running head: barley HvKCS6 controls Bgh germination

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Evolutionary conserved function of barley and Arabidopsis 3-KETOACYL-CoA SYNTHASES in providing wax signals for germination of powdery mildew fungi

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One-sentence summary:
An orthologous pair of genes from barley and Arabidopsis is involved in production of a very long chain fatty acid signaling required for germination of conidia from distantly related powdery mildews.
footnotes:

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Abstract

For plant pathogenic fungi such as powdery mildews, which survive only on a limited number of host plant species, it is a matter of vital importance that their spores sense that they landed on the right spot to initiate germination as quick as possible. We investigated a barley (Hordeum vulgare) mutant with reduced epicuticular leaf waxes on which spores of adapted and non-adapted powdery mildew fungi showed reduced germination. The barley gene responsible for the mutant wax phenotype was cloned in a forward genetic screen and identified to encode a 3-KETOACYL-CoA SYNTHASE (HvKCS6), a protein participating in fatty acid elongation thus and required for synthesis of epicuticular waxes. GC-MS analysis revealed that the mutant has significantly less aliphatic wax constituents with a chain length above C24. Complementation of the mutant restored wild type wax and overcame germination penalty, indicating that wax constituents less present on the mutant are a crucial clue for spore germination. Investigation of Arabidopsis (Arabidopsis thaliana) transgenic plants with sense silencing of AtCUT1, the HvKCS6 ortholog, revealed the same germination phenotype against adapted and non-adapted powdery mildew fungi. Our findings hint to an evolutionary conserved mechanism for sensing of plant surfaces among distantly related powdery mildews which is based on KCS6-derived wax components. Perception of such a signal must have been evolved before the monocot-dicot split took place approximately 150 million years ago.
Introduction

Aerial parts of plants are usually covered with a cuticle. This interface between an organism and the environment has developed in ancient times as a prerequisite for pioneering plants when leaving their water home and occupying dry land (Bargel et al., 2004). Besides its unquestionably important function in protecting plants from desiccation, cuticles represent also the outermost barrier which shelter plants against pathogen and pest attacks. The cuticle has a water repellent effect which, from the physical point of view, enhances slipperiness and thereby impedes the ability of non-specialized microbes to get in touch with a potential host (Howe and Schaller, 2008). Thus, in the course of evolution, pathogens had to elaborate strategies to cope with the cuticle barrier in order to become a successful invader. During this process, however, some pathogens might have started to utilize cuticle-derived signals in a more sophisticated fashion as a clue to initiate accelerated germination only in the presence of potential plant hosts (Lapin and Van den Ackerveken, 2013).

Generally, the cuticle is composed of the cutin polymer matrix and cuticular wax. Cuticular wax is embedded within the cutin polymer (intra-cuticular wax) and on the outer surface, (epicuticular wax). Epicuticular wax often, but not in all species, forms three-dimensional crystallites as e.g. in barley (Jetter et al., 2000; Kunst and Samuels, 2003; Bargel et al., 2004). Cutin is a covalently cross-linked polymer made of saturated C16 hydroxy- and partially unsaturated C18-hydroxy and -epoxy fatty acids (Bargel et al., 2004). Cuticular waxes, by contrast, are complex, organic solvent-extractable, mixtures of monomeric C20 to C60 aliphatics that may include triterpenoids, phenylpropanoids and flavonoids (Samuels et al., 2008). The composition of intra- and extracellular waxes is extremely variable and may differ between plant species, between organs within a given species, and also between developmental stages of plant organs (Post-Beittenmiller, 1996; Bargel et al., 2004). Wax biosynthesis starts in leucoplasts, the photosynthetically inactive plastids of the epidermis, with de novo synthesis of C16 and C18 fatty acids. These are subsequently elongated and modified in the endoplasmic reticulum at first to very-long-chain fatty acids (VLCFAs, C20 to C34) and then to alcohols, aldehydes, esters, alkanes and ketones (Samuels et al., 2008). The plastidial fatty acid synthesis as well as the extension to VLCFAs is carried out by multi-enzyme complexes termed fatty acid synthases (FASs) and fatty acid elongases (FAEs), respectively, both consisting of four dissociable enzymes catalyzing consecutive enzymatic reactions (Samuels et al., 2008). For elongation, C2 units derived from malonyl-CoA are
added to fatty acids or VLCFAs by the FAE complex, involving a condensation, reduction, dehydration and a second reduction step. Several cycles are needed to yield chain lengths of e.g. C24 to C34. Each of those cycles seems to be carried out by different FAE complexes, distinct by individual β-KETOACYL-CoA SYNTHASEs (KCS) as their specificity lies in the KCS condensing enzyme (Samuels et al., 2008; Chen et al., 2011; Haslam and Kunst, 2013). Therefore, it is not surprising that a large family of KCSs exists, e.g. 21 KCS-like sequences have been identified in Arabidopsis thaliana (Arabidopsis) (Kim et al., 2013). However, only for two of these enzymes (AtKCS1 and AtCER6/AtCUT1/AtKCS6) the function in elongation of epicuticular wax VLCFAs was experimentally verified so far (Millar et al., 1999; Todd et al., 1999; Fiebig et al., 2000).

Our current understanding of the wax biosynthetic pathway and the precise function of particular enzymes is still limited, although genetic as well as biochemical approaches had been used widely. Thereby, forward genetic screens profited from the easy to score visual phenotype of mutants with little or no wax coating on aerial plant organs which appear glossy or glaucous. In barley, these mutants are termed eceriferum (Latin: cera = wax and ferre = to bear) and cer is used as symbol for the respective gene loci (Lundqvist and von Wettstein, 1962). The Arabidopsis community followed this terminology and today 85 and 22 cer loci have been identified in barley and Arabidopsis, respectively (Kunst and Samuels, 2003). However, since these mutants are rather affected in their total wax load then in differences among wax components, it is unlikely that they cover the whole wax biosynthetic pathway (Post-Beittenmiller, 1998). In Arabidopsis, this gap was closed by reverse genetic approaches which enabled the identification of additional genes involved in cuticular wax biosynthesis (Kunst and Samuels, 2003). Sequence homologies to some of these genes were used to clone candidate genes of barley (Richardson et al., 2007). However, since forward and reverse genetic data are missed, gene-function relationships in barley are still speculative.

Airborne plant pathogens, regardless whether they are dispersed by water or wind, have to cope with the problem of adhesion to the waxy surface of their host plants. In case of water-disseminated pathogens such as Magnaporthe oryzae or Colletotrichum graminicola this is achieved by release of glue, whereas for wind-disseminated pathogens like powdery mildews the initial contact can be secured by hydrophobic interactions between conidiospore and leaf surface (Epstein and Nicholson, 2006). Nevertheless conidial extracellular material might also help in this case to attach a conidiospore to the plant surface (Wright et al., 2002). Upon attachment, pathogens have to overcome the cuticle and the epidermal cell wall barriers to
reach the nutrient pool of the plant interior. This process is referred to as penetration that might involve the generation of high turgor pressure in dedicated cells, so-called appressoria, to mechanically breach the cell wall, eventually supported by the activity of hydrolytic enzymes (Tucker and Talbot, 2001). For example in *Blumeria graminis* f. sp. *hordei* (*Bgh*), the barley powdery mildew fungus, a combination of turgor-driven and enzymatic-supported penetration has been suggested (Pryce-Jones et al., 1999). Generally, powdery mildews are highly specialized biotrophic pathogens infecting and reproducing only on living tissue of a limited range of plant species (Both and Spanu, 2004). The disease cycle of *Bgh*, which uses barley as sole host, starts with the attachment of a conidiospore to the leaf surface and the formation of a primary germ tube (Carver et al., 1995). In addition, an appressorial germ tube is formed giving rise at its tip to an appressorium from which the fungus penetrates the underlying tissue. In case of success, the fungus invades an epidermal cell and forms his feeding organ, the so-called haustorium, while leaving the plasma membrane intact. Thereafter, the pathogen develops secondary hyphae on the leaf surface and continues to penetrate neighboring epidermal cells. The fungus completes its life cycle by building conidial mother cells and emergence of novel conidiospores (Eichmann and Hückelhoven, 2008). Right after initial contact, *Bgh* conidiospore prepare an ‘infection court’ at the interface to their host and Epstein and Nicholson (2006) speculated that at this space of tight adherence the concentration of lytic enzymes such as cutinases (Pascholati et al., 1992) effectively could be maintained at higher levels. This enzymatic activity may lead to the release of monomeric or oligomeric degradation products, e.g. cutin monomers, which can act as damage-associated molecular patterns (DAMPs) and trigger defense responses (Schweizer et al., 1996; Tucker and Talbot, 2001). The involvement of epicuticular wax components in defense was shown in a recent study in which silencing of a cytochrome P450 gene, involved in generation of VLCFA derivatives such as secondary alcohols and -ketones, diminished penetration resistance of barley against *M. oryzae* (Delventhal et al., 2011). By contrast, cutin monomers may also contribute to disease susceptibility as they e.g. in *Bgh* and *Ustilago maydis* play a role in appressorial germ tube and appressorium formation, respectively (Francis et al., 1996; Mendoza-Mendoza et al., 2009). For the wheat powdery mildew fungus, it was demonstrated that the expression of a secreted lipase involved in release of epicuticular wax components from infected wheat leaves was important for fungal adhesion and development (Feng et al., 2009). Further fatty acid components supporting pathogen development rather than acting as DAMPs are long-chain and very long-chain alcohols and aldehydes from the epicuticular wax layer which play a crucial role in spore germination of *Bgh* (Hansjakob et al., 2010), *Puccinia*
emaculata, Phakopsora pachyrhizi and Colletotrichum trifolii (Uppalapati et al., 2012). For the latter three pathogens, a gene involved in generation of respective wax compounds was cloned from Medicago truncatula and turned out to be a transcription factor affecting wax biosynthetic genes. For Bgh, however, no wax biosynthetic pathway gene involved in spore germination has been cloned so far from its intrinsic barley host. Thus, evidence for the involvement of VLCFA derivatives in Bgh germination comes from experiments with nonhost wax-mutant plants such as maize or Arabidopsis and from chemical complementation with particular VLCFA derivatives (Hansjakob et al., 2010; Weis et al., 2014). An important issue that therefore could not be touched so far is the question whether the reduced Bgh conidial germination rate affects disease severity.

Here, we close this long-lasting gap by identifying the barley 3-KETOACYL-CoA SYNTHASE gene HvKCS6 as being required for germination of Bgh conidiospores on its host. We verified by genomic complementation of the respective mutant and GC-MS analysis of wax components, that this gene encodes a condensing enzyme which is part of the fatty acid elongation complex and has a presumed specificity for elongation of C24 to C26 VLCFAs. Comparative analyses with Arabidopsis revealed a conserved function of the orthologous gene in providing essential signals for germination of conidiospores from different powdery mildew species. Using compatible host-pathogen combinations, we showed that on barley and Arabidopsis wax-mutant plants a reduced germination rate of powdery mildew conidiospores finally resulted in less frequently formed disease symptoms, thus open the road to a novel breeding trait.

Results

Germination of Bgh conidiospores is compromised on barley mutant emr1

The barley mutant emr1 (enhanced Magnaporthe resistance gene 1), generated in our lab, was identified in a suppressor screen for restoration of resistance against Magnaporthe oryzae in the hypersusceptible genetic background Ingridmlo5 (Jansen et al., 2007). Based on visual scoring of disease symptoms, no phenotypic response was observed against other important barley leaf pathogens as e.g. powdery mildew, net blotch, leaf scald or rust (Jansen and Schaffrath, 2009). For powdery mildew, however, the presence of the strong resistance allele mlo5 could have masked potential effects. Therefore, we re-evaluated the interaction between Bgh and emr1 mutant plants using a microscopic assay and analyzed the formation of initial
infection structures as depicted in Supplemental Figure S1. In this experiment, the percentage of conidiospores which did not germinate on leaves of emr1 mutant plants was almost twice as high (33%) in comparison to leaves of its ancestor Ingridmlo5 (18%) (Figure 1A). Germinated conidiospores gave rise to mature appressoria at a similar rate on both genotypes, i.e. on Ingridmlo5 plants 82% germination and 72% appressoria and on emr1 mutant plants 67% germination and 55% appressoria. Thus, apart from compromised germination, no further differences were found in the pre-penetration process of Bgh. These results demonstrate that the emr1 mutant exhibits two different phenotypes, one of which is the enhanced resistance against M. oryzae and the other a reduction in the germination frequency of Bgh conidiospores. The following experiments were designed to answer the question whether both phenotypes are conferred by the same mutation and to identify the underlying gene(s).

**emr1 is depleted in leaf surface waxes**

During inoculation a higher capacity for water retention was observed on leaves of the emr1 mutant in comparison to other barley cultivars (Figure 2A). This observation together with a glossy appearance of emr1 leaves was reminiscent of barley eceriferum (cer) mutant plants with altered leaf wax coating (Post-Beittenmiller, 1996). We applied scanning electron microscopy (SEM) to further investigate this phenomenon and found a strong reduction of wax crystals on the leaf surfaces of emr1 mutants compared to those of Ingridmlo5 plants (Figure 2B). Taking a closer look, it appears as if particular, smooth platelet-like, structures of wax crystals are absent on mutant leaves. Applying gaschromatographic-mass spectrometry analysis (GC-MS), the difference between Ingridmlo5 and emr1 mutant plants in total content of cuticular leaf wax could be quantified to 10 µg cm⁻² and 2.4 µg cm⁻², respectively (Figure 2C).

Based on the SEM pictures, we speculated that emr1 mutants lack particular components of the epicuticular leaf wax. We followed this thought in more depth by detailed GC-MS analysis of individual wax components. Strikingly, we determined that the most abundant component among barley cuticular leaf waxes, the C26 alcohol (hexacosanol), was reduced by 90% on primary leaves of emr1 mutant plants compared to IngridMLO and Ingridmlo5 plants, respectively (Figure 3, see Supplemental Figure S2). Similar to the decrease in hexacosanol content also the amount of the C26 aldehyde (hexacosanal) dropped from 0.3 µg cm⁻² on Ingridmlo5 plants to 0.007 µg cm⁻² on emr1 plants. Concomitant with the reduction in
wax components with C26 chain lengths an increase of the C24 alcohol (tetracosanol) was measured on *emr1* (Figure 3), suggesting a block in elongation of VLCFAs from C24 to C26 chain lengths in *emr1* plants. This hypothesis is supported by the observation that C26-based components were also absent in wax esters of *emr1* mutant plants (see Supplemental Figure S2). Interestingly, *emr1* plants seem to compensate the lack of C26 constituents in esters by forming novel alcohol-acid combinations to keep the total chain length (as sum of alcohol and acid). For example in Ingrid*mlo5* plants the ester with a chain length of C46 is built from the C26 alcohol and the C20 acid while in *emr1* mutant plants the C46 ester is made from the C24 alcohol and the C22 acid or the C22 alcohol and the C24 acid (see Supplemental Figure S2).

**Mapping and in silico identification of the gene responsible for the wax phenotype**

On the basis of the observation that *emr1* leaves are depleted in cuticular VLCFA derivatives and in the light of work published by Hansjakob and co-workers who demonstrated that the presence of C26 alcohols and C26 aldehydes, severely affects the ability of *Bgh* to germinate *in vitro* and *in vivo* (Hansjakob et al., 2010; Hansjakob et al., 2011), we decided to follow the reduced wax phenotype in a mapping approach and to analyze thereafter whether the gene mutation identified would explain the *Bgh* phenotype.

For mapping, we used the cross between *emr1* and Grannenlose Zweizeiligem*mlo11* that we described previously in Jansen et al. (2007). During genetic segregation analysis among F2 individuals and derived F3 offspring, fifteen individuals were found in which higher water retention and enhanced *Magnaporthe* resistance, *emr1 sensu stricto*, did not co-segregate, indicating two independent gene loci. For practical reasons, we designated the gene locus responsible for the wax phenotype as LOW WAX 1 (*LWA1*) and the mutant allele accordingly as *lwa1*. Plants homozygous for one of these two mutations and bearing the wild type allele for the other one were selected from the F3 offspring. Inoculation of these plants with *Bgh* conidiospores revealed that the *lwa1* but not the *emr1* allele is responsible for the impaired *Bgh* germination (Figure 1B). Segregation of *lwa1* was followed in 92 F2 plants by water-spraying and differentiation between retention of small or big water droplets on the leaf surface (Figure 2A). The *emr1* phenotype was followed by inoculation of plants with *M. oryzae*. For single nucleotide polymorphism (SNP) genotyping, genomic DNA was extracted from all 92 F2 plants and subjected to a 384 multiplex SNP GoldenGate VeraCode barley assay. After genotyping, a linkage map of chromosome 4H containing *lwa1*, *emr1* and linked SNP markers was constructed (Figure 4, see Supplemental Table S1). SNP markers suggested
map positions for the *lwa1* and *emr1* gene loci on different arms of barley chromosome 4H. For further characterization of the *LWA1* gene, we employed the co-linearity in the grass genomes (Bennetzen and Freeling, 1997). Using the web-tool GenomeZipper (Mayer et al., 2009) the rice gene Os03g0219900 (RAP Os-ID) was identified as being syntenic to the closest barley iSELECT marker 4139-888 (syn. BOPA 1_0606). The rice gene next to Os03g0219900 is Os03g0220100 (LOC_Os03g12030) encoding a protein with a predicted 3-KETOACYL-CoA SYNTHASE activity. The syntenic region of barley 4H for this gene was identified as locus 1751 (flcDNA, AK252279.1). This sequence corresponds to the barley high confidence gene MLOC_51583 that was previously referred to as *HvCUT1.3* (Richardson et al., 2007). Sequencing of a PCR product amplified from genomic DNA from *Ingridmlo5* or *lwa1* plants with primers specific for the *LWA1* gene identified a single nucleotide acid polymorphism in the mutant genotype which led to an amino acid change in the deduced protein at position L136F (see Supplemental Figure S3). Data base analysis revealed a predicted function for the *LWA1* gene as 3-KETOACYL-CoA SYNTHASE and therefore the gene *HvCUT1.3* was renamed as *HvKCS6*.

**Complementation of *lwa1***

Complementation of the *lwa1* mutant was undertaken to determine whether the mutant phenotype could be rescued by constitutive overexpression of the wild type *HvKCS6* coding sequence from *Ingridmlo5* plants and for verification of the predicted enzymatic function of the HvKCS6 protein. Therefore, pre-cultured immature embryos of *lwa1* mutant plants were inoculated either with *Agrobacterium* strain AGL-1 which harbors a plasmid containing the wild type *LWA1* gene under control of the constitutive maize UBIQUITIN-1 promoter or the *GFP* reporter gene under control of the same promoter as a control. Selection of putative transformants was done on hygromycin containing media and twenty-nine regenerants were obtained in genotype *MLO/lwa1* and ten regenerants in genotype *mlo5/lwa1*, respectively. All *LWA1* PCR-positive primary transformants, hemizygous for the transgene, displayed a restored wild type epicuticular wax layer as verified by monitoring of water retention. In contrast, the transgenic *GFP* event, which did not harbor the *HvKCS6* transgene, behaved like the *lwa1* mutant. Based on DNA gel blot-analysis, three independent integration events were selected for both genomic backgrounds (*MLO/lwa1* and *mlo5/lwa1*) (see Supplemental Figure S4). GC-MS profiling of wax components revealed that the amount of the C26 alcohol was restored in these regenerants to the level in *IngridMLO* or *Ingridmlo5* plants (Figure 3). The
amount of the C26 aldehyde was on average higher for complemented plants in comparison to wild type plants which might be due to the strong constitutive expression of HvKCS6 and a preferential conversion of the C26 alcohol to the corresponding aldehyde. Interestingly, the amount of C24 alcohol was also elevated in complemented plants as compared to lwa1 mutant plants which might also be an effect of the strong overexpression of HvKCS6.

In parallel to the GC-MS analysis, a Bgh infection assay was performed on detached leaves of the primary transformants. A representative result of a single event for each group of transformed genotypes (MLO/lwa1 or mlo5/lwa1) is shown in Figure 1C and compared to the GFP-transgenic control. Germination of Bgh conidiospores was compromised on the transgenic GFP event to a similar level as on lwa1 mutant plants (Figure 1A). By contrast, plants transformed with wild type HvKCS6, both in a MLO and mlo5 genetic background, showed a significantly lower frequency of not germinated Bgh conidiospores (10%) which was even lower than on Ingridmlo5 plants (Figure 1A). Taken together, our results obtained by complementation verified that the lack of a functional HvKCS6 allele is responsible for the altered epicuticular wax composition and the compromised germination of Bgh conidiospores on lwa1 mutant plants.

**Introggression of the lwa1 allele in the MLO genetic background**

A matter of discussion which could not be answered till then addresses the question whether the presence of the lwa1 allele only leads to a reduced germination or above that does also lower powdery mildew disease severity. This was difficult to examine because the strong powdery mildew resistance allele mlo5 present in lwa1 mutant plants makes a macroscopic evaluation of mature powdery mildew pustules impossible. To assess this important issue, we used a cross between Ingridmlo5/emr1/lwa1 and IngridMLO/EMR1/LWA1 that we described in a previous study (Jansen et al., 2007). From this cross, we retrieved plants homozygous for both, the wild type MLO and the mutant lwa1 allele. Inoculation of these plants with Bgh revealed a strong reduction in powdery mildew disease symptoms (76% less infected leaf area) on lwa1 mutant plants (Figure 5). Detailed cytological analysis at 48 h p.i. demonstrated that on plants of the MLO/LWA1 genotype only 5% of Bgh conidiospores did not germinate while this frequency was 55% for MLO/lwa1 plants (Figure 1D). This result confirmed the data received with the lwa1 mutant allele in a mlo5 genetic background. The significantly lower germination rate of Bgh conidiospores on MLO/lwa1 plants resulted in less frequently formed appressoria and haustoria (see Supplemental Figure S5A). However, when compared
to the percentage of germinated conidiospores there was no substantial difference between both genotypes as half of all germinated conidiospores had established a haustorium at that time point. At these successful infection sites, a significantly lower number of haustoria per colony was observed on MLO/lwa1 plants as compared to MLO/LWA1 plants at 72 h p.i. (see Supplemental Figure S5B). This ostensible fitness penalty did not result in differences in colony size as evidenced by a similar number of epidermal cells covered by individual Bgh colonies on both genotypes. Similarly, no differences were found in Bgh colony size on both genotypes at later stages of the infection process (96 h p.i.) and they did also not differ in the number of conidiophores formed per colony (see Supplemental Figure S5C). From this comprehensive microscopic analysis it must be concluded, that the reduced number of Bgh pustules on MLO/lwa1 plants is a direct consequence of the severely reduced germination rate of Bgh conidiospores.

The lwa1 mutant allele also affects germination of non-adapted powdery mildew fungi

Our next goal was to test whether the altered epicuticular wax composition of the lwa1 mutant affects pre-penetration infection structures of powdery mildews in general. Thus, we inoculated lwa1 mutant plants with conidiospores of the wheat powdery mildew fungus Blumeria graminis f. sp. tritici (Bgt) which is a nonhost pathogen of barley and therefore unable to complete its life cycle. Microscopic analysis revealed a severe reduction in germination of Bgt conidiospores on lwa1 leaves as compared to Ingridmlo5 leaves (Figure 6A). Thereafter, and similar to the results obtained for Bgh, most of the germinated conidiospores developed mature appressoria on both genotypes, indicating that the lwa1 mutation does not affect the efficiency of appressorium formation. Next, we investigated whether also powdery mildew fungi more distantly related to Bgh than Bgt were similarly affected in their conidial germination rate on lwa1 mutant plants. Accordingly, we inoculated the lwa1 mutant with Erysiphe pisi and Golovinomyces orontii, the powdery mildews of pea and Arabidopsis, respectively. Both fungi exhibited reduced germination of conidiospores on lwa1 mutant plants in comparison to Ingridmlo5 plants as evidenced by microscopic analysis (Figure 6A).

AtCUT1 was identified as functional ortholog of HvKCS6
Driven by the observation that germination of conidiospores of adapted as well as non-adapted powdery mildew fungi was affected by the altered composition of epicuticular waxes on the *lwa1* mutant, we hypothesized that wax components such as hexacosanal are a general requirement for regular germination of powdery mildew conidiospores. Blast analysis revealed that the gene At1g68530 (*AtCER6/AtCUT1/AtKCS6*) is the Arabidopsis homolog to *HvKCS6* and phylogenetic analysis supported this finding (see Supplemental Figure S6). We used transgenic Arabidopsis plants with reduced *AtCUT1* expression due to a co-silencing phenomenon after introduction of a *35S:AtCUT1* construct for further analysis (Millar et al., 1999). In the latter study it was reported that epicuticular waxes are severely reduced on stems of these transgenic plants and primary alcohols with a chain length above C26 and C28 are less abundant. We inoculated these *AtCUT1*-silenced plants with non-adapted powdery mildew fungi, such as *Bgt, Bgh* or *E. pisi*, which resulted in each case in a lower germination rate of conidiospores as compared to wild type *Col-0* Arabidopsis plants (Figure 6B). Again, no effect on the rate of successfully formed appressoria could be determined. Similarly, inoculation of *AtCUT1*-silenced plants with the Arabidopsis-adapted powdery mildew *G. orontii* revealed a lower conidial germination rate and no influence on appressorium formation (see Supplemental Figure S7A). Moreover, and in accordance with our results received for *Bgh* on *lwa1* barley plants, a lower germination rate of conidiospores at 16 h p.i. resulted in reduced disease severity on *AtCUT1*-silenced plants as evidenced by less infection sites with elongated secondary hyphae at 72 h p.i. (see Supplemental Figure S7B). Altogether, these results suggest that *AtCUT1* and *HvKCS6* are functional orthologs with respect to their role in pathogenicity of adapted and non-adapted powdery mildew fungi.

**Discussion**

With the exception of Arabidopsis for which a genetic toolbox is widely available, identification of genes causing a mutant phenotype is still challenging especially in cereals with rather complex genomes like barley or wheat. The most severe drawback which limits the power of forward genetic screens with these important crop plants is the time-consuming step of generating mapping populations and the subsequent map-based cloning. Certainly, progress made in gene sequencing technologies (next generation sequencing, NGS) and bioinformatics which enable direct comparison of mutant and wild type genomes looks promising and might remarkably shorten the time required (Zuryn et al., 2010). However, although this method was recently successfully applied to rice (Nordstrom et al., 2013), its distribution among a wide range of different plant species is still not achieved. Here, we
report on the identification of a gene causing a reduced wax phenotype in barley by simultaneous SNP mapping, an integrated method at the transition between classical and new gene discovery technologies.

Some years ago, we selected the barley emr1 mutant in a screen for genotypes with enhanced resistance against Magnaporthe oryzae (Jansen et al., 2007), an upcoming threat causing head blast in barley and wheat cultivation (Lima and Minella, 2003; Urashima et al., 2004). During mutant characterization, we identified a second phenotype which was the enhanced capacity of emr1 mutant plants to retain water on their leaves (Figure 2A). This observation together with a glossy appearance of leaves reminded us of the pioneering work of Diter and Penny von Wettstein on eceriferum (cer) barley mutant plants, all of which had defects in wax load of aerial plant organs (Lundqvist and von Wettstein, 1962; von Wettstein-Knowles, 2001). Indeed, SEM and GC-MS analysis of emr1 primary leaves revealed a decrease in total cuticular waxes (Figure 2B and C). On wild type barley plants hexacosanol (C26 alcohol), comprising 75% of extractable cuticular wax, is by far the most abundant constituent (Richardson et al., 2005). Performing detailed GC-MS profiling of individual wax components, we discovered that hexacosanol was reduced on emr1 mutant leaves by 90% in comparison to the respective control, i.e. Ingridmlo5 plants (Figure 3, see Supplemental Figure S2) and is therefore among the most severe waxless phenotypes reported for barley mutants so far.

Since it is known that cuticle components such as 1-hexadecanol or 1,16-hexadecanediol are crucial, at least on rice, for appressorium formation of M. oryzae (Gilbert et al., 1996), we inquired whether the reduced wax coverage is responsible for enhancement of resistance against this fungus. Investigation of a cross between emr1 and a distantly related barley genotype (Grannenlose Zweizeiligemlo11) led to the identification of fifteen F2-segregants in which emr1-dependent resistance against M. oryzae did not co-segregate with the wax phenotype. This irrefutably showed that both phenotypes are caused by independent mutations. Inoculation of segregants with low wax phenotype, termed lwal, with the barley powdery mildew fungus revealed a significant reduction in the germination rate of conidiospores which was independent of the presence or absence of emr1 or EMR1 alleles (Figure 1B). This was a novel finding since a powdery mildew phenotype was not reported for any of the barley cer-mutants so far. To analyze whether or not the lwal mutation is responsible for the Bgh germination phenotype, a SNP-based mapping approach was performed which finally placed the lwal allele on the long arm of chromosome 4H (Figure 4).
Fortunately, a gene in the syntenic region of rice next to the closest marker was annotated as a wax biosynthetic pathway gene with a predicted enzymatic function in fatty acid elongation. The respective barley gene was identified as the MLOC_51583 gene of the recently released barley genome (Consortium, 2012). Sequencing confirmed a single nucleotide polymorphism between the MLOC_51583 sequences of lwa1 and Ingridmlo5 leading to a lysine to phenylalanine amino acid substitution (see Supplemental Figure S3). Also the barley LWA1 protein has a predicted 3-KETOACYL-CoA SYNTHASE (KCS) activity. Such KCS activity is required for the elongation of VLCFAs and thus, a loss-of-function mutation would sufficiently explain the lwa1 phenotype of being depleted in hexacosanol (Figure 3). We validated the predicted enzymatic function of LWA1 by complementation of lwa1-mutant plants with the LWA1 wild type coding sequence and subsequent GC-MS profiling of wax components (Figure 3). The MLOC_51583 gene was thereafter renamed as HvKCS6. Based on the GC-MS data which revealed that the depletion in C26 VLCFA derivatives on lwa1 plants was accompanied by an increase in C24 VLCFA derivatives and their restoration to wild type levels in HvKCS6-complemented transgenics, it became evident that HvKCS6 exhibits specificity for the elongation step from C24 to C26 wax constituents. Thereby, we functionally proved that HvKCS6 is a condensing enzyme of the FAE complex and confirmed its specificity in chain length elongation. Besides complementation of the waxless phenotype, germination of Bgh conidiospores did also return to wild type rates on the regenerants (Figure 1C), indicating that HvKCS6-derived VLCFAs have a crucial role in Bgh pathogenicity.

A function as KSC in elongation of VLCFAs was also shown for the Arabidopsis gene At1g68530 (AtCER6/AtCUT1/AtKCS6) (Millar et al., 1999). Based on blast analysis, Li et al. (2013) suggested barley EST DQ646644 (named in this study HvCER6, identical to HvCut1.3 (Richardson et al., 2007)) as a potential homolog for At1g68530. In their study, the authors placed 27 barley cer-loci, none of which have been cloned so far, on a barley consensus map. Based on complete linkage, HvCER6 became a prime candidate for CER-ZG. The localization of cer-zg on barley chromosome 4H might imply that cer-zg and lwa1 are identical loci, however, neither the reported phenotype of wax depletion only on upper leaves for cer-zg mutant plants nor re-sequencing of HvKCS6 in the cer-zg mutant background supported this assumption.

An involvement of C26 VLCFA derivatives, i.e. hexacosanol and hexacosanal, as clue for germination of Bgh conidiospores had already been suggested by Tsuba et al. (2002). Zabka et al. (2008) showed that glass slides covered with hexacosanal were effective in supporting
germination of \textit{Bgh} conidiospores. Hexacosanol, by contrast, was less supportive in this assay. Testing a set of different aldehydes, it became evident that \textit{n}-octacosanal (C28) followed by \textit{n}-tetracosanal (C24) and \textit{n}-docosanal (C22) in decreasing order also stimulate germination of \textit{Bgh} conidiospores (Hansjakob et al., 2010). It must be noted, however, that from the latter, less efficient compounds, concentrations of one to two orders of magnitude higher were needed to induce hexacosanal comparable germination rates. This concentration dependency might explain why on hexacosanal-depleted \textit{lwa1} plants, although having a bit higher rate of \textit{n}-tetracosanal (see Supplemental Figure S2), nevertheless a significant reduction in germination of \textit{Bgh} conidiospores was found. Hansjakob et al. (2011) performed \textit{in vivo} experiments on maize mutants depleted in total leaf cuticular waxes and showed that chemical complementation of mutant leaves with hexacosanal elevated the germination rate of \textit{Bgh} conidiospores. Integrating these data from the literature with the results obtained in the present study, it became most evident that the depletion in hexacosanal, and not the 90\% reduced amount of hexacosanol, is responsible for the germination penalty observed for \textit{Bgh} on \textit{lwa1} mutant plants. Also rust fungi from different genera depend on fatty acid-derived signals for differentiation of pre-penetration infection structures or stomata sensing (Rubiales et al., 2001; Uppalapati et al., 2012). This suggests a common mechanism by which ancient fungi adapted to plants as forthcoming hosts before ascomycetes split from basidiomycetes approx. 400 Myr ago, (Taylor and Berbee, 2006). Inoculation of \textit{lwa1} mutant plants with \textit{Puccinia hordei} or \textit{Phakopsora pachyrhizi} did not reveal any influence of the altered epicuticular wax composition on the infection process (see Supplemental Figure S8), pointing to specific wax components rather than the general amount of waxes as signal in rust pathogenicity. Alternatively, it could also be that those wax components affected by the \textit{lwa1} mutation do not affect the infection process of rust pathogens under investigation.

We broadened our analysis with \textit{lwa1} mutant plants with a set of different powdery mildew species which all form a nonhost type of interaction with barley. In each case, a significant reduction in germination of conidiospores was found, irrespective of whether or not the pathogens came from monocot or dicot host plants (Figure 6A). Based on quantitative microscopic evaluation no influence on the formation of other pre-penetration infection structures was observed. We further extended this analysis by performing a complementary series of experiments with Arabidopsis plants transcriptionally silenced for \textit{AtCUT1}. This was done because SEM pictures revealed the absence of smooth platelet-like, structures among epicuticular wax crystals from stems of these \textit{AtCUT1}-silenced plants (Millar et al., 1999), which was reminiscent of the same phenotype on leaves of \textit{lwa1} barley mutants, suggesting a
conserved function of *HvKCS6* and *AtCUT1* in VLCFA biosynthesis. Since no powdery mildew disease phenotype was reported for the latter Arabidopsis genotype, we started a series of experiments and inoculated these *AtCUT1* silenced plants with monocot and dicot powdery mildew pathogens. Again, this demonstrated the relation of VLCFA derivative-availability and full-extended germination of conidiospores (Figure 6B). Since germination of conidiospores from *Bgt* and *G. orontii* was compromized on Arabidopsis *AtCUT1* and barley *lwa1* mutant plants, it could be speculated that the missed VLCFA-derived signal is identical on both plants. Our results confirmed that *HvKCS6* and *AtCUT1* are functional orthologs with respect to their biosynthetic function and their role in powdery mildew conidiospore germination. A conserved function of genes from barley and Arabidopsis in powdery mildew pathogenicity was also observed for *HvROR2/AtPEN1* and *HvMLO/AtMLO2*, gene-pairs involved in penetration or post-penetration resistance (Collins et al., 2003; Consonni et al., 2006). The pair *HvKCS6/AtCUT1* is a further example for such orthologous genes.

**Conclusions**

Our results demonstrate a conserved requirement of plant cuticle-associated signals in pathogenicity of powdery mildew pathogens under investigation. This is remarkable because these pathogen species comprised dicot and monocot powdery mildews which build host and nonhost interactions with the plants species tested. Given the fact that powdery mildews are separated from each other because of their host specificity, a KCS6-derived germination signal must have been adopted by an ancient powdery mildew which was pathogenic on the monocot-dicot progenitor. Since it is known that powdery mildew spores are short-lived, a quick germination on the right surface could have been a crucial step during adaptation of a would-be pathogen to its forthcoming host. In this ancient scenario, VLCFA-derived signals are crucial clues for host identification which might have been orchestrated later on by other thigmo- or chemotactic signals. It is interesting that powdery mildews kept VLCFA-derived signals even after speciation and host range expansion. During co-evolution with their hosts, however, some powdery mildews might have acquired additional VLCFA derivatives as signal for accelerated germination, a process which would not astonish as the wax composition varies between plant species. Our results demonstrate the conservation of ancient pathogenicity determinants in modern powdery mildew pathogens for more than 140-150 Myr, a time at which the monocot-dicot split is expected to have taken place (Chaw et al., 2014).
Modifying the epicuticular wax signature might therefore be a promising approach towards plant protection.

Materials and Methods

Plant material and fungal infection assays

The barley mutant *lwa1* was created by chemical mutagenesis (*NaN₃*) of the near-isogenic backcross line *Ingridmlo5* (obtained from the Max-Planck-Institute for Plant Breeding Research, Cologne, Germany) as described in Jansen et al. (2007). Barley plants segregating for *lwa1* and *emr1* alleles were recruited from the cross *emr1 x GZmlo11* (‘Grannenlose Zweizeiligemlo11’, obtained from the Max-Planck-Institute for Plant Breeding Research, Cologne, Germany). Plants homozygous for the wild type *MLO* and the mutant *lwa1* allele were retrieved from a cross between *Ingridmlo5/emr1/lwa1* and *IngridMLO/EMR1/LWA1* which was also described in Jansen et al. (2007). Barley plants were grown in a growth chamber (16 to 18 °C, 50 to 60% relative humidity and a 16 h photo-period, 210 µmol m⁻² s⁻¹). Infection assay with *M. oryzae* was done as described in Jansen et al. (2007).

*Arabidopsis thaliana* plants used in this study were transcriptionally silenced for *AtCUT1* due to sense suppression by the transgene 35S:*AtCUT1* (Millar et al., 1999) and the respective wild type accession Col-0. Plants were sown in soil, stratified for 2 days at 4°C in darkness and grown for 2 weeks in a phytotron (22°C, 60% humidity and a 8.5 h photoperiod, 120 µmol m⁻² s⁻¹). Thereafter, seedlings were transferred to long-day conditions for four weeks (22 °C, 50 to 60% humidity and a 18 h photoperiod, 110 µmol m⁻² s⁻¹).

*Blumeria graminis* f. sp. *hordei* (Bgh) race K1 (Hinze et al., 1991) was kindly provided by Prof. Schulze-Lefert (Max-Planck-Institute for Plant Breeding Research, Cologne, Germany) whereas *Blumeria graminis* f. sp. *tritici* (Bgt) was a field isolate collected near Aachen. Both species were propagated in separate plant cabinets (18 °C, 65% humidity and a 16 h photoperiod, 130 to 150 µmol m⁻² s⁻¹), Bgh on barley cv. *IngridMLO* and Bgt on wheat cv. Feldkrone, by weekly inoculation of 7-days-old seedlings. One day before inoculation, old conidiospores were removed from infected plants by shaking. Adaxial surfaces of 7-days-old primary barley leaves or 6-weeks-old *Arabidopsis* plants were inoculated by shaking infected plants over horizontally fixed leaves or over detached leaves on kinetin-agar (0.8% agar-agar, 2.5 µg ml⁻¹ kinetin) in a settling tower at an average spore density of 1 to 15 conidiospores.
mm$^{-2}$. After settling of conidiospores for 1 h, plants were returned to growth cabinets and cultivated under conditions described above.

*Golovinomyces orontii* and *Erysiphe pisi* f. sp. *medicaginis* were kindly provided by Prof. Panstruga (RWTH University, Institute for Biology I, Aachen, Germany). *G. orontii* was maintained on *Col-0* plants (20°C, 55 to 60% humidity and a 8 h photoperiod, 100 µmol m$^{-2}$ s$^{-1}$). Ten to eleven days post inoculation conidiospores from infected plants were used to inoculate test plants by brush inoculation. *E. pisi* was propagated on susceptible *Pisum sativum* plants (22°C, 50 to 60% humidity and a 10 h photoperiod, 160 µmol m$^{-2}$ s$^{-1}$). Adaxial surfaces of either 7-days-old primary leaves of barley or 6-weeks-old Arabidopsis plants were inoculated by shaking infected plants (10 d p.i.) over them. Inoculated plant material was incubated under conditions as described above.

*Puccinia hordei* (barley rust) was a field isolate collected near Aachen and sub-cultivated on barley cv. IngridMLO in a plant cabinet (18°C, 50 to 60% humidity and a 16 h photoperiod, 140 µmol m$^{-2}$ s$^{-1}$). For inoculation, 7-days-old barley primary leaves were sprayed with a suspension of uredospores in freon (1,1,2-trichloro-1,2,2-trefluoroethan) as described in Jansen et al. (2007). Inoculated plants were incubated in a dark moist chamber for 24 h and then kept under the same conditions as described above (see Supplemental Figure S8).

*Phakopsora pachyrhizi* was isolated from infected plant material collected in Brazil and maintained on the soybean cv. Petrina as described in Loehrer et al. (2008). Uredospore suspension for inoculation was generated by washing an infected soybean leaf in 0.1% (v/v) Tween-20 in distilled water. Primary leaves of 7-days-old barley plants were inoculated by spraying and placed in a dark moist chamber for 24 h. Thereafter, plants were kept under normal growth conditions (see Supplemental Figure S8).

**Microscopic and macroscopic analysis**

For analysis of individual spores by light microscopy (Nikon Eclipse 50i, Duesseldorf, Germany), leaves were cleared with their inoculated surface up on Whatman paper (3 MM) moistened with ethanol:acetic acid (3:1, v/v) to avoid displacement of not germinated conidiospores (Lyngkjaer and Carver, 1999). Fungal structures were stained with ink-acetic acid solution (10% ink in 25% acetic acid, v/v). Images of infection structures (see Supplemental Figure S1) were taken with a Leica DMR microscope (Wetzlar, Germany) equipped with a JVC digital camera KY-F75U 3-DDC (Friedberg, Germany) and software...
DISKUS version 4.81.1027 (Koenigswinter, Germany) for image acquisition. Quantification of infected leaf area by image processing was performed with Adobe Photoshop CS2, version 9.0.

**Determination of HvKCS6 mapping position**

Phenotyping was done with F2 individuals and derived F3 offspring from the cross between *emr1* and Grannenlose Zweizeiligmlo11 described in Jansen et al. (2007). Therefore, water retention was monitored after spraying of 2-weeks-old plants with water and, in addition, disease severity was measured after inoculation with *M. oryzae* using a detached leaf assay also described in Jansen et al. (2007). Genomic DNA was isolated from each of these F2 individuals using 30 to 50 mg leaf material harvested from the 2-weeks-old seedlings. The material was homogenized using a TissueLyser II (Qiagen, Hilden, Germany) and extraction was carried out using the BioSprint DNA Plant Kit and the BioSprint 96 workstation from the same supplier. Isolated DNA was dissolved in distilled water and, based on agarose gel electrophoresis, DNA concentration of all samples was adjusted to approximately 25 ng µl⁻¹.

SNP genotyping was carried out at KWS LOCHOW (Einbeck, Germany) using a 384 iSELECT VeraCode barley chip. After genotyping of 92 F2 plants, a linkage map was constructed with JoinMap 4.0 (Kyazma B.V., Wageningen, Netherlands (Van Ooijen, 2006)). First, genotype data were transformed into the mapping data format (‘AHB’ with A = homozygous *emr1*, H = heterozygous and B = homozygous ‘Grannenlose Zweizeiligmlo11’). To calculate the genetic linkage map with 92 individuals and 161 polymorphic SNPs, the population type and the LOD threshold of linkage were set to F2 and ≥ 2.0, respectively. Using these parameters, a linkage map of chromosome 4H containing *lwa1*, *emr1* and linked SNP markers was constructed.

**Construction of the transformation vector and generation of transgenic plants**

The genomic HvKCS6 sequences of Ingridmlo5 and lwa1 mutant plants were amplified with forward primer 5'-TAGGATCCCAGCACGGCTGAACGCATAC-3' containing a BamHI restriction site and reverse primer 5'-ATGAATTCTGGTTAATTTGGTGCGGGGC-3' containing an EcoRI restriction site and cloned into intermediate vector pUBI-ABM (DNA-Cloning-Service, Hamburg, Germany) between maize *UBIQUITIN-1* promoter with first intron and *NOPALINE SYNTHASE* termination sequence of *Agrobacterium tumefaciens*. 

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Correct clones were verified by sequencing and the whole expression cassette was subcloned into binary vector p6int-UBI (DNA-Cloning-Service, Hamburg, Germany) via SfiI restriction sites. The binary vector was introduced into *A. tumefaciens* strain AGL-1 by electroporation as described elsewhere (Hensel et al., 2009).

Stable transgenic barley plants were obtained by *Agrobacterium*-mediated gene transfer following a method described previously (Hensel et al., 2009) with slight modifications. Briefly, immature embryos of both genotypes were pre-cultured for five days on BCIM with dicamba at a final concentration of 5 mg l\(^{-1}\) at 24°C in the dark (Hensel et al., 2009). After agroinfection pre-cultured immature embryos were co-cultured at stacks on moistened filter paper (300 µL BCCM, (Hensel et al., 2009)) in 5.5 cm diameter petri dishes for two days in the dark. Callus induction was performed on BCIM with either 20 or 50 mg l\(^{-1}\) hygromycin B (Roche Diagnostics, Mannheim, Germany), respectively. Regeneration took place on BRM medium with 25 mg l\(^{-1}\) hygromycin B at 24°C in the light (Hensel et al., 2009). In contrast to the previously published method, all regenerants were further processed excepting that some of the primary transgenic plants may be derived from the same transformation event. Rooting and transfer into soil were the same as described before.

Transgenic events were identified and confirmed by PCR based on primer pairs specific for the junction between *ZmUBI-1:hpt* (5′-TTTAGCCCTGCCTTCATACG-3′, 5′-GATTCCTTGCGGTCCGAATG-3′) and *ZmUBI-1:HvKCS6* (5′-TTTAGCCCTGCCTTCATACG-3′, 5′-TGCACCAGCCTGTACTTGG-3′) using 100 ng genomic DNA isolated following the protocol of Pallotta et al. (2000). In order to identify siblings, DNA gel blot analyses were conducted using an *hpt*-specific hybridization probe, constructed with the same primers as for PCR and with a DIG-PCR Labelling Kit (Roche Diagnostics, Mannheim, Germany). Genomic DNA (30 µg) were digested with *Hind*III and separated by agarose gel electrophoresis. After transfer on a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) blots were hybridized and processed following the manufacturer instructions (see Supplemental Figure S4).

**Wax extraction and GC-MS analysis**

For wax analysis of barley genotypes Ingrid*MLO*, Ingrid*mlo5* and *emr1*, 7-days-old primary leaves were cut off and immediately immersed in chloroform for 10 s at room temperature. From transgenic plants, either the third or fourth leaf of each individual (5-weeks-old) was
used for wax analysis. The corresponding leaf area was determined by scanning of dipped leaves and pixel quantification using Adobe Photoshop CS2, version 9.0. The resulting solution containing the cuticular waxes was spiked with 10 µg of tetracosane (Sigma-Aldrich, Munich, Germany) as an internal standard. The solvent was evaporated under a stream of nitrogen, and compounds containing free hydroxyl and carboxyl groups were converted into their trimethylsilyl ethers and esters, respectively, with bis-(N,N-trimethylsilyl)-trifluoroacetamide (Machery-Nagel, Dueren, Germany) in pyridine for 40 min at 70°C before GC-MS analysis. Wax constituents were identified by their electron-impact MS-spectra after GC-MS analysis and quantified using an identical GC-system equipped with a flame ionization detector (FID) as described previously (Richardson et al., 2005).

**Phylogenetic analysis**

Protein sequences for barley KCS members were obtained from the EnsemblPlants database (Kinsella et al., 2011) and those for Arabidopsis from the ‘Arabidopsis Information Resource’ (TAIR) homepage (Lamesch et al., 2012), respectively. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013) by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992) with 1000 replicates to obtain bootstrap values. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. Codon positions included were 1st+2nd+3rd+Non-Coding. All positions containing gaps and missing data were eliminated. There were a total of 225 positions in the final dataset. The underlying protein alignment was produced by using the ClustalW-method implemented in MEGA6 (see Supplemental Figure S6).

**Accession numbers**

Sequence data from this article can be found in the EMBL/GenBank data libraries or the Arabidopsis Genome Initiative under following accession numbers: barley 3-KETOACYL-CoA SYNTHASE HvCER6/HvCUT1.3/HvKCS6, barley EST DQ646644/flcDNA AK252279.1/MLOC_51583; Arabidopsis 3-KETOACYL-CoA SYNTHASE AtCER6/AtCUT1/AtKCS6, At1g68530; rice gene syntenic to closest barley iSELECT marker 4139-888 (syn. BOPA 1_0606), Os03g0219900; rice 3-KETOACYL-CoA SYNTHASE
Os03g0220100 (LOC_Os03g12030). Accession numbers of barley and Arabidopsis KCS sequences used for phylogenetic analysis are provided in Supplemental Figure S6.

**Supplemental Material**

Supplemental Figure S1: Powdery mildew infection structures on barley and Arabidopsis.

Supplemental Figure S2: Detailed analysis of cuticular wax profiles from different barley genotypes.

Supplemental Figure S3: Comparison of *HvKCS6* nucleotide sequences from Ingrid*mlo5* (*mlo5*) and *lwa1* barley genotypes.

Supplemental Figure S4: Southern-blot confirmation of T-DNA integration pattern.

Supplemental Figure S5: Microscopic analysis of post-penetration developmental stages of *Blumeria graminis* f. sp. *hordei*.

Supplemental Figure S6: Phylogenetic tree of barley and Arabidopsis KCS protein families.

Supplemental Figure S7: Microscopic analysis of the development of *Golovinomyces orontii* on Arabidopsis transgenic plants (*35S:AtCUT1*) with sense silencing of *AtCUT1*.

Supplemental Figure S8: Pre-penetration development of a host and nonhost rust-species on primary leaves of the *lwa1* mutant.

Supplemental Table S1: Genotype data for 21 informative SNPs from chromosome 4H and for *emr1* and *lwa1* loci across 92 F2 offspring originating from an *emr1* x *GZmlo11* cross.

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infection assay on Arabidopsis. We thank Burkhard Schmidt (RWTH Aachen University) for assistance with GC-FID analyses of cuticular waxes.

**Authors Contribution**

DW performed most of the experimental work, analyzed data and helped in writing the manuscript. MJ performed plant crosses and participated in the analysis of wax components. SU and IJ helped with infection assays of powdery mildew or rust pathogens on barley. RBF performed the GC-MS work. WW together with VK performed the SNP-genotyping and analyzed together with KP data derived from that experiment. GH performed the plant transformation work. RP provided the SEM pictures. KP, LS, RBF and JK helped in analyzing data. US designed the experiments, analyzed data and wrote the paper.

**Literature cited**


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Figure Legends

Figure 1: Investigation of interaction sites of Blumeria graminis f. sp. hordei (Bgh) with different barley genotypes. Primary leaves of barley plants were inspected at 16 h p.i. Progression of pre-penetration infection stages was analyzed for each conidiospore and assigned to different categories as indicated. (A) Frequency of different infection stages is given for the interaction of Bgh with Ingridmlo5 (mlo5/EMR1/LWA1) or mutant emr1 (mlo5/emr1/lwa1). (B) Germination rates of Bgh conidiospores on barley plants segregating for lwa1 and emr1 alleles. (C) Frequency of different Bgh infection stages on lwa1 mutant plants complemented with the wild type LWA1 allele in IngridMLO or Ingridmlo5 genetic background. Regenerants transformed with a GFP-construct served as control. (D) Pre-penetration development of Bgh on barley genotypes bearing the LWA1-wild type or lwa1-
mutant allele in the IngridMLO genetic background. Columns represent mean values (n = 3) ± SD with 100 interaction sites inspected per genotype and per leaf. Asterisk indicates significant differences (P < 0.7) determined in a Student’s t-test. The experiment was repeated with similar result in triplicate (A), once (B, D) or with different leaves of individual events (C). h.p.i., hours post inoculation

Figure 2: Leaves of the barley mutant emr1 display an altered epicuticular wax layer. (A) Water retention of primary leaves is strongly enhanced on emr1 mutant plants compared to Ingridmlo5. (B) Scanning electron microscopy (SEM) of adaxial leaf surfaces of the barley genotypes Ingridmlo5 and emr1 (scale bars = 1 µm). (C) Total cuticular waxes were extracted with chloroform from leaves of Ingridmlo5 or emr1 plants and derivatized with bis-(N,N-trimethylsilyl)-trifluoroacetamide. Wax components were quantified by GC-FID and the total amount of waxes was calculated as the sum of single components. Columns represent mean values of three independent measurements (n = 3) ± SD with 5 primary leaves measured per genotype and replicate; means differ significantly between both genotypes (t-test, P < 0.001).

Figure 3: Quantification of cuticular wax components in different barley genotypes. Genotypes exhibit the wild type-LWA1 or mutant-lwa1 allele in either the IngridMLO or Ingridmlo5 genetic background as indicated. Transgenic plants complemented with the wild type-LWA1 allele in a lwa1-genetic background are marked with ‘compl”’. Plants transformed with GFP served as control. Total cuticular wax was extracted from leaves by dipping in chloroform without hydrolyzation. For this analysis primary regenerants from independent transformation events selected after DNA gel-analysis were used and wax was extracted from a single leaf of selected regenerants. Wax molecule content was determined by GC-MS and only those showing significant differences between genotypes are presented.

Figure 4: Linkage map of chromosome 4H based on the F2 population ‘emr1’ x ‘Grannenlose Zweizeiligemlo11’, including 21 segregating SNP markers as well as lwa1 and emr1. The marker positions in cM and the SNP names are displayed left and right to the map, respectively. In the genetic linkage map gene loci of lwa1 and emr1 do not cosegregate. The lwa1 locus was mapped close to the iSELECT marker 4139-888 (syn. BOPA 1_0606).
Figure 5: Development of powdery mildew disease symptoms on barley plants differing in LWA1/lwa1 alleles. (A) Picture of Bgh-infected primary leaves of barley cv. IngridMLO (MLO/LWA1) or lwa1 mutant plants (MLO/lwa1) at 6 d p.i. (B) Quantification of infected leaf area by image processing. Data are given as mean ± SD (n = 5). Asterisk indicates significant difference (P = 0.008) determined in a Student’s t-test. The experiment was repeated twice with similar result. d p.i., days post inoculation

Figure 6: Pre-penetration development of different powdery mildew species on barley or Arabidopsis. The interactions of lwa1 barley mutant plants (A) or Arabidopsis 35S:AtCUT1 sense silenced plants (B) with different species of powdery mildew fungi were microscopically investigated at 16 h p.i. Interaction sites were grouped into different categories from which only those are displayed that showed significant differences. Columns represent mean values (n = 3) ± SD with 100 interaction sites inspected per genotype and per leaf. Asterisks indicate significant differences (P ≤ 0.043) determined in a Student’s t-test. The experiment was repeated once with similar result. Plant genotypes: Col-0, Arabidopsis ecotype Col-0; mlo5, Ingridmlo5; lwa1, barley mutant lwa1; pathogens: Bgh, Blumeria graminis f. sp. hordei; Bgt, Blumeria graminis f. sp. tritici; E. pisi, Erysiphe pisi; G. orontii, Golovinomyces orontii; h p.i., hours post inoculation
Figure 1: Investigation of interaction sites of *Blumeria graminis* f. sp. *hordei* (*Bgh*) with different barley genotypes. Primary leaves of barley plants were inspected at 16 h p.i. Progression of pre-penetration infection stages was analyzed for each conidiospore and assigned to different categories as indicated. (A) Frequency of different infection stages is given for the interaction of *Bgh* with *Ingrid* *mlo5* (*mlo5*/EMR1*/LWA1*) or mutant *emr1* (*mlo5*/emr1*/lwa1*). (B) Germination rates of *Bgh* conidiospores on barley plants segregating for *lwa1* and *emr1* alleles. (C) Frequency of different *Bgh* infection stages on *lwa1* mutant plants complemented with the wild type *LWA1* allele in a *Ingrid* *MLO* or *Ingrid* *mlo5* genetic background. Regenerants transformed with a *GFP*-construct served as control. (D) Pre-penetration development of *Bgh* on barley genotypes bearing the *LWA1*-wild type or *lwa1*-mutant allele in the *Ingrid* *MLO* genetic background. Columns represent mean values (n = 3) ± SD with 100 interaction sites inspected per genotype and per leaf. Asterisk indicates significant differences (P < 0.7) determined in a Student’s t-test. The experiment was repeated with similar result in triplicate (A), once (B, D) or with different leaves of individual events (C). h p.i., hours post inoculation.
Figure 2: Leaves of the barley mutant *emr1* display an altered epicuticular wax layer. (A) Water retention of primary leaves is strongly enhanced on *emr1* mutant plants compared to *Ingridmlo5*. (B) Scanning electron microscopy (SEM) of adaxial leaf surfaces of the barley genotypes *Ingridmlo5* and *emr1* (scale bars = 1 µm). (C) Total cuticular waxes were extracted with chloroform from leaves of *Ingridmlo5* or *emr1* plants and derivatized with bis-(N,N-trimethylsilyl)-trifluoroacetamide. Wax components were quantified by GC-FID and the total amount of waxes was calculated as the sum of single components. Columns represent mean values of three independent measurements (n = 3) ± SD with 5 primary leaves measured per genotype and replicate; means differ significantly between both genotypes (t-test, P < 0.001).
Figure 3: Quantification of cuticular wax components in different barley genotypes. Genotypes exhibit the wild type-LWA1 or mutant-lwa1 allele in either the IngridMLO or Ingridmlo5 genetic background as indicated. Transgenic plants complemented with the wild type-LWA1 allele in a lwa1-genetic background are marked with ‘compl’. Plants transformed with GFP served as control. Total cuticular wax was extracted from leaves by dipping in chloroform without hydrolyzation. For this analysis, primary regenerants from independent transformation events selected after DNA gel-analysis were used and wax was extracted from a single leaf of each regenerant. Wax molecule content was determined by GC-MS and only those showing significant differences between genotypes are presented.
Figure 4: Linkage map of chromosome 4H based on the F2 population ‘emr1’ x ‘Grannenlose Zweizeiligemlo11’, including 21 segregating SNP markers as well as lwa1 and emr1. The marker positions in cM and the SNP names are displayed left and right to the map, respectively. In the genetic linkage map gene loci of lwa1 and emr1 do not cosegregate. The lwa1 locus was mapped close to the iSELECT marker 4139-888 (syn. BOPA 1_0606).
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