

Research Paper

Characterization and genetic mapping of *eceriferum-ym* (*cer-ym*), a cutin deficient barley mutant with impaired leaf water retention capacity

Chao Li^{1,2}), Cheng Liu²), Xiaoying Ma¹), Aidong Wang¹), Ruijun Duan¹), Christiane Nawrath³), Takao Komatsuda²) and Guoxiong Chen^{*1,2})

¹) Laboratory of Plant Stress Ecophysiology and Biotechnology, Cold and Arid Regions Environmental and Engineering Research Institute, Chinese Academy of Sciences, Lanzhou 730000, China

²) National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan

³) Department of Plant Molecular Biology, University of Lausanne, Lausanne CH-1015, Switzerland

The cuticle covers the aerial parts of land plants, where it serves many important functions, including water retention. Here, a recessive cuticle mutant, *eceriferum-ym* (*cer-ym*), of *Hordeum vulgare* L. (barley) showed abnormally glossy spikes, sheaths, and leaves. The *cer-ym* mutant plant detached from its root system was hypersensitive to desiccation treatment compared with wild type plants, and detached leaves of mutant lost 41.8% of their initial weight after 1 h of dehydration under laboratory conditions, while that of the wild type plants lost only 7.1%. Stomata function was not affected by the mutation, but the mutant leaves showed increased cuticular permeability to water, suggesting a defective leaf cuticle, which was confirmed by toluidine blue staining. The mutant leaves showed a substantial reduction in the amounts of the major cutin monomers and a slight increase in the main wax component, suggesting that the enhanced cuticle permeability was a consequence of cutin deficiency. *cer-ym* was mapped within a 0.8 cM interval between EST marker AK370363 and AK251484, a pericentromeric region on chromosome 4H. The results indicate that the desiccation sensitivity of *cer-ym* is caused by a defect in leaf cutin, and that *cer-ym* is located in a chromosome 4H pericentromeric region.

Key Words: *eceriferum*-mutant, cuticle, cutin, desiccation resistance, genetic mapping, wax.

Introduction

Water availability is one of the most critical requirements for the survival of terrestrial plants and water deficit is a common cause of decreased productivity and restricted growth (Boyer 1982). In this regard, the formation of an extracellular hydrophobic cuticle, which coats the outer surfaces of land plant aerial organs, thereby limiting water loss, has been a crucial evolutionary innovation (Goodwin and Jenks 2005). In addition, the cuticle provides protection against a range of abiotic and biotic stresses and has an important influence on plant growth and development (Yeats and Rose 2013).

The structure and properties of the cuticle vary considerably among plant species, organs and growth stages, but it mainly consists of two hydrophobic components, cutin and waxes (Jeffree 1996). The cutin matrix is a polyester composed of inter-esterified oxygenated C16 and C18 fatty

acids and glycerol, and typically comprises 40–80% of the cuticular weight (Heredia 2003, Nawrath 2006). Waxes embedded in the cutin matrix (intracuticular) or deposited on its surface (epicuticular) are mostly composed of an aliphatic mixture of very long chain (VLC) fatty acids and their derivatives, including alkanes, alcohols, aldehydes, ketones and alkyl esters (Buschhaus and Jetter 2011, Nawrath 2006). The cutin matrix thus provides the framework for wax deposition and its structural organization and size may affect the fine structure and water-barrier functions of the waxes. Unlike the distinct patterns of regulation associated with different wax components, a study of *Arabidopsis thaliana* leaf cuticles revealed an indiscriminate increase in the abundance of all cutin monomers by 65% following exposure to water stress (Kosma *et al.* 2009). This may suggest that the total amount of polymeric cutin is more important for water retention than the relative proportion of any specific cutin monomer. It has been demonstrated that the transcription factor *WIN1/SHN1*, an important regulator of cutin synthesis, controls cuticular water permeability by directly targeting the *long-chain acyl-CoA synthetase 2* (*LACS2*), which is responsible for the synthesis of cutin

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*Corresponding author (e-mail: guoxiong@lzb.ac.cn)

intermediates (Kannangara *et al.* 2007). Moreover, cutin deficiency has been associated with substantial increases in the permeability of cuticles that show no major qualitative or quantitative changes in the constituent waxes in a number of mutants, such as the *A. thaliana* double mutant *glycerol-3-phosphate acyltransferase 4 (gpat4)/gpat8* (Li *et al.* 2007), *defective cuticular ridges (dcr)* (Panikashvili *et al.* 2009), the *Oryza sativa* (rice) *wilted dwarf and lethal 1 (wdll1)* (Park *et al.* 2010) and the *Solanum lycopersicum* (tomato) *cutin deficient 1 (cd1)* mutant (Isaacson *et al.* 2009) and the *Hordeum vulgare* (barley) mutants *eibi1* (Chen *et al.* 2011) and *eceriferum-zv (cer-zv)* (Li *et al.* 2013).

More than 20 genes that participate in cutin synthesis, transportation, polymerization and regulation have been identified and characterized (Yeats and Rose 2013), most of which are from *A. thaliana*. However, other plant species are providing valuable experimental systems, such as barley, where a collection of 1,580 cuticle *eceriferum (cer)* mutants with glossy leaves, sheaths and/or spikes have been generated as a consequence of chemical and physical mutagenesis, and the underlying mutations have been ascribed to 79 distinct genetic loci (Lundqvist and Lundqvist 1988). Some of these loci have been roughly mapped (Druka *et al.* 2011, Franckowiak 1997), but none of the mutated genes has been cloned or fine mapped to date. The single recessive *cer* mutants *eceriferum-ym (cer-ym)* and *cer-zv* fall into the category of plants with glossy spikes, sheaths and leaves (Franckowiak and Lundqvist 2012). The *cer-ym* and *cer-zv* mutations have been localized to similar genomic regions, 72.70–101.44 cM and 47.80–107.76 cM, respectively, on chromosome 4H (Druka *et al.* 2011).

In this study, we report the characterization of *cer-ym* and the genetic mapping of *cer-ym* locus, and we report that *cer-ym* is a cutin defective mutant.

Materials and Methods

Plant materials

The barley mutant line *cer-ym.753* was originally induced in the cultivar ‘Bonus’ by ethylene oxide (Franckowiak and Lundqvist 2012). Seeds of Bowman-Near isogenic line carrying *cer-ym.753* (GSHO 2217, BC₄), named hereafter BW-NIL (*cer-ym.753*), and Bowman (PI 483237) were obtained from the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) (http://www.ars-grin.gov/npgs/acc/acc_queries.html). The BC₆ generation of GSHO 2217 was termed as BW144 in Druka *et al.* (2011) and not identical with GSHO 2217. *Hordeum vulgare* subsp. *spontaneum* accession ‘OUH602’ (wild barley) was obtained from the Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan.

Water loss measurements

A leaf-drying assay was conducted by sampling 2 cm sections of fully expanded leaves from BW-NIL (*cer-ym.753*) and Bowman at the one-leaf stage and keeping

them under laboratory conditions for 3 h. To test the leaf water loss rate, 8 cm lateral sections of fully expanded leaves were collected from BW-NIL (*cer-ym.753*) and Bowman at the end of the tillering stage, and kept under laboratory conditions. Leaf weight was recorded at 30 min intervals for 2 h and the percentage loss in fresh weight was presented as means ± SD for three biological replicates. To compare the water loss at the whole plant level, roots were removed from BW-NIL (*cer-ym.753*) and Bowman at the end of the tillering stage and the remaining shoot was kept under laboratory conditions for 24 h.

Transpiration rate assay

Distal 10 cm sections from fully expanded leaves were collected from BW-NIL (*cer-ym.753*) and Bowman at the end of the tillering stage. The cut end was immediately immersed in distilled water in a 2 mL tube and the junction between the leaf sample and the tube was sealed with Parafilm (American National Can). The leaf samples were placed at 26°C, 60% relative humidity in the light (108 μmol quanta s⁻¹ m⁻²) for 3 h, and then in darkness for 6 h. The combined weight of the tube, water and leaf was measured before and after different light conditions and the water loss by transpiration was calculated based on the weight loss. Leaf area was determined by analyzing leaf photographs taken after the treatments using Image J software (National Institutes of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>). The detached-leaf transpiration of each sample was expressed as weight (g) of water loss per unit area (m²) per hour (g water m⁻² h⁻¹) and shown as means ± SD for either three or four biological replicates.

Toluidine blue (TB) test of cuticle permeability

Approximately 2 cm long segments from fully expanded leaves of BW-NIL (*cer-ym.753*) and Bowman at the end of tillering stage were sampled and placed in 0.05% (weight/volume) aqueous toluidine blue (TB) stain (Solarbio) at room temperature for 3 h. After washing with distilled water, the leaf segments were imaged with a digital camera (Cannon PC1587).

Cutin monomer and wax analysis

Cuticular lipids from the middle 6 cm segments of fully expanded mature leaves of BW-NIL (*cer-ym.753*) and Bowman at the tillering stage were analyzed by gas chromatography-mass spectrometry/flame ionization detector (GC-MS/FID) and presented as means ± SD for four biological replicates. The leaf area was calculated using ImageJ software to evaluate digital photographs. Leaf waxes were extracted by immersing the leaf sample in chloroform for 20 s, before drying and derivatization for GC-MS/FID as described by Greer *et al.* (2007). The cutin components were prepared for GC-MS/FID analysis from the dewaxed samples by extended delipidation, depolymerization, and derivatization with N,O-bis(trimethylsilyl) trifluoroacetamide as described by Bessire *et al.* (2007).

Genetic mapping

A set of 123 F₂ plants from the cross OUH602 × BW-NIL (*cer-ym.753*) (GSHO 2217) were analyzed by exploiting a set of markers (AK358684, AK370363, AK248269, AK364461, AK251484 and AK364819) flanking the *cer-zv* locus on chromosome 4H (Li *et al.* 2013). Segments of detached leaves from the F₃ generation (16–20 progenies per F₂ line) of the mapping population were phenotyped for the severe-desiccation trait as described in the leaf drying assay. The genotype on *cer-ym* locus for each F₂ line was deduced from the phenotype of its F₃ progenies. Linkage map was constructed using Map Maker3 (Lander *et al.* 1987) and the Kosambi's mapping function (Kosambi 1944).

Results

cer-ym is sensitive to desiccation treatment

The BW-NIL (*cer-ym.753*) exhibited a semi-dwarf phenotype and a substantial reduction in visible wax crystals in leaf (Fig. 1A), similar to the phenotype of Bowman-near isogenic line carrying *cer-zv.268* allele (GSHO 2207, BC₄), named hereafter BW-NIL (*cer-zv.268*), which is a known cutin related recessive mutant that has a reduced water-retention capacity in its leaves (Li *et al.* 2013). We therefore hypothesized that the water-retention capacity might be similarly impaired in BW-NIL (*cer-ym.753*), and this was tested using several different experiments. When detached leaves were placed under laboratory conditions, the mutant leaves exhibited a severely desiccated appearance after 3 h of dehydration (Fig. 1B). The fresh weight of BW-NIL (*cer-ym.753*) leaves decreased by 41.8 ± 3.1% after 1 h and 69.9 ± 3.8% after 2 h of dehydration, compared to the Bowman leaf weight that only decreased by 7.1 ± 0.8% and 12.7 ± 1.9%, respectively (Fig. 1C). The BW-NIL (*cer-ym.753*) shoots from which the roots had been removed at the ending of the tillering stage also showed far more extensive desiccation (Fig. 1D) after 24 h of dehydration compared to the equivalent Bowman shoots, further indicating the hypersensitivity of *cer-ym* to desiccation.

cer-ym has a defective leaf cuticle

The transpiration rates of detached BW-NIL (*cer-ym.753*) and Bowman leaves were reduced substantially, but to a similar extent, in the absence of light (Fig. 1E), suggesting that the stomata of BW-NIL (*cer-ym.753*) leaves function normally. Leaf transpiration occurs primarily through the cuticle when stomata are closed in the dark (Niederl *et al.* 1998), and we observed that BW-NIL (*cer-ym.753*) leaf transpiration rate were considerably higher than those of Bowman leaves under both dark and light conditions (Fig. 1E), supporting the idea that *cer-ym* has a defective leaf cuticle. Increased permeability of BW-NIL (*cer-ym.753*) cuticles was further evidenced by strong staining of BW-NIL (*cer-ym.753*) leaves with TB after a 3 h of incubation, while Bowman leaves showed not such staining (Fig. 1F).

The *cer-ym* leaf cuticle shows a major reduction in the levels of cutin monomers

The cutin and waxes of the cuticle of mature leaves at the tillering stage were extracted and analyzed to help establish whether the absence of any particular component might explain the increased cuticle permeability of the *cer-ym* mutant. The four main cutin monomers in wild type barley leaves are: omega-hydroxy-9, 10-epoxy octadecanoic acid (ωOH-9, 10 epoxy C18); 9(10), 16-dihydroxyhexadecanoic acid (9(10), 16-OH C16); omega-hydroxyoctadecanoic acid (ωOH C18:1); and omega-hydroxyhexadecanoic acid (ωOH C16). We determined that the levels of all four monomers were substantially reduced (60%–90%) in BW-NIL (*cer-ym.753*) leaves compared to Bowman leaves (Fig. 2A). In contrast, the levels of 1-hexacosanol, the main wax component, which is thought to be an important marker for wax deposition, were significantly higher (~16%) in BW-NIL (*cer-ym.753*) leaves compared to Bowman (Fig. 2B). Based on these results we inferred that the increased permeability of BW-NIL (*cer-ym.753*) leaves is caused by an overall reduction in cutin polymer deposition.

cer-ym and *cer-zv* map to the same pericentromeric region

A set of *cer-zv* locus-flanking markers, positioned in the pericentromeric region of chromosome 4H, were used to locate the *cer-ym* locus. It was shown that *cer-ym* mapped within a 0.8 cM region between AK370363 and AK251484, and co-segregated with AK248269 and AK364461 (Fig. 3).

Discussion

The reduced leaf water-retention capacity of *cer-ym* can be attributed to a defective cuticle

The *cer-ym* leaves exhibited a substantially higher transpiration rate regardless of whether their stomata, which functioned normally, were open or closed, and the mutant leaves were more readily stained by TB than those of Bowman plants. In addition, the levels of the main cutin monomers showed a major decrease in the mutant leaves, while the primary wax component was slightly more abundant. Taken together, these results suggest that the reduced capacity of *cer-ym* leaves to retain water is caused by a severe reduction in the amount of leaf cutin.

The barley cutin mutants *eibil* and *cer-zv* have been reported to be hypersensitive to desiccation (Chen *et al.* 2011, Li *et al.* 2013). The desiccation-sensitive trait of *eibil* is associated with a cutin deficiency, which is caused by a lesion in a gene encoding an ABCG31 transporter (Chen *et al.* 2011). The *cer-ym* mutant has been thought to be distinct from *eibil* and to be caused by a mutation at a different locus from *eibil* (which is located on chromosome 3H) (Druka *et al.* 2011). Indeed, the monomer profiles of the two mutants are different since the main cutin monomer, ωOH-9, 10 epoxy C18, was reported to be reduced by only 50% in *eibil* (Chen *et al.* 2011), but it shows a more substantial decrease (~90%) in BW-NIL (*cer-ym.753*) (Fig. 2A).

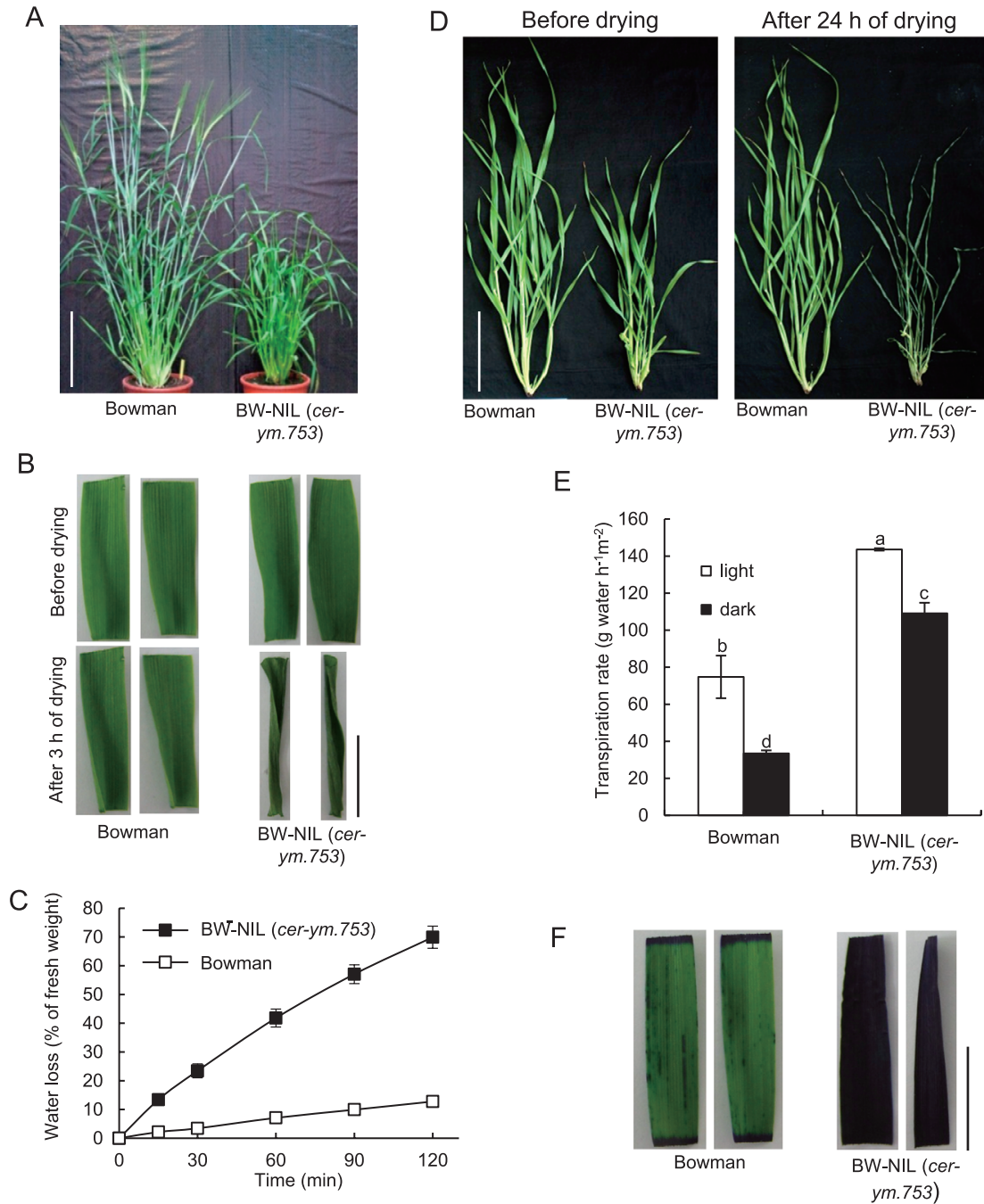


Fig. 1. Defective BW-NIL (*cer-ym.753*) leaf cuticle. (A) Contrasting morphologies of BW-NIL (*cer-ym.753*) (GSHO 2217) and Bowman. Scale bar, 10 cm. (B) Desiccation sensitivity of BW-NIL (*cer-ym.753*) and Bowman under laboratory conditions. Detached leaves after 3 h of drying. Scale bar, 1 cm. (C) Water loss rates of detached leaves. Error bars represent S.D. of biological replicates ($n = 3$). (D) Plants with the roots removed after 24 h of drying. Scale bar, 10 cm. (E) Average transpiration rate of detached leaves from BW-NIL (*cer-ym.753*) and Bowman under light and dark conditions. Different letters denote significant differences at $P < 0.05$. Error bars represent S.D. of biological replicates ($n = 3$ or 4). (F) Staining of leaf segments from BW-NIL (*cer-ym.753*) and Bowman with 0.05% (w/v) toluidine blue solution at room temperature. Scale bar, 1 cm.

BW-NIL (*cer-ym.753*) showed a similar degree of desiccation sensitivity to BW-NIL (*cer-zv.268*), which has previously been characterized as a desiccation sensitive mutant with impaired cutin deposition (Li *et al.* 2013). We found that the relative profiles of leaf cutin components of BW-NIL (*cer-ym.753*) and BW-NIL (*cer-zv.268*) are essentially

indistinguishable: the levels of ω OH-9, 10 epoxy C18, 9(10), 16-OH C16, ω OH C18:1 and ω OH C16, the four main leaf cutin monomers, were reduced by 91%, 78%, 70% and 63%, respectively, in BW-NIL (*cer-ym.753*), and were reduced by approximately 92%, 82%, 72% and 61%, respectively, in BW-NIL (*cer-zv.268*). Although the desiccation

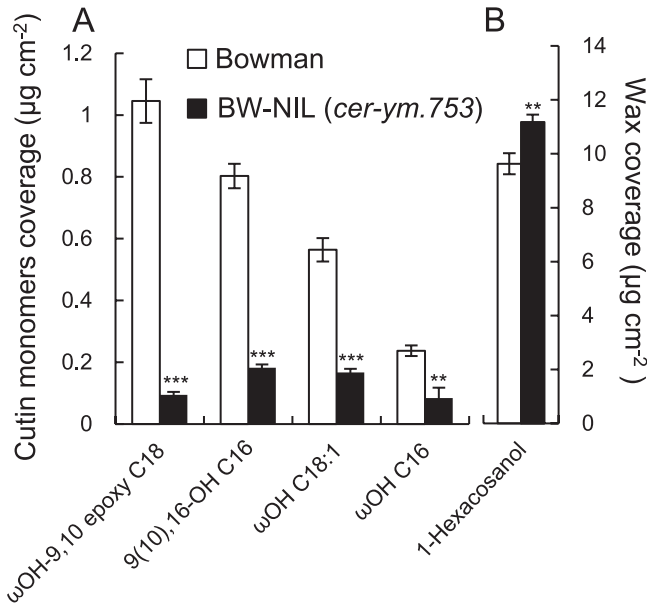


Fig. 2. Major cutin monomers (A) and wax component (B) of BW-NIL (*cer-ym.753*) and Bowman leaves. Error bars represent S.D. of biological replicates (n = 4). Two and three asterisks denote significant differences of means at P < 0.01 and P < 0.001, respectively, between Bowman and the mutant as determined by a student’s test.

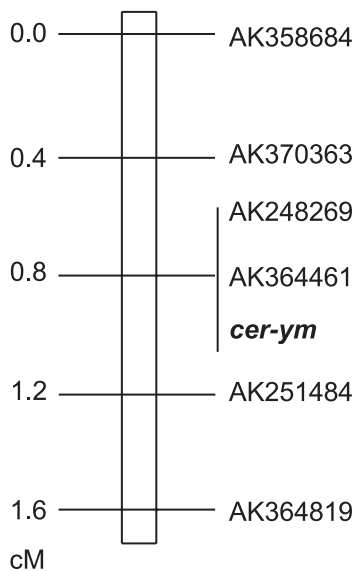


Fig. 3. Genetic map of the *cer-ym* locus. A set of 123 OUH602 × BW-NIL (*cer-ym.753*) F₂ progenies were analyzed with 6 genetic markers, which were previously positioned in pericentromeric region of chromosome 4H (Li *et al.* 2013). The genotype on *cer-ym* locus for each F₂ line was deduced from the phenotypes of its F₃ progenies.

sensitivity and cutin monomers contents were similar between *cer-zv* and *cer-ym* mutants, the former plants were weaker than the latter ones; most of the tillers were higher and stronger in *cer-ym* plants than in *cer-zv* plants (Supplemental Fig. 1). These results are consistent with that the *cer-zv* mutant shows more severe growth retardation than the *cer-ym* mutant (Franckowiak and Lundqvist 2012);

cer-zv plants are semidwarf and very weak (BGS 439 in <http://wheat.pw.usda.gov/ggpages/bgn/42/BGS478-571.htm>), while *cer-ym* plants are semidwarf and weak (BGS 526 in <http://wheat.pw.usda.gov/ggpages/bgn/42/BGS572-684.htm>).

The association of cutin component contents or organization with desiccation sensibility is also found in cuticle mutants of other species. Arabidopsis *lacs2* mutant leaves exhibit marked reductions in both C16 and C18 cutin monomers, and leaf desiccation sensitivity is much higher in *lacs2-3* than in the wild type (Bessire *et al.* 2007, Weng *et al.* 2010). Arabidopsis *dcr* mutant flowers display almost undetectable 9(10),16-dihydroxy-hexadecanoic acid, the main cutin monomer, and increased sensitivity to desiccation (Panikashvili *et al.* 2009). Tomato *cdl* mutant shows largely decreased levels in all cutin monomers and severe symptoms of desiccation (Isaacson *et al.* 2009). Rice *wall* mutant with no significant changes either in the composition and levels of waxes and cutin monomers shows a loose packing of the cuticle and increased rates of water loss (Park *et al.* 2010). Further studies are required to determine whether the *cer-ym* cuticle structure is also affected.

cer-ym mapped to a chromosome 4H pericentromeric region

cer-ym and *cer-zv* are two of the 79 *eceriferum* loci, which are confirmed by allelic tests (Lundqvist and Lundqvist 1988). Both the two mutant loci have been mapped to chromosome 4H (Druka *et al.* 2011, Li *et al.* 2013). In this current study, *cer-ym* were mapped co-segregated with AK364461, a marker that co-segregates with *cer-zv*, in the pericentromeric region where showed a very low recombination frequency (Mayer *et al.* 2011), suggesting an important role of this region for cuticle development.

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