Pak. J. Bot., 46(2): 639-643, 2014.

DEVELOPMENT AND CHARACTERIZATION OF THE ASIATIC DESI COTTON (*GOSSYPIUM ARBOREUM* L.) LEAF EPICUTICULAR WAX MUTANTS

MUHAMMAD YOUNAS KHAN BAROZAI[1,2] AND TAYYAB HUSNAIN[2]

[1]*Department of Botany University of Balochistan Quetta, Pakistan.*
[2]*National Centre of Excellence in Molecular Biology (CEMB) 87 West Canal Bank Road Thokar Niaz Baig Lahore, Pakistan.*
**Correspondence author e-mail: barozaikhan@gmail.com*

Progress

Abstract

Epicuticular wax covers plant aerial organs to protect them from various biotic and abiotic stresses. Wax mutants have fundamental role in understanding the mechanism of production and deposition of these waxes. In the present study three epicuticular wax mutant plants were developed through chemical mutagens; diethyl sulfate (DES) and ethyl methane sulphonate (EMS) in diploid Asiatic desi cotton (*Gossypium arboreum*) which is known for its tolerance against various biotic and abiotic stresses. The epicuticular wax appears as smooth stripy layers in wild type *G. arboreum* plant leaf under scanning electron microscopy. The GaWM1 (*Gossypium arboreum* Wax Mutant1), GaWM2 and GaWM3 showed altered wax morphology from smooth strips to embedded tubules/fibers, irregular patches and non-stripy smooth layers, respectively. The wild type *G. arboreum* plant has 183.7 ± 8.72 µgcm^{-2} total wax loads that reduced to 66.79% on GaWM1, 59.50% on GaWM2 and 49.29% on GaWM3. These mutants will be help full to understand the mechanism of epicuticular wax deposition and production at molecular level.

Introduction

Cotton (*Gossypium* spp.) is a member of the family *Malvaceae* that belongs to genus *Gossypium*. *Gossypium* has 45-50 species. Majority of these species are diploid (2n=26) and others are tetraploid. Ninety eight percent (98%) world's cottons are produced from the tetraploid species, *G. hirsutum* and *G. barbadanse*, and just two percent (2%) by diploid species *G. arboreum* & *G. herbaceum* (Azmat & Khan, 2010). Although the diploid species share just 2% to the world cottons but are the vital source of important biotic and abiotic resistant genes. Among the diploid species, especially, Asiatic desi cotton (*G. arboreum*) has built-in desirable resistant genes for various biotic and abiotic stresses like; drought, root rot, CLCuV (Cotton Leaf Curl Virus) and insect pests (bollworms and aphids) (Mansoor *et al.,* 2003; Barozai & Husnain, 2011; Rahman *et al.,* 2012).

Abiotic stresses have very adverse effect on the plant life (Barozai & Wahid, 2012; Barozai *et al.,* 2012; Barozai, 2013; Barozai *et al.,* 2013). Epicuticular wax is one of the special features developed by plants to seal and protect the aerial organs from various biotic and abiotic stresses. It serves as a first line of defense and to avoid non-stomatal destructive water loss. The epicuticular waxes also contribute to other additional functions in plant protection like; reflect ultraviolet (UV) light, restrict the attachment and growth of insects, increase plant resistance against pathogens like bacteria and fungi and decrease water deposition on the surface of the plant thus reducing retention of dust, pollen and air pollutants (Kerstiens, 1996; Kakani *et al.,* 2003).

The epicuticular wax mutant plants have shown great contributions in the identification of genes involved in the production and deposition of the epicuticular waxes (Kunst & Samuels, 2003). Deficiencies in the wax layer are easily visible because mutant plants have a glossy appearance. This trait has become a precise genetic marker, and several mutants affected in wax biosynthesis and deposition have been isolated and studied in different plant species. Various physical and chemical mutagens have been used to develop wax-deficient mutants in various plant species like, *Arabidopsis* (Koornneef *et al.,* 1989) *sorghum bicolor* (Jenks *et al.,* 1994), barley (*Hordeum vulgare*) (Von, 1987) and maize (*Zea mays*) (Bianchi *et al.,* 1978).

The above review demonstrated that Asiatic desi cotton (*G. arboreum*) is a potential plant against biotic and abiotic stresses, and the epicuticular wax is the key player to protect the plants from such stresses (Barozai & Husnain, 2011). There is no previous study of the epicuticular waxes in the Asiatic desi cotton (*G. arboreum*). This generates a thrust to focus and study the epicuticular wax in Asiatic desi cotton (*G. arboreum*). The epicuticular wax was studied in various plant species through wax mutants (Jenks *et al.,* 1994; Von, 1987; Bianchi *et al.,* 1978). The epicuticular wax mutants are not only the source to study the wax morphology, deposition pattern and chemical composition through comparative approaches. They also have potential to identify the genetic resources of epicuticular wax. To date, leaf epicuticular wax mutants in cotton with visible altered morphology have not been reported. This particular study is focused on the Asiatic desi cotton (*G. arboreum*) epicuticular wax mutant development and characterization. The Asiatic desi cotton (*G. arboreum*) leaf epicuticular wax mutants are the key sources to analyze and understand the leaf epicuticular wax physical appearance, texture, structure and chemical nature. They can also be utilized for the identification of the molecular mechanism of the epicuticular wax production and deposition in the Asiatic desi cotton (*G. arboreum*). All such knowledge will be a base to improve the cotton plant against the biotic and abiotic stresses.

In the present study, three leaf epicuticular wax mutants are reported for the first time in diploid Asiatic desi cotton (*G. arboreum*). The three leaf epicuticular wax mutants are developed through the chemical mutagens and are characterized through Scanning Electron Microscopy (SEM) and Gas Chromatography and Mass Spectrometry (GC-MS).

Materials and Methods

Development of wax mutants: Pure inbred seeds of Asiatic desi-cotton (*Gossypium arboreum*) variety FDH-786 were obtained from local germplasm center Ayub Agriculture Research Institute (AARI, Faisalabad). Concentrated H_2SO_4 was used for delinting of seeds. Seeds were continuously stirred with the help of a spatula for 10-15 min until the surface of seeds became shiny. Some water was added and stirred again; seeds were washed five times with tap water to remove the acid completely. The seeds floated at the surface of water were removed.

Mutation induction: The chemical (ethyl methane sulphonate [EMS], diethyl sulfate [DES] and sodium azide [SA]) and physical mutagens (gamma rays) were used as mutagens to induce mutation in *G. arboreum* seeds. The seeds were irradiated with Gamma rays (source Co-60) at Pakistan Radiation Services (PARAS) Lahore. The various combinations of chemical and physical mutagens with different pre-soaking time (PST) were used.

Seeds of each mutagenic treatment and wild plants were sown in a separately single tray with dimensions (76.2cm×121.9cm×15.24cm), filled with composite soil (peat, sand, soil, 1:1:1) in Centre of Excellence in Molecular Biology (CEMB) green house at temperature 25±2°C, and relative humidity near 50%. Metal halide illumination lamps (400 W) were used to supplement natural radiation. Light radiation reached a maximum of 1,500µmpl m^2s^{-1} at the top of canopy at midday. The plants were regularly watered with tap water on alternate day.

After initial growth, the M1 plants were transferred to CEMB's cotton field. Each flowering bud was covered in paper envelop for self pollination to get homogenize plants in M2.

Wax mutant selection: M2 seeds were sown, same as mentioned earlier. The M2 plants were screened and initial potential wax mutant candidates were selected on the basis of traits reported in wax mutants like; Glossy leaves appearance; a phenotypic genetic marker for wax mutants, Infertility; a pleotropic marker for wax mutant in water stressed condition (Koornneef *et al.,* 1989), Curved/wrinkled leaves and flowers size were slightly smaller than wild type (Aharoni *et al.,* 2004), Fusion of aerial parts in mutants plant (Chen *et al.,* 2003) and Rapidly chlorophyll extraction in 80% ethanol. Further gravimetric wax analysis, extraction and quantification by immersing leaf in non-polar solvent like hexane and comparing it to wild type *G. arboreum* and stomatal indices comparison; as increased stomatal indices is reported in wax mutants (Holroyd *et al.,* 2002) were done on these selected initial potential wax mutant candidates.

The flowers of the selected initial potential wax mutant candidate plants were covered with paper envelop for self pollination to get homogenize seeds. M3 seeds of the selected lines were sown. The final potential wax mutant candidates in M3 on basis of the above mentioned wax mutants' traits were selected for further confirmation and characterization.

Wax mutant confirmation: For confirmation, the final potential wax mutant candidate plants selected in M3 were analyzed by Scanning Electron Microscopy (SEM) and Gas-Chromatography - Mass Spectrometry (GC-MS).

Scanning electron microscopy (SEM): The wild type *G. arboreum* and final potential wax mutant candidates' leaves from their plants were collected at vegetative growth stage after 35 days of germination (just before flowering) and processed according to Jenk *et al.,* (1995). Briefly, the excised leaves were air dried for seven (7) days in desiccators containing silica at room temperature. Each sample was mounted on aluminum stubs and sputter coated with gold using 120-s bursts at 40A, twice from the sputter coater (SPI MODULE). Coated surfaces were viewed using a JEOL JSM-5910 scanning electron microscope (JEOL) equipped with a tungsten cathode at 10 KV.

Chemical analysis: Leaves from final potential wax mutant candidate and wild type *G. arboreum* plants were cut and immediately immersed in hexane for 60 s at room temperature. The resulting solution of cuticular waxes was concentrated by keeping at 40ºC, and compounds containing free hydroxyl and carboxyl groups were converted into their trimethylsilyl ethers and esters, respectively, with bis-(N,N-trimethylsilyl)-trifluoroacetamide (BSTFA) (Machery-Nagel) in pyridine for 40 min at 70°C before GC-MS analysis. Wax constituents were identified by their electron-impact MS spectra (70 eV, m/z 50 to 700) after capillary GC (DB-5, 30 m × 0.35 mm, 0.1 µm [J&W]) on an Agilent 6890N gas chromatograph combined with a mass-selective detector 5973N (Agilent Technologies). Samples were injected into the column at 50°C, held at 50°C for 2 min, and then desorbed by increasing the temperature according to the following profile, 40°C/min to 200°C, 2 min at 200°C, 3°C/min to 310°C, and 30 min at 310°C. The flow rate of Helium (carrier gas) was maintained at 2 mL/min. Two microlitre of the solutions were analyzed and quantified with respect to an internal standard (10µg tetracosane), which was added to the wax samples before GC-MS. The quantitative composition of the mixtures was studied by capillary GC (Agilent; 30 m HP-1, 0.32-mm i.d., df = 1µm) and flame ionization detection under the same GC conditions as above but Helium (carrier gas) inlet pressure was programmed for 50 kPa at injection, held for 5 min, then raised with 3 $kPa.min^{-1}$ to 150 kPa and held for 40 min at 150 kPa. Single peaks were quantified against the internal standard by manually integrating peak areas (Aharoni *et al.,* 2004). Components were identified by the help of NIST library, 2005. The extracted leaf area was determined by scanning the leaves with the help of scanner (hp scanjet 8200) and leaf area measuring software Compu Eye (Bakr, 2005).

Genetic analyses: The final confirmed wax mutants were backcrossed and F1 plants were studied in terms of their phenotypic characters to elucidate the genotype of the mutants. Further, The F2 seeds from confirmed F1 plants were harvested, planted in 100 pots and grown in the same greenhouse under the conditions described earlier. For the statistical analysis a Chi-square analysis for

goodness of fit of the F2 distributions to a 3:1 was conducted. Since this study involved in F2 population with only two phenotypes being compared, unpaired t tests, between the two classes for the traits measured, were conducted.

Results

Wax mutants selection: In the present study physical mutagen (Gamma rays) and chemical mutagens (EMS, DES & SA) were used to develop the wax mutants in Asiatic desi cotton (*G. arboreum*). A number of wax-deficient mutants were developed by various physical and chemical mutagens in other plant species. Total forty nine (49) initial potential wax mutant lines on the basis of aerial fusion, glossy leaves, curved/wrinkle leaves, rapidly chlorophyll extraction, water stressed infertility, small flower size, increased stomatal indices and gravimetric epicuticular wax analysis were selected in the M2 plants.

These initial potential wax mutant plants in M2 were covered with paper envelope to get more homogenize seeds in M3. Almost 3000 seeds of M3 were sown and screened for wax mutants' traits. The plants showed wax mutants' traits were selected as final potential wax mutant candidates. These final potential wax mutant candidates were analyzed in triplicate by SEM and GC-MS. Three plants were confirmed as wax deficient mutants by SEM and GC-MS in M3. The three mutants are resulted from the chemical mutagens, designated as GaWM1, 2 and 3 (*Gossypium arboreum* Wax Mutant 1, 2 & 3). Two are developed by EMS (GaWM1 and GaWM3) and one is by DES (GaWM2).

SEM confirmation of the epicuticular wax mutants: As previously no single study of desi cotton (*G. arboreum*) leaf epicuticular wax has been reported so, a hexane treated (epicuticular wax removed); leaf was visualized by SEM as a reference, (Fig. 1a), to better understand the desi cotton (*G. arboreum*) leaf epicuticular wax morphology and deposition pattern. In wild type *G. arboreum* plant the leaf epicuticular wax appear as smooth stripy layers, (Fig. 1b). The absence of these stripy layers in hexane treated leaf, confirmed them as an epicuticular wax. The layer appearance is dominant over stripy. These epicuticular wax stripy layers have also showed sharpness and glossiness under SEM.

In all the three wax mutants, these sharp, shiny and stripy layers are absent and modified in a new morphological pattern. The GaWM1 showed epicuticular wax morphological pattern as embedded dull thick fibers (Fig. 1c). These embedded dull thick fibers are much less in density than the wild stripy layers. The GaWM2 showed epicuticular wax morphology as irregular dispersed patches (Fig. 1d). These patches showed less shine than the wild type wax stripy layers but more than the GaWM1 embedded thick fibers. The patches density is also less. The GaWM3, (*Gossypium arboreum* Wax Mutant 3) displayed epicuticular wax morphology as thin layers, (Fig. 1e). The layers are much less dense than GaWM1, GaWM2 and wild type plant. Little shine could be seen in these thin layers of wax.

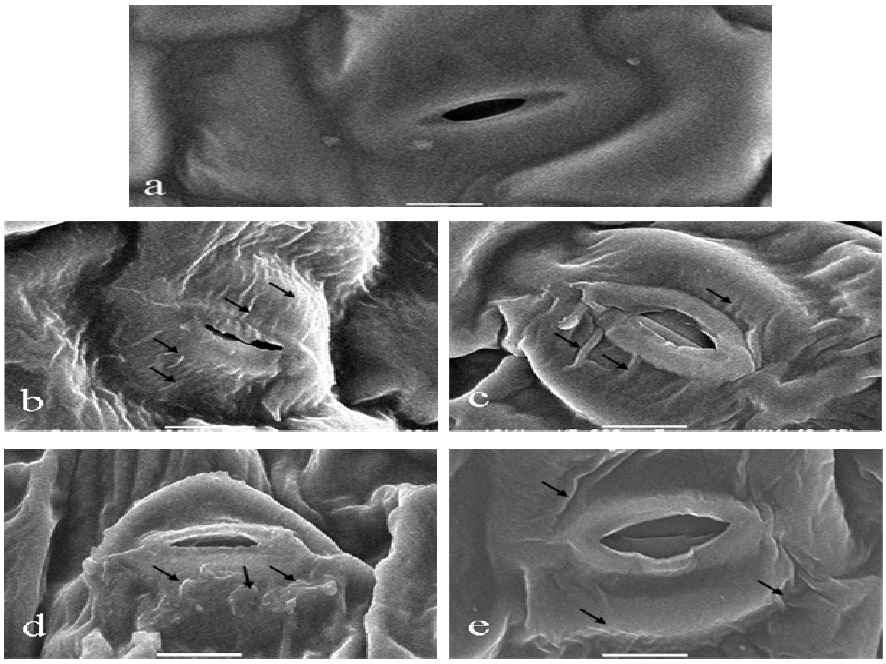

Fig. 1. Cotton (*G. arboreum*) hexane treated (wax removed), wild type *G. arboreum* and wax mutants leaves epicuticular wax morphology and patterns of deposition. Scanning electron micrograph of adaxial leaf surfaces of hexane treated (as reference) (a), wild type *G. arboreum* (b), GaWM1 (c), GaWM2 (d) and GawM3 (e) were taken at 5,000X magnification with scale bar representing 5 µm. The epicuticular wax, indicated by arrows appears as smooth stripy layers in wild type *G. arboreum* (b) which are absent in hexane treated reference (a). The cotton wax mutants have altered wax morphology from smooth strips to embedded tubules/fibers (c), irregular patches (d) and non-stripy smooth layers (e).

Chemical analyses: GC-MS is a very crucial platform to validate the epicuticular wax mutants. The hexane extracted cuticular waxes of wild type *G. arboreum* and mutant plants were analyzed by GC-MS that validate the scanning electron microscopy (SEM) results.

The total wax loads, determined from the GC-MS analyses for leaf cuticular waxes, are 183.7±8.7 μgcm^{-2}, 122.7±6.4 μgcm^{-2}, 109.31±7.4 μgcm^{-2} and 90.54±5.9 μgcm^{-2} in wild type *G. arboreum*, wax mutants, GaWM1, GaWM2 and GaWM3 respectively.

The total wax load was determined 66.79% on GaWM1, 59.50% on GaWM2 and 49.29% on GaWM3 as compared to wild type *G. arboreum*. It means there are 33.21%, 40.50% and 50.71% reduction in total wax load respectively. The chemical analyses revealed that the dominated wax class is alkane. Other major wax classes are acids, esters, and alcohols respectively (Table 1). More alkane concentration (136.7±9.0μgcm^{-2}) is observed in the wild type *G. arboreum*. Wax mutant (GaWM2) shows dominancy in Acid (9.7±2.4 μgcm^{-2}) and Ester (3.4± O.3 μgcm^{-2}). Aldehyde, alcohol and unidentified classes have shown more wax concentration in wild type *G. arboreum* plant (Table 1).

Table 1. Leaf cuticular wax classes in wild type *G. arboreum* and wax mutants (GaWM1, GaWM2, GaWM3), data are shown as mean ± standard error in μgcm^{-2} of wax classes; Alkane, Acid, Ester, Aldehyde, Alcohol and unknown for wild and wax mutants *G. arboreum* leaf cuticular wax.

Cotton (*G. arboreum*)	Wax classes and their concentrations (μgcm^{-2})					
	Alkane	**Acid**	**Ester**	**Aldehyde**	**Alcohol**	**Unknown**
WILD	136.7 ± 8.98	6.4 ± 2.76	1.2 ± 0.1	0.2 ± 0.04	3.5 ± 1.21	35.7 ± 4.35
GaWM1	95.7 ± 6.83	7.4 ± 1.87	2.2 ± 0.4	0.2 ± 0.07	0.5 ± 0.35	16.7 ± 3.87
GaWM2	90.3 ± 9.32	9.7 ± 2.43	3.4 ± O.3	0.1 ± 0.03	3.2 ± 1.08	12.6 ± 8.23
GaWM3	70.2 ± 10.32	2.4 ± 0.30	0.2 ± 0.07	0.1 ± 0.01	0.35 ± 0.02	17.39 ± 10.12

Genetic analysis of inheritance: The F1 resulted from the backcrossing of the mutants plant displayed normal wax characteristics as the wild type *G. arboreum* showed, indicating the recessive nature of the gene mutation in the GaWM1, GaWM2 and GaWM3. The F2 resulted from the self pollination of the F1 applying Chi-square analysis showed 1.33, 0.28 and 13.81 values at $p<0.01$ confirming 3:1 phenotypic segregation for GaWM1 and GaWM3, and a deviation from 3:1 in GaWM2 respectively, suggesting that the alteration in the epicuticular wax in GaWM1 and GaWM3 is due to the single recessive nuclear gene mutation and in the GaWM2 is due to the more than one recessive nuclear gene mutation.

Discussion

Presently no studies for Asiatic desi cotton (*G. arboreum*) leaf epicuticular wax deposition and morphology are reported. The aim of the present study is to understand and reveal the *G. arboreum* leaf epicuticular wax morphology and development of the wax mutants and their characterization. These mutants are the valuable resources for the identification of genes involved in the epicuticular wax production, deposition and their metabolic pathways.

Total three wax deficient mutants were confirmed by SEM and GC-MS. All the mutants are resulted from the chemical mutagens. Two are developed by EMS (GaWM1 and GaWM3) and one is by DES (GaWM2). Koornef *et al.,* (1989) developed a number of wax deficient mutants in *Arabidopsis thaliana* by EMS. The wax mutant varieties in *sorghum bicolor* (L.) were also produced by diethyl sulfate (DES) and ethyl methane sulphonate (EMS) (Jenks *et al.,* 1994). The most loci involved in wax biosynthetic pathway were identified in barley, which are 85. Next is *Arabidopsis*, with total 25 known wax mutants showing different degrees of waxlessness is reported.

The epicuticular wax appeared as smooth stripy layers in wild type *G. arboreum* plant adaxial leaf under scanning electron microscope. Similar finding was observed in *Arabidopsis* leaf (Jenks *et al.,* 1995). Jenks *et al.,* (1996) reported crystal less smooth epicuticular wax in wild-type *Arabidopsis* adaxial and abaxial leaf surfaces. The *G. hirsutum* leaf epicuticular wax showed fine, slender striations morphology in SEM (Bondada & Oosterhuis, 2000). It suggests a variation in epicuticular wax morphology among species within a genus. As *G. arboreum* is more biotic and abiotic stress tolerant than *G. hirsutum* so, it also suggests that smooth waxy layers might have role in sufficient protection of plant from these stresses. The *Arabidopsis* stem showed epicuticular wax as crystalline tubules instead of smooth layers.

The wild type *G. arboreum* total wax load is almost six fold to the *Arabidopsis* stem total wax load. As *G. arboreum* has proven more tolerant to biotic and abiotic stresses, as compare to *Arabidopsis*, so it may be due to the over wax deposition on the leaves of *G. arboreum*. In the new developed *G. arboreum* wax mutants the wax deposition was reduced from 33% to 51%. The *Arabidopsis* Cer (wax deficient) mutants have total wax load reduction in a range of 10 -90% to wild. The Cer1, Cer2, Cer3 and Cer6 have only 13%, 25%, 20% and 10% total wax load as compared to wild (Rowland *et al.,* 2007).

Alkane is found the dominant wax class in G. arboreum followed by acids, esters, and alcohols respectively. The alkane is also dominant wax component class on the leaves and stem epicuticular waxes of *Arabidopsis* and *G. hirsutum* leaves (Bondada *et al.,* 1996) with 58% and 42.65% to total wax load respectively. The alcohol is the second dominant class with 24% followed by alkane in *Arabidopsis* leaf epicuticular wax (Jenks *et al.,* 1996; Suh *et al.,* 2005). Cameron *et al.,* (2006) observed alkane as a dominant wax chemical compositional class with 80±2 % following by 4% alcohol to total wax load in tobacco tree (*Nicotiana*

glauca L.). The barley (*Hordeum vulgare*) and maize (*zea maize*) juvenile epicuticular waxes have primary alcohol as dominant wax compositional class with 63% and 75-89% concentrations to total wax load respectively (Bianchi *et al.,* 1978). The three wax mutants GaWM1, GaWM2 and GaWM3 have shown variation in the members of the alkane and acid classes of wax. Similar findings were reported in *Arabidopsis* wax mutants where decrease and increase in alkane series were observed (Jenks *et al.,* 1995).

Conclusions

Total three Asiatic desi cotton (*Gossypium arboreum* L.) wax deficient mutants are developed and characterized. All the three mutants are resulted from the chemical mutagens; two are developed by EMS (GaWM1 & GaWM3) and one is by DES (GaWM2). There is 33.21%, 40.50% and 50.71% reduction in total wax load was observed in GaWM1, GaWM2 and GaWM3 respectively. They are the best resources to understand the epicuticular wax deposition at molecular level.

Acknowledgments

The authors would like to acknowledge for the chemical analysis assistance by Dr. Zia-ur-Rehman, Pakistan Council of Scientific and Industrial Research (PCSIR) Lahore and for the scanning electron microscopy assistance by Dr. Riaz, central resource lab (CRL) University of Peshawar Pakistan. This work is supported by a PhD scholarship awarded by the Higher Education Commission, Pakistan.

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(Received for publication 13 April 2012)