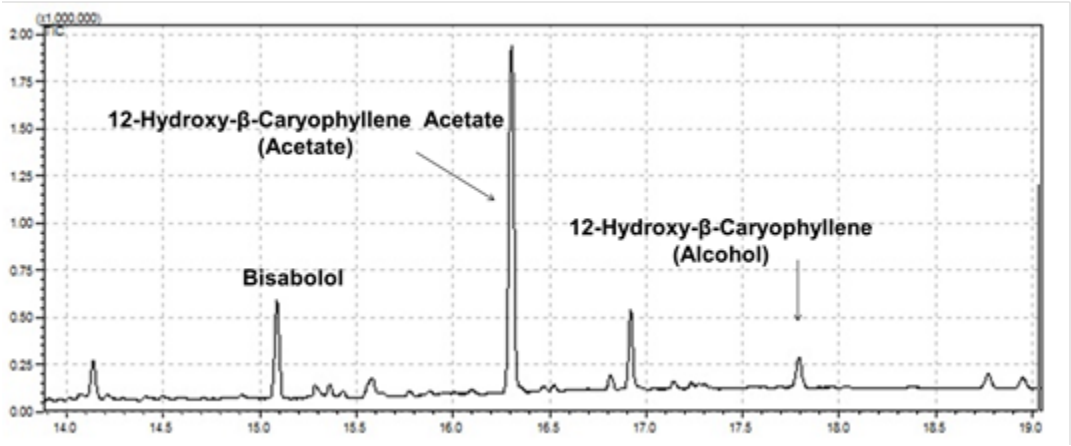

Figure 3. Chromatogram of a tri-species hybrid illustrating the presence 12-hydroxy-^{2}-caryophyllene derivatives.

Results and Discussion

The initial analysis of a *G. armourianum* plant confirmed the presence of 12-hydroxy-^{2}-caryophyllene acetate. In comparison, this acetate derivative was not found in the commercial upland cotton cultivar (Fig. 4).

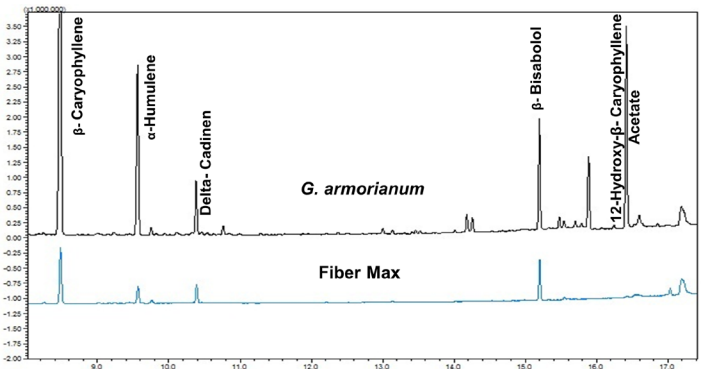

Figure 4. Chromatogram illustrating the presence 12-hydroxy-^{2} -caryophyllene acetate in *G. armourianum* and its absence in commercial upland cotton.

Table 1. GCMS screening results for ^{2} - caryophyllene derivatives in cotton tri-species breeding lines grown in College Station, TX. Result presented as percentage of plants having or lacking specific ^{2} - caryophyllene derivatives.

Row	Row ID	Gen.	n	β-Caryophyllene (%)	Acetate Only (%)	Alcohol Only (%)	Acetate & Alcohol (%)	No Ac. or Alc. (%)
1	13-3 HAA (x)	BC_2S1	34	100	14.7	23.5	23.5	38.3
2	13-3 HAA (x)	BC_2S1	42	100	23.8	30.9	11.9	33.4
3	13-4 HAA (x)	BC_2S1	38	100	39.4	26.3	12.1	22.2
4	13-5 HAA (x)	BC_2S1	36	100	16.6	33.3	16.6	33.5
5	13-9 HAA (x)	BC_2S1	43	100	30.2	18.6	27.9	23.3
6	13-27 HHA (x)	BC_2S1	43	100	44.1	4.5	2.3	49.1
7	13-6 HAT (x)	BC_2S1	44	100	0	63.6	0	36.4
8	13-8 HAT (x)	BC_2S1	31	100	0	64.5	0	35.5
9	13-7 HAT (x)	BC_2S1	30	100	0	70	0	30
10	13-7 HAT (x)	BC_2S1	37	100	0	70.2	0	29.8
11	14-16 HAT (x)	BC_2S1	46	100	0	0	0	100
12	14-16 HAT (x)	BC_2S1	44	100	0	0	0	100
13	16-28 HAT (x)	BC_2S1	48	100	31.2	22.9	29.1	16.8
14	BAR-32 x 13-3	BC_3	48	100	8.3	39.5	6.2	46
15	BAR-32 x 13-4	BC_3	41	100	14.6	24.3	2.4	58.7
16	BAR-32 x 13-5	BC_3	39	100	15.3	25.6	2.5	56.6
17	BAR-32 x 13-9	BC_3	39	100	12.8	23.1	12.8	51.3
18	BAR-32 x 13-27	BC_3	49	100	0	6.1	4.1	89.8
19	BAR-32 x 14-4	BC_3	49	100	14.2	22.4	12.2	51.2
20	BAR-32 x 14-8	BC_3	48	100	16.6	35.4	12.5	35.5
21	BAR-32 x 16-12	BC_3	37	100	2.7	48.6	18.9	29.8
22	BAR-32 x 16-26	BC_3	48	100	12.5	35.4	10.4	41.7
23	BAR-32 x 16-28	BC_3	45	100	17.7	24.4	13.3	44.6
24	BAR-32 x 16-7	BC_3	49	100	18.36	32.6	4.1	44.94

The results of the screen are presented as percent of plants containing the particular metabolite.

The results of the screening of field grown lines are presented in Table 1. ^{2} -caryophyllene was present in all plants analyzed. Conversely, depending on the breeding line, the caryophyllene derivatives were either present or absent. It is also important to note that some lines in the field trial were replicated, and the data presented in Table 1 shows similarities in percentages of metabolites between rows, indicating that the analytical method is reliable and reproducible. Furthermore, the sensitivity of this methods is illustrated by the fact that only one terminal leaf from each plant is utilized from analysis. The analytical method described in this study offers the advantage of being simple, sensitive, and accurate. Additionally, compared to genetic analytical methods it is also considerably less expensive to perform. Genetic analytical methods such as PCR can give misleading results, due to the fact that while certain genes may be expressed, translation of the desired trait may not always occur. Western blotting is much more accurate for the analysis of translated traits, but it is considerably much more time consuming and expensive compared to our method. Results of this investigation indicate that chemotypic analysis of leaf tissue (via GC-MS) is a simple, sensitive and reproducible approach for determining which progeny may possess a desired

trait. As such, this technique may be used to expedite breeding efforts by selecting only plants that express the desired chemical profile needed for resistance

Conclusions

The GC-MS analytical method presented here is a reliable and reproducible tool that can be used to expedite breeding efforts to produce cotton cultivars that are resistant to a variety of pests and are agronomically desirable. Currently there are many insect and microbial pest plaguing the cotton industry. As such, there is an urgency to develop cotton cultivars that have more resistance to both biotic and abiotic.

References

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