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Identification and Characterization of Reduced Epicuticular *Wax* Mutants in Rice

Thomas H. TAI

(*United States Department of Agriculture-Agricultural Research Service, Crops Pathology and Genetics Research Unit, Department of Plant Sciences, Mail Stop 1, University of California, Davis, California 95616, USA*)

Abstract: Epicuticular wax forms the outermost protective barrier of the aerial surfaces of land plants, working in concert with other components of the plant cuticle to prevent uncontrolled loss of water and to provide protection against an array of external environmental stress. In this study, chemically-mutagenized populations of rice (*Oryza sativa* L.) derived from approximately 4 750 M_2 families were screened for adhesion of water droplets resulting in a wet leaf/glossy (wlg) phenotype. Mutants were identified in 11 independently-derived M_2 families. Scanning electron microscopy analysis confirmed the association of the wlg phenotype with changes in the epicuticular wax crystals of these plants. The phenotypes of five mutants (7-17A, 26.1, 524.2, 680.2, and 843.1) were confirmed to be the result of single recessive gene mutation. Evaluation of mutants from 3 (6-1A, 7-17A, and 11-39A) of 11 M_2 families revealed significant reductions (> 50%) in surface wax content and increases in cuticle membrane permeability.

Key words: chemical mutagenesis; cuticle wax; forward genetics; membrane permeability; rice; scanning electron microscopy

The plant cuticle is a hydrophobic layer produced by and deposited on the outer surface of the epidermal cells of land plants. Considered to be the single most important adaptive trait for the terrestrialization of plant life (Yeats and Rose, 2013), the cuticle functions primarily to prevent uncontrolled diffusion of water and gases (Kerstiens, 1996). The two major components of the cuticle are cutin and wax, both of which are polymers of fatty acids. Together these forms the cuticular membrane, which provides protection from an array of environmental stress including, but not limited to UV irradiation, pathogens, and insects (Jenks et al, 1994; Riederer, 2006; Yeats and Rose, 2013; Serrano et al, 2014). Studies have also shown that the cuticle plays an important role in plant development by inhibiting organ fusion (Lolle et al, 1992; Sieber et al, 2000) and mediating pollen-stigma interactions (Preuss et al, 1993; Fiebig et al, 2000).

Epicuticular waxes are typically found as microscopic crystals on the outer surface of the cutin polymer matrix. These wax crystals, which exist in various shapes and sizes (Baker, 1982), refract light resulting in a glaucous (grayish green/blue) appearance of aerial surfaces (Clark and Lister, 1975). Plants with mutations in genes affecting the accumulation of epicuticular waxes are characterized by the glossy, bright green appearance. This trait can be easily detected through visual inspection, which has enabled the identification of many cuticular *wax* mutants in monocots such as *Zea mays* (Post-Beitenmiller, 1996) and in dicots such as *Arabidopsis thaliana* (Jenks et al, 1995). Epicuticular waxes also strongly influence leaf wettability. Wax-deficient mutants have more hydrophilic leaves. As a result, water droplets adhere to leaf surfaces of these mutants. In rice (*Oryza sativa* L.), the leaf cuticle is not smooth and the glossy phenotype is not observed without water adhesion (Qin et al, 2011).

In contrast to the wealth of cuticular *wax* mutants in

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Corresponding author: Thomas H. TAI (Thomas.Tai@ars.usda.gov)

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maize and *Arabidopsis*, relatively few have been identified and characterized in rice. Jung et al (2006) reported the cloning of the *Wax-deficient anther1* (*Wda1*) gene, which was identified by characterization of a T-DNA insertion male sterile line. This mutant exhibits severely defective microspore development, epicuticular wax-deficient anthers, and altered wax composition (i.e., significant reductions in fatty acids, alkanes, alkenes, and primary alcohols). The *wax crystal-sparse leaf1* (*wsl1*) rice mutant was also isolated from a T-DNA insertion population, and was originally identified due to its reduced stature (Yu et al, 2008). Cloning of the gene revealed it to be homologous to the 3-ketoacyl-CoA synthase gene family in *Arabidopsis* and other plants.

Islam et al (2009) reported the identification of 11 *Glossy 1* (*GL1*)-like gene homologues in rice and the function of the *OsGL1-2* gene through the generation and analysis of transgenic plants over-expressing the gene and the characterization of a T-DNA insertion mutant. Over-expression of *OsGL1-2* results in increased cuticular wax and enhanced drought tolerance, and the *Osgl1-2* mutant exhibits reduced cuticular wax synthesis and increased sensitivity to drought stress. More recently, two groups reported the characterization of cuticular wax-deficient mutants derived from spontaneous and ethylmethane sulfonate (EMS)-induced mutations (Qin et al, 2011; Mao et al, 2012). Positional cloning revealed that both mutations are in the *OsGL1-1* gene. The *Osgl1-1* mutant (also known as *wsl2*) shows significant reduced cuticular wax and increased drought sensitivity. Compositional analysis of the wax from the *wsl2* mutant revealed significant reductions in the C_{22} to C_{32} fatty acids, suggesting a role in the elongation very long chain fatty acid (Mao et al, 2012).

To date, rice mutants have been employed to characterize four cuticular wax genes, *Wda1* (*OsGL1-5*), *Wsl1*, *OsGL1-2*, and *OsGL1-1* (*Wsl2*). This suggests that forward and reverse genetic approaches have been underutilized in the genetic dissection of cuticle wax metabolism in rice. In this study, 11 independent induced mutants with reduced epicuticular wax were identified using a forward genetics screen of rice populations derived from sodium azide mutagenesis. Ten of these mutants exhibited the wet leaf/glossy (wlg) phenotype previously described for the *wsl* mutants (Yu et al, 2008; Mao et al, 2012). The reduction in wax crystals of all 11 mutants was confirmed by scanning electron microscopy. Genetic analysis indicated that at least five mutants (7-17A, 26.1, 524.2, 680.2, and 843.1) exhibited inheritance patterns consistent with single recessive gene mutation. Further characterization of individuals from three mutant families (6-1A, 7-17A, and 11-39A) confirmed the association of their cuticle wax phenotypes with increased membrane permeability as evaluated by non-stomatal water loss and chlorophyll leaching. Those mutants represented valuable resources for the genetic dissection of cuticular wax metabolism in rice.

MATERIALS AND METHODS

Plant materials, mutagenesis, and screening

All mutants in this study were derived from Sabine, a long-grain tropical japonica variety developed by the United States Department of Agriculture-Agricultural Research Service (Anna McClung) from the cross RU9302165/Dixiebelle. Mutant populations were developed by sodium azide mutagenesis of foundation seeds of Sabine (2009 and 2011 years) that were provided by David MACKILL (Mars, Inc.). Briefly, seeds (M_0 generation) were soaked in distilled water for 16–20 h, drained, and then treated with 1–2 mmol/L sodium azide in 0.1 mol/L sodium phosphate buffer, pH 3.0. After incubation in the sodium azide solution for 3 h, seeds (M_1) were rinsed three times with distilled water, sown in soil and then grown to maturity in a greenhouse. Seeds (M_2) were harvested from M_1 plants with the seeds from each M_1 panicle (typically 1–2 per plant) kept separately. Two or three M_2 seeds per panicle were sown to produce M_3 seeds for phenotyping. For screening, five M_3 seeds were planted and the resulting plants were screened at 3–5 weeks post emergence by watering the leaves of the plants using a 'shower'-type water nozzle that produced water droplets. Plants with leaves to which water droplets adhered resulting in a wlg appearance were identified as putative cuticle *wax* mutants. The same phenotyping method was employed to evaluate available M_3 populations to determine inheritance patterns of various mutations identified. Segregation ratios observed in those populations were subjected to Pearson's chi-square test for goodness-of-fit to the single recessive gene mode of inheritance. Mutants were identified from two populations (Sabine in 2009 and 2011) generated from three different seed mutagenic treatments (Table 1).

Scanning electron microscopy (SEM)

Tissue from fully expanded leaves (1–2 leaves from

Table 1. Genetic and physical characteristics of reduced epicuticular *wax* mutants.

M_1 line	M_2 line	Seed source	Mutagenesis	Phenotype of M_3	Single gene recessive mutation	Mutant designation
6-1	6-1A	2009	1 mmol/L NaN_3 / 16 h presoak	Wet leaf/glossy segregating	Nd	*wsl3*
6-1	6-1B	2009	1 mmol/L NaN_3 / 16 h presoak	Wet leaf/glossy	Nd	
7-17	7-17A	2009	1 mmol/L NaN_3 / 16 h presoak	Wet leaf/glossy segregating	Yes	*wsl4*
11-39	11-39A	2009	1 mmol/L NaN_3 / 20 h presoak	Wet leaf/glossy	Nd	*wsl5*
26	26.1	2011	2 mmol/L NaN_3 / 16–20 h presoak	Wet leaf/glossy segregating	Yes	*wsl6*
264	264.2	2011	2 mmol/L NaN_3 / 16–20 h presoak	Wet leaf/glossy segregating	Nd	*wsl7*
524	524.2	2011	2 mmol/L NaN_3 / 16–20 h presoak	Wet leaf/glossy segregating	Yes	*wsl8*
680	680.2	2011	2 mmol/L NaN_3 / 16–20 h presoak	Wet leaf/glossy segregating	Yes	*wsl9*
843	843.1	2011	2 mmol/L NaN_3 / 16–20 h presoak	Wet leaf/glossy segregating	Yes	*wsl10*
1064	1064.2	2011	2 mmol/L NaN_3 / 16–20 h presoak	Wet leaf/glossy	Nd	*wsl11*
1086	1086.2	2011	2 mmol/L NaN_3 / 16–20 h presoak	Wet leaf/glossy	Nd	*wsl12*
1558	1558.1	2011	2 mmol/L NaN_3 / 16–20 h presoak	Intermediate	Nd	
1558	1558.2	2011	2 mmol/L NaN_3 / 16–20 h presoak	Intermediate	Nd	*wsl13*

Phenotypic ratios of segregating M_3 populations were evaluated using Pearson's Chi-square test for goodness-of-fit to the 3:1 (wild-type:mutant) ratio expected for single gene recessive mutations.

Nd, Not determined; *wsl*, Wax crystal-sparse leaf.

the youngest leaf) of 4–5 weeks old plants and flag leaves of booting-stage plants were cut into small pieces (≤ 1 cm in length) and immersed in modified Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.06 mol/L Sorensen's phosphate buffer, pH 7.3). Fixing was assisted using a Pelco 34700 BioWave (Ted Pella, Inc., Redding, California, USA) allowed to proceed at room temperature for 1–2 h followed by overnight incubation at 4 °C. After dehydration in a graded ethanol series (30%–100%), samples were subjected to critical point drying in a Tousimis® 931.GL Supercritical Autosamdri (Tousimis Research Corp., Rockville, Maryland, USA) and sputter coated with gold using a Pelco Auto Sputter Coater SC-7 (Ted Pella, Inc., California, USA). Samples were observed and images were taken using a Philips XL30 TMP (F.E.I. Co., Hillsboro, Oregon, USA). SEM analysis was performed at the Electron Microscopy Laboratory, Department of Pathology and Laboratory Medicine, University of California, Davis, California, USA.

Total wax content by weight

The total wax content was determined using the weight method as described by Zhou et al (2013) with minor modification. Two grams of leaf blades from flag leaves of booting and early heading (i.e. panicle exserted but prior to grain filling) tillers of mutants (M_3 generation) and wild-type plants were harvested and cut into approximately 3 cm lengths. The tissue was transferred to a pre-weighed test tube (25 mm × 150 mm), and 30 mL hot chloroform (60 °C) was added to extract the cuticle wax from the leaf surfaces. Leaves were removed after 30 s and the wax content was determined by re-weighing the tubes on an analytical balance after complete evaporation of the chloroform. Total wax content (mg/g) for each individual was determined from two independent extractions except where noted due to insufficient material.

Cuticle membrane permeability

Water loss measurements were taken using a detached leaf assay. The second leaves from the top of three booting-stage tillers per plant were detached by cutting below the auricles (i.e. interface between the leaf blade and sheath) and submerged in distilled water in the dark for more than 2 h. All subsequent manipulations were conducted in a darkened room. The leaves were removed, blotted dry, and cut at the auricle prior to weighing using an analytical balance. Leaf blades were weighed at 0, 0.5, 1.0, 1.5, 2.5, and 3.0 h. Leaves were kept in the dark at room temperature between measurements. The percentage of weight loss was determined based on the initial leaf blade weight. Two M_3 mutants from each line and one wild-type were assayed in two independent experiments (total of six leaf blades per plant).

The chlorophyll leaching assay was performed as described by Islam et al (2009) with minor modifications. Briefly, the third leaf from the top was sampled from each tiller of booting stage plants and the leaf was cut into segments (about 3 cm) and immersed in 30 mL of 80% ethanol at room temperature with gentle shaking in the dark. Three milliliters aliquots were removed from each sample after 1, 3, 5, 7, 9, and 24 h. Readings were taken at 647 and 664 nm using a SpectraPlus^{384} spectrophotometer (Molecular Devices, Sunnyvale, California, USA) and aliquots were returned

to their respective samples after each measurement. Chlorophyll concentration was determined as described by Lolle et al (1997). The micromolar concentration of total chlorophyll per gram of fresh weight tissue was calculated using the equation: Total micromoles chlorophyll = $7.93 \times A_{664} + 19.53 \times A_{647}$. Chlorophyll efflux at each time point was expressed as a percentage of total chlorophyll extracted after 24 h. Measurements were taken from three leaves (i.e. blades) per plant with two M_3 plants per mutant family and one wild-type plant. The experiment was performed twice (i.e., a total of 6 leaves for the wild-type and 12 leaves for each mutant family/line).

Statistical analysis

The means, standard deviation (SD), and significant differences (*t*-test for unequal variance) between wild-type and mutants for total wax and membrane permeability measurements were determined using Excel 2010 (Microsoft, Redmond, Washington, USA).

RESULTS

Identification of putative cuticle *wax* mutant

To identify putative cuticle *wax* mutants, the leaves of M_3 plants from approximately 4 750 M_2 individuals (1 450 and 3 300 from Sabine 2009 and 2011 populations, respectively) were watered with a 'shower'-type spray nozzle. Using this simple screening method, plants exhibiting a wlg phenotype due to the adherence and coalescence of water droplets on the leaf surfaces were identified (Fig. 1-A). Evaluation of the M_3 plants derived from mutagenesis of Sabine 2009 seeds resulted in the identification of four M_2 lines (6-1A, 6-1B, 7-17A, and 11-39A) with the wlg phenotype. Of these, 6-1A and 6-1B were derived from sibling M_2 plants (i.e., M_2 seeds from one panicle of the same M_1 individual) while the other two M_2 lines were derived from different M_1, and therefore, a total of three presumably independent mutation events were represented. Segregation of the wlg phenotype was observed in the 6-1A and 7-17A M_2 families. Of the five M_3 individuals screened for 6-1A, three exhibited the mutant phenotype while two of the five M_3 individuals screened for 7-17A exhibited the mutant phenotype. All the M_3 plants in both the 6-1B and 11-39A M_2 families (three in each case) displayed the wlg phenotype.

To further investigate the inheritance pattern of these mutant phenotypes, additional M_3 plants from each of the M_2 families were phenotyped. Due to a limited number of seeds, only 15 M_3 were planted for each M_2 family. Thirteen M_3 of the 6-1A M_2 family were germinated and six plants displayed the mutant phenotype. Thus 9 out of a total of 18 M_3 plants were mutant. This was not consistent with a single recessive gene mutation ($\chi^2 = 6.000$, $df = 1$, $P = 0.0143$). For the 6-1B M_2 family, six seeds were germinated and all of the M_3 were mutant, indicating that the mutation is homozygous in this line. All 15 M_3 seeds of the 7-17A M_2 family produced plants, 2 of which exhibited the mutant phenotype. Thus out of 20 total M_3 plants, 16 wild-type and 4 mutants were observed. This ratio is consistent with a single recessive gene mutation ($\chi^2 = 0.267$, $df = 1$, $P = 0.6056$). Five of the M_3 seeds from the 11-39A M_2 family produced plants, all of which had the wlg phenotype, indicating that the mutation is

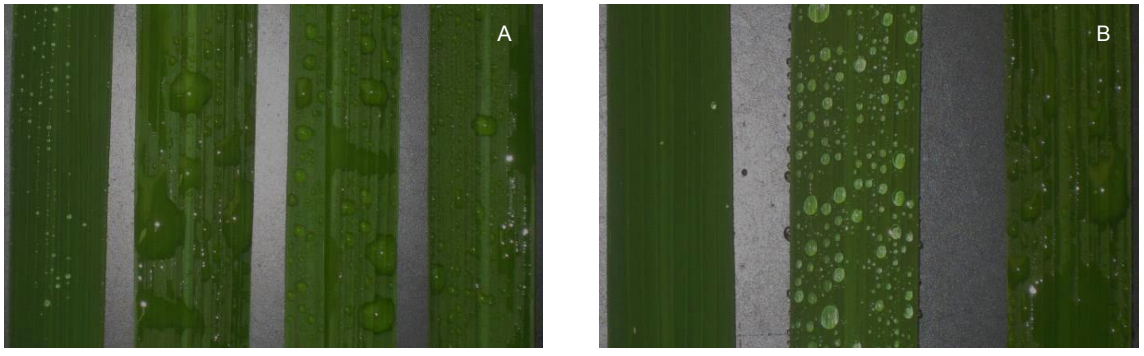

Fig. 1. Wet leaf/glossy and intermediate-type mutant phenotypes.

Leaves were subjected to a fine misting with water from a spray bottle. Water droplets adhere to the leaves of mutants and coalesce resulting in a wet, glossy appearance.

A, Comparison of wild-type Sabine and the three mutants 6-1A, 7-17A, and 11-39A. Booting-stage flag leaves are shown; B, Comparison of the wild-type Sabine and the mutants 1558.2 and 6-1A. Fifth fully-expanded leaves from the base are shown. Unlike the other mutants, water droplets adhere to 1558.2 but do not coalesce and the leaves do not appear wet or glossy.

homozygous in this line.

Screening of M_2 families from the Sabine 2011 population resulted in the identification of seven additional lines (26.1, 264.2, 524.2, 680.2, 843.1, 1064.2, and 1086.2) with the wlg phenotype (Table 1). Five of these M_2 families exhibited segregation of the phenotype among the M_3 plants (26.1, 264.2, 524.2, 680.2, and 843.1) while all the M_3 plants of two of the M_2 families, 1064.2 and 1086.2, displayed the mutant phenotype. In addition to the wlg phenotype, two sibling derived M_2 families (1558.1 and 1558.2) were identified as exhibiting an altered phenotype with regard to water droplet adherence. Unlike wild-type Sabine, all the M_3 plants examined (five per family) exhibited the adherence of water droplets to their leaves. These droplets did not coalesce and the leaves did not appear wet or glossy in contrast to the wlg phenotype of the other mutants (Fig. 1-B).

Of the five Sabine 2011 M_2 families exhibiting segregation among the M_3 plants, the inheritance patterns of the mutant phenotype were further examined in 26.1, 524.2, 680.2 and 843.1. For 26.1, 680.2, and 843.1, an additional 72 M_3 seeds were sown and the resulting plants were scored for the wlg phenotype. In the case of 524.2, an additional 90 M_3 seeds were sown for phenotyping. Results of the evaluation of these M_3 populations are shown in Table 2. The χ^2 goodness-of-fit tests of the ratios of wild-type to mutant observed for each of the M_3 populations are consistent with a 3:1 segregation indicative of a single gene recessive mutation being responsible for the mutant phenotype.

Analysis of epicuticular wax by scanning electron microscopy

To determine if the wlg and water droplet adherence phenotypes were associated with altered epicuticular wax in those mutants, SEM was performed on leaf samples from 4–5 weeks old M_3 mutants representing each of the 11 M_2 families. In the wild-type Sabine plants, both the adaxial (Fig. 2) and abaxial (not shown) leaf blade surfaces were covered with a dense layer of platelet-type wax crystals typical of rice (Yu et al, 2008). SEM images of the mutants clearly showed a significant reduction in the size and density of the epicuticular wax crystals as well as possible differences in their shape. Among the ten mutants identified as exhibiting a wlg appearance, there appeared to be qualitative differences with the samples from 524.2 and 1086.2 having more crystals than the other mutants. Consistent with the distinct water droplet phenotype observed for 1558.2, SEM analysis revealed a greater density than those in the other mutants. In addition, the platelet-type crystals observed in 1558.2 appeared to be larger than the wild-type Sabine although the much higher density in the wild-type may be obscuring the actual size of those individual platelets. Given the similarity of these mutants with two previously reported *wsl* mutants (Yu et al, 2008; Mao et al, 2012), these mutants were designated as *wsl3* to *wsl13* (Table 1).

Characterization of total epicuticular wax content and cuticle membrane permeability

The epicuticular wax content and cuticle membrane permeability of the flag leaves of reproductive stage mutants were examined. At the time of this study, reproductive stage M_3 plants were available for three of the M_2 families (6-1A/B, 7-17A, and 11-39A). The weight method was employed to determine the total epicuticular wax content (Zhou et al, 2013). Results are shown in Table 3. The wax content of the wild-type Sabine was similar to the reported wild-type Zhonghua 11 variety examined by Zhou et al (2013) using this method. All the three mutants exhibited significant reductions in epicuticular wax content relative to wild-type ranging from 54.15% to 77.41%.

To assess the cuticle membrane permeability of these mutants, water loss from detached leaves was measured over time as shown in Fig. 3-A. Significant differences in rate of water loss were observed between the mutants and wild-type Sabine for all the time points. Among the mutant lines, 7-17A exhibited the greatest water loss, consistent with the epicuticular

Table 2. Segregation of wet leaf/glossyphenotype in four M_3 populations.

Population	M_3 seeds planted [a]	M_3 plants phenotyped [a]	Segregation ratio (wild-type:mutant)	Pearson's Chi-square test (3:1)	
				χ^2	*P* value
26.1	77	57	40:17	0.708	0.400
680.2	77	61	43:18	0.661	0.416
843.1	77	73	53:20	0.224	0.636
524.2	95	68	53:15	0.314	0.575

[a] includes initial screening of M_3 plants.

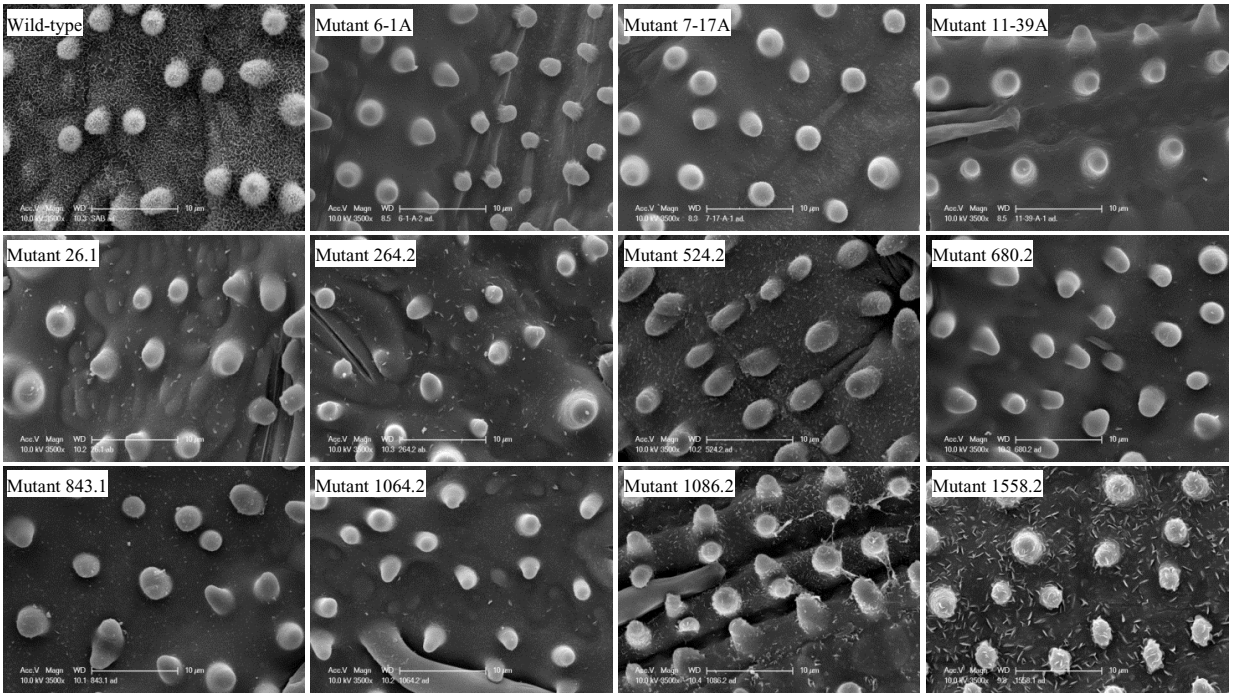

Fig. 2. Scanning electron micrographs of wild-type Sabine and 11 reduced epicuticular *wax* mutants.
Adaxial leaf blade surface shown (knob-like structures are papillae). Scale bars, 10 μm.

wax content analysis. For the first three time points, there was no significant difference in water loss rate between 7-17A and 6-1A plants. In contrast, water loss of 7-17A was significantly greater than both the wild-type and 11-39A over the entire time course examined (*t*-test, $P < 0.01$). Comparison of the water loss by 6-1A and 11-39A revealed no significant differences over the time course despite differences in their total epicuticular wax content (Table 3).

In addition to water loss measurements, chlorophyll leaching assays were conducted to evaluate cuticular membrane permeability in M_3 mutants from the three mutant families. All the three mutant lines exhibited more rapid and greater loss of chlorophyll than the wild-type over the first three time points (Fig. 3-B; *t*-test, $P < 0.05$). While this trend continued for the duration of the assay for the 6-1A and 7-17A lines, the mutants from 11-39A line were not significantly different from wild-type after 5 h. This observation was consistent with the finding that 11-39A mutants exhibited the lowest reduction in epicuticular wax (Table 3).

Table 3. Total epicuticular wax content of wild-type and *wsl* mutant lines by weight method.

Line	Wax content (mg/g)	Reduction in wax content (%)
Sabine (wild-type)	3.01 ± 0.49	
6-1A/B [a]	0.91 ± 0.31 **	69.77
7-17A	0.68 ± 0.42 **	77.41
11-39A	1.38 ± 0.10 **	54.15

Values are given as mean ± *SD* with four replicates except where noted.

[a], Due to insufficient tissue from 6-1A M_3 mutants, additional samples were taken from 6-1B M_3 mutants and content was determined using a total of five replicates (three from 6-1A plants, two from 6-1B plants included).

** means significant difference between mean values at $P < 0.01$ by *t*-test between the wild-type and each of the mutant line.

DISCUSSION

Cuticular waxes are a major component of the cuticle membrane. The crystalline waxes found on the outer surface of the cuticle represent the first barrier to various environmental stress (Post-Beittenmiller, 1996). Due to the light refracting properties of those crystals, mutants with altered/reduced epicuticular waxes typically exhibit a glossy, bright green appearance easily detected in many plants. Numerous examples have been reported in barley (*Hordeum vulgare*), maize (*Zea mays*), *Arabidopsis thaliana* and other species (Post-Beittenmiller, 1996; Jenks et al, 2002). Genetic analysis of those mutants, particularly in *Arabidopsis*, has contributed immensely to our understanding of the wax biosynthetic pathways in plants as well as to their transport and the regulation

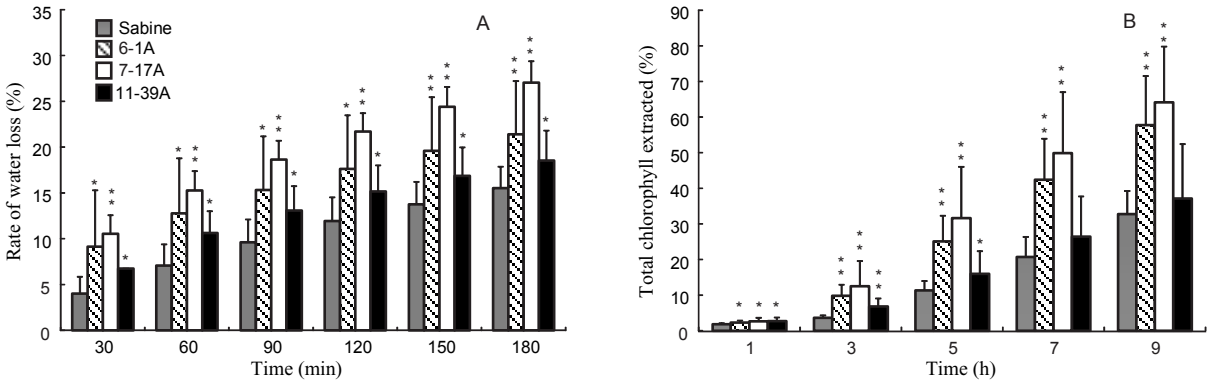

Fig. 3. Cuticle membrane permeability of three *wax* mutants.

A, Water loss rate from detached leaves over time; B, Chlorophyll loss over time.

Each bar represents the mean ± *SD* of two independent assays (two replicates for wild-type and four replicates for each mutant). Levels of significance between wild-type Sabine and each mutant were determined by *t*-test for unequal variance; * and ** mean significant difference from wild-type at $P < 0.05$ and $P < 0.01$, respectively.

of their biosynthesis (Post-Beittenmiller, 1996; Samuels et al, 2008; Yeats and Rose, 2013).

Epicuticular wax-deficient mutants in rice do not exhibit a glossy appearance without water adhesion (Qin et al, 2011). In this study, populations derived from chemical mutagenesis were screened to identify mutants that exhibited a wlg appearance after spraying their leaves with water. Using this simple approach, eleven M_2 mutant families with the wlg phenotype and two M_2 families which exhibited an intermediate-type phenotype (i.e. water droplet adhesion but no wlg appearance) were identified. Together, those 13 M_2 families represented 11 independent mutations (two of the wlg families were derived from the same M_1 as both the intermediate-type families). SEM analysis of all the mutants confirmed significant changes in the amount and, in some cases, morphology of the epicuticular wax crystals compared to wild-type. The intermediate-type mutant exhibited more (and larger) crystals than the other mutants. Those crystals also appeared to be larger than the ones observed in wild-type. The results of SEM analysis were consistent with those mutants derived from independent mutation events.

Of the 11 mutants, 7 were identified in segregating M_3 populations (i.e., M_2 families). Analysis of the M_3 from six of the seven segregating populations indicated that single gene recessive mutation underlie the phenotypes observed in the 7-17A, 26.1, 524.2, 680.2, and 843.1 M_2 families. The results of the analysis of 18 M_3 individuals from the 6-1A M_2 family were not supportive of a single recessive gene mutation model. The observed phenotypic ratio (9 wild-type to 9 mutant) is consistent with either the 1:1 or 9:7 genetic ratio. As rice is an inbreeding species, the 1:1 genetic ratio is not readily explainable. A ratio of 9:7 (i.e., complementary gene action) indicates the involvement of two genes, both of which would be required for normal epicuticular wax content. Studies are underway to determine if this is the case or if the observed phenotypic ratios are due to subtle differences in the phenotypes or incomplete penetrance of the mutation.

As the mutants described in this study were identified over several months, only those from three of the M_2 families (6-1A, 7-17A, and 11-39A) were available (i.e. of the appropriate developmental stage and with sufficient tissue) for additional analyses including determination of the epicuticular wax content and the assessment of cuticle membrane permeability. The results of those evaluations reinforced the initial phenotyping and SEM analysis with regard to the reductions in wax content (Table 3) which were consistent with changes in cuticle permeability as determined by non-stomatal water loss measurements and chlorophyll leaching. More detailed characterization of those and the remaining mutants await the availability of additional materials (e.g., M_4 and backcross lines). In addition to compositional analysis of the cuticular wax using gas chromatography-mass spectrometry analysis, the response of the mutants to drought conditions will be determined.

The first cuticular wax mutant in rice was identified fortuitously following the characterization of male sterile and reduced stature mutants from a T-DNA insertion population. The utility of T-DNA insertion mutants for gene cloning using both forward and reverse genetics approaches has long been established.

More recently, spontaneous and chemically-induced cuticular wax-deficient rice mutants were identified and subsequent positional cloning revealed mutations in the *GL1-1* homologue in rice (also referred to as *wsl2*) in both cases (Qin et al, 2011; Mao et al, 2012). While this traditional effective forward genetics approach employing induced mutations is generally time-consuming and resource-intensive. Recently strategies have been proposed and employed to rapidly identify causative mutations using whole-genome sequencing (Abe et al, 2012; Nordstrom et al, 2013; Schneeberger, 2014). Application of this fast-forward genetics (Schneeberger and Weigel, 2011) strategy to the four mutants with sufficiently large segregating populations (Table 3) is underway. The rapid identification of the underlying mutant alleles will increase interest in forward genetic screening and enable resources to be directed to more detailed analyses of novel mutants/mutations.

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