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MOLECULAR BIOLOGY AND PHYSIOLOGY

Differential Expression of Trichomes on the Leaves of Upland Cotton (*Gossypium hirsutum* L.).

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ABSTRACT

The close relationship between *Gossypium* species (Malvales) and *Arabidopsis* (Brassicales) determined by molecular phylogeny might facilitate the identification of regulatory mechanisms for trichome development in cotton leaves. Focus of this study is on the evaluation and quantification of differences in trichomes on cotton leaves with varying degrees of leaf pubescence. Trichomes were evaluated using scanning electron and light microscopy on both fixed and live samples. Trichome quantity and complexity were measured on mainstem leaves and usually found to increase on new leaves as they develop progressively up the plant. Glandular trichomes (GTs) were found on all mainstem leaves examined and were not affected by various loci that either increased or inhibited the expression of non-GTs (NGTs or hairs). Neither GTs nor NGTs on Pilose leaves were affected by the expression of N_1N_1 genotype, which removes approximately 75% of all lint and 100% of all fuzz on cotton ovules. The abaxial side of the leaf usually had more GTs and NGTs. These data indicate that at least two regulatory networks exist in cotton leaves for trichome initiation, one for GTs and the other for NGTs. A third regulatory network is likely for the initiation of ovule trichomes; however, this third network might share genetic components with the NGTs. Future studies will use four very pubescent cotton lines identified in this survey for further genetic evaluations by crossing with three fiberless ovule lines.

Trichomes are defined as hair- or scale-like protuberances of the epidermis that form an assortment of single and multicellular structures with a diverse array of functions including, but not exclusive to, plant cooling, protection, and seed dispersal (Evert, 2006; Werker, 2000). Werker designated the leaf trichomes as either glandular (GT) or non-glandular (NGT), which will be adopted in this study. In cotton (*Gossypium hirsutum* L.), the GTs are short stemmed with a multicellular, globular head whereas, the NGTs comprise a large, variable group of hair-like structures ranging from the simple single hair to the complex dendritic type (Bryson et al., 1983; Oosterhuis and Jernstedt, 1999). Elucidating the regulatory network responsible for trichome development in cotton leaves would be useful in identifying shared components for trichome development in cotton ovules.

Ovular trichomes, or cotton lint (fiber), are the most economically valuable trichomes in the world and drive a multi-billion dollar worldwide textile industry (National Cotton Council, 2008). Fibers are single cells that originate from the epidermal layer of ovules and grow as long as 3 cm (Basra and Malik, 1984). The development of fibers has been well documented into four morphologically distinct stages: initiation, elongation, secondary cell-wall thickening, and maturation or drying of the fiber (Basra and Malik, 1984; Naithani et al., 1982). Identifying genes controlling these processes has been elusive. If these genes could be identified, it could lead to molecular approaches to modify both fiber initiation and development including improving yields and fiber quality.

In 1957, the first smooth-leaf cotton cultivar was released (Smith, 1964). This event marked a paradigm shift from using pubescent leaf cultivars to the development of smooth-leaf cultivars, which were beneficial in the ginning of lint with less trash (Lee, 1984b; Smith, 1964). After many years of trying to reduce leaf smoothness, Lee (1971, 1984a, 1984b, 1984c) reported that deficits in lint percentage could be an inherent problem in cotton lines with increased leaf smoothness. Subsequently, the smooth-leaf alleles T_2^{arm} and T_1^{sm} were associated with small and significant deficits in lint percentage, respectively (originally reported as S_1^{sm} and Sm_2 by Lee, 1984a; new nomenclature in Lee, 1985). These findings sug-

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gest possible genetic linkages between the regulatory mechanisms of leaf and ovular trichomes.

A few different regulatory networks responsible for NGT initiation in leaves have been reviewed in plants (Serna and Martin, 2006). Leaf trichome initiation in Snapdragon (*Antirrhinium*) and Solananaceous species appears to be induced by *MIXTA* or *MIXTA-like* genes (Serna and Martin, 2006), whereas in *Arabidopsis*, there appears to be four genes involved in trichome initiation, including *GLABROUS1* (*GL1*), *TRANSPARENT TESTA GLABRA1* (*TTG1*) and *GLABRUS3*/*ENHANCER OF GLABRA3* (*GL3*:*EGL3*) (Marks et al., 2009). Leaf trichome development in *Arabidopsis* is presently the best understood model with currently 40 genes reported to be involved in trichome development (Marks et al., 2009). Fortuitously, *Gossypium* species (Malvales) have been determined by molecular phylogenetic analysis to be closely related to Brassicales (*Arabidopsis*) in the Eurosid II clade (Bausher et al., 2006; Lee et al., 2006; Soltis et al., 1999). This could indicate that the regulatory mechanism for trichome initiation in *Arabidopsis* might also exist in cotton. One possible evidence of this similarity is that the trichome initiation gene *GL1* was recently mapped to the Pilose locus, which substantially increases trichomes in leaves, stems, and even capsules (bolls) in *G. hirsutum* (Desai et al., 2008; Lee, 1984a).

A review of the Mississippi Obsolete Variety Collection (MOVC) in the National Collection of *Gossypium* Germplasm revealed that large differences in pubescence exist among cotton leaves (Percival, 1987). This study originally began as a survey to confirm the ratings reported in the MOVC of leaf pubescence of specific cotton lines. As the study continued it incorporated identifying changes in leaf pubescence during plant growth (for genetic evaluation of populations) and ascertained which cotton lines to use in future genetic studies. Subsequently, other cotton lines that were known to carry loci for mutations that either increased or decreased leaf trichome numbers, or eliminated ovule fuzz production were included in these studies. This is the first study reporting that at least two regulatory networks for trichome initiation occur in the same leaf tissue at the same developmental stage. There is also evidence that these networks function more efficiently on the abaxial surface of the leaf for the expression of GTs and NGTs compared to the adaxial surface. There is no evidence that the dominant fuzzless seed allele N_1, which removes approximately 75% of all lint and 100% of all fuzz on cotton ovules (Turley and Kloth, 2002) has any effect on leaf trichome expression in the Pilose line.

MATERIALS AND METHODS

Plant Materials. Thirty-four inbred lines were used in this study including 31 allotetraploid *G. hirsutum* lines and three diploid lines. Twenty-three inbred *G. hirsutum* lines used in this study were obtained from the MOVC (Percival, 1987). These lines included a mixture of pubescent and glabrous (smooth) leaf lines designated 117 (piloseT_1), 143, 156, 166 (T_1^h), 208, 220, 243, 396, 397, 398, 504 (SL1-7-1; Turley and Kloth, 2008), 596, 628, 859, 903, 905, 982, 985, 998, 999, 1000, 1054 (resembles T_1^h), and 1055. The other eight lines include: MD17 fls (Turley, 2002; Turley and Kloth, 2002), XZ142w (fiberless; Zhang and Pan, 1991), XZ142 (wildtype) from Dr. Tianzhen Zhang, DP5690 and near isogenic lines Stv213T_1^{sm} and Stv213t_1^{sm} were gifts from Dr. Bill Meredith, and two very pubescent lines were derived as BC_1F_2 plants from a Pilose//Pilose/243N_1 cross. Selections were made from the F_2 progeny and checked for the homozygous expression of either N_1N_1 (linted, fuzzless seed) or n_1n_1 (linted, fuzzy seed).

Three inbred diploid lines, *G. arboreum* L. (A2-77), *G. herbaceum* L. (A1-18) and *G. raimondii* Ulbr. (D5-36) were evaluated for their trichome profiles. Two of these lines were probable ancestral progenitors of *G. hirsutum*. Small-expanding mainstem leaves (2×10^{-2} m in length) from *G. arboreum* and *G. herbaceum* were harvested from node 22 of field-grown plants. The *G. raimondii* is photoperiodic and does not flower in the Stoneville, MS location during the summer, therefore, a 2×10^{-2} m leaf (measured by mainvein length) was collected from a branch off the mainstem of a greenhouse grown plant. The diploid cottons were included to verify presence of GTs and NGTs on the leaves.

Twenty inbred lines were evaluated in the greenhouse for development of trichomes in the spring and summer of 2009 including: Pilose (117 in Table 1), 156, 166, 208, 220, 596, 628, 859, 903, 905, 982, 985, 998, 999, 1000, 1054, 1055, BC_1F_2-Pilose-fuzzless, BC_1F_2-Pilose-fuzzy, and DP5690. These experiments focused on counting the GTs and NGTs on mainstem leaves at nodes 1, 3, 5, 7, 9, 11, 13, and 15. The cotyledonary node was designated as node 0, thereby mandating that the first true leaf is at node 1. Leaf samples were not harvested until the

mainstem leaf positioned on the node directly above the sample reached full size. Due to the increasing complexity of the NGTs on the leaves progressing up the plants and resulting time constraints, the GTs were counted only on leaves from nodes 1, 3, 5, 7, and 9 (partially). These same lines and mainstem leaves were evaluated in the field in the summer of 2009. In the winter and spring of 2010, another set of smooth leaf lines were evaluated including lines 396, 397, 398, 998, 999, 1000, Stv213T_1^{sm}, and Stv213t_1^{sm}. The NGTs were counted on the designated mainstem leaves as listed above. Three leaves were collected and evaluated from the same node and variety in all studies listed in this manuscript.

The field experiment during the summer of 2010 was modified by selecting 11 of the previously evaluated lines, including PiloseT_1, 166T_1^h (Endrizzi and Ramsay, 1983), 1054 (unknown pubescence locus), BC_1F_2-fuzzless ($T_1T_1N_1N_1$), BC_1F_2-fuzzy ($T_1T_1n_1n_1$), DP5690t_3, 243N_1, 998 (unknown smooth leaf locus), 999 (unknown smooth leaf locus), Stv213T_1^{sm}, and Stv213t_1^{sm}. The GTs and NGTs were counted on the mainstem leaves from five nodes— 1, 5, 9, 13, and 17—from plants grown in the field in 2010 in a randomized block design. This selection included the most and least pubescent cotton lines in the study and included four different leaf pubescence mutations and one fuzzless seed allele.

Table 1. Evaluation of leaf pilosity in 20 selected lines of cotton. A list of the Mississippi Obsolete Variety Collection (MOVC) numbers, accession numbers, variety names, leaf pubescence ratings (all from Percival, 1987).

MOVC Number	Accession Number	Variety Name	Leaf Pubescence Rating	Ovule Phenotype
117	PI 528521	Pilose	6	Fuzz/Fiber
143	PI528543	Mexican Fuzzless Seed UA3-3	3	Fuzzless (n_2n_2)
156	PI 528555	Person American UA 7-39	5	Fuzz/Fiber
166	PI 528560	M.U.8B UA 7-44	6	Fuzz/Fiber
208	PI 528587	G hirsutum Tashkent	5	Fuzz/Fiber
220	PI 528594	K-2102 Var.182 AK-Djura	5	Fuzz/Fiber
243	PI528610	Ballard Fuzzless Seed	2	Fuzzless (N_1N_1)
396	PI528718	Super Round	1	Fuzz/Fiber
397	PI528719	Crenate	1	Fuzz/Fiber
398	PI528720	Okra round	1	Fuzz/Fiber
504	PI528807	SL1-7-1 (fiberless)	3	Fiberless
596	PI 528877	CB 2630	5	Fuzz/Fiber
628	PI 528880	CB 2554	5	Fuzz/Fiber
859	PI 528960	Wields Cleveland	5	Fuzz/Fiber
903	PI 528991	PI 1944833 KP28	5	Fuzz/Fiber
905	PI 528993	PI 194831, BP52, MB2	5	Fuzz/Fiber
982	PI 529051	5143	5	Fuzz/Fiber
985	PI 529054	A7215	5	Fuzz/Fiber
998	PI529067	DES716	1	Fuzz/Fiber
999	PI529068	DES717	1	Fuzz/Fiber
1000	PI529069	DES723	1	Fuzz/Fiber
1054	PI 529123	M4	6	Fuzz/Fiber
1055	PI 529124	M100	5	Fuzz/Fiber
DP5690	PVP9100116	DP5690	1	Fuzz/Fiber
MD17	PI616493	Mississppi Delta 17 (fiberless)	NR	Fiberless
XZ142w	NR	Xuzhou 142w (fiberless)	NR	Fiberless
XZ142Fuzz	NR	Xuzhou 142 (wildtype)	NR	Fuzz/Fiber

Field plots were single row, 5 m long and spaced 1.02 m apart. Where applicable, plots were overseeded and after the plants reached the first true leaf stage, seedlings were thinned to 6.5 plants m^{-2}. In the greenhouse, plants were grown in Metro Mix 360 in 5-gallon pots. These plants were watered daily, or as often as needed, and fertilized every 2 wk with a tablespoon of All Purpose plant food (Miracle Grow). Weeds and insects were managed using standard agronomic practices for the Mississippi delta for both field and greenhouse experiments.

Scanning Electron Microscopy (SEM). Leaf discs and mainstem sections were removed from node 13 mainstem leaves of Pilose and DP5690 plants. Leaf discs were removed from both sides of the midvein and the closest lateral vein by the convergence of the veins adjacent to the petiole (diagramed by Bryson et al., 1983). A rubber stopper was placed under the leaf and a cork borer (1.2×10^{-2} m diameter) was used to cut the disks. Concurrently, cotton mainstem sections were harvested and cut into 5×10^{-3} m whole mainstem segments and were split vertically through the pith and vascular tissue to produce two semicircular (180°) mainstem blocks. Both the leaves and mainstems were then placed in 5 mL of 6% glutaraldehyde in 50 mM Pipes (pH 7.4) overnight at 4°C. Samples were rinsed twice in 10 mM cacodylate (pH 7.2) for 15 min. An additional two rinses were performed with deionized water. The samples were then dehydrated in an ethanol series: 25% for 15 min, 50% for 15 min, and 75% for 15 min. The 75% solution was then decanted, 100% ethanol was added, and the samples were dehydrated overnight at 4°C. The ethanol was removed and the leaves and stem samples were critical point dried, substituting carbon dioxide to remove all traces of water. Leaf disc sections were mounted on aluminum stubs with either the abaxial or adaxial leaf surface or the flat cut stem surface was attached, then samples were sputter coated (Hummer X Coater, Anatech LTD., Denver, NC) with 1.8×10^{-8} m of 100% gold-palladium to a thickness of 1.5×10^{-8} m. Observations were made using a JEOL (Japan Electronics Corporation, Tokyo, Japan) JSM-840 SEM operating at 15 kV. Images were collected with a 4PI digital acquisition program.

Light Microscopy (LM). LM was used to evaluate both thin sections and live leaf tissue. For the first project, small leaves ($< 2 \times 10^{-2}$ m) were dissected with a razor blade into small pieces and placed in 25-mL glass vials with 5 mL of 3% (v/v) glutaraldehyde and 0.1% Tween 20 (polyoxyethylenesorbitan monolaurate) in 0.05 M PIPES buffer (pH 7.4) for 2 h at 24°C. These samples were then washed twice with 0.1% Tween 20 in 0.025 M PIPES buffer followed by one wash with 0.025 M PIPES. The samples were then dehydrated in an ethanol series consisting of 30, 50, 75, and 100% ethanol at intervals of 2 h at 4°C. Upon reaching 100% ethanol, the samples were transferred to -20°C for 24 h. Samples were then drained and dehydrated with an additional 100% ethanol at -20°C for 24 h. LR White (Polysciences, Warrington, PA) was then added in 25% increments at 4°C for 24 h each until 75% resin was achieved (3 d total). The resin was then removed and the samples were transferred to 3 mL of 100% resin and stored at -20°C for 24 h. The resin was removed from the vial, the vial refilled with 3 mL of another exchange of 100% resin, and then stored at -20°C for another 24 h. The samples (no exchange of resin) were placed on a rocking shaker for 24 h at 24°C. Samples were transferred into the detached lids of 2.6×10^{-2} m polypropylene embedding pucks (Ted Pella, Inc., Redding, CA) that were then slightly overfilled with LR White to form a convex surface on the puck. ACLAR® Embedding Film (Ted Pella, Inc., Redding, CA) was cut into 6.3×10^{-2} m^2, which were placed on top of the pucks, forming an oxygen-proof seal over the specimens in LR White.

Specimens were cut from polymerized LR White blocks, oriented on acrylic stubs to obtain cross-sections of the leaves. These blocks were trimmed using a razorblade into rhombosoids and sections (3.5×10^{-7} m) were collected using a Delaware Histo-knife (Delaware Diamond Knives, Wilmington, DE) and a Reichert Ultracut Ultramicrotome (Cambridge Instruments, Buffalo, NY). Sections were dried on chrome-alum-coated slides on a slide-warming tray. Sections were stained for 10-30 s with 1% (w/v) toluidine blue in 1% (w/v) sodium borate on the warming tray, washed with distilled water from a wash bottle, dried with compressed air, mounted with Permount (Fisher Scientific, Pittsburgh, PA) and observed with a Zeiss Axioskop (Zeiss, Oberkochen, Germany). An Olympus Q-Color 3 digital camera (Olympus Corp., Tokyo, Japan) was used to record images.

For the second project, six leaf disc samples from three plants as described above (SEM methods) were harvested from various mainstem leaves (nodes indicated above) and examined with a Keyence VHX-600 (Keyence, Osaka, Japan) digital microscope using an ultra-small high performance lens (20 – 200 X) with an adjustable illumination adaptor. This scope has two advantages for photographing trichomes with

(1) real-time depth composition and (2) the use of the adjustable illumination adaptor that reflects light horizontally across the surface of the epidermis.

Cotton leaf trichomes were categorized into two major types: multicellular GTs and diverse NGTs. The NGTs were further categorized as simple (single hair), simple dendritic (branches radiating from a central symmetrical point), or complex dendritic (branches radiating from nonsymmetrical points and planes). Simple dendritic trichomes were designated as forked (two branches), tripartite (three branches), and stellate (4 branches); whereas complex dendritic were classified as any trichome with five or more branches. These complex trichomes resemble more of a bush than a tree.

Two leaf discs (1.2 X 10^{-2} m diameter) were removed from each leaf between the mainvein and the next closest lateral vein. Using the "Measure" setting, four adjoining 1 X 10^{-6} m^2 squares were delineated per leaf disc on the computer screen for ease of counting. The method of selecting areas for counting was to leave the scope slightly out of focus, move the leaf into the viewing frame, and then focus. Counting trichomes on the major veins was avoided. Each 1 X 10^{-6} m^2 square was counted separately for GTs and NGTs per leaf (4 X 10^{-6} m^2 per disc/8 X 10^{-6} m^2 per leaf) giving eight different counts. For the NGT graph, each group of trichomes— single, forked, tripartite, stellate and complex—were grouped from all eight squares from one leaf. The different types of trichomes from the three sets of leaves per line were averaged/8 X 10^{-6} m^2 and graphed. For the listing of NGTs in Table 2 each trichome was counted (no designation of type) for each leaf then the average number of trichomes per mm^2 was determined. The most prevalent trichome types are listed in Table 2. For the GTs, the average number of trichomes were determined for each leaf and then the overall average was determined. These averages were calculated from both the abaxial and adaxial side of each leaf. These were graphed and standard mean error was determined using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA).

Table 2. Evaluation of leaf pubescence on node 17 mainstem leaves of 24 selected lines of cotton. Average number of glandular trichomes (GTs) and non-glandular trichome (NGTs) were calculated for the adaxial and abaxial leaf surfaces. Mississippi Obsolete Variety Collection (MOVC) numbers from Percy (1987).

MOVC Number	Adaxial GTs per mm^2	Abaxial GTs per mm^2	Adaxial GTs/mm^2; most common NGT	Abaxial NGTs/mm^2; most common NGT
143	8.67±0.44	10.58±0.68	None	1.00±0.26; Forked
156	7.83±0.15	9.17±0.11	None	0.96±0.17; Stellate
166	6.59±0.06	10.88±0.38	0.08±0.04; Single	2.92±0.04; Single
208	8.25±0.19	11.13±0.51	None	1.25±0.40; Forked
220	9.17±0.40	10.54±0.51	None	0.79±0.04; Forked
243	7.96±0.79	10.83±0.4	None	None
396	7.67±0.30	10.13±0.14	None	None
397	8.17±0.34	10.46±0.65	None	None
398	8.71±0.22	10.00±0.25	None	None
504	8.25±0.19	9.54±0.53	None	1.00±0.31; Single
596	8.25±0.57	12.33±0.36	0.13±0.07; Single	1.38±0.33; Single
628	7.88±0.13	10.17±0.40	0.04±0.04; Single	0.68±0.13; Single
859	7.46±0.29	10.29±0.11	1.25±0.22; Single	2.25±0.07; Single
903	7.75±0.13	9.17±0.11	None	1.25±0.13; Forked
905	8.39±0.29	11.13±0.22	0.46±0.15; Single	1.67±0.04; Single
982	7.92±0.33	9.13±0.19	1.25±0.19; Single	1.75±0.13; Single
985	8.96±0.25	9.75±0.56	1.46±0.27; Single	3.79±0.15; Single
998	6.25±0.36	10.92±0.55	None	None
999	6.38±0.89	10.30±0.37	None	None
1000	8.17±0.42	10.58±0.41	0.04±0.04; Single	1.00±0.00; Stellate
1055	7.54±0.25	9.71±0.48	0.29±0.04; Single	2.36±0.22; Forked
MD17	8.17±0.42	10.58±0.40	None	None
XZ142w	8.25±0.19	10.41±0.30	1.13±0.14; Single	3.42±0.4; Singles
XZ142Fuzz	8.58±0.41	10.625±0.33	None	0.42±0.15; Forked

RESULTS

Cotton Accessions and Cultivars. Table 1 lists most of the lines used in this study, along with the MOVC number, accession number, variety name, leaf pubescence rating, and boll phenotype. The leaf pubescence rating is based on a rating system where 1 is a smooth leaf (very few trichomes) with pubescence increasing incrementally to 6, which is extremely pubescent (Percival, 1987). No information is provided as to how this ranking was obtained. Also included in this table are three fiberless lines, i.e., MD17, SL1-7-1 (#504), and XZ142w; accompanied by XZ142 (wildtype) and two fuzzless seed lines, i.e., 143 and 243. Near isogenic lines Stv213T_1^{sm}, Stv213t_1^{sm}, BC_1F_2-Pilose-fuzzless, and BC_1F_2-Pilose-fuzzy were not included in this table because these lines have no preliminary data available.

SEM and LM. The leaf surfaces and stems of Pilose and DP5690 were evaluated with SEM. Figure 1 includes the SEM view of both the abaxial and adaxial surfaces of Pilose and DP5690 leaves and the Pilose mainstem. These views include: 1A) four complex dendritic and two GTs (arrow) on the abaxial surface of a Pilose leaf; 1B) two single trichomes (arrows), one forked and stellate trichomes with two GTs on the adaxial surface of a Pilose leaf; 1C) an enlarged view of a multicellular GT; 1D) two forked and one GT on the Pilose mainstem; 1E) seven GTs on the abaxial side of a DP5690 leaf, and 1F) five GTs on the adaxial side of a DP5690 leaf. The GTs often appear sunken, usually in close proximity to the vascular tissue on the abaxial leaf side and are approximately 3.0×10^{-5} m in diameter.

LM of thin sections was used to assess the cellular structure of GTs and NGTs (Fig. 2A, B). These sections were from young, expanding Pilose leaves approximately 2.0×10^{-2} m in length. These leaves were fully covered in NGTs, insomuch that the surface of the leaf was not visible. As can be seen in Fig. 2A, the four groups of dendritic trichomes appeared to be comprised of single cells. No septated (multicellular) single hairs were ever observed in these studies. The GTs in Fig. 2B are usually comprised of 1- or 2-ft cells, one stalk cell, and two to three tiers of head cells as reported by Wall (1970). Any apparent difference between the description and Fig. 2B might be due to the angle of the section cut and further work and comparisons would need to compare multiple lines, including those used by Wall (1970).

LM was used to evaluate quickly all trichomes on the surface of the leaves. The viewing of these trichomes was enhanced by directing light horizontally across the surface of the leaf and using the microscope's 3-D program, which captures multiple focal points of the specimen and composes these into a single image. This process will sometimes give the NGTs an artificial fluorescent appearance as in Fig. 2C, D. A common phenomena on the adaxial surface (exposed to direct sunlight) is that the GTs are a reddish color (Fig. 2D, E, F). Red GTs are rarely seen on the abaxial side of field-grown plants and were never observed on either side of the leaves on greenhouse-grown plants (Fig. 2C). Also, GTs appeared to have a limited life span on leaves as shown in Fig. 2F. On a young leaf the GTs are either red or clear. During the course of this study it was observed that as the leaf matures the GTs will often change from red to yellow to a dark color. It is difficult to find GTs on very old leaves.

Diploids *G. arboreum*, *G. herbaceum*, and *G. raimondii* were compared to *G. hirsutum*. The abaxial sides of leaves of *G. arboreum* (Fig. 3A), *G. herbaceum* (Fig. 3B), and *G. raimondii* (Fig. 3C) have distinguishable NGTs and the small white spots (arrows) are the GTs. Only a few GTs could be seen through the thick covering of NGTs on the abaxial side of *G. raimondii* leaves, therefore, the adaxial side was photographed (Fig. 3D). One note is that no red GTs were observed on the adaxial side of the young *G. raimondii* leaves (Fig. 3D).

Development of Leaf Trichomes and Leaf Mutations. The evaluation of trichome development included as many of the known leaf trichome alleles as we have collected from the cotton germplasm. PiloseT_1, 166T_1^h, 1054 (unknown pubescence locus) were rated 6 in MOVC evaluation. The Pilose allele T_1 is confirmed by the expression of trichomes on the boll (Fig. 4B). The boll trichomes are similar to the trichomes on *Arabidopsis* (Folkers et al., 1997) except that cotton boll trichomes are rarely branched (Fig. 4C, D). Lines 166 and 1054 did not produce trichomes on their bolls (Fig. 4A). Lines 166 and 1054 also produced less complex and fewer leaf trichomes than Pilose (Fig. 5, Table 2, [Suppl. Mat.1-A]). Only Pilose (Fig. 5A, B, [Suppl. Mat1.-A]) and 1054 (Fig. 5C, D, [Suppl. Mat.1-A]) adaxial and abaxial leaf trichome counts are shown for comparison. The adaxial leaf surfaces (Fig. 5A, [Suppl. Mat.1-A]) of the Pilose leaves produced single hairs and forked trichomes abundantly with a scattering of tripartite,

stellate, and complex dendritic trichomes. Line 1054 (Fig. 5C, [Suppl. Mat.1-A]) produced mostly single hairs with no complex dendritic trichomes observed. Difference were also noted in the abaxial surfaces of the leaves with Pilose leaves producing more forked, stellate and complex dendritic trichomes (Fig. 5B, [Suppl. Mat.1-A]), whereas, 1054 produced single hair and forked trichomes abundantly (Fig. 5D, [Suppl. Mat.1-A]). Line 166 was similar to line 1054 but had a minor reduction of leaf trichomes (Table 2, Fig. 5C, D, [Suppl. Mat.1-A, D]).

Comparisons were made also between two pubescent lines derived as homozygous plants from a selection of BC_1F_2 families designated BC_1F_2-fuzzless ($T_1T_1N_1N_1$) and BC_1F_2-fuzzy ($T_1T_1n_1n_1$). These lines theoretically consisted of 75% of the Pilose genotype with one line homozygous for the dominant fuzzless seed N_1N_1 allele and the other line homozygous for the wild-type n_1n_1. These two lines appear identical both in phenotypes and in measurements of trichomes [Suppl. Mat. 1-B, C]. Therefore the homozygous expression of the N_1N_1 allele that removes approximately 75% cotton lint and 100% of seed fuzz does not affect the leaf trichome development in BC_1F_2 Pilose-fuzzy leaves.

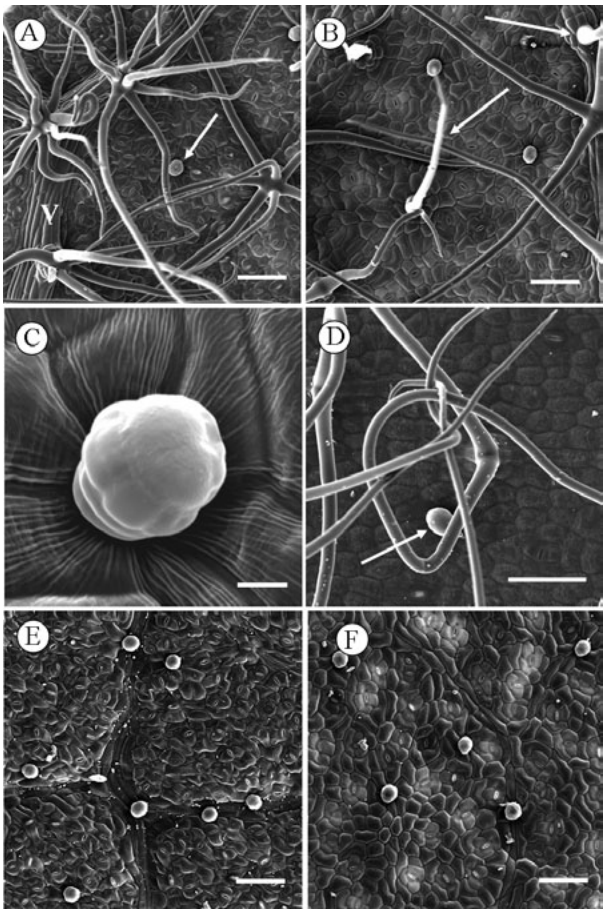


Figure 1. Scanning electron micrographs of the adaxial and abaxial surfaces of fully expanded pilose (A-C) and DP5690 smooth leaves (E-F) and a pilose stem (D). These panels include: A) Abaxial view of a pilose leaf, V labels the leaf vein and the arrow points to a GT. B) Adaxial viewe of a pilose leaf, arrows points to single celled non-glandular hairs. C) Magnified view of a GT. D) Stem trichomes on the pilose line, arrow points to a GT. E) Abaxial surface of the DP5690 smooth leaf. F) Adaxial surface of DP5690, note that only GTs are present on both sides of the leaf. Magnification bars in A, B, D, E, F are equal to 100 µm and for C is equal to 10 µm.

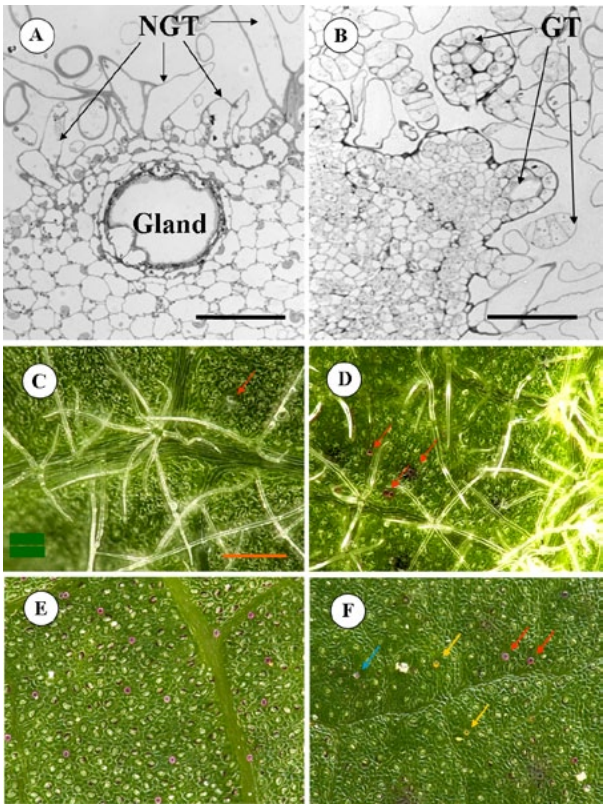


Figure 2. Light micrographs of cotton leaves. A) Cross section of a Pilose leaf and multicellular NGTs. B) Cross section of the Pilose leaf and multicellular GTs. C) Light micrograph of the abaxial surface of a Pilose leaf. Note the complex trichomes and red arrow that points to a GT. D) Light micrograph of the adaxial surface of a Pilose leaf. Note the complexity of the NGTs, the red arrows point to the red GTs. E) Light micrograph of the adaxial surface of the line 243 leaf. Note the lack of NGTs and numerous red GTs. F) Light micrograph of the adaxial surface of a DP5690 leaf. Note the lack of NGTs, with red arrows point to the red GTs, yellow arrows point to yellow aging GTs and a light blue arrow pointing to a blackish GT. Magnification bars in A and B are equal to 30µm, and in panel C equal to 300µm. Panels C through F are of equivalent leaf areas.

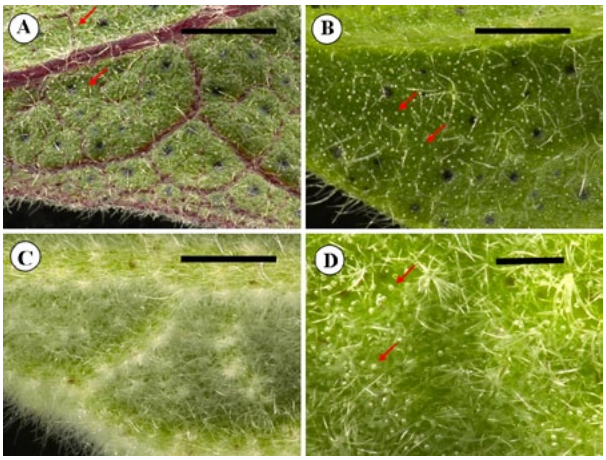


Figure 3. Light micrographs of the abaxial surface of three diploid cotton leaves, including: A) *G. arboreum*, B) *G. herbaceum*,and C) *G. raimondii*. Panel D) is a close up of the adaxial surface of the *G. raimondii* leaf for easy viewing of the GTs. Magnification bars in A, B, C are equal to 2 mm, whereas in panel D the bar equals 400µm.

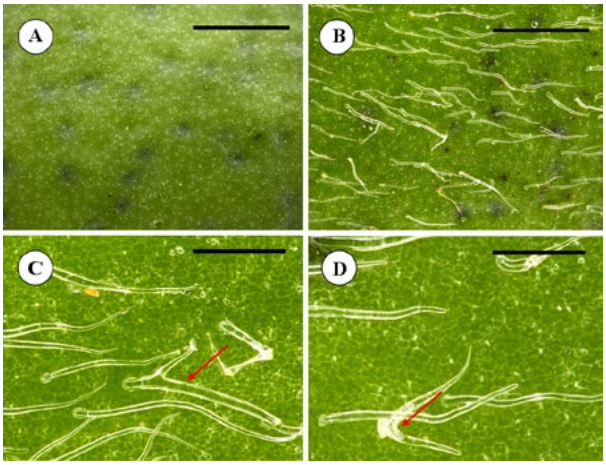


Figure 4. Light micrographs of the surface of cotton bolls from the smooth leaf cotton DP5690, Pilose and two BC_1F_2 progeny of a Pilose X (Pilose X 243N_1). Panel A) DP5690 boll— smooth surface. B) Pilose, note simple hairs. C) BC_1F_2 progeny expressing N_1N_1, note the branched trichome (red arrow) and D) BC_1F_2 progeny expressing n_1n_1, note the branched trichome (red arrow). Magnification bars in A and B are equal to 2 mm and in C and D are equal to 0.5mm.

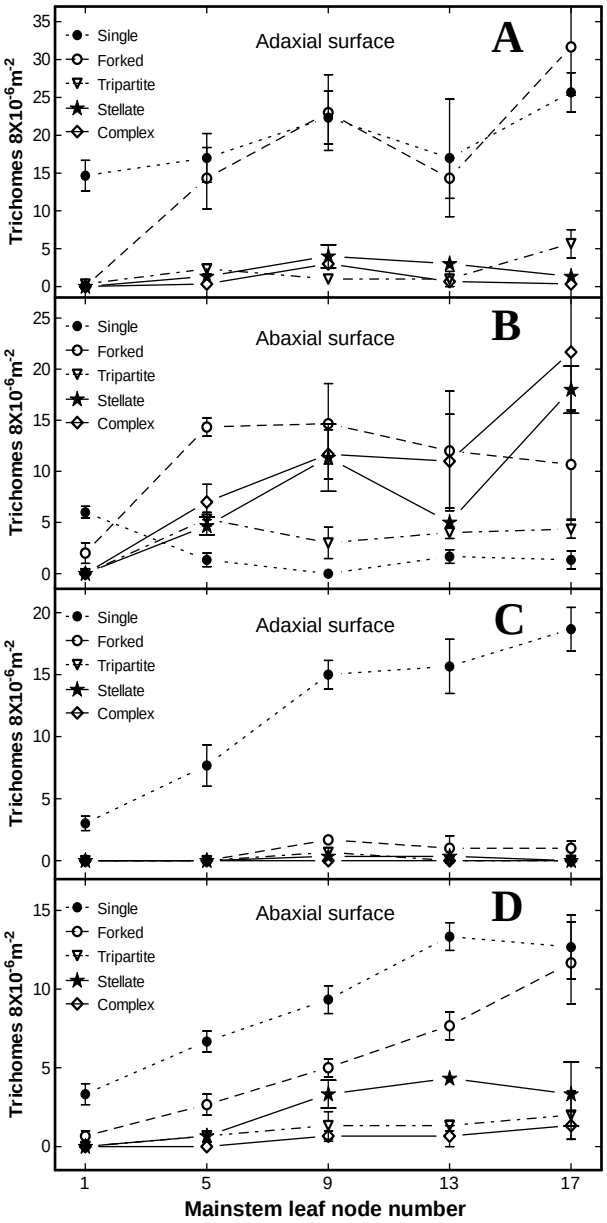


Figure 5. Graphed comparisons of the trichome counts on the adaxial and abaxial surfaces of the leaves of Pilose (A and B) and 1054 (C and D). Five types of trichomes were measured including single hairs, forked (two branches,), tripartite (three branches), stellate (four branches) and complex trichomes. Complex trichomes had five or more branches.

Trichome data were not graphed for the smooth leaf lines Stv213T_1^{sm}, Stv213t_1^{sm}, DP5690t_3, 243, 998, and 999 because only rarely was a NGT counted on fully expanded leaves. Differences between lines Stv213T_1^{sm}, Stv213t_1^{sm}, and DP5690t_3 (Fig. 6A, B, C) and lines Pilose, 166, and 1054 (Fig. 6D, E, F) can easily be visualized. The pubescence of these leaves is exaggerated by collecting small nonexpanded leaves (approximately 2 X 10^{-2} m long). It is apparent how different these lines are in trichome production. This comparison shows a difference between lines Stv213T_1^{sm} and Stv213t_1^{sm} that was impossible to quantify in the field with fully expanded leaves. The low production of NGTs on these smooth leaf lines were verified in greenhouse experiments.

Interestingly, the expression of GTs in the pubescent or smooth leaf lines on the either the adaxial or the abaxial surface was not affected by the high or low expression of NGTs. The developmental pattern for GTs from four lines carrying pubescence/smoothness alleles was graphed (Fig. 7). Lines Stv213T_1^{sm}

and DP5690t_3 carried alleles for smooth leaves and PiloseT_1 and 1054 (allele unknown) carried the alleles for extreme pubescence. The trend with these four lines and all others in this experiment is that more GTs were seen on the abaxial side of leaves.

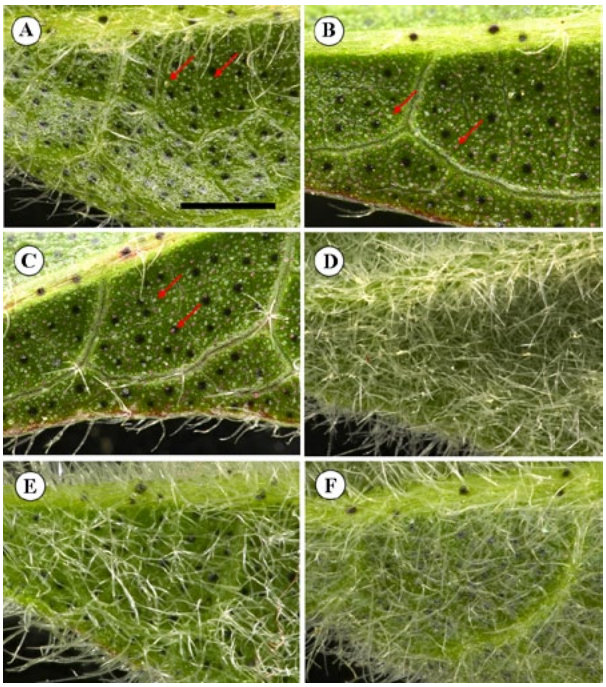


Figure 6. Light micrographs of three smooth leaf lines Stv213T_1^{sm} (A), Stv213t_1^{sm} (B, near isogenic lines) and DP5690 (C) and three pubescent leaf lines Pilose (D), 166 (E) and 1054 (F). Magnification bar in A equals 2 mm and is representative of all panels.

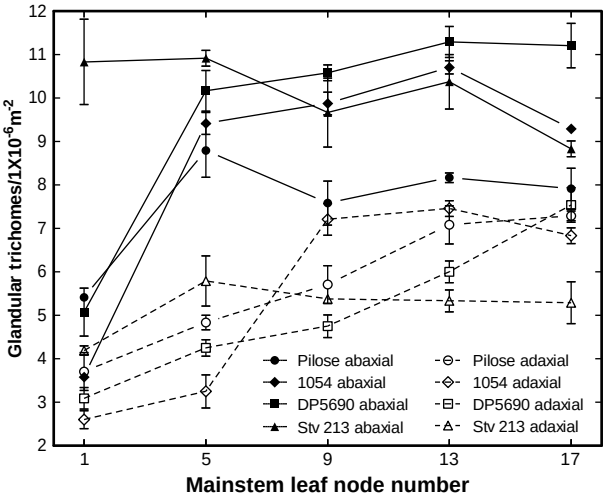


Figure 7. Graphed comparisons of the GTs on the adaxial and abaxial surfaces of smooth leaf lines DP5690 and Stv213 and the pubescent leaf lines Pilose and 1054.

Survey of Additional Inbred Cotton Lines. Table 2 summarizes the other cotton lines evaluated in this study. Many of the lines listed in Table 2 were evaluated developmentally by counting trichomes on mainstem leaves progressively up the plants [Supp. Mat. 2A-I]. These plants included lines that were listed as very pubescent (5 on the pubescent scale), four lines that were reported as smooth (1 on the pubescent scale), and the fuzzless and the fiberless seed lines. Interestingly, the XZ142w fiberless seed line would likely be rated as a 6 on the pubescent scale. Many of the plants listed as very pubescent in the MOVC, such as, 156, 208, 220, and 903 did not produce a significant number of trichomes on their adaxial surface (Table 2, [Suppl. Mat. 2A-I]). Line 166 was rated as more pubescent than line 985 in the MOVC. In this study it was determined that line 985 had more NGTs on both the adaxial and abaxial surfaces of their leaves than line 166. Also no cotton line evaluated in this study comes close to the pubescence or complexity of dendritic NGTs as seen in the Pilose line.

DISCUSSION

SEM and LM of cotton leaves have been reported in earlier studies (Bryson et al., 1983; Hornbeck and Bourland, 2007; Kosmidou-Dimitropoulou et al., 1980; Wise et al., 2000). This report differs from these previous reports in that it focuses on the development and complexity of leaf trichomes during the growth of the cotton plants and the differences in complexity on the adaxial and abaxial surfaces of the leaf. This study also establishes the differences between cotton GTs and the various types of NGTs, which were designated as single hairs, forked, tripartite, stellate, and complex dendritic hairs from observations made in Pilose leaves. Pilose had the most diverse array of trichomes in this study and many of its trichome types were not observed in other pubescent leaf lines. A few of the complex dendritic types in Pilose had up to 18 branches. These dendritic types as well as forked, tripartite, and stellate were each counted as one trichome in this study. It appears that most NGTs and all GTs are multicellular structures with various functions in cotton leaves. The most likely function would be leaf protection. Unlike GTs, the NGTs in both Pilose and DP5690 recently have been found to have cell walls permeated with lignin and xylans (Cochran et al., Vaughn and Turley, unpublished), major components of vascular tissue and wood. Also the GTs were reported to contain phenolic compounds including isoquercitin and therefore would also benefit cotton in a protective way (Kosmidou-Dimitropoulou et al., 1980).

Cotton is relatively closely related to the model plant *Arabidopsis* in the Eurosid II clade (Bausher et al., 2006; Lee et al., 2006; Soltis et al., 1999). This could indicate that many of the same genes responsible for leaf trichome development in *Arabidopsis* could also be functioning in cotton leaves. The cotton *GL1* homolog, reported in *Arabidopsis* to be required for trichome initiation (Marks et al., 2009), was recently mapped to the PiloseT_1 locus in *G. hirsutum* (Desai et al., 2008). A promoter region for a *GaMYB2*, a functional homolog of *GL1*, was also used to express a GUS gene in trichomes of *Arabidopsis* and in GTs cells of tobacco (Shangguan et al., 2008). Other cotton homologs for *Arabidopsis* trichome development genes could also rescue the wild-type phenotype in trichome mutants in *Arabidopsis* biotypes, including *TTG1* (Humphries et al., 2006) and *Glabra 2* (Guan et al., 2008). These data do not indicate that these genes function in cotton to initiate and develop leaf trichomes, however, due to the phylogenetic closeness between cotton and *Arabidopsis*, the expression of these genes should be evaluated further in cotton leaves.

Another fortuitous event in cotton research, is that leaf pubescence/smoothness became an area of concerted investigations with release of the smooth-leaf varieties. This allowed multiple alleles for both pubescent leaf and smooth-leaf phenotypes to be identified and characterized in cotton (Lee, 1985). During these studies Lee (1971, 1984a, 1984b, 1984c) reported that deficits in lint percentage could be an inherent problem in cotton lines with increased leaf smoothness. Subsequently the smooth-leaf alleles T_2^{arm} and T_1^{sm} were associated with small and significant deficits in lint percentage, respectively (Lee, 1984a; 1985). With the possibility that leaf trichome development share a few common genes with lint development on cotton ovules, a genetic study using the very pubescent lines, i.e., 166, 859, 1054, and Pilose crossed by the two fuzzless and three fiberless lines, MD17 (Turley, 2002; Turley and Kloth; 2002), SL1-71 (Turley and Kloth, 2008) and XZ142w (Zhang and Pan, 1991) might allow the linkage(s) to be established. This linkage with the knowledge that cotton leaf trichomes share a similar regulatory network with *Arabidopsis* trichomes, would give a foothold in identifying the regulatory network controlling cotton fiber initiation. Preliminary genetic studies identifying this linkage indicated this could be a good approach.

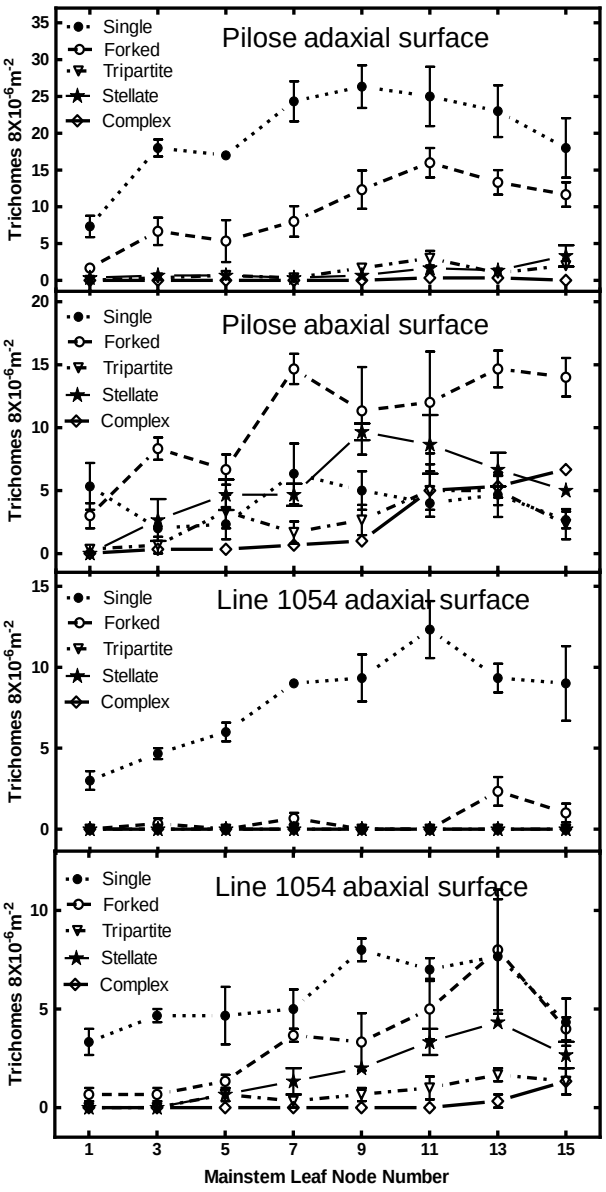
Comparing GT and NGT data indicated that the pubescence alleles that either increase (Pilose) or decrease (smooth-leaf) NGTs have no apparent effect on the expression of GTs. The GTs were observed in the mainstem leaf at the first node, whereas, in almost every other line in this report no NGTs were ever observed. Usually the exception occurred in Pilose, Pilose-derived, 166, 859, or 1054 lines and usually then only a few NGTs were ever observed on first node, mainstem leaves. These observations indicate that at least two regulatory mechanisms exist for trichome development in the cotton leaf. One regulatory network would be responsible for the initiation and development of GTs and another responsible for the initiation and development of NGTs. It is possible that these two networks might share components. Only two of the five pubescent loci, i.e., t_1 and t_3 (Lee, 1985), were represented in this study. The other locus (Lee, 1985), t_2, will also need to be evaluated to identify any possible linkages. We are presently trying to obtain the lines that carry different alleles to these loci. Also a general rule in this study and other reports (Kosmidou-Dimitropoulou et al., 1980) is that both the number of GTs and NGTs usually increased on the abaxial side of the leaf progressing up the plant. In many cases, an increase in the complexity of NGTs was also observed on the abaxial side of the leaf. These data indicate that not only can these different regulatory networks exist within a few cells of each other in young developing leaves, but that they can also have increased expression on the abaxial surface of the leaf. In cotton leaves it is common that trichomes form from adjacent epidermal cells.

A comparison of the diploids *G. arboreum*, *G. herbaceum*, and *G. raimondii* was made to verify possible differences in the expression of GTs and NGTs with the allotetraploid *G. hirsutum* lines. The allotetraploid *G. barbadense* L. has also been reported to have GTs and NGTs (Wise et al., 2000). *Gossypium arboreum* and *G. raimondii* are likely descendents of early progenitors of *G. hirsutum* (Brubaker et al., 1999) and therefore any differences in trichomes could be used as another tool in the understanding of leaf trichome development in cotton. Another reason to include the diploid cotton lines is for a comparison of the trichomes to the diploid *Arabidopsis*. *Arabidopsis* does not produce GTs (Belstein et al., 2006) and only produces single-celled, branching NGTs that are unlike anything that were observed on a cotton leaf. Pilose boll trichomes

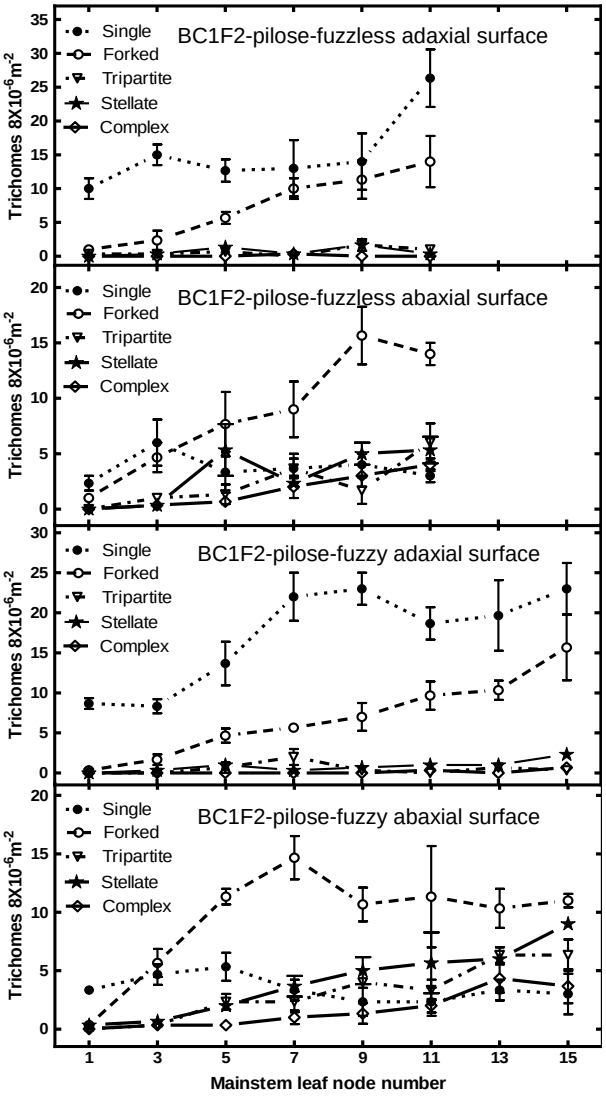
(Fig. 4) resemble a less branched version of *Arabidopsis* leaf trichomes. There are only a few of the Brassicales that produce both GTs and NGTs on their leaves (Belstein et al., 2006). With the model that two independent regulatory networks function to produce these two types of trichomes, GTs and NGTs, further work in the Brassicales may provide additional insights.

The data in Table 2 indicate complexity in the expression of leaf trichomes in cotton. Differences of expression between the abaxial and adaxial leaf surfaces and differences between the major trichome expressed raise questions about how do these trichomes develop and are regulated. It was noted early on that 243 and MD17 (both N_1 carrying lines) had very smooth-leaves. The BC_1F_2 families from the cross of (243 X Pilose) X Pilose indicated that this smooth leaf phenotype is not the result of the expression of N_1. Other observations are that the most predominant trichomes on node 17 leaves (Table 2) are either single or forked. On two occasions stellate trichomes were the most prevalent trichomes on the abaxial surface (lines 156 and 1000). A problem that becomes apparent from Table 1 and Table 2 is that the rating system used in the MOVC has flaws. Many of the lines with a pubescence rating of 5 (highly pubescent) did not have measurable numbers of trichomes on the adaxial leaf surface.

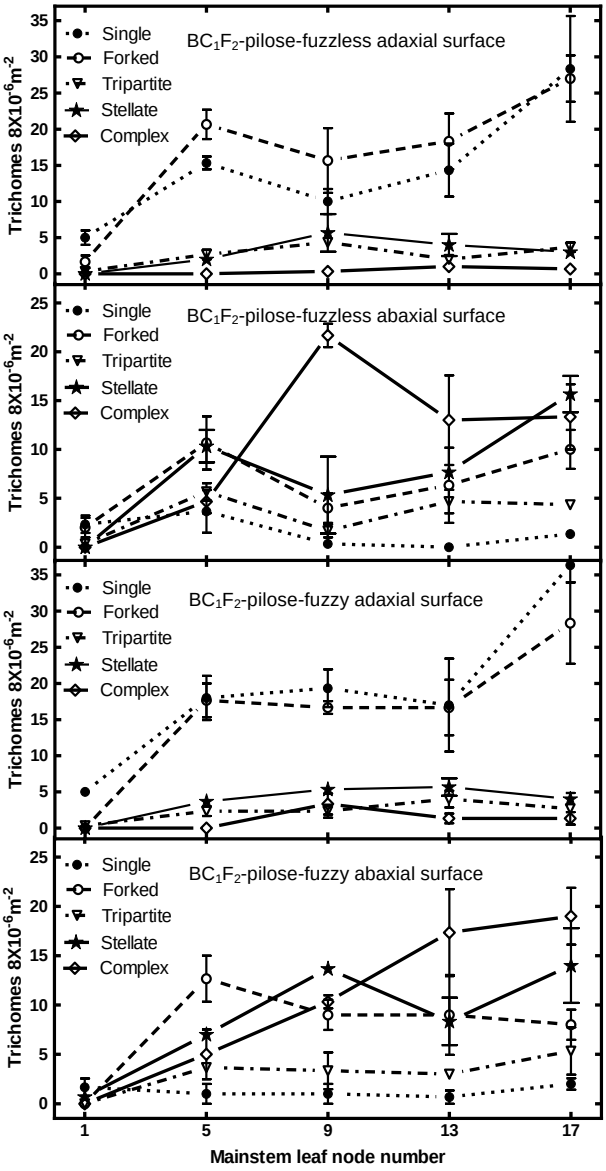
Originally this project was implemented to identify cotton lines which could be used in a genetic study, with an emphasis on using mainstem leaves at specific nodes to identify the different trichome phenotypes in a segregating population. As found in this study, determining segregation patterns can be easily accomplished either with small leaves (2 cm in length harvested from a determined upper node close to the apical meristems as is shown in Fig. 6) or the use of leaf discs from leaves harvested at specific nodes higher up the plant (Fig. 5). Counting leaf trichomes can be cumbersome when using large populations, and therefore, using 2-cm leaves with a visual type rating system may be ideal. Other rating system show great promise (Bourland et al., 2003; Hornbeck and Bourland, 2007). Also, segregation of populations, incorporating Pilose as a parent, could be visually evaluated for the expression of complex dendritic trichomes. The genetics of Pilose has been well established (Kloth, 1985a, 1985b). Trichomes in other populations would need to be counted. Molecular mapping of leaf and stem pubescence has been reported (Desai et al., 2008; Lacape and Nguyen, 2005; Wright et al., 1999). These mapping methods will be useful in determining chromosomal locations and provide an additional method to evaluate segregation. These will also be useful in establishing any linkages between mutations in leaf and ovular trichome development.



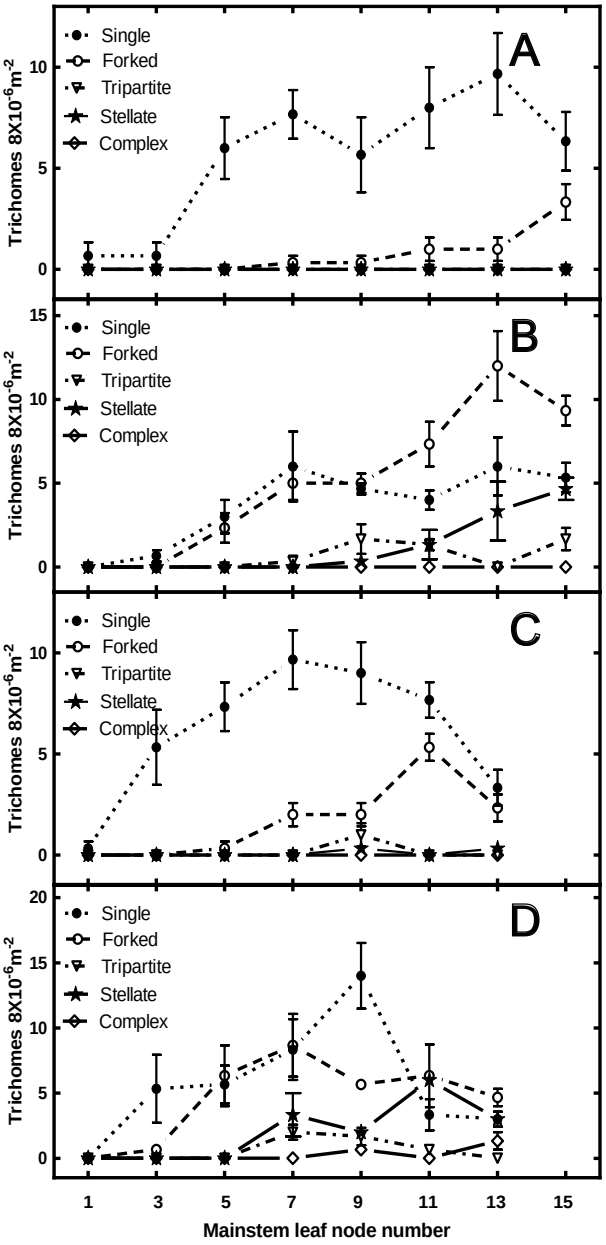
Supplemental Material 1-A. Comparison of the trichome counts on the adaxial and abaxial sides of leaves from lines Pilose and 1054 grown in the greenhouse.



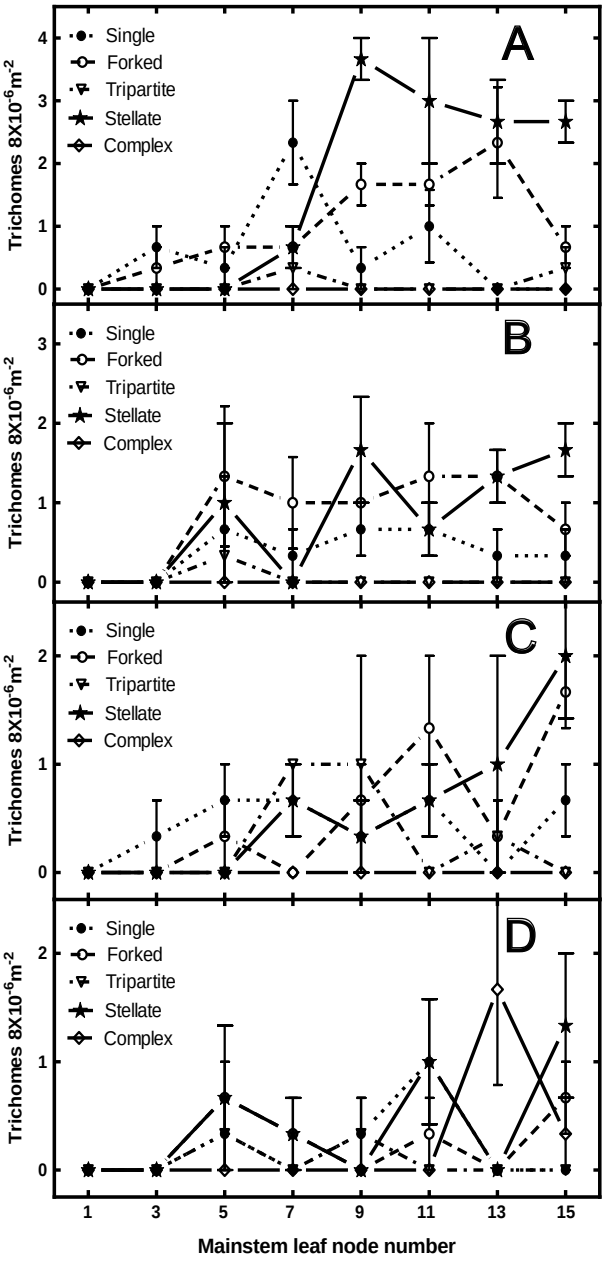
Supplemental Material 1-B. Comparison of the trichome counts on the adaxial and abaxial sides of leaves from lines BC1F2 Pilose-Fuzzless and BC1F2 Pilose-Fuzzy grown in the greenhouse.



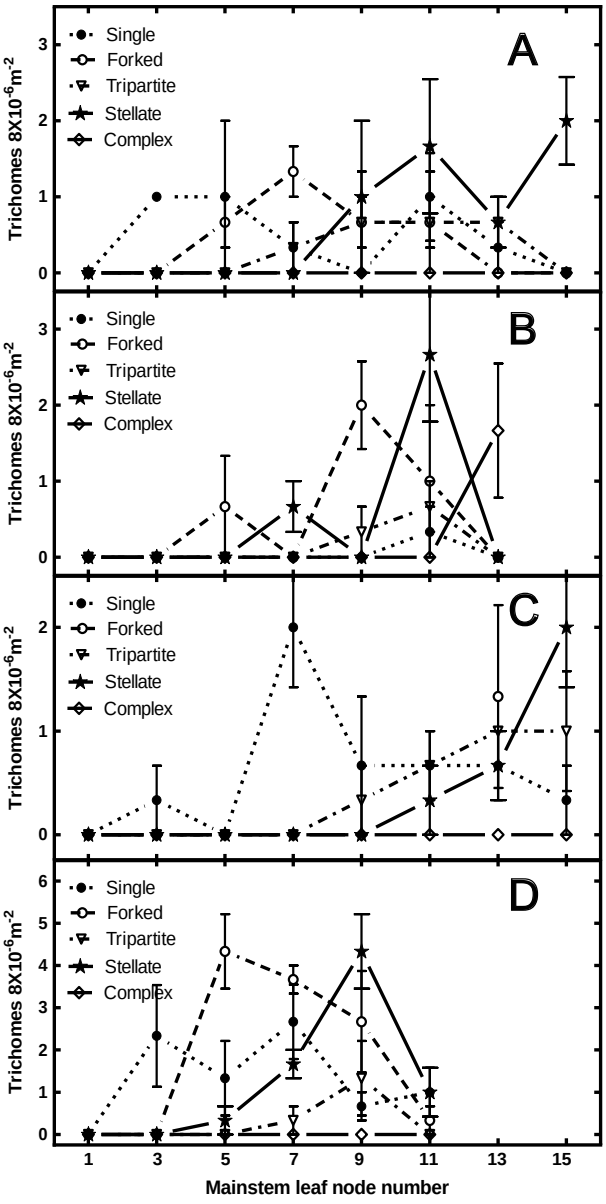
Supplementary Material 1-C. Comparison of the trichome counts on the adaxial and abaxial sides of leaves from lines BC1F2 Pilose-Fuzzless and BC1F2 Pilose-Fuzzy grown in the field.



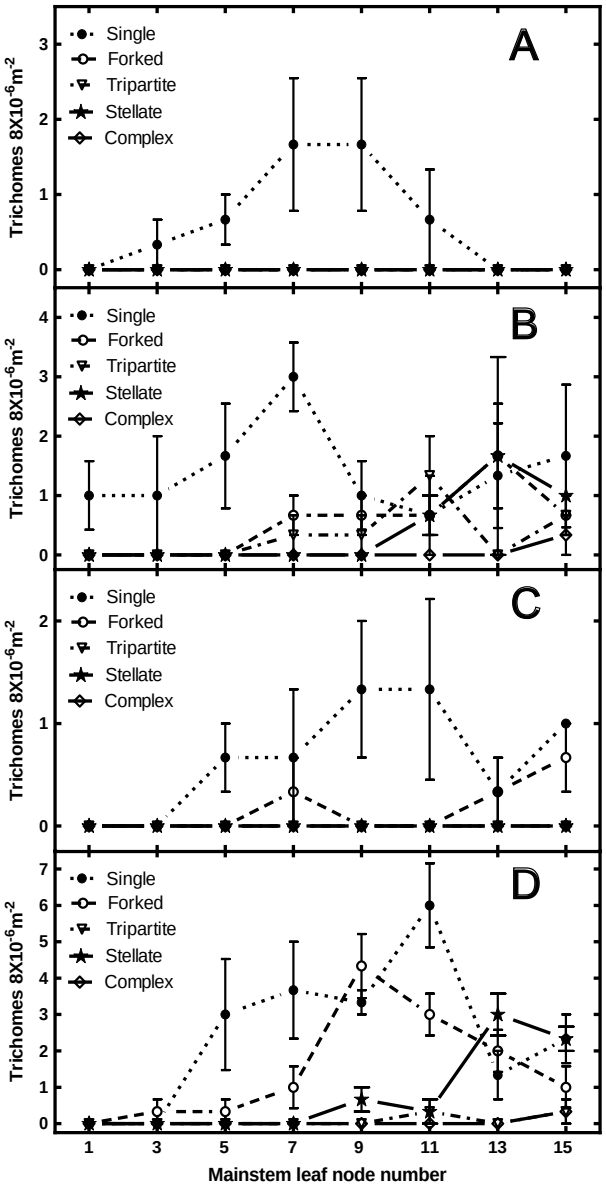
Supplementary Material 1-D. Comparison of the trichome counts on the adaxial (A and C) and abaxial (B and D) sides of leaves from line 166 grown in the greenhouse (A and B) and the field (C and D).



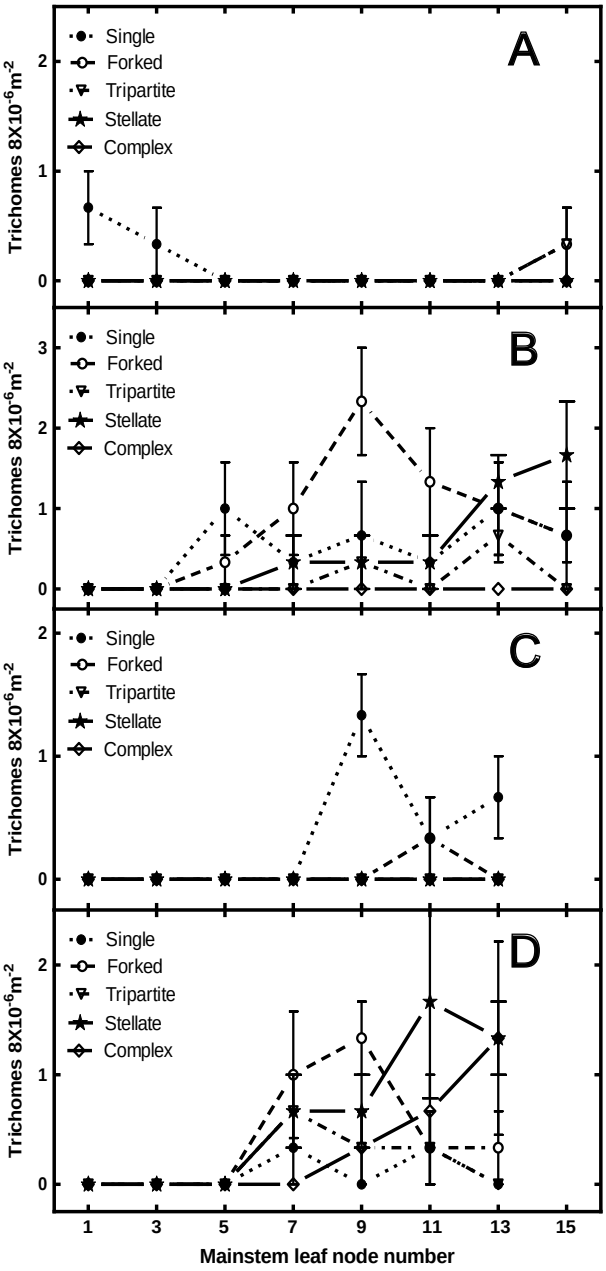
Supplementary Material 2-A. Comparison of the trichome counts on the abaxial sides of green-house (A and C) and field grown leaves (B and D) on lines 156 (A and B) and 208 (C and D). No trichomes were counted on the adaxial sides of these leaves.



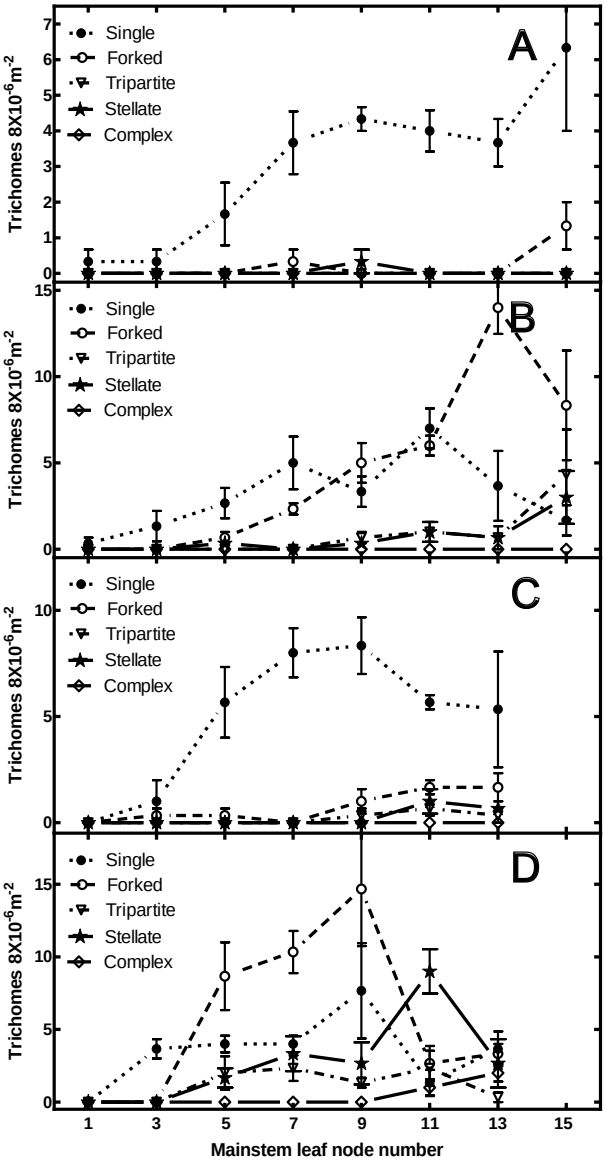
Supplementary Material 2-B. Comparison of the trichome counts on the abaxial side of greenhouse (A and C) and field grown leaves (B & D) on lines 220 (A and B) and 903 (C and D). No trichomes were counted on the adaxial sides of these leaves.



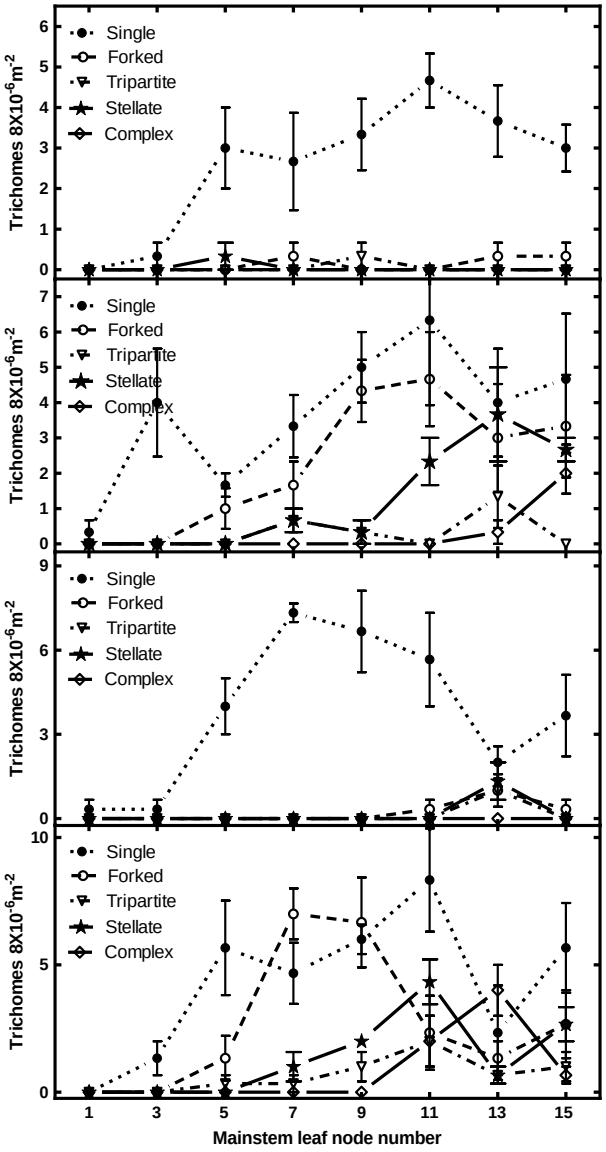
Supplementary Material 2-C. Comparison of the trichome counts on the adaxial (A and C) and abaxial (B and D) sides of leaves from line 596 grown in the greenhouse (A and B) and the field (C and D).



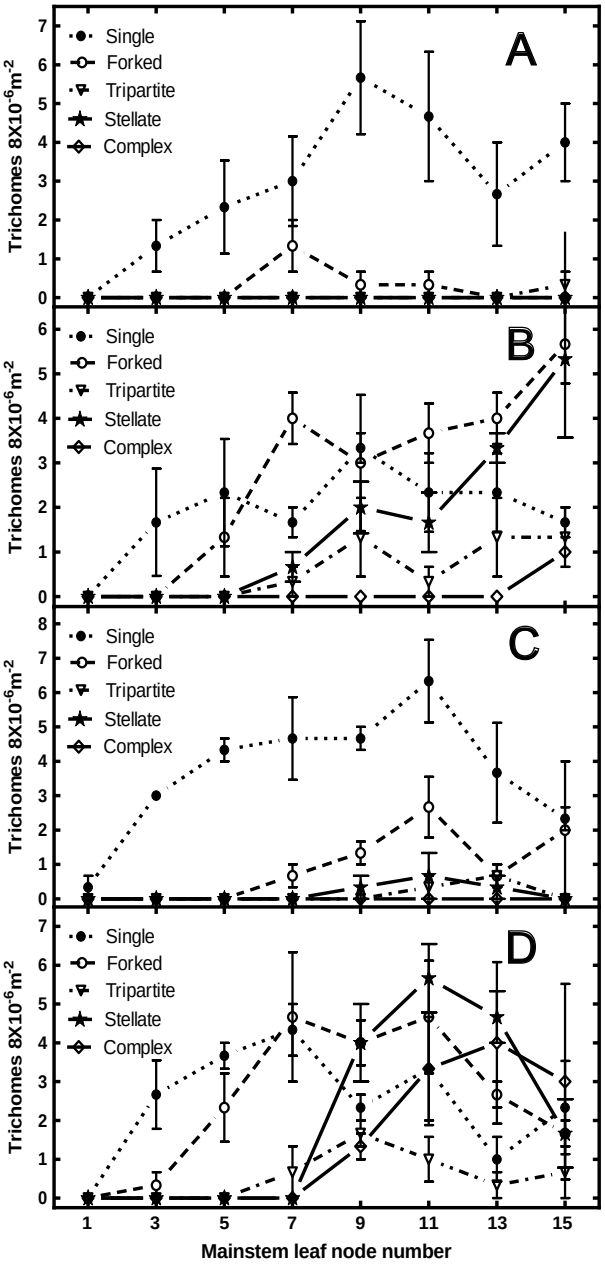
Supplementary Material 2-D. Comparison of the trichome counts on the adaxial (A and C) and abaxial (B and D) sides of leaves from line 628 grown in the greenhouse (A and B) and the field (C and D).



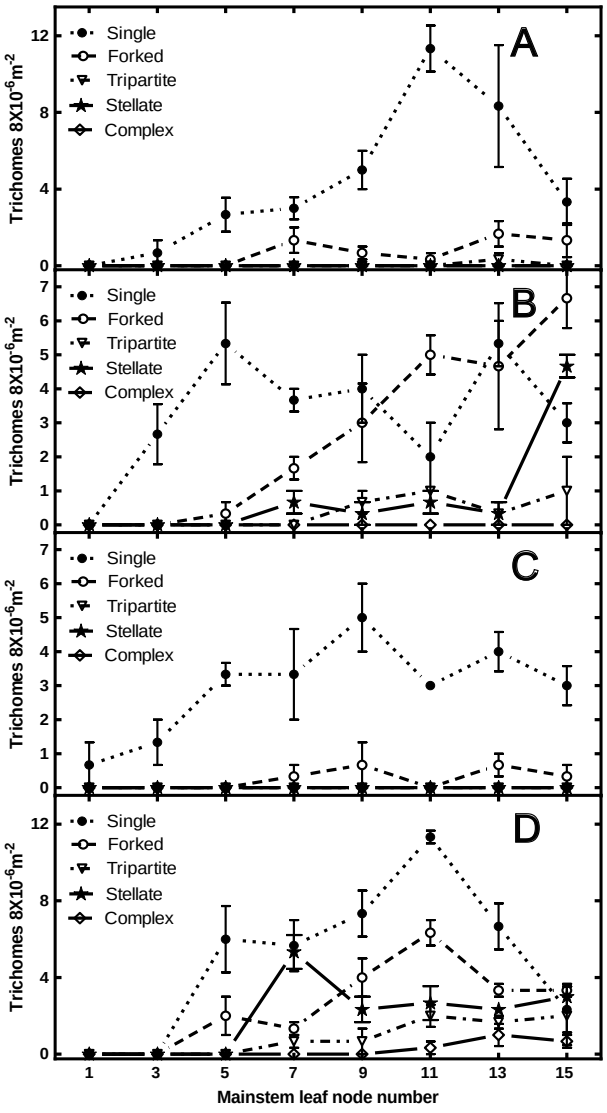
Supplementary Material 2-E. Comparison of the trichome counts on the adaxial (A and C) and abaxial (B and D) sides of leaves from line 859 grown in the greenhouse (A and B) and the field (C and D).



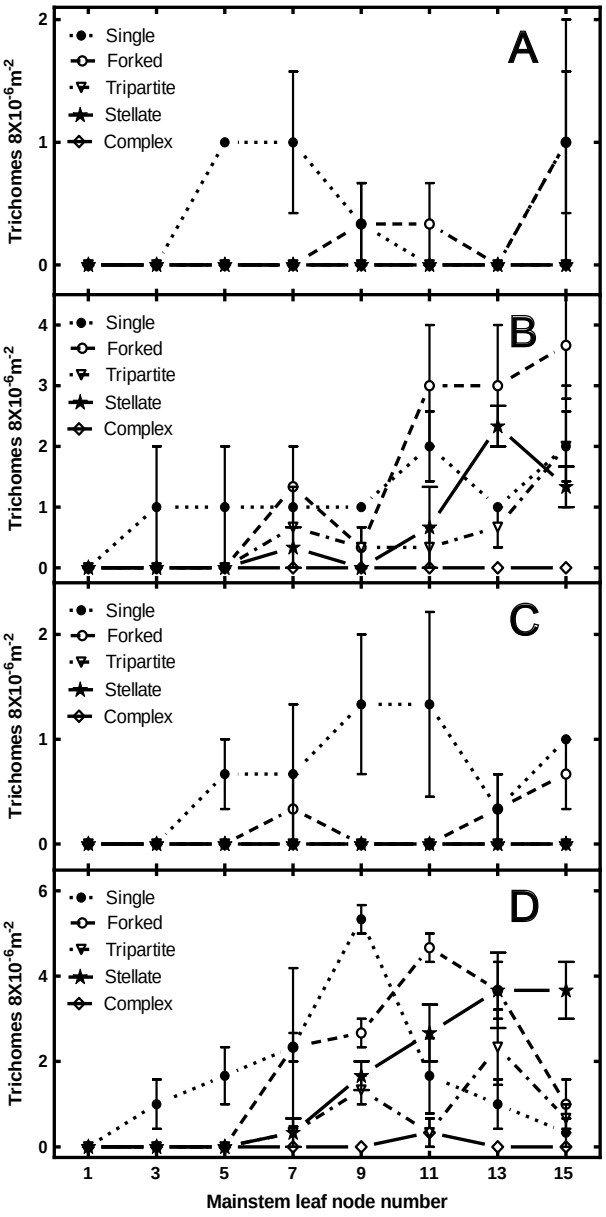
Supplementary Material 2-F. Comparison of the trichome counts on the adaxial (A and C) and abaxial (B and D) sides of leaves from line 905 grown in the greenhouse (A and B) and the field (C and D).



Supplementary Material 2-G. Comparison of the trichome counts on the adaxial (A and C) and abaxial (B and D) sides of leaves from line 982 grown in the greenhouse (A and B) and the field (C and D).



Supplementary Material 2-H. Comparison of the trichome counts on the adaxial (A and C) and abaxial (B and D) sides of leaves from line 985 grown in the greenhouse (A and B) and the field (C and D).



Supplementary Material 2-I. Comparison of the trichome counts on the adaxial (A and C) and abaxial (B and D) sides of leaves from line 1055 grown in the greenhouse (A and B) and the field (C and D).

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DISCLAIMER

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