Leaf surface wax composition of genetically diverse mulberry (*Morus* sp.) genotypes and its close association with expression of genes involved in wax metabolism

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Silkworm (*Bombyx mori*), the primary producer of silk, has strong feeding preference for most turgid and hydrated mulberry leaves. In a previous study, we showed positive correlation between moisture retention capacity (MRC) of the mulberry leaf and leaf surface wax amount. In the present study, we examined wax constituents in genotypes that exhibited a wide range of leaf surface wax amount and MRC. Gas chromatographic analysis revealed that acids, alkanes, aldehydes, primary alcohols, iso-alkanes, triterpenoids, esters were among mulberry waxes identified and the major being alkanes. The highest total leaf wax amount was 1006.8 μg dm^{-2} in the V1 genotype whereas S-36 had the least wax at 436.9 μg dm^{-2}. The alkanes were dominated by those having 25, 27, 29, 31 and 33 carbons, with C_{31} compounds being the most abundant. RNA-blot hybridization performed with 13 wax biosynthesis associated genes using heterologous probes revealed a close association between surface wax composition and expression levels of genes associated with wax elongation (*CUT1, KCS1*). The association was also established with homologous probes of *KCS1, WAX2, CUT1* and *LTP1*-like genes. In summary, regulation of early wax precursor metabolism is a likely contributor to the variation observed in leaf wax composition in mulberry.

Keywords: Cuticular wax, moisture retention capacity, mulberry, silkworm, wax genes.

NATURAL silk production is a multi-billion dollar industry world-wide, and its main feedstock is silk produced by the silkworm (*Bombyx mori*), an insect with highly specific feeding preferences for certain species of mulberry (*Morus*). A previous study demonstrated that the amount of total leaf surface wax (cuticular waxes extracted by immersion in organic solvent) on mulberry leaves was correlated with water loss rate[1], a condition that has a major influence on the palatability of leaves by the silkworm. Whether certain other mulberry leaf wax characteristics, such as wax chemical composition, might also influence *B. mori* feeding behaviours (either as feeding stimulants/deterrents or barriers to water loss) has not been explored. It is well known that wax composition can play a significant role in plant–host selection by various insects[2]. It is also known that certain leaf cuticular wax components can play an important role in water retention, mainly by regulating cuticular transpiration. Moreover, plants adapted to arid environments often have heavy wax depositions in their cuticles, and define one of the many adaptations that limit plant water loss[3]. Mulberry, a somewhat drought-tolerant species, has a relatively heavy wax deposit on its leaves when compared to other plant species that are not adapted to drought. In addition to leaf surface wax amount, epicuticular wax crystallization patterns have been associated with water loss rates[1]. Information on mulberry cuticular wax chemical composition is needed to further shed light on the role of mulberry waxes in cuticular permeability to water and associated leaf water retention, as well as possible chemico-ecological effects on feeding behaviours. The chemical composition of leaf waxes on mulberry has not been reported previously. Typical plant cuticular waxes are a mixture of different chemical compounds, often including *n*-alkanes (C_{21}–C_{35}), secondary alcohols (C_{21}–C_{35}), ketones (C_{21}–C_{35}), fatty alcohols (C_{22}–C_{34}), fatty acids (C_{16}–C_{34}), aldehydes (C_{21}–C_{35}) and wax esters (C_{32}–C_{64})[4]. Composition of waxes varies from genotype to genotype, as well as due to environmental conditions and development[5]. Many genes associated with biosynthesis of plant waxes have been characterized[6]. Today, two main

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branches of the wax metabolic pathway are postulated, the so-called decarbonylation pathway, which leads to production of alkanes and aldehydes, and the acyl reduction pathway that produces primary alcohols and esters[3]. Synthesis of major wax components occurs via sequential elongation of acyl-CoA by two-carbon units derived from malonyl-CoA[7]. Many of the key genes encoding metabolic or transport proteins in wax biosynthesis, including *ACC* (ref. 8), *CER1*, *CER2* (ref. 9), *CER26* (ref. 10), *GL* (ref. 11), *CER4*, *CER5*, *CER6*, *CER9* (ref. 12), *KCS1* (ref. 13), *WAX2* (ref. 14), *KCR* (ref. 15), *HCD* (ref. 16), *ECR* (ref. 17), *LACS1*, *LACS2* (ref. 18) and *MAH1* (ref. 19) have been identified and characterized. Genes encoding putative regulatory proteins like *CER7* (ref. 20), *RST1* (ref. 21), *WIN1*/*SHN1* (ref. 22), *WXP1/WXP2* (ref. 23) and *MYB96* (ref. 24) are also known to be associated with surface wax production.

In a previous report, we examined 291 mulberry genotypes and described the high correlation between leaf post-harvest water loss rates and leaf surface wax amounts[1]. The four genotypes having the highest amount of surface wax and the four with lowest wax amounts (determined using a colorimetric assay) were examined further in this study using gas chromatography-mass spectrometry (GC-MS) for leaf wax chemical composition. Mulberry wax composition has not been previously reported. To assess potential gene involvement in the observed variation in leaf waxes of these mulberry lines, we examined the transcript abundance of 13 genes previously associated with plant wax biogenesis. In addition to shedding new light on mulberry leaf wax composition, describing variation in waxes on diverse mulberry genotypes, the role of gene expression in that observed variation and implications for the commercial silk production industry are also discussed.

Materials and methods

Plant materials

A collection of mulberry germplasm, maintained by the Central Sericultural Research and Training Institute (CSR&TI) in Mysuru (India), representing accessions from around the world, was utilized in this study. These include *Morus alba*, *Morus indica* and *Morus nigra* cultivars that are best adapted for Indian conditions. The germplasm collection was clonally propagated stock established in the mid-1980s. The plants were pruned every year to get fresh canopy, and uniform leaf samples were collected for this study at 63 days after pruning. All replicates in the study represent genetically and developmentally identical comparisons. From this collection, four accessions having the highest total wax amount per leaf area, and four having the lowest wax per leaf area, were selected for detailed chemical analysis of leaf surface (cuticular) waxes, and associated expression of selected genes.

Leaf samples were selected from the outermost canopy from south facing side of the specimen and among those having full sun exposure. The leaf development stage was standardized as being newly, fully expanded leaves and all leaves were collected at 63 days after pruning. The replicated samples for each genotype were taken from three different clonal plants of the same genotype. Cuticular waxes were extracted from leaves by their submersion for 45 s in gas chromatography (GC) grade hexane[25] in a 20 ml scintillation vial. Immediately after leaf surface wax extraction, leaf area was measured using the leaf area meter (Delta T system, UK) to express wax quantity on a surface area basis. Leaf surface extracts contained waxes from both abaxial and adaxial leaf surfaces. Wax compositional analysis was performed according to Jenks *et al.*[25]. The hexane-soluble cuticular wax extracts were evaporated to dryness under a nitrogen stream, and the dried residue was prepared for GC by derivatization using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide. Derivatization was for 15 min at 100°C. After surplus *N*,*O* bistrifluoroacetamide was evaporated under nitrogen, the sample was re-dissolved in hexane for analysis (Hewlett-Packard 5890 series II GC, equipped with a flame ionization detector, Palo Alto, CA). The GC was programmed with an initial temperature of 80°C and increased at 15°C min^{-1} to 260°C, and held for 10 min. The temperature was again increased at 5°C min^{-1} to 320°C, and held for 15 min. Quantification was based on flame ionization detector peak areas and the internal standard hexadecane. Specific correction factors were developed from external standards and applied in a standard manner as described by Jenks *et al.*[26]. The total amount of cuticular wax was expressed per unit leaf surface area. Selected sub-samples were used for injection in a GC-MS (Finnigan MAT/Thermo spray Corp., San Jose, CA) to produce electron ionization mass spectra to verify the identities of all components.

Expression of cuticular wax-associated genes in selected mulberry genotypes

Total genomic DNA was isolated from mulberry (*M. alba* L.) by modified CTAB method[27] using young leaves. A PCR-based approach was used to amplify wax-associated genes using gene specific primers (Table 1) designed from conserved regions of selected genes. The amplified gene fragments were cloned into T/A cloning vector[28] sequenced (ABIPRISM, USA), and analysed using existing sequences in the NCBI database (http://www.ncbi.nlm.nih.gov).

Northern blot hybridizations were carried out in the four selected genotypes according to Sambrook and Russell[29] on newly-fully-expanded leaves. RNA (20 μg) from

each sample was separated on formaldehyde denaturing gel and transferred to Hybond nylon membrane and fixed by UV (1200 J for 60 s) in a UV cross-linker. The blots were probed with either wax-associated genes as heterologous probes obtained from RIKEN, Japan (http://www.brc.riken.jp) or homologous mulberry probes (DX913094.1, DX913095.1, DX913093.1 and EI811359.1) (Table 1). The specific probes were prepared by labelling with ^{32}P dCTP[30]. Pre-hybridization was carried out at 42°C for 2 h and hybridization at 60°C overnight with blocking solution (0.5 M sodium phosphate buffer, pH 7.2, 1 mM EDTA and 7% w/v SDS). The blots were processed, air-dried, and exposed to imaging plate in a light-tight cassette, and processed further using phosphorimager (Fujifilm Imager Analyser, FLA 5000, Japan) according to the manufacturer's instruction.

RT-PCR was used to verify Northern blot results for all selected genes in the four mulberry genotypes. For RT-PCR analyses, 5 µg of total RNA from newly, fully expanded leaves were reverse-transcribed using MMLV reverse transcriptase (MBI Fermentas) using random primers (Table 2). Using cDNA, PCR was performed in 20 µl reaction volume containing cDNA, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.05 µM each primer and 1 unit of *Taq*-DNA polymerase. The PCR condition was 94°C for 4 min, 94°C for 1 min, 65°C for 1 min, 72°C for 2 min, with the final extension step at 72°C for 8 min, with 25 cycles of amplification. For quantitative RT-PCR (qPCR) analysis, 20 µl reactions containing cDNA, 1× SYBR-Green qPCR master mix (DyNAmo SYBR-Green qPCR Kit FINNZYMES, Finland, www.finnzymes.fi) and 250 nM each of primer. Each primer pair was designed to yield a PCR product ranging from 150 to 250 bp. The house keeping gene *actin* was used as an internal control, and used for normalization of transcript abundance[31].

Table 1. Primers used to amplify candidate wax biosynthetic genes from mulberry. The primers were designed using conserved regions of the genes

Primer name	Primer sequence (5′–3′)
KCS1 forward	TCCAAGTTCACCCCAACTCGA
KCS1 reverse	CGACGTGGACGAGGCGGTACTT
WAX2 forward	AAGCTCTTTACATTTGTCGCCGT
WAX2 reverse	TCCGGAGATGTTGCACCACAA
CUT1 forward	GGTCATCAACAAGTACAAGCTG
CUT1 reverse	GCAGAAGTGCTCAAAGGCTG
LTP1 forward	GTACACCCCATCGTTTCATTCTCTTCAC
LTP1 reverse	GCCGGAGTGCTCAAGTAAGTG

Table 2. Primers used for gene expression studies of leaf surface wax biosynthesis genes in four mulberry genotypes by qPCR

Primer name	Sequence (5′–3′)
KCS1 forward	GCCATGCTCCTCCCCAACTGC
KCS1 reverse	ACG GTATAATGGCCACAAAACCA
WAX2 forward	GTATGAAACAGAAGAGGAGTTG
WAX2 reverse	GGGAAAGCTCTTTACATTTGTCGCCGG
CUT1 forward	GCTTCAACCTCTCCGGCATGGGC
CUT1 reverse	GTCCAGGTTCTTCTGCAGCTCG
LTP1 forward	TGGAGTGATGAAGTTGGCATGC
LTP1 reverse	AGTAGCATCATCTCACCGTTGC
Actin forward	TCCATAATGAAGTGTGATGT
Actin reverse	GGACCTGACTCGTCATACTC

Silkworm bioassay

The silkworm bioassay was done at CSR&TI Mysuru, for selected mulberry accessions varying for MRC and wax load. The bioassay was done by feeding the silkworm breed CSR2XCSR4 with the foliage of contrasting accessions from the first instar larvae to last instar larvae (Anon., hand book of mulberry, CSR&TI, Mysuru). Along with this, larval and cocoon parameters like number of cocoons per 10,000 larvae, single cocoon weight (g) and single shell weight (g) were measured to compare the contrasting accessions for final cocoon yield.

Statistical analysis

The cuticular wax composition and silkworm bioassay data between two different groups were analysed using *t*-test in SAS PROC GLM (SAS version 9.3, SAS Institute Inc., Cary, NC, USA).

Results and discussion

Analysis of wax composition

Eight mulberry (*Morus* sp.) genotypes, representing the highest and lowest wax amounts determined in a previous screen of 291 accessions[1], were examined for leaf surface wax chemical composition. Wax composition was dominated by acids, alkanes, aldehydes, alcohols, iso-alkanes, terpenoids, esters and some unknown compounds (Figure 1). In low wax load mulberry genotypes, average amounts for each wax constituent class were measured on a leaf area basis in µg dm^{-2} and total wax composition in percentage as 68.1 (13.5), 5.9 (1.2), 93 (18.4), 163.5 (32.3), 1.9 (0.4), 138.2 (27.3), 20.5 (4.1) and 14.9 (2.9) of acids, aldehydes, primary alcohols, alkanes, iso-alkanes, terpenoids, esters and unknowns respectively (Figure 1). By comparison, high surface wax types averaged in µg dm^{-2} (percentage of total wax) as 138.6 (16.6), 5.4 (0.6), 122.4 (14.6), 272.4 (32.6), 2.6 (0.3), 238.3 (28.5), 24.4 (2.9), and 32 (3.8) for acids, aldehydes, primary alcohols, alkanes, iso-alkanes, terpenoids, esters and unknowns respectively (Figure 1). In all studied genotypes, alkanes were the most abundant class, and showed the greatest total and proportional variation (Table 3). The alkane homologues possessed total carbon atoms from C_{25} to C_{33}

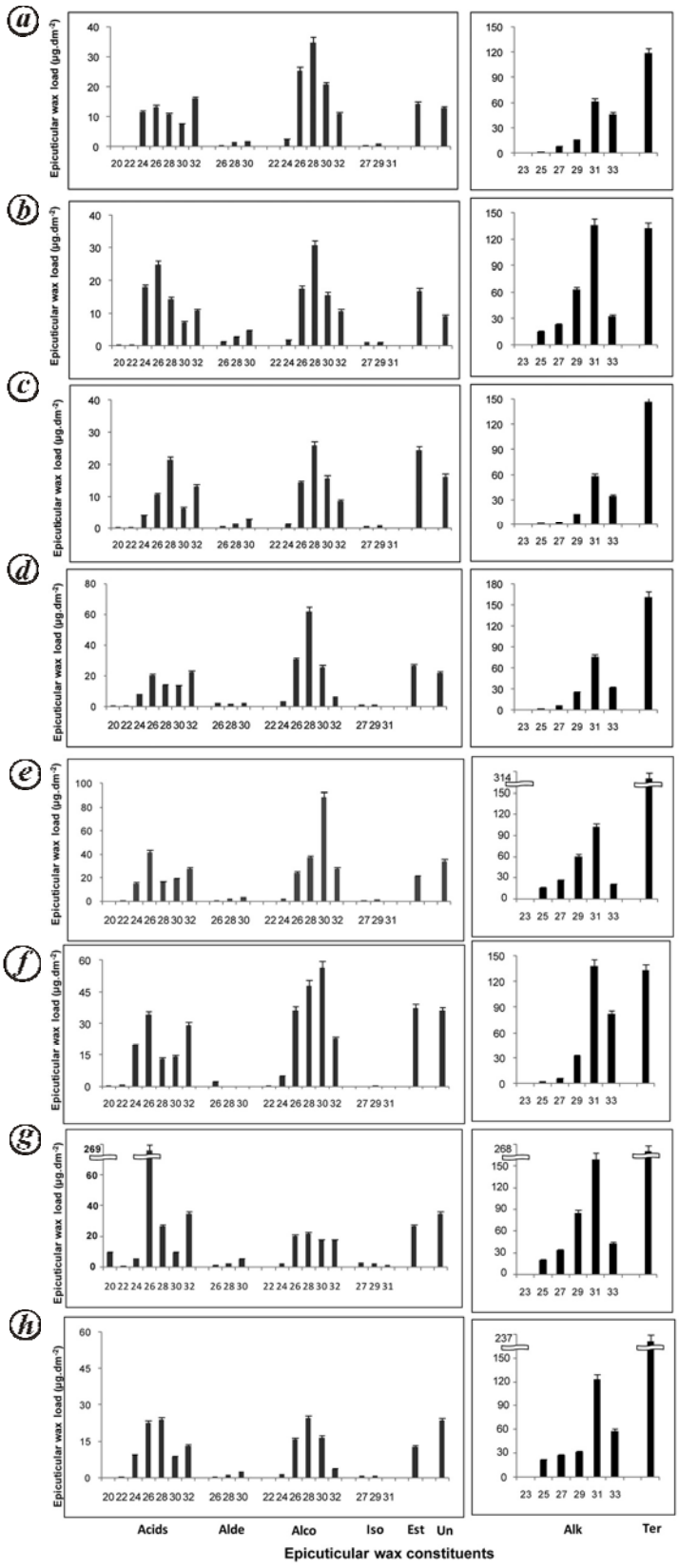

Figure 1. Cuticular wax composition analysis by GC-MS in contrasting groups of mulberry for post harvest water loss and cuticular wax amount. The genotypes (***a***–***d***) represents low wax load group and (***e***–***h***) represents high wax load group. ***a***, *S-36*; ***b***, *M. nigra 287*; ***c***, *S-642*; ***d***, *Khurkul*; ***e***, *Muki*; ***f***, *Batatul*; ***g***, *V1*; ***h***, *Rotundifolia*. The result is the mean of three independent analysis for each sample: alde – aldehydes, alco – alcohols, iso – isoalkanes, est – esters, un – unknowns, alk – alkanes, ter – terpenoids. The values in the *X*-axis indicate carbon chain length of each compound.

Table 3. Analysis of leaf epicuticular wax composition in contrasting genotypes of mulberry. The data indicate mean values (μg dm^{-2}) standard error from 3 replicate plants of each cultivar. Genotypes *S-36*, *M. nigra 287*, *S-642* and *Khurkul* represent low wax load group whereas *Muki*, *Batatul*, *V1* and *Rotundifolia* belong to high wax group

Mulberry genotypes	Total	Acids	Aldehydes	Alcohols	Alkanes	Iso alkanes	Terpenoids	Esters	Unknowns
S-36	436.9 ± 2.1	59.2 ± 2.6	3.8 ± 0.6	93.9 ± 2.1	132.7 ± 2.0	1.6 ± 0.4	118.6 ± 2.1	14.3 ± 1.7	12.8 ± 1.2
M. nigra 287	592.4 ± 4.0	76.7 ± 4.2	8.7 ± 1.1	76.5 ± 1.3	270.6 ± 2.3	2.1 ± 0.5	131.7 ± 2.8	16.9 ± 1.5	9.1 ± 1.7
S-642	432.7 ± 3.1	56.4 ± 2.3	4.7 ± 1.2	73.5 ± 3.2	109.9 ± 2.9	1.4 ± 0.3	146.9 ± 5.0	24.4 ± 3.2	16.2 ± 1.3
Khurkul	562.5 ± 3.9	80.1 ± 4.4	6.7 ± 1.1	128.1 ± 2.6	140.9 ± 4.8	2.8 ± 1.8	155.6 ± 3.7	26.5 ± 3.1	21.8 ± 2.0
Muki	905.2 ± 5.2	121.8 ± 2.7	7.2 ± 1.8	180.2 ± 4.3	224.0 ± 1.3	2.4 ± 0.4	314.3 ± 4.5	21.1 ± 2.3	34.2 ± 2.2
Batatul	750.4 ± 4.5	110.9 ± 3.2	2.1 ± 0.5	168.9 ± 3.6	261.0 ± 3.7	0.6 ± 0.1	133.4 ± 3.9	37.5 ± 2.6	36.1 ± 1.8
V1	1006.8 ± 3	243.2 ± 3.4	7.9 ± 1.3	78.2 ± 2.7	342.2 ± 4.3	5.9 ± 1.3	268.8 ± 2.9	26.4 ± 1.3	34.2 ± 1.1
Rotundifolia	681.6 ± 2.6	78.4 ± 2.7	4.4 ± 1.2	62.1 ± 1.8	262.2 ± 1.9	1.6 ± 0.3	236.7 ± 3.0	12.7 ± 1.0	23.5 ± 2.0
t-test value		2.42	2.08	0.72	4.08	1.66	4.87	1.21	7.86
P significance level		(<0.05)	(<0.05)	(<0.05)	(<0.01)	(<0.05)		(<0.05)	

Table 4. Genes involved in leaf surface wax biosynthesis obtained from RIKEN, Japan. The genes involved in different functions of wax biosynthesis, their MIPS accession numbers and reported putative function of the gene are presented

Wax gene	MIPs acc. no.	Putative function	Reference
CER6	At1g68530	Condensing	35
CER60	At1g25450	Condensing	36
KCS1	At1g01120	Condensing	13
TSC13	At3g55360	Fatty acid elongation	37
LTP1	At2g38540	Lipid transport	38
LTP2	At2g38530	Lipid transport	38
LTP6	At3g08770	Lipid transport	38
LTP7	At2g15050	Lipid transport	38
FAR	At4g33790	Fatty alcohol production	39
YBR 159	At1g67730	Fatty acid elongation	15
LACS2	At1g49430	Fatty acid transport from plastid to endoplasmic reticulum	18
WAX2	At5g57800	Dehydrogenation	14
CER5	At1g51500	Lipid transport	40

with C_{31} compounds being the most abundant. Previous reports[3] indicated that alkane amount is negatively associated with water loss from leaves in *Arabidopsis thaliana*, and likewise, we showed a similar association of alkane content and moisture retention capacity (MRC) in mulberry leaves[1]. This adds to the findings of Mamrutha *et al.*[1] showing that MRC was highest in the higher wax load accessions. Recently, it has been shown that a higher ratio of alkanes to terpenoids contributes for lower rates of water loss[32].

Expression of cuticular wax-associated genes in selected mulberry genotypes

The expression patterns of genes associated with surface wax production by newly, fully expanded leaves were analysed by RNA-blot hybridization and qPCR in the high and low wax mulberry genotypes. For gene expression studies using northern blots, 13 genes associated with wax biosynthesis were obtained from RIKEN (Table 4) and used as heterologous probes. Genes possibly involved in wax transport (*CER5*, *LTP1*, *LTP2*, *LTP6* and *LTP7*), decarbonylation (*WAX2*) and enoyl reduction (TSC13) did not show large variation in their expression between contrasting genotypes (Figure 2). However, there were significant differences in the expression of putative biosynthetic genes such as *CUT1*, *CER60*, *KCS1*, *FAR* and *AtYBR* (Figure 2). The abundance of *KCS1* and *CUT1* transcripts, that encode primary proteins that elongate wax precursors for later modification into alkanes, were significantly higher in genotypes having more abundant leaf waxes. Interestingly alkanes were the most abundant wax class, and responsible for most of the difference in total wax load on high and low wax load leaves, and the gene presumed associated with alkane synthesis, *WAX2*, did not show higher expression in high wax genotypes.

To validate results from the northern blots and the role of these genes in mulberry surface wax production, we identified two genes that exhibited high variation in heterologous gene expression (*MaCUT1* and *MaKCS1*), and two that did not (*MaWAX2* and *MaLTP1*). The mulberry *KCS1* (*MaKCS*, DX913094.1) and the mulberry *WAX2* (*MaWAX2*, DX913095.1) genes showed 100% and 93% homology with *Arabidopsis KCS1* (At1g01120) and *WAX2* (At5g57800) respectively. Similarly, the mulberry *CUT1* homologue (*MaCUT1*, DX913093.1) showed 85%

homology with *AtCUT1* (At1g68530), whereas the partial mulberry *LTP1* homologue (*MaLTP1*, EI811359.1) showed 95% homology with the *AtLTP1* (At2g38540). Total expression of *MaWAX2* and *MaLTP1* gene transcripts in young expanding mulberry leaves did not show any trend in expression between two distinct groups. There was significant difference in the expression of both *MaCUT1* and *MaKCS1* transcripts (Figure 3) between two distinct mulberry groups under study. Gene expression studies using semi-quantitative RT-PCR assay showed similar results (data not shown). The transcript abundance of *MaCUT1* and *MaKCS1* was significantly higher in high wax load leaves of Muki and Batatul genotypes, than in leaves of the low wax types, *M. nigra* and Khurkul (Figure 4). The transcript abundance of *MaCUT1* and *MaKCS1* was 1.2 and 1.5 fold higher in high wax types than low wax types. Higher levels of expression of these genes in high wax types suggest that condensing enzyme encoding genes, with their functions located early in the wax synthetic pathway, are important determinants of variation in total wax amount in mulberry[10]. Although other studied wax-associated genes did not show differential expression associated with cuticle wax composition, it cannot be ruled out that transcriptome analysis at other developmental time points, such as at early leaf emergence, would not in fact show higher

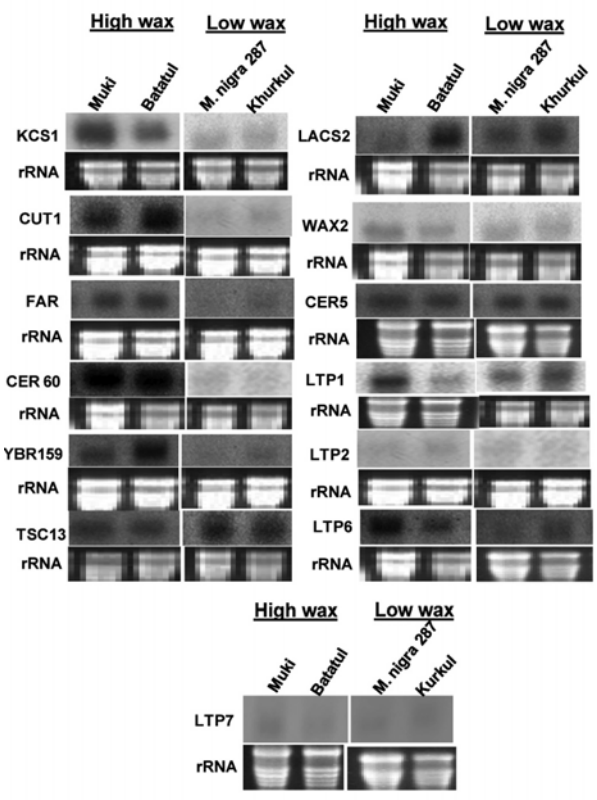

Figure 2. Northern blot to study the expression of genes involved in leaf surface wax synthesis in contrasting (high and low wax) accessions of mulberry using heterologous probes. Different genes involved or related to wax biosynthesis and deposition (*CUT1*, *CER60*, *KCS1*, *FAR*, *AtYBR*, *WAX2*, *TSC13*, *CER5*, *LTP1*, *LTP2*, *LTP6*, *LTP7*) were used as probes.

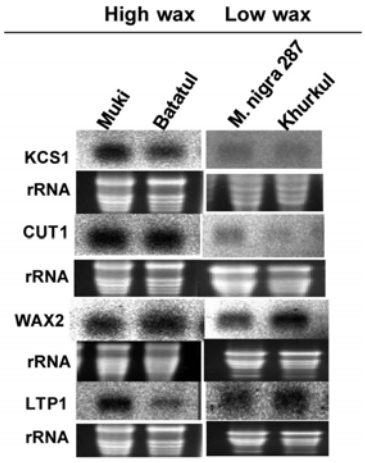

Figure 3. Northern blot to study the expression of genes involved in leaf surface wax synthesis in contrasting (high and low wax) accessions of mulberry using homologous probes. *KCS1*, *CUT1*, *WAX2* and LTP1 were used as probes.

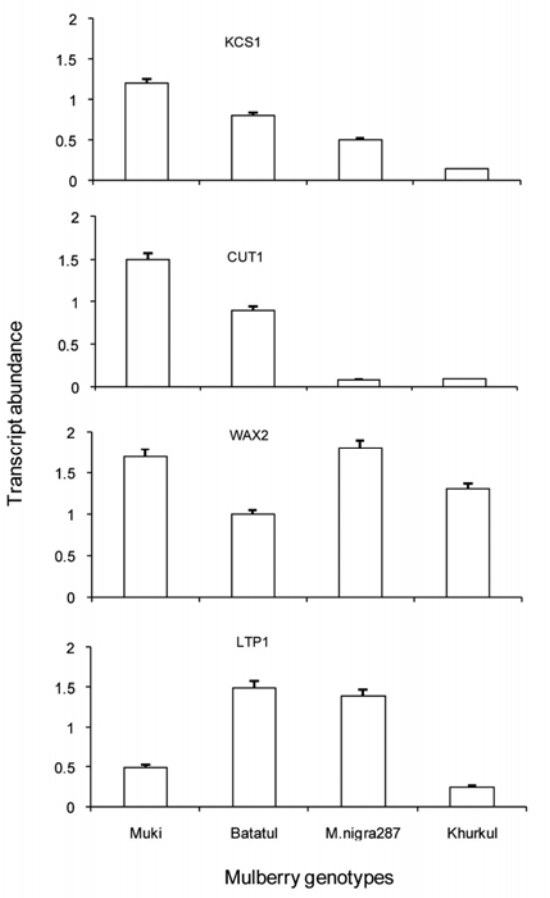

Figure 4. Expression analysis of wax biosynthesis genes by quantitative RT-PCR in the contrasting accessions of mulberry. *Muki* and *Batalul* represent high wax load cultivars, whereas *M. nigra 287* and *Khurkul* represent low wax load cultivars. Results are presented as transcript abundance by normalizing with reference gene and the data represents the mean ± SE of three replications.

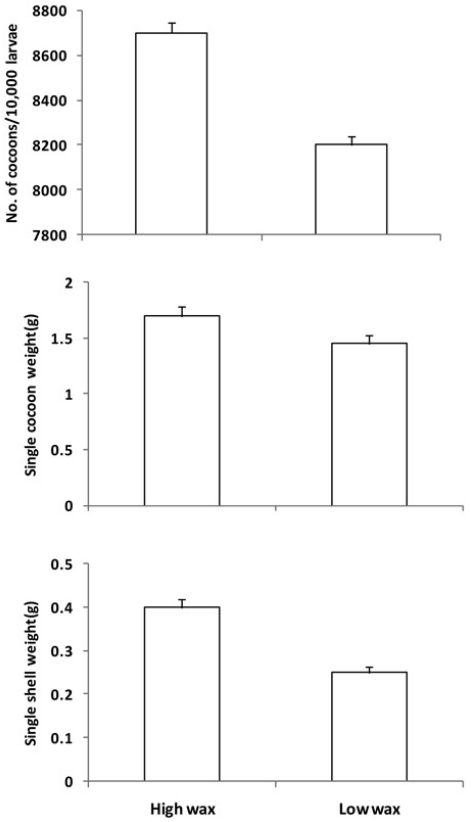

Figure 5. Silkworm bioassay using contrasting mulberry accessions differing in leaf surface wax content. The average values of contrasting groups are considered for each trait.

expression for genes such as *CER1* or *CER3* associated with the proportionally higher alkane contents on the high wax cultivars.

Silkworm bioassay

A silkworm bioassay was carried out by feeding the foliage of contrasting accessions of mulberry to silkworms, from first instar to last instar larvae and several cocoon and larval parameters were recorded. The cultivars *V1*, *Batatul*, *Muki* and *Rotundifolia* were high MRC and high wax category accessions, whereas the cultivars S-36, *M. nigra 287*, *S-642* and *Khurkul* were considered as low MRC and low wax accessions (Figure 5). Cocoon weight per 10,000 larvae was 14 kg for high MRC high wax accessions, and the value was 12 kg for low MRC low wax accessions. There was variation in single cocoon weight, and the average was 1.7 g for high MRC high wax accessions, compared to 1.45 g for low MRC low wax accessions. The single shell weight (i.e. the weight of cocoon after removing the pupae) was 0.4 g for high MRC high wax accessions but was 0.25 g for low MRC low wax accessions. The cocoon shell ratio was 23% and 20% for high MRC high wax accessions and low MRC low wax accessions respectively. All these parameters are statistically significant at $P < 0.05\%$, as indicated by *t*-test. Higher leaf moisture content is known to increase the amount of ingestion and digestion capacity of silkworms[33]. Because moisture acts as olfactory and gustatory stimulant[34], appetite of silkworms was decreased when withered leaves were fed. As such, low leaf moisture affects the growth and development of silkworm[34]. The silkworm has biting and chewing mouthparts, and prefers to feed on high wax genotypes due to absence of trichomes in leaf which otherwise hinders its biting capacity.

Conclusion

In this study, we analysed the components of leaf cuticle waxes in contrasting genotypes of mulberry, which in a previous report were shown to exhibit variations in leaf surface wax amount and post-harvest water loss. Significant variation in total wax amount was associated primarily with variation in alkane content in these contrasting genotypes. Gene expression studies presented here revealed an association between total leaf surface wax amount on newly expanded leaves and the expression levels of *CUT1* and *KCS1* genes. These results provide evidence that genes encoding wax pathway condensing enzymes, those involved in elongating wax precursors, are important contributors to the variation observed in total wax and alkane amounts on young mulberry leaves. These results shed light on new possibilities for using condensing enzymes, such as *KCS1* and *CUT1* in mulberry genetic selection and improvement strategies, exploiting either natural variation or the use of transgenic approaches. Applying these findings towards the improvement of leaf water retention capacity of mulberry leaves used as feed for silkworms could make an important contribution to improved efficiencies in commercial silk production.

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