Theor Appl Genet (2013) 126:637–646
DOI 10.1007/s00122-012-2007-3

ORIGINAL PAPER

# An *eceriferum* locus, *cer-zv*, is associated with a defect in cutin responsible for water retention in barley (*Hordeum vulgare*) leaves

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Received: 21 June 2012 / Accepted: 13 October 2012 / Published online: 4 November 2012


**Abstract** Drought limits plant growth and threatens crop productivity. A barley (*Hordeum vulgare*) ethylene imine-induced monogenic recessive mutant *cer-zv*, which is sensitive to drought, was characterized and genetically mapped in the present study. Detached leaves of *cer-zv* lost 34.2 % of their initial weight after 1 h of dehydration. The transpiration was much higher in *cer-zv* leaves than in wild-type leaves under both light and dark conditions. The stomata of *cer-zv* leaves functioned normally, but the cuticle of *cer-zv* leaves showed increased permeability to ethanol and toluidine blue dye. There was a 50–90 % reduction in four major cutin monomers, but no reduction in wax loads was found in the *cer-zv* mutant as compared with the wild type. Two $F_2$ mapping populations were established by the crosses of 23-19 × *cer-zv* and *cer-zv* × OUH602. More polymorphisms were found in EST sequences between *cer-zv* and OUH602 than between *cer-zv* and 23-19. *cer-zv* was located in a pericentromeric region on chromosome 4H in a 10.8 cM interval in the 23-19 × *cer-zv* map based on 186 gametes tested and a 1.7 cM interval in the *cer-zv* × OUH602 map based on 176 gametes tested. It co-segregated with EST marker AK251484 in both maps. The results indicated that the *cer-zv* mutant is defective in cutin, which might be responsible for the increased transpiration rate and drought sensitivity, and that the $F_2$ of *cer-zv* × OUH602 might better facilitate high resolution mapping of *cer-zv*.

Communicated by P. Langridge.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00122-012-2007-3) contains supplementary material, which is available to authorized users.

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## Introduction

Drought is a key ecological factor influencing food production and agro-ecosystems (Boyer 1982). Breeding for drought-tolerant cultivars is an approach that can increase overall food yields in an increasingly dry climate. Well-developed cuticles become determinant factors making plants survive in dry environments (Riederer and Schreiber 2001; Schulze et al. 1980). Plant cuticles not only provide a protective layer that helps plants defend against biotic and abiotic stresses, like drought and pathogens, but also play important roles in plant growth and development (Nawrath 2006). A plant cuticle consists of a cutin matrix, which is cross-linked to the polysaccharides of the epidermal cell wall, intracuticular waxes embedded in cutin matrix, and wax crystals deposited on the outside of the cutin layer (Nawrath 2006). The cuticular wax is primarily composed of very long chain (VLC) fatty acids and its derivatives

such as primary and secondary alcohols, aldehydes, alkanes, and ketones (Samuels et al. 2008). The typical cutin monomers are hydroxy and hydroxy epoxy C16 and C18 fatty acids (Pollard et al. 2008).

It was suggested that water deficit can induce the deposition of waxes and cutins, which in turn reduces the transpiration rates in some plants (Kosma et al. 2009; Shepherd and Wynne Griffiths 2006). Pea plants with more epicuticular waxes showed higher rain-fed harvest indices under drought conditions (Sánchez et al. 2001). A large number of genes involved in cuticle formation have been identified (Li et al. 2010), and overexpression of some of these genes in *Arabidopsis* and crop species exhibit a promising way to provide drought tolerance for transgenic plants. In *Arabidopsis*, overexpressing three *Arabidopsis thaliana* (At) transcriptional factor (TF) *SHN* clade genes *AtSHN1/2/3*, *A. thaliana* wax synthesis gene *ECERIFERUM1*(*CER1*), and *Medicago truncatula* (Mt) TF genes *MtWXP1* and *MtWXP2* all trigger wax synthesis and improve water deficit resistance (Aharoni et al. 2004; Bourdenx et al. 2011; Zhang et al. 2007). In crop species, overexpression of *MtWXP1* and *MtWXP2* in alfalfa (*Medicago sativa*) showed increased wax loads and enhanced drought tolerance (Zhang et al. 2005). Overexpression of *AtSHN2* gene, *Oryza sativa* (Os) *SHN*-like gene *OsWR1*, and VLC fatty acid synthesis gene *OsGLOSSY1-2* in rice all increased wax amounts and the capacity for drought resistance (Islam et al. 2009; Trijatmiko et al. 2005; Wang et al. 2012). But some studies failed to find a significant relationship between leaf cuticular wax and leaf transpiration rate (Larsson and Svenningsson 1986; Ristic and Jenks 2002). Fifteen barley *eceriferum* mutants of a cultivar Bonus with various amounts of epicuticular waxes showed similar transpiration rates (Larsson and Svenningsson 1986). The cuticular wax load of two maize lines did not show correlation with epidermal transpiration rates (Ristic and Jenks 2002). Cutins are suggested critical factors for water preservation in barley leaves of *eibi1* mutant (Chen et al. 2011), though it has been demonstrated that the cutin synthesis is also triggered in transgenic *Arabidopsis* plants when over-expressing *AtSHN1* (Kannangara et al. 2007), and *Arabidopsis* glycerol-3-phosphate acyltransferase gene *GPAT4* and *GPAT8* (Li et al. 2007). However, the relationship between cutin deposition and drought tolerance is largely unknown.

Thirty-seven genes directly involved in cutin deposition have been identified (Beisson et al. 2012): genes functioning in the synthesis of cutin monomers: *GPAT4, GPAT6*, *GPAT8, long chain acyl-coA synthetase 1* (*LACS1*), *LACS2*, *aberrant induction of type three gene 1* (*ATT1*), *LACERATA* (*LCR*), and *HOTHEAD* (*HTH*) (Kurdyukov et al. 2006b; Li-Beisson et al. 2009; Li et al. 2007; Lü et al. 2009; Schnurr et al. 2004; Wellesen et al. 2001; Xiao et al. 2004); genes in cutin monomer transport: ATP-binding cassette (ABC) transporter gene *ABCG11*, *ABCG13,* and *ABCG32/HvABCG31* (Bessire et al. 2011; Chen et al. 2011; Panikashvili et al. 2007, 2011); genes in cutin polymerization: *BODYGUARD* (*BDG*), *DEFECTIVE IN CUTICULAR RIDGES* (*DCR)* and *WILTED DWARF and LETHAL 1* (*WDL1*) (Kurdyukov et al. 2006a; Panikashvili et al. 2009; Park et al. 2010); genes in regulation: *ABNORMAL LEAF SHAPE 1* (*ALE1*), *ALE2,* and *CURLY FLAG LEAF 1* (*CFL1*) (Tanaka et al. 2001, 2007; Wu et al. 2011). Most of these genes were identified and characterized in *Arabidopsis*.

A total of 1,580 barley *eceriferum* (*cer*) mutants, expressing reduced or absent epicuticular wax crystals, were assigned to 79 loci (Lundqvist and Lundqvist 1988; Lundqvist and von Wettstein 1988), among which 27 were roughly mapped (Franckowiak 1997), but none of these genes were identified or even finely mapped. The mutant *cer-zv* showed glossy leaves, sheaths, and spikes, semi-dwarfed and very weak plants (Lundqvist et al. 1997). Here we report the characterization and genetic mapping of the *cer-zv* mutant.

## Materials and methods

### Plant materials

Seeds of Bowman and *cer-zv.268* isogenic line in Bowman (GSHO2207, $BC_4F_3$) were obtained from the United States Department of Agriculture, Agricultural Research Service (USDA-ARS). The *cer-zv.268* mutant was originally an ethylene imine-induced mutant in Foma. The *cer-zv.268* in Bowman is referred to as *cer-zv* throughout this report. Seeds were germinated in Petri dishes with wetted filter paper and kept at room temperature in the dark for 3–5 days, then transplanted to 2.5 l soil-filled pots, and grown to maturity in a greenhouse. Crosses were made between *cer-zv* and two wild barley accessions, 23-19 from the Institute of Evolution, University of Haifa, Israel, and OUH602 from the Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan. Dehusked caryopses of $F_1$–$F_3$ and parental lines were incubated in 70 % ethanol for 5 min, 1 % $H_2O_2$ for 5 min, wetted filter paper (4 °C in the dark) for 3–5 days, then transplanted to 2.5 l soil-filled pots and grown to maturity in a greenhouse.

### Leaf drying test

A 2 cm long leaf segment from the tip was dried under laboratory conditions at one-leaf stage. Leaf segments of the wild-type and heterozygous $F_2$ plants stayed fresh, but

leaf segments of *cer-zv* mutant-type $F_2$ plants desiccated after 90 min drying (Chen et al. 2004). About 8 cm long, newly expanded leaf segments were sampled from *cer-zv* and Bowman for leaf water loss rate assay. The weight of the leaf fragments was measured at designated times, following the procedure described by Chen et al. (2004). Each mean was calculated from four replicates.

### Whole plant drought tolerance test

One seedling of *cer-zv* and one seedling of Bowman were transplanted together in a 0.5 l soil-filled pot after germination, and grown to a three-leaf stage in the greenhouse with nine replicates. Afterwards, seedlings were subjected to drought stress by withdrawing water until all the leaves of the *cer-zv* were severely dehydrated, then re-watered, and observed for recovery.

### Transpiration rate assay

The distal 10 cm long segments of newly expanded leaves of *cer-zv* and Bowman were detached and the cut ends immediately inserted into 2 ml tubes filled with distilled water and sealed with Parafilm, and the tubes were placed under the following conditions: 26 °C, 60 % relative humidity, 108 (light) or 0 (dark) μmol quanta $s^{-1}$ $m^{-2}$. Water loss by transpiration was estimated by reduced weight from the tubes and was recorded at half an hour intervals. Finally, leaf area was measured by taking pictures, and area calculation was performed using Image J software (Gao et al. 2011). Detached-leaf transpiration was expressed as weight (g) of water lost per unit area ($m^2$) per hour (g water $m^{-2}$ $h^{-1}$), and each mean was calculated from four replicates.

### Chlorophyll leaching assay

About 10 cm long segments were taken from newly expanded leaves of *cer-zv* and Bowman, and leaf area was measured using the method described in the above transpiration rate assay. A pool of five leaf segments per line and three replicates were used. Leaves were inserted into 50 ml tubes filled with 80 % ethanol. Tubes were agitated gently for 24 h on a platform shaker. Aliquots of 1,000 μl were used for chlorophyll quantification at intervals of 10 min, 30 min, 60 min, 120 min, 180 min, and 24 h after initial immersion. The solution was measured at wavelengths 647 and 664 nm using a 752N UV–visible spectrophotometer (Huanghe, Shanghai) and calculated following the method described by Lolle et al. (1998). Chlorophyll efflux at each interval was expressed as a percentage of total chlorophyll extracted at 24 h.

### Toluidine blue (TB) test

About 2 cm long segments were taken from newly expanded leaves of *cer-zv* and Bowman. The segments were immersed in 0.05 % (weight/volume) aqueous TB (Solarbio) for 3 h. Then, leaf segments were washed with distilled water and photographed using a laser scanner.

### Cutin and wax content analysis

The middle 6 cm long segments of fully extended mature leaves of plants at the tillering stage were used for cutin and wax measurements with four replicates. The area of the leaf segments was measured using digital images, and then the materials were immersed in chloroform and agitated for 20 s. The extract was dried for derivatization and GC–MS/flame ionization detector (FID) analysis followed the method described by Greer et al. (2007). After wax extraction, the remaining leaf matter was delipidated, depolymerized, and derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide to allow GC–MS/FID analysis as described by Bessire et al. (2007).

### DNA isolation, PCR conditions

DNA was extracted from fresh leaves following the previously described method (Komatsuda et al. 1998). Each PCR mixture (10 μl) contained 20 ng of genomic DNA as the template, 1× Takara ExTaq buffer, 2.0 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.3 μM of each primer, and 0.25 U of ExTaq DNA polymerase (Takara, Tokyo) (0.6 μl DMSO if needed). PCR began with an incubation at 94 °C for 5 min, followed by 30 or 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 or 60 s, and a final extension of 72 °C for 5 min. Fragments were checked by standard agarose gel electrophoresis through 1–3 % (w/v) agarose (Iwai Kagaku, Tokyo).

### Molecular marker development and identification of chromosome location

Primer sets of 40 length polymorphism markers and 7 CAPS markers (6–7 loci per chromosome, 1–2 loci per 30–50 cM interval) of a barley high-density transcript linkage map (Sato et al. 2009) were applied to detect single nucleotide polymorphisms (SNPs) between 23-19 and *cer-zv*. The amplicons with no length polymorphisms between parents were sequenced to find Indels and SNPs for designing markers. Length polymorphism markers were applied to a 30 mutant phenotype (M-type) $F_2$ population of 23-19 × *cer-zv* to identify the chromosomal location of *cer-zv* locus. Another set of CAPS and dCAPS makers were designed from EST sequences assigned in chromosome 4H

centromeric region in a high-throughput SNP map (Close et al. 2009). The molecular markers used in the present study are listed in Table S1 (Online Resource 1).

### Genetic mapping of *cer-zv*

The markers assigned on chromosome 4H were first applied for mapping the *cer-zv* locus using 93 $F_2$ progenies of 23-19 × *cer-zv*. The phenotype of a *cer-zv* mutant-type $F_2$ plant was characterized by a desiccated leaf segment (a detached leaf segment after 90 min drying under laboratory conditions) at one-leaf stage, and confirmed by a dwarf plant, glossy spike, sheath, and leaf at the flowering stage. The genotype of *cer-zv* locus of an $F_2$ plant was analyzed by the leaf drying test with its $F_3$ plants. *cer-zv* was also mapped with 88 $F_2$ progenies of *cer-zv* × OUH602. Linkage maps were constructed using Map Maker3 (Lander et al. 1987), and Kosambi's mapping function was used to convert recombination frequencies into map distances. The genetic maps were constructed by MapChart (Voorrips 2002).

## Results

### *cer-zv* was sensitive to drought stress

*cer-zv* was a recessive mutant first induced in Foma by ethylene imine, characterized as semi-dwarf, totally absent epicuticular wax crystals in sheaths, leaves and spikes (Lundqvist et al. 1997). *cer-zv* was backcrossed to Bowman to generate a near isogenic stock GSHO 2207 (Lundqvist et al. 1997), referred to as *cer-zv* throughout this report. The *cer-zv* leaves were glossy and the *cer-zv* plants were weak (Fig. 1), based on what we expected—that mutant leaves have a lower capacity to retain water. A leaf drying assay was conducted to test this expectation. The results revealed that the mutant leaves were drying faster than Bowman leaves (Fig. 2a). The detached leaves from *cer-zv* plants lost 34.2 % of their initial weight after 1 h of dehydration, while those of Bowman lost only 3.2 %. And after 3 h of drying, *cer-zv* leaves showed severe desiccation (Fig. 2a, b).

The decreased water retention ability in mutant leaves might lead to a decreased drought tolerance in mutant plants. To do the drought tolerance assay, one mutant and one Bowman seedlings were grown together in a 0.5 l pot in a greenhouse with nine replicates. Mutant seedlings withered 3 days earlier than Bowman after withholding water. During 18 days of drought stress, mutant leaves showed more severe dehydration than Bowman (Fig. 2c). Leaves of mutant seedlings still withered while the leaves of Bowman were restored the next day after rehydration (Fig. 2d). The results showed that *cer-zv* mutant plants were more sensitive to drought stress than Bowman.

**Fig. 1** The plants of the *cer-zv* mutant (*right*) and its isogenic line Bowman (*left*)

### *cer-zv* was defective in leaf cuticle

Plant leaves transpire through the stomata and cuticle. Transpiration rates of detached leaves of *cer-zv* and Bowman decreased in response to darkness and increased in reactions to light within 30 min (Fig. 3a), meaning that *cer-zv* leaves had functional stomata. However, the transpiration rate of mutant leaves was much higher than that of wild-type leaves in both light and dark (Fig. 3a), suggesting a dysfunctional cuticle in mutant leaves. The transpiration assay indicated that the *cer-zv* leaves were defective in cuticle, which could explain the faster drying of mutant leaves as compared with wild-type leaves.

To confirm the defect in the cuticle of *cer-zv*, the mutant leaves were subjected to a chlorophyll leaching assay. The chlorophyll efflux rate of mutant leaves in 80 % ethanol was much higher than wild-type leaves (Fig. 3b). Mutant leaves had lost about 67 % of their chlorophyll, while the wild-type only lost about 3 % after 3 h of incubation. The great difference suggested that mutant leaves had a dramatic increase in the permeability of their cuticle.

A TB test was used to confirm the higher permeable cuticle in *cer-zv*. Mutant leaves were totally stained after 3 h of incubation in 0.05 % TB, while Bowman leaves had not been dyed (Fig. 3c), indicating easier penetration of TB

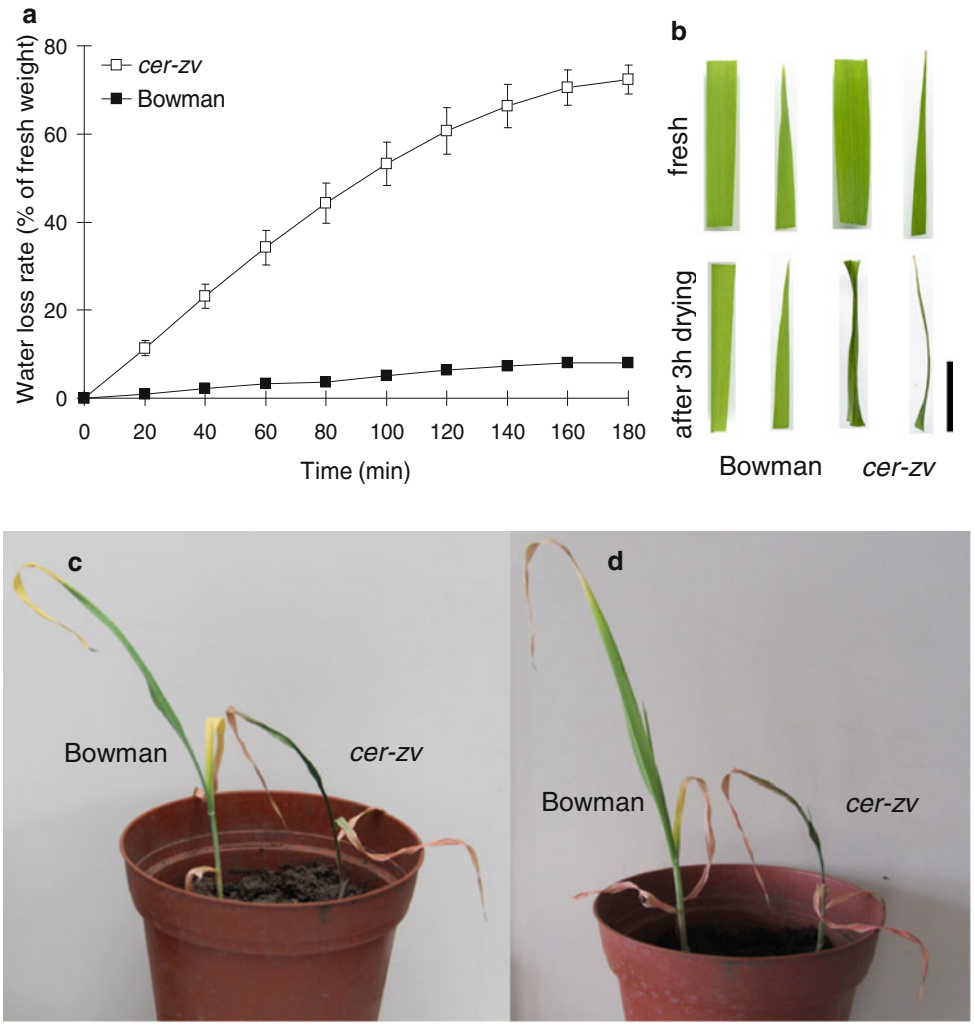


**Fig. 2** Drought sensitivity of *cer-zv* and Bowman. **a** Water loss rates of detached leaves from *cer-zv* and Bowman. Fragments of fully expanded leaves from seedlings of *cer-zv* and Bowman were cut from the distal part of the leaf blades. The fragments were dried abaxially side up on a laboratory bench under laboratory conditions. *Bars* represent mean ± SD ($n = 4$). **b** Detached leaves of *cer-zv* and Bowman after 3 h drying on a laboratory bench under laboratory conditions. *Scale bar* 1 cm. **c** *cer-zv* and Bowman seedlings after withholding water for 18 days in a greenhouse, a representative of nine pots. **d** The seedlings in **c** 1 day after rehydration

molecules through the mutant cuticle than the wild-type cuticle. Thus, the above results indicated that *cer-zv* was defective in leaf cuticle.

## *cer-zv* cuticle had a lower amount of cutin

Plant cuticle is composed of cuticular wax and cutin. We analyzed leaf wax and cutin composition in the mutant and wild type. Our results showed that leaf cutin monomers were altered in the *cer-zv* leaves (Fig. 4a). The most abundant cutin monomer in *barley* leaves was omega-hydroxy-9, 10-epoxy octadecanoic acid (ωOH-9, 10 epoxy C18) followed by 9(10),16-dihydroxyhexadecanoic acid (9(10), 16-OH C16), omega-hydroxyoctadecenoic acid (ωOH C18:1), and omega-hydroxyhexadecanoic acid (ωOH C16). Compared with wild-type leaves, the total cutin content per unit area was 80 % reduced, and all cutin monomers were 50–90 % reduced in *cer-zv* leaves. As to cuticular wax coverage (a total of 16 identified wax components), no significant differences were found between the *cer-zv* and Bowman leaves (Fig. 4b). Out of the 16 wax components identified in the mature leaves, 1-hexacosanol, the main barley cuticular wax component and a marker for estimating wax deposition (Richardson et al. 2005), and another nine minor wax components presented no significant differences between the *cer-zv* and Bowman, whereas the other six minor components showed higher loading in *cer-zv* than Bowman (Fig. S1). These results suggested that a *cer-zv* mutation had wide influence on cutin formation as well as the loading of some minor wax components.

## The *cer-zv* gene was located on chromosome 4H

*cer-zv* is a monofactorial recessive (Lundqvist et al. 1997) gene, which was confirmed by the leaf drying test of $F_2$ seedlings derived from the cross of 23-19 × *cer-zv* and *cer-zv* × OUH602 (Table 1). Homozygous mutant-type leaves were dried, but heterozygous and homozygous type leaves stayed fresh after 90 min drying under laboratory conditions. The segregation of this leaf's drying trait fitted

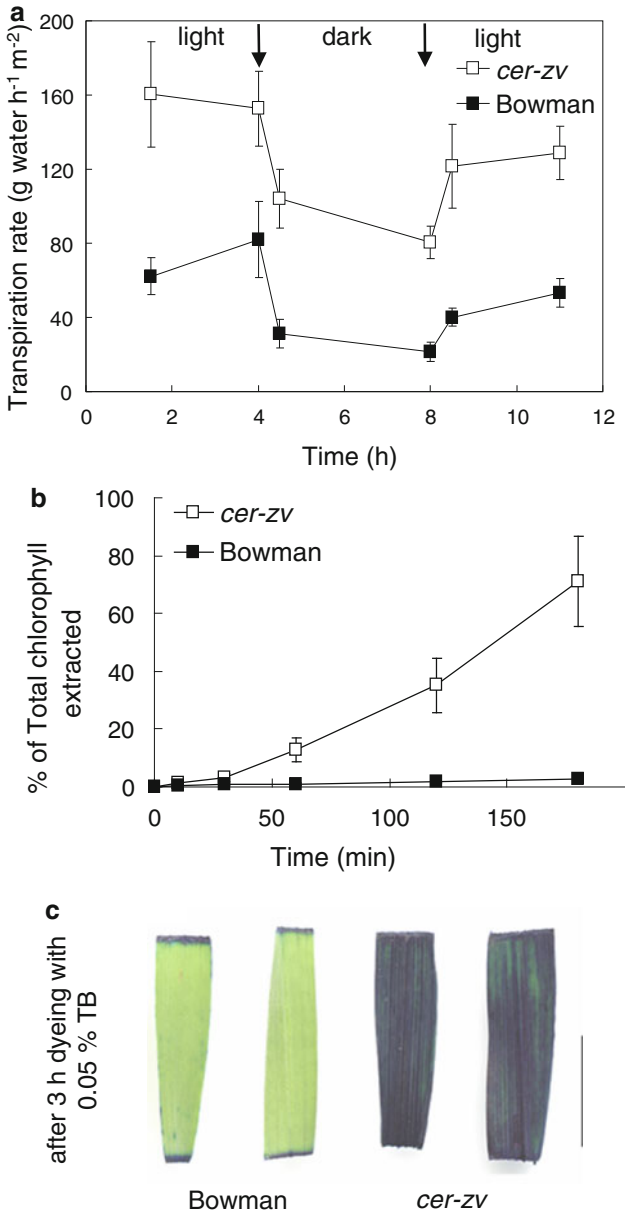


**Fig. 3** Defective cuticle in *cer-zv*. **a** Transpiration rate of detached leaves of *cer-zv* and Bowman. The distal parts of leaves were cut from *cer-zv* and Bowman plants and cutting ends were immediately inserted in distilled water in 2 ml tubes. The weight of a tube with a detached leaf was measured at designated times under light (0–4.0 h), dark (4.0–8 h), and light (8–11.5 h) sequentially. *Bars* represent mean ± SD ($n = 4$). **b** Chlorophyll extraction of *cer-zv* and Bowman leaves. Detached leaves were immersed in 80 % ethanol, and aliquots were removed at designated times to analyze the chlorophyll content extracted. *Bars* represent mean ± SD ($n = 4$). **c** Leaf segments of *cer-zv* and Bowman were stained for 3 h with 0.05 % (W/V) toluidine blue aqueous solution. *Scale bar* 1 cm

Mendelian expectation for a single-locus recessive trait, indicating that the *cer-zv* mutant was caused by a single recessive nuclear mutation. The *cer-zv* mutant is a dwarf. The dwarfism and cuticle phenotypes co-segregated in the $F_2$ generation.

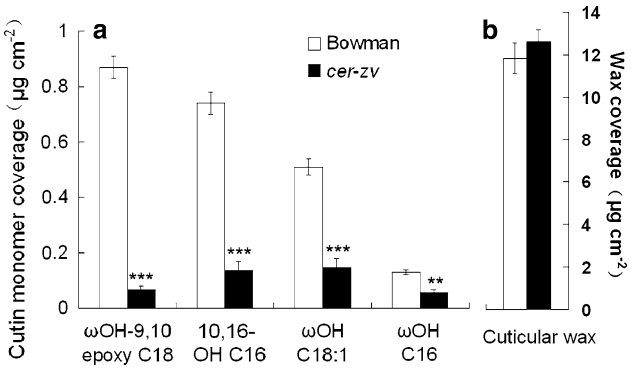


**Fig. 4** Major cutin monomers and cuticular wax coverage in *cer-zv* and Bowman leaf blades. *Bars* represent mean ± SD ($n = 4$). Two and three *asterisks* denote significant differences at $P < 0.01$ and $P < 0.001$, respectively, between wild type and mutant as determined by student's tests

**Table 1** Segregation of plants with the *cer-zv* phenotype in the $F_2$ of 23-19 × *cer-zv* and *cer-zv* × OUH602

| Crosses | Wild type | Mutant type | $\chi^2$ for 3:1 | *P* value |
|---|---|---|---|---|
| 23-19 × *cer-zv* | 141 | 49 | 0.063 | 0.80 |
| *cer-zv* × OUH602 | 105 | 39 | 0.333 | 0.56 |

**Table 2** Genetic linkage between *cer-zv* and EST markers selected from seven chromosomes

| Chromosome | Length-polymorphism markers[a,b] | Genotype[c] | | | $\chi^2$ for 1:2:1 |
|---|---|---|---|---|---|
| | | AA | AB | BB | |
| 1H | AK357398[a] | 10 | 8 | 11 | 5.90 |
| 2H | AK356967[a] | 6 | 9 | 15 | 10.2 |
| 3H | AK251437[a] | 11 | 9 | 10 | 4.87 |
| 4H | AK364371[b] | 0 | 2 | 28 | 74.8*** |
| 5H | BJ475227[a] | 8 | 11 | 11 | 2.73 |
| 6H | AK355622[a] | 9 | 15 | 5 | 1.14 |
| 7H | AK370329[a] | 7 | 8 | 14 | 10.0 |

Thirty individuals of homozygous *cer-zv* were selected from $F_2$ population of the cross between 23-19 and *cer-zv*. The population was the same as the 23-19 × *cer-zv* population presented in Table 1

*** Significant at 0.1 % probability level

[a] Derived from the barley high-density transcript linkage map (Sato et al. 2009), [b] derived from the present works, [c] AA homozygous 23-19, BB homozygous *cer-zv*, AB heterozygous

To locate *cer-zv* on a barley chromosome, one length polymorphism marker per chromosome was selected to genotype 30 mutant-type $F_2$ individuals derived from the 23-19 × *cer-zv* cross (Table 2). AK364371 near the centromeric region from chromosome 4H (Sato et al. 2009) showed a linkage with *cer-zv*, indicating that *cer-zv* mutation occurred on barley chromosome 4H.

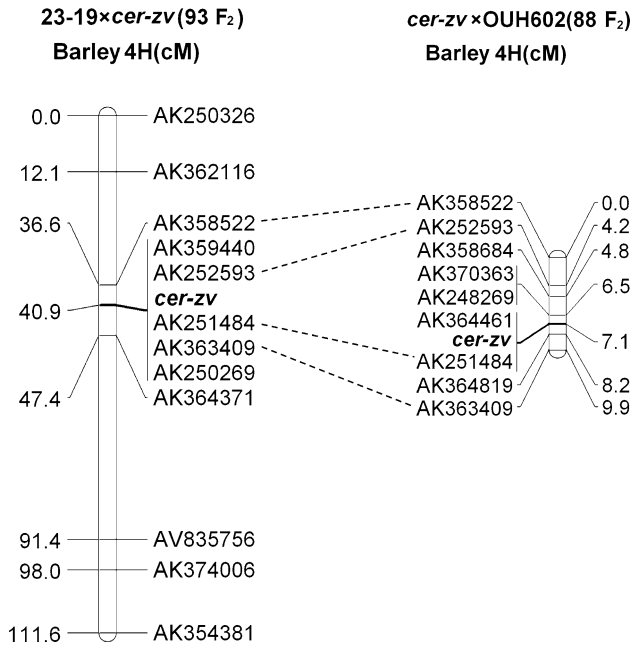


**Fig. 5** Molecular mapping of *cer-zv*. *cer-zv* was mapped on chromosome 4H pericentromeric region using 93 $F_2$ progenies of 23-19 × *cer-zv* (*left*) and 88 $F_2$ progenies of *cer-zv* × OUH602 (*right*)

EST markers surrounding AK364371 on chromosome 4H were selected from Sato et al. (2009) and Close et al. (2009) for mapping *cer-zv*. With 93 $F_2$ progenies of 23-19 × *cer-zv*, the *cer-zv* locus was mapped within 10.8 cM between AK358522 and AK364371, and co-segregated with AK359440, AK252593, AK251484, AK363409, and AK250269 (Fig. 5).

To confirm the genetic location of *cer-zv*, 88 $F_2$ progenies of *cer-zv* × OUH602 were used for mapping *cer-zv*. *cer-zv* was located within 9.9 cM between AK358522 and AK363409. AK364371 was not mapped due to monomorphism between *cer-zv* and OUH602. *cer-zv* was further mapped within 1.7 cM between AK370363/AK248269 and AK364819 in the $F_2$ progenies, and co-segregated with AK364461 and AK251484 (Fig. 5).

AK251484 co-segregated with *cer-zv* in both maps, indicating consistent genetic location of *cer-zv* in barley genetic maps. Both AK252593 and AK363409 recombined with *cer-zv* in *cer-zv* × OUH602 map separating the two markers by 5.7 cM, whereas they co-segregated with *cer-zv* in 23-19 × *cer-zv* map, indicating a higher recombinant rate in *cer-zv* × OUH602 cross than 23-19 × *cer-zv* cross in the *cer-zv* region. In addition, out of 21 ESTs used for developing markers, 15 were found polymorphic between OUH602 and *cer-zv*, while only 5 showed polymorphism between 23-19 and *cer-zv*. In this sense, OUH602 and *cer-zv* had higher polymorphism than 23-19 and *cer-zv*.

## Discussion

### The drought sensitivity of *cer-zv* plants is attributed to a defective cutin

The *cer-zv* plants were found sensitive to drought stress which could possibly be explained by excessive water loss from the leaves (Fig. 2). The increased transpiration in *cer-zv* leaves was caused by a dysfunctional cuticle, which was confirmed by the increased permeability to ethanol and dye in *cer-zv* leaves (Fig. 3). The amount of cutin was dramatically decreased but the total amount of 16 identified wax components was not significantly changed in *cer-zv* leaves (Fig. 4). One may infer that the *cer-zv* mutant is defective in a cutin matrix. Out of 16 identified wax components, 6 minor components increased (Fig. S1), which might lead to the disorganization of cuticular waxes. Considering the weak relationship between transpiration rates and cuticular waxes in 15 barley *cer* mutants of a cultivar Bonus (Larsson and Svenningsson 1986), the drought sensitivity of *cer-zv* plants might be attributed to a defective cutin.

The *eibi1* mutant is the first cutin mutant identified in barley (Chen et al. 2011). The *cer-zv* mutant is a new barley cutin mutant because *eibi1* and *cer-zv* are located on different barley chromosomes, 3H and 4H, respectively. The two barley cutin mutants show different degrees of cutin deficiency: ωOH-9, 10 epoxy C18 acid, the main cutin monomer, displayed a 90 % reduction in *cer-zv*, much more than the 50 % reduction in *eibi1*. However, the cuticle permeability is higher in *eibi1* than *cer-zv* mutant: *eibi1* and *cer-zv* detached leaves lose water about 65 and 35 % of fresh weight after 1 h of dehydration, respectively.

In the other monocot plants, a few cutin mutants have been identified. The *Sorghum bicolor bloomless2* (*bm2*; previously "*bm22*") mutants display a reduced cuticle thickness and increased water loss (Jenks et al. 1994; Peters et al. 2009). The maize *GLOSSY1* (*gl1*) mutant cuticle thickness is reduced by about 50 %, and the cuticle proper is almost absent (Sturaro et al. 2005). However, cuticle permeability is not affected by the *gl1* mutations. The rice *wilted dwarf and lethal 1* (*wdl1*) mutant has a loosely packed cuticle and an irregular thickness of the cell wall, which is associated with increased water loss from *wdl1* mutant leaves (Park et al. 2010).

In dicot plants, mutants with decreased cutin and increased cuticle permeability in leaves are found in *Arabidopsis*. For example, *lacs2* mutant leaves show a reduced thickness of the cutin layer on the abaxial surface, an increased permeability to chlorophyll in 80 % ethanol (Schnurr et al. 2004), a strong reduced content of cuticular polyester, and an enhanced water loss (Bessire et al. 2007). The *dcr* mutant leaves are absent in the major cutin

monomer, 9(10), 16-dihydroxy-hexadecanoic acid, and show enhanced permeability to water (Panikashvili et al. 2009) and toluidine blue (Tanaka et al. 2004). Double knockouts *gpat4/gpat8* are strongly reduced in cutin aliphatic monomer content, leading to a fourfold greater water loss rate for *gpat4/gpat8* compared with WT (Li et al. 2007). *gpat4/gpat8* mutant had a strong increase in cuticle permeability to toluidine blue and displayed an increased sensitivity to the necrotrophic fungal pathogen *Alternaria brassicicola*. These results as well as our results proved that the cutin layer is crucial to a functional cuticle and to water retention in plant leaves.

Epicuticular wax crystals appear absent in the *cer-zv* mutant, showing a glossy phenotype on the leaf, the spike, and the stem under the field conditions (Lundqvist et al. 1997). The Bowman leaves, before boot stage, were also showing a similar glossy phenotype as *cer-zv* leaves, when the plants were growing in a greenhouse (the leaves on the young tillers in Fig. 1). The wax content analysis was conducted at the tillering stage, at which stage the glossy phenotype was not significantly different between *cer-zv* and Bowman (Fig. S2). Out of the 16 identified wax components, not one component decreased in the *cer-zv* mutant, only 6 minor components increased in the *cer-zv* mutant compared with the wild type (Fig. S1). The similar amount of wax between *cer-zv* and Bowman leaves (Fig. 4) might account for the similar glossy phenotype between *cer-zv* and Bowman at tillering stage. The decreased cutin loading might lead to a defective cuticle structure, which might affect surface wax coating in *cer-zv* mutant, leading to a glossy phenotype even after booting stage (Fig. 1). The wax amount of rice *wdl1* mutant cuticle is the same as that of its wild-type cuticle, so are the wax components, but the *wdl1* mutant has a loose and irregular cuticle and disorganized epicuticular wax coating (Park et al. 2010). Cutin and other covalently bound lipids attached to the cell wall also show no significant changes between *wdl1* and wild type. It is proposed that *WDL1* is involved in cutin organization, affecting depolymerizable components (Park et al. 2010). Sometimes, increased wax deposition causes a glossy phenotype. For example, overexpression of *SHN1/WIN1*, *SHN2*, *SHN3*, and *WXP1* dramatically enhanced wax accumulation in transgenic plants and resulted in a strikingly glossy leaf phenotype (Aharoni et al. 2004; Broun 2004; Zhang et al. 2007). Therefore, factors other than wax levels, possibly its composition or alteration of the overall surface structure, result in the glossy leaf surface phenotype. To understand the glossy phenotype in *cer-zv*, we should do detailed scanning electron microscopy analysis of leaf surface in a further study.

### *cer-zv* maps to the centromeric region of chromosome 4H

In the *cer-zv* × OUH602 map (Fig. 5), the co-segregating marker AK364461 and the flanking markers AK370363/AK248269 and AK364819 correspond to the markers 2_0831, 2_0853/2_1442 and 3_0605, which are all located at 48.72 cM in the genetic centromeric region on chromosome 4H in the barley genome zipper (Mayer et al. 2011). However, the second co-segregating marker, AK251484, corresponds to the marker 1_1042 which is located at 51.3 cM out of the genetic centromeric region. One might infer that *cer-zv* may be located in the pericentromeric region of chromosome 4H.

*cer-zv* co-segregated with AK251484 on chromosome 4H in two independent maps (Fig. 5), but in a large interval (10.8 cM) in 23-19 × *cer-zv* map, as against a small interval (1.7 cM) in *cer-zv* × OUH602 map. AK252593 and AK363409 co-segregated with *cer-zv* in the former map, but recombined with *cer-zv* in the latter one. Furthermore, increased polymorphism was found in EST sequences between *cer-zv* and OUH602 rather than between *cer-zv* and 23-19. Therefore, the $F_2$ of *cer-zv* × OUH602 might better facilitate high resolution mapping and identification of *cer-zv*.

**Acknowledgments** We thank Drs. Y. Nagamura, J. Song and Mrs. Y. Ma for their helpful and constructive contributions. We thank Mrs. R. Permut for English editing. This research was supported by the One Hundred Talents Project of the Chinese Academy of Sciences (O827751001) and the National Natural Science Foundation of China (30970449, 31170369, and 31160036), and Genomics for Agricultural Innovation of the Ministry of Agriculture, Forestry, and Fisheries of Japan (TRG1007), as well as by the Swiss National Science Foundation (grant 31003A-125009 to C.N.).

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