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MOLECULAR BIOLOGY & PHYSIOLOGY

Microscopic Methods to Evaluate Gland Initiation and Development in Cotton Ovules

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ABSTRACT

Gossypol is a terpenoid aldehyde found in cotton (*Gossypium hirsutum* L.) glands that are located throughout the plant and seed, where it serves a protective function against pests and pathogens. Cottonseed use is limited mainly to cattle feed because gossypol is toxic to most animals except ruminants. Lowering gossypol content in the seed would increase the possible uses for cottonseed. Developing new strategies to modify gossypol in cottonseed requires a better understanding of the development of gossypol containing glands. The first step is to determine when gossypol glands are initiated and filled with gossypol. Gland development was investigated using microscopic images of developing seeds from 10 glanded and two glandless cotton lines. A digital microscope with a VH-Z20R (20X to 200X) lens was used to capture developing ovule (seed) images at 14 to 22 d after flowering (DAF). One boll per plot was imaged for each DAF time point and five different sets of time intervals were collected in each of two years. Imaging revealed empty glands forming as early as 14 DAF and as late as 20 DAF. For most of the entries, some glands were filling by 18 DAF and as early as 16 DAF for ultra early lines (≤ 110 d to maturity).

Glands are found in the seed and on all parts of the cotton (*Gossypium hirsutum* L.) plant. These glands contain gossypol and other terpenoid aldehydes that act as a defense mechanism against pests and pathogens (Bell and Stipanovic, 1977; Scheffler et al., 2012). Cottonseed is an excellent source of oil (21%) and high quality protein (23%) (Lusas and Jividen, 1987); however, the value of cottonseed as a by-product of fiber production is limited. Gossypol is the predominant terpenoid aldehyde in cottonseed glands and its toxicity to non-ruminant animals and humans limits its use mainly to cattle feed, because only ruminants can tolerate the toxic effects of gossypol (Kim et al., 1996; Santos et al., 2003). In non-ruminants such as pigs or chickens or fish, gossypol can inhibit weight gain and decrease reproductive capabilities (Randel et al., 1992).

There are several strategies available to mitigate the antinutritional effects of gossypol in the seed, including mechanical processes to remove gossypol from cottonseed products (Damaty and Hudson, 1975; Gardner et al., 1976; Mayorga et al., 1975), using naturally occurring glandless mutants (Hess, 1977; McMichael, 1959, 1960), or selectively inhibiting gossypol production only in the seed using RNAi techniques (Rathore et al., 2012; Sunilkumar et al., 2006). Each strategy has drawbacks. Mechanical treatments add cost and reduce the nutritional value of the resulting cottonseed meal (Lusas and Jividen, 1987). Glandless lines have been unsuccessful commercially because they are more susceptible to disease and insect predation (Hess, 1977; Lusas and Jividen, 1987). Two genes (GL_2, GL_3) differentially control glanding, and GL_2 is the main gene controlling gland formation in seed (Lee 1962, 1965; McCarty et al., 1996). This differential control of the glanded trait, has been exploited to develop semi-glanded lines with low seed gossypol and near normal glanding on the rest of the plant (Romano and Scheffler, 2008; Scheffler et al., 2012). It should be possible to develop even better lines if the genes and associated underlying biochemical pathways and mechanisms were better understood. In glandless mutants, no gossypol is produced. There are some Australian diploid relatives of cotton in which the mature seed has undeveloped, unfilled glands observable only under the microscope. Within 2 to 3 d after germination, the cotyledon and hypocotyl glands in these diploids fill with gossypol and become visible to the naked eye (Brubaker et al., 1996). These examples indicate a relationship between biochemical mechanisms underlying gland formation and gossypol biosynthesis. Ultra low gossypol cottonseed (ULGCS) from plants generated using RNAi

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technologies have glands that are mostly unfilled (Rathore et al., 2012; Sunilkumar et al., 2006). Currently, material developed with this technique must pass expensive regulatory hurdles. The high cost of developing genetically modified organisms versus the economic benefit has hindered their development. Other options are needed, but developing strategies to modify gossypol in cottonseed first requires a better understanding of the development of gossypol-containing glands and determination of the stage at which gossypol is present in the gland.

Technologies are now available to study gene expression and elucidate the underlying mechanisms for a specific developmental stage or physiological condition. For example, RNA sequencing (RNA-Seq) methods have become standard for use in transcriptome profiling (Wang et al., 2009, 2010). A transcriptome profile is identification by sequencing all the transcripts in a cell or tissue and measuring the amount of each transcript present. The transcriptome creates a "snapshot" for a specific tissue or time point. RNA-Seq technology uses high through-put DNA sequencing to map or quantify transcriptomes. To use this technology to study gland development or gossypol synthesis effectively, the correct developmental stage or time point needs to be determined.

The presence of glands on cotton plants and gossypol in cottonseed was first reported in the late 1800s (Carruth, 1918), and there is a wealth of information on glands and gossypol in mature seed (Bell and Stipanovic, 1977). However, few reports cover gland development that occurs during embryogenesis. An early study by Gallup (1928) reported that gossypol, using an ether extraction and aniline assay, was not detected at 16 or 24 d after flowering (DAF), but was detected at 32 DAF (0.23% of the dry-matter weight). He also reported that the amount had tripled by 46 DAF (0.63%) and then decreased at 52 DAF (0.54%). A 1932 study attempted to evaluate developing embryos with indirect chemical tests, but chemical detection of gossypol was not yet reliable and their results were inconclusive (Reeves and Valle, 1932). Reeves and Beasley (1935) conducted one of the most comprehensive studies using fixed sections and light microscopy. The study used field grown plants, but only one cultivar: Startex. They reported first observing "resin" pre-glands at "15 days after fertilization allowing 36 hours for fertilization to be completed," which equates to 16.5 DAF by our measurements, which start at day of flowering (0 DAF). They also added sulfuric acid to the contents of the young glands and found that at 15 d (16.5 DAF) the pre-glands did not change color, but at 18 d (19.5 DAF) addition of sulfuric acid turned the gland contents red just as it did in mature embryos. They speculated that there was gossypol or a related substance in the glands at 18 d (19.5 DAF). Reeves and Beasley (1935) noted that although the stages of development were consistent, there was a great deal of variability in the samples evaluated and concluded it was "impossible to state the exact age at which cotton embryos reached any particular stage." Subsequent chemical analyses have used mature or germinating seed that did not include the ovule stage of development (Ihle and Dure, 1972; Turley and Chapman, 2010). Beasley (1975) used scanning electron microscopy to document the developmental process from flower development through pollination and fiber development. However, as with most other studies of seed development, it concentrated on fiber initiation and development on the outer epidermis and no mention was made of glands (Pearson, 1939; Romano et al., 2011; Stewart, 1975).

Cadinene synthase catalyzes a key reaction at the branch point for biosynthesis of gossypol and related compounds. As part of a study evaluating (+)-δ-cadinene synthase (CDN) activity, samples were collected in the greenhouse from the cultivar Sumian-6 at 20, 27, 35, 40, and 60 d after anthesis (DAA) (Meng et al., 1999). The authors assayed for gossypol using a phloroglucinol thin-layer chromatography method and reported that gossypol was first detected at 35 DAA and continued to increase until 60 DAA. Martin et al. (2003) evaluated the efficacy of plants transformed with an antisense CDN construct, and included a test studying the concentration of gossypol at 10 time points from 5 to 50 days postanthesis (dpa). Individual seeds from the greenhouse grown cultivar Coker 312 were analyzed by high performance liquid chromatography (HPLC). They reported that "accumulation of gossypol began between 25 and 33 dpa and reached a maximum at 45 dpa".

A number of previous studies have reported that the rate of development for bolls or seed was influenced by temperature (Burke and Wanjura, 2010; Reddy et al., 1993, 1995; Yeates et al., 2013). Temperature also can affect ovule gland development and needs to be taken into consideration when evaluating gland formation and gossypol deposition in the glands.

Studies designed to determine the genes and mechanisms underlying gland development or gossypol synthesis require accurate determination of the

correct developmental stage to target, as well as a guide to when the stage occurs. Previous studies indicate there is no clear agreement on when glands appear, when gossypol begins to accumulate, if the genotype used influences development, or how environment can affect the rate of development. This study visually evaluated developmental stage using two-dimensional microscopy of fresh, untreated ovules or embryos to determine the number of DAF when glands were first visible, when gossypol was first noted, and the progression of gland morphogenesis in the developing ovule. To assess variation due to the genotype used, multiple-glanded genotypes were compared with glandless mutant lines used as negative controls. To evaluate the effect of environment, the study covered three years in the field and one season in the greenhouse.

MATERIALS AND METHODS

Plant Material. The study included a range of cotton types and maturities as well as three lines reported as having no seed glands (glandless) (Table 1). *G. barbadense* L. (Pima) and *G. hirsutum* L. Upland and Acala types with maturities ranging from 108 to 170 d were evaluated (Table 1). Four of the lines were classified as ultra early lines (UEL) with maturities of 110 d or less. These lines were developed in Uzbekistan (latitude 40° N), which is at the same latitude as Pittsburgh, PA, in comparison to Stoneville, MS (latitude 34° N). The short growing season in Uzbekistan requires early maturing cotton varieties and these might have a faster rate of gland development than later maturing lines.

Table 1. Plant material used for the study.

Entry	Scientific Name/ Type	[y]2009 Field	2009-10 Green house	2010 Field	UEL Field 2010 and 2011	2011 Field
H1220	*G. hirsutum* upland	X	X	X		X
MD51ne	*G. hirsutum* upland	X	X	X		X
STV gl	*G. hirsutum* upland, glandless	X	X	X		X
STV GL	*G. hirsutum* upland, Glanded	X	X	X		X
Maxxa GL	*G. hirsutum* Acala, Glanded	X	X	X		X
Maxxa gl	*G. hirsutum* Acala, glandless		X	X		X
JACO gl	*G. hirsutum* upland, glandless		X			
JACO GL	*G. hirsutum* upland, Glanded		X			
PHY 485	*G. hirsutum* upland			X		
PIMA S7	*G. barbadense* Pima			X		X
PHY 810	*G. barbadense* Pima			X		X
PHY 72	*G. hirsutum* Acala			X		X
Acala 1517	*G. hirsutum* Acala			X		X
L2 [z]UEL	*G. hirsutum* upland, Glanded				X	X
L3 UEL	*G. hirsutum* upland, Glanded				X	
S9 UEL	*G. hirsutum* upland, Glanded				X	
N77 UEL	*G. hirsutum* upland, Glanded				X	
GVS 5069	*G. hirsutum* upland, Glanded					X

[z]UEL = ultra early line (≤110 d to maturity)

[y]X = entry was used in a particular year

Sample Collection. Field experiments were conducted at Stoneville, MS in 2009, 2010, and 2011. The 2009 test had five entries (Table 1) and was designed based on information from two ancillary studies. Each plot was a single 18-m row with five (2009), 11 (2010), or 16 (2011) plots of each line. In all three years, the first flowers began mid to late June with mid-flower occurring 10 to 15 July depending on the line and year. White flowers were tagged in the early afternoon on the day of anthesis, starting early July until early August (Table 2). Whenever possible, first position bolls were sampled; however, at the later sampling dates some second position bolls were tagged and any boll just below the tagged flower was removed to decrease the possibility of flower abortion. Flowers were tagged on at least 10 different days each year, and at each tagging date 16 to 20 bolls were tagged per variety to guarantee sufficient boll harvest for each DAF time point. Each date was marked using a different color and/or shape tag to distinguish each date in the field. For each sampling time point (12, 14, 16, 18, 20, 22, or 24 DAF), six to 10 replications were collected. The time points varied depending on the year. For each time point, two bolls per line were collected in the field, placed on ice, and taken to the laboratory for dissection and imaging. In 2010 and 2011, a separate replicated study in the same field evaluated four lines (Table 1) classified as UEL (≤ 110 d to maturity) at six time points (12, 14, 16, 18, 20, 22 DAF).

An additional replicated experiment with eight lines (Table 1) and 20 pots of each line was conducted in the greenhouse to have a controlled environment for comparison to the field data. The lines were grown in 4-gallon pots with Metro Mix 360 (Sun-Gro, Agawam, MA) and fertilized monthly. Five pots of each line were planted at 2-wk intervals from the first week in October through the end of November for a total of 20 pots. Conditions in the greenhouse were 16 h day/night under artificial light and 32° C/21° C day/night temperature. The first flowers were observed approximately 50 d after emergence. Flowers were tagged on a plant for up to one month after first flower. First or second position flowers were tagged, sampled, and imaged using the same protocol as for the field experiments.

Digital Imaging. For each line collected in the field, one of the two bolls was selected randomly and two ovules (subsamples) were removed. A razor blade was used to cut along two sutures of the boll (ovary) tissue to reveal two locule chambers. Two ovules were removed with a small spatula, one from the middle of each locule. The two ovules were blotted with a Kimwipe™ to remove excess moisture followed by dissection under a dissecting microscope (National, Schertz, TX) with a handheld double-edge blade (Electron Microscopy Sciences, Hatfield, PA). The ovule was cut in half longitudinally along the ridge and the embryo removed from the seed coat (integuments) by gently scooping with a small curved spatula. One of the two halves of each embryo was imaged using a VHX-600 Keyence Digital Microscope (Keyence Inc., Osaka, Japan), with a VH-Z20R (20X-200X) lens following the manufacturers protocol for calibration at 30X (Fig. 1). For each embryo half, the outer surface and the inner side were carefully examined at 30X magnification for gland formation, followed by zooming up to 200X for closer examination, and given a score of 1 (no glands), 2 (empty, unfilled glands), or 3 (glands filling with gossypol). As glands were visible first along the edge of the outer surface of the embryo, summaries and statistical analyses reported here are based on outer side scores. The inner half scores were consistent, normally delayed by two DAF. The scoring system was based on detailed observations made in preliminary tests from 2008 and 2009 field and greenhouse data. In these tests, we observed that the gland always appeared first as unfilled, with the yellow gossypol beginning to fill the gland two or more days later. All the images were saved to an external hard drive for future observation or analysis.

Table 2. Summary of planting, flowering and sampling for studies conducted from 2009 through 2011.

Year	Planting date	First Flowers Observed			First flowers tagged	Last flowers tagged	Sampling time point range ([y]DAF)
		[z]UEL	Upland/ Acala	PIMA			
2009	April 28		June 27	July 3	July 14	August 2	14-60
2010	April 29	June 14	June 21	June 29	July 9	August 6	18-24
2011	April 29	June 16	June 23	June 30	July 8	August 3	12-24

[z] UEL = ultra early line (≤110 d to maturity)

[y] DAF = days after flowering, 64 days were mature seed

Figure 1. Keyence digital microscope for fresh tissue imaging.

As part of a separate 2011 study, additional flowers were tagged at the same times and in the same plots used for the microscopic observations. Ovules were removed from bolls collected at 14, 16, 18, 20, and 64 DAF and gossypol quantified using the HPLC method described by Scheffler and Romano (2008). The results are included as confirmation that the visual appearance of gossypol in the glands coincided temporarily with detection of gossypol using HPLC analysis.

Statistical Analyses. Ovule data were analyzed in a completely randomized design using the SAS GLM procedure (SAS version 9.2, SAS Institute Inc., Cary, NC). Variables included entry and DAF with ovules as subsamples nested within DAF to determine significance levels ($p \leq 0.05$) for the *F*-test. Comparisons between varieties were made using the Tukey-Kramer method for LS-means. Cluster analysis (SAS PROC CLUSTER) was performed to group lines with similar "DAF to filled glands". Distances between clusters were calculated by average linkage. SAS PROC TREE was used to create the dendrogram.

RESULTS AND DISCUSSION

Sampling tissues for gene expression studies requires accurate methods to estimate the correct stage or state of the tissue under investigation. This is true whether the method is microarrays, quantitative-PCR, or newer technologies such as high-throughput sequencing or RNA sequencing (RNA-Seq). Our research goal is to study genes expressed in the formation of glands and synthesis of gossypol in the developing ovule (seed). This requires being able to collect ovule tissue that has no glands, unfilled glands, or filled glands. A series of preliminary tests were conducted in 2005 to determine when gossypol was first detected in developing ovules using an HPLC analysis method (Scheffler and Romano, 2008). Table 3 summarizes the results and shows that in all five lines assayed, no gossypol was detected at 18 DAF, but all had detectable gossypol by 24 DAF. The amount of gossypol increased sharply until 32 DAF and then plateaued. No gossypol was detected in the ovules of the glandless line STV gl at any of the stages sampled (Table 3).

Using this information, the first microscopic evaluation was initiated in 2009 with a field test and a greenhouse experiment with controlled temperature and light. In the field, unfilled glands were first detected at 18 DAF for one boll from H1220, but the mean for H1220 unfilled glands was 20 DAF. Filled glands for H1220 first appeared at 22 DAF. For all other lines, the mean appearance of unfilled glands (22 DAF) and filled glands (25 DAF) was the same. The number of glands did not appear to increase after 28 DAF. Gossypol appeared as a bright yellow liquid and was restricted to the glands. In the ovule, gossypol was never observed outside of the glands. The contents of the gland turned its characteristic brownish purple color only after the seed had matured and dried ($\geq$ 64 DAF), which coincided with the outer seed coat (hull) turning brown. The STV gl (glandless) line was sampled from 14 to 60 DAF and unfilled glands were never observed in ovules from that line, even at 200X magnification. In the greenhouse experiment, unfilled glands first appeared on H1220 and Maxxa GL at 20 DAF and filled glands 24 DAF. By 28 DAF 100% of all the samples from all the lines had filled glands (data not shown).

The 2010 field experiment evaluated 11 lines and based on the 2009 results, initially sampled at 18, 20, 22, and 24 DAF (Tables 1 and 2). Because some of the lines had unfilled glands at 18 DAF, 16 DAF bolls were sampled and no glands were observed even at 200X magnification. By 24 DAF all the lines had filled glands (Table 4). A separate study evaluated four UEL ($\leq$ 110 d to maturity) at six time points (12, 14, 16, 18, 20, 22 DAF). Unfilled glands first appeared in all the lines by 16 DAF and 100% had filled glands by 20 DAF (data not shown).

Table 3. Gossypol in developing ovules from 7 to 64 DAF.

Entry	[z]DAF	Total % gossypol	Ratio (+) to (-) gossypol
[x]DES 119	7	[y]BDL	
DES 119	7	BDL	
DES 119	7	BDL	
DES 119	14	BDL	
DES 119	14	BDL	
DES 119	25	0.90	62 to 38
DES 119	35-38	2.00	61 to 39
FM 832	14	BDL	
MD51ne	14	BDL	
MD51ne	14	BDL	
SG 747	14	BDL	
SG 747	14	BDL	
SG 747	14	BDL	
STV gl	14	BDL	
STV gl	14	BDL	
TM-1	14	BDL	
TM-1	14	BDL	
TM-1	14	BDL	
FM 832	18	BDL	
FM 832	18	BDL	
MD51ne	18	BDL	
MD51ne	18	BDL	
STV gl	18	BDL	
SG 747	18	BDL	
TM-1	18	BDL	
FM 832	24	0.40	61 to 39
MD51ne	24	0.48	66 to 34
SG 747	24	0.87	60 to 40
SG 747	24	0.81	57 to 43
STV gl	24	BDL	
TM-1	24	0.80	67 to 33
FM 832	32	1.09	55 to 45
FM 832	32	1.19	52 to 48
MD51ne	32	1.39	61 to 39
SG 747	32	1.41	60 to 40
SG 747	32	1.31	56 to 44
STV gl	32	BDL	
TM-1	32	1.98	62 to 38
FM 832	36	1.10	57 to 43
MD51ne	36	1.27	63 to 37
MD51ne	36	1.72	62 to 38
SG 747	36	1.20	55 to 45
SG 747	36	1.68	56 to 44
STV gl	36	BDL	
TM-1	36	1.60	63 to 37
FM 832	46	0.89	55 to 45
FM 832	46	1.14	54 to 46
MD51ne	46	1.42	61 to 39
MD51ne	46	1.75	61 to 39
SG 747	46	1.38	57 to 43
SG 747	46	1.44	55 to 45
STV gl	46	BDL	
TM-1	46	1.35	59 to 41
TM-1	46	1.81	63 to 37
DES 119	64	1.98	62 to 38
DES 119	64	1.79	64 to 36
FM 832	64	1.12	59 to 41
FM 832	64	1.18	58 to 42
MD51ne	64	2.06	64 to 36
MD51ne	64	1.89	63 to 37
SG 747	64	1.42	58 to 42
SG 747	64	1.49	60 to 40
STV gl	64	BDL	
STV gl	64	BDL	
TM-1	64	2.05	64 to 36
TM-1	64	2.15	65 to 35

[z] DAF = days after flowering, 64 days were mature seed

[y] BDL = below detectable limit, multiply by 10 to convert to ug/mg

[x] DES 119 (PI606809), FM 832 (FIBERMAX 832 PVP 9800259),

MD51ne(CV-103, PI 566941), SG 747(Sure-Grow 747 PVP 9800118),

STV gl (Romano et al. 127:619-624), TM-1(Crop Science 10:670-671)

Table 4. Comparison of means for entries in the field 2010 test.

2010 Entry	[z,y]DAF 18 Score	DAF 20 Score	DAF 22 Score
Acala 1517	2.17 bc	2.94 a	2.94 a
H1220	2.33 bc	2.79 ab	3.00 a
MAX GL	2.50 ab	2.86 a	3.00 a
MD51ne	2.17 bc	2.86 a	3.00 a
PHY 485	2.92 a	3.00 a	3.00 a
PHY 72	2.92 a	2.94 a	3.00 a
PHY 810	2.30 bc	3.00 a	2.94 a
PIMA S7	1.83 c	2.30 b	2.67 b
STV GL	2.42 ab	2.82 ab	3.00 a

[z] Ovules were rated 1 (no glands) 2 (empty glands), or 3 (glands filling)

[y] Means within a column followed by the same letter are not significantly different at $p \geq 5\%$

The 2011 field test included the same entries as 2010, except there was insufficient seed for PHY 485 and it was replaced with GVS 5069. Based on the 2010 results, the range of sampling time points was increased to cover 12 DAF through 24 DAF (Table 2). The range of time points used for the statistical analyses was 14 DAF to DAF 22. There were no glands at 12 DAF or 14 DAF except for a few samples of the UEL L2 entry (Table 5, Fig. 2). For the other entries, the first unfilled glands were observed at 16 to 18 DAF and all the entries had filled glands by 22 DAF (Figs. 3-5). Statistical analysis of the data indicated variation between entries in both years 2010 ($p \leq 0.0001$) and 2011 ($p \leq 0.0001$) (Tables 4 and 5). Differences were not significant between ovule subsamples in either year ($p \geq 0.82$). The number of DAF until unfilled glands were observed did not differ significantly among the four UEL lines (Table 1) ($p \geq 0.49$). Cluster analysis showed that the Pima lines were consistently later and UEL L2 earlier, but the Acala and Upland types were intermixed (Fig. 6). Testing of ovules collected from the same plots indicated gossypol detection by HPLC analysis coincided with the appearance of gossypol in the glands (Table 6).

To evaluate the development of the ovule, we first observed and imaged ovules and embryos at 8 DAF. At this stage, the embryo was virtually undetectable and the ovule inner space was filled with clear liquid endosperm (Fig. 7). By 22 to 28 DAF, the inner ovule cavity was mostly filled with folded cotyledons and the embryo meristem and

root were visible as depicted in Fig. 7 for 22 DAF. Empty glands consistently appeared first on the edges of the developing cotyledons and proceeded to cover the entire embryo by 24 to 28 DAF. The glands began filling with gossypol within 4 d after they were first observed, which coincided with gossypol first being detected by HPLC tests. In the glandless controls, the characteristic yellow gossypol was never observed in the embryos (Fig. 7), although HPLC analysis of young roots from the same entries showed there was gossypol present (data not shown). The root samples for STV gl (glandless) ranged from 2.6 to 5.4 ug/mg and were comparable to its glanded near-isogenic line STV GL (3.2-4.8 ug/mg). The gossypol in the roots was not confined in visible glands. This indicated that the glandless entries still had the ability to produce gossypol, but when glands were not present, gossypol was not detected in the ovules (Table 3). Gland initiation started later in 2009 compared to 2010 or 2011, which could be due to season-long lower temperatures (Fig. 8). A number of studies have linked rate of boll development to temperature (Burke and Wanjura, 2010; Reddy et al., 1993, 1995), and our study also indicated that ovule development and consequently gland development was affected by temperature.

In this study, there were differences within years between genotypes for when glands were observed and when gossypol was detected, but little sampling variation within a genotype. Across years there were differences in the number of days to glands first observed. These findings confirm the prediction by Reeves and Beasley (1935) that although the steps in development were the same, the rate was variable and might be affected by environment and other factors. Our results showed that gossypol was first detected in the glands at 18 to 20 DAF, earlier than reported by two previous studies: Meng et al (1999) and Martin et al. (2003). The phloroglucinol thin-layer chromatography method used by Meng et al. (1999) is not as sensitive as HPLC and might not have been able to detect the low levels of gossypol present in the earlier stages of development. Although Martin et al. (2003) used HPLC, the plants were grown in a greenhouse, where in our experience, greenhouse plants often develop 1 to 2 d later than the field. Another factor might have been that the HPLC analysis was done on an individual seed (20-40 mg), which is less sensitive than the bulk sample (100 mg) method used in our analysis.

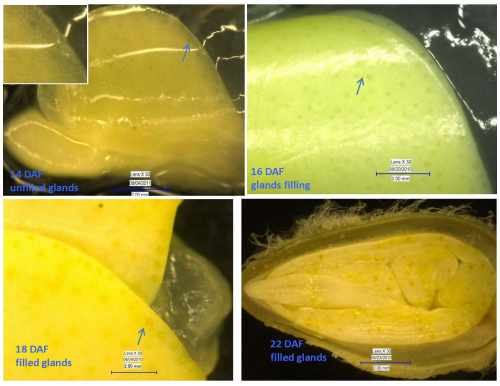


Figure 2. L2 (ultra early line) ovules from 14 to 22 DAF, arrows indicate a gland.

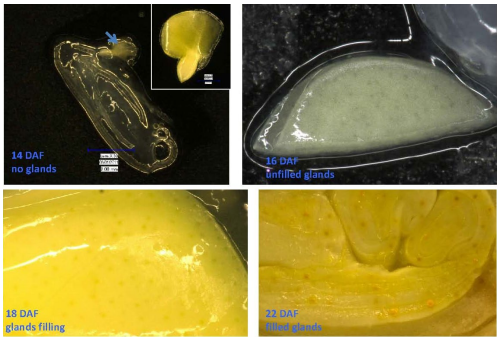


Figure 3. H1220 ovules from 14 to 22 DAF.

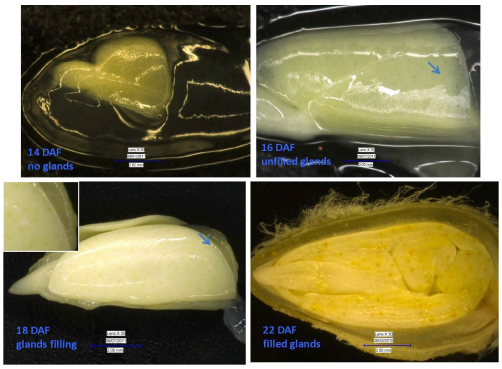


Figure 4. STV GL ovules from 14 to 22 DAF, arrows indicate a gland.

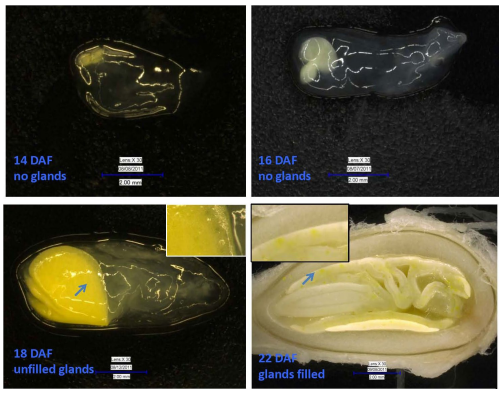


Figure 5. PIMA S7 ovules from 14 to 22 DAF, arrows indicate a gland.

Table 5. Comparison of means for entries in the field 2011 test.

2011 Entry	[z,y]DAF 14 Score	DAF 16 Score	DAF 18 Score	DAF 20 Score	DAF 22 Score
Acala 1517	1.00 a	1.63 bcde	2.13 bcd	2.86 a	3.00 a
GVS 5069	1.00 a	2.13 abc	2.94 a	3.00 a	3.00 a
H1220	1.00 a	2.21 ab	2.69 ab	3.00 a	3.00 a
L2	1.43 b	2.44 a	2.88 a	3.00 a	3.00 a
MAX GL	1.00 a	1.86 abcd	2.50 abc	3.00 a	3.00 a
MD51ne	1.00 a	1.44 de	2.07 bcd	2.75 a	3.00 a
PHY 72	1.00 a	1.50 cde	2.29 abcd	2.86 a	3.00 a
PHY 810	1.00 a	1.00 e	1.63 d	2.13 b	2.86 a
PIMA S7	1.00 a	1.00 e	2.00 cd	2.21 b	3.00 a
STV GL	1.00 a	1.94 abcd	2.71 ab	2.86 a	3.00 a

[z] Ovules were rated 1 (no glands) 2 (empty glands), or 3 (glands filling)

[y] Means within a column followed by the same letter are not significantly different at $p \geq 5\%$

Table 6. Gossypol content of developing ovules from the field 2011.

Entry	DAF 14 Gossypol [z](ug/mg)	DAF 16 Gossypol (ug/mg)	DAF 18 Gossypol (ug/mg)	DAF 20 Gossypol (ug/mg)	DAF 64 Gossypol (ug/mg)
Acala 1517	[y]BDL	BDL	BDL	2.1	12.7
GVS 5069	BDL	BDL	2.7	4.6	14.1
H1220	BDL	BDL	1.3	4.9	17.2
L2	BDL	BDL	3.2	4.8	11.1
MAX GL	BDL	BDL	BDL	3.2	16.2
MD51ne	BDL	BDL	BDL	2.4	18.9
PHY 72	BDL	BDL	BDL	2.2	12.9
PHY 810	BDL	BDL	BDL	BDL	13.1
PIMA S7	BDL	BDL	BDL	BDL	13.7
STV GL	BDL	BDL	1.6	2.1	16.0

[z] Divide (ug/mg) by 10 to convert to % gossypol

[y] BDL = below the detectable limit

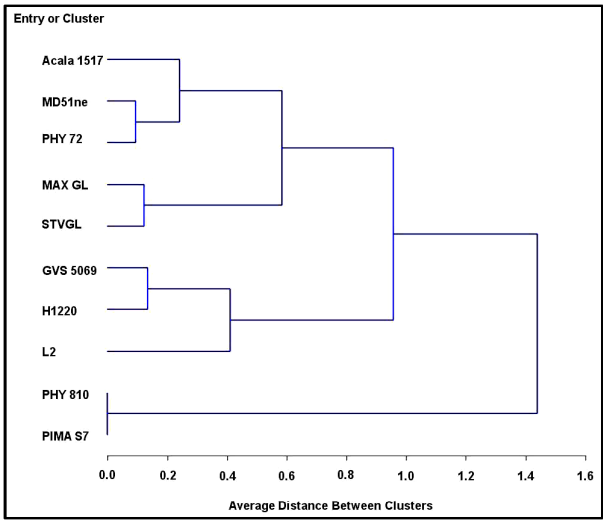


Figure 6. Dendrogram of mean glanding score at 16 DAF.

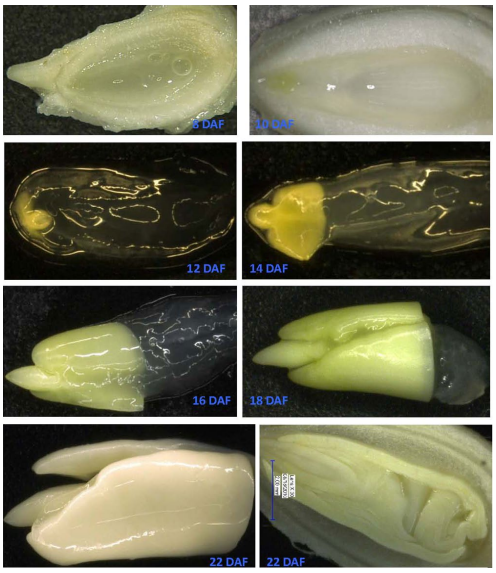
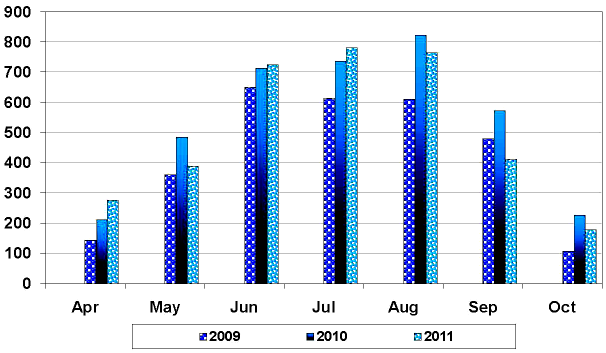


Figure 7. Ovule development of STV gl (glandless) from 8 to 22 DAF.



Source www.deltaweather.msstate.edu

Figure 8. Monthly cumulative degree days (DD60) for growing seasons 2009 to 2011.

CONCLUSIONS

The results from this series of experiments showed that gland formation in developing seed occurred as soon as there was an organized cell structure to support the creation of the gland. Depending on the genotype, temperature (cumulative degree days [DD60]), and possibly other modifying environmental conditions, the number of DAF that the first glands were observable could vary from 14 to 20 DAF. The first detectable gossypol was two to four days after gland formation. In glandless mutants as those reported by McMichael (1960), no glands were ever observed and no gossypol was detected in the ovule or mature seed. No significant variation was observed among ovules from bolls of one variety tagged at the same time, nor was there variation among ovules in different locules of the same boll. Our results indicate that collecting samples for gene expression studies, when specific ovule developmental stages are being targeted, requires determination of the stage of development before tissue collection. Our microscopic evaluation method can be done first on a small subsample of tagged bolls and when the desired stage is identified, tissue from the other bolls tagged on the same date can be collected for processing. If only a small amount of tissue is required, other ovules from the same sampled boll could be used. This method can also be used for targeting other genes that are expressed in a particular developmental stage(s).

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