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Molecular characterization of *mlo* mutants in North American two- and six-rowed malting barley cultivars

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SUMMARY

Barley lines PRU1, URS1 and URS2 represent three candidate *mlo* mutants induced in either the two-rowed cultivar Prudentia or the six-rowed cultivar Ursula. Both Prudentia and Ursula are North American malting barley varieties with specific malting properties. Here, we analysed the three candidate mutants at the molecular level. We identified lesions in the *Mlo* gene of all three lines, causing either a premature stop codon (PRU1), a shift in the reading frame (URS1) or a single amino acid replacement (URS2). In a transient gene expression assay, the URS2 *mlo* allele fails to complement a barley null mutant genotype, indicating that URS2 is a genuine *mlo* mutant (here designated as *mlo*-33). The MLO-33 mutant variant accumulates to similar levels as the wild-type MLO protein in Arabidopsis protoplasts, suggesting that MLO-33 is stable *in planta*. We show that the *mlo*-33 allele can be readily detected in barley genomic DNA by a cleaved amplified polymorphic sequence marker, rendering this allele particularly suited for marker-assisted breeding.

INTRODUCTION

Worldwide, barley represents an important crop primarily used in stock farming (feeding barley) or for brewing beer and whisky (brewing barley). In Europe, the prevalent malting varieties are two-rowed cultivars, whereas in North America brewers prefer six-rowed barley. In recent years, American-style beers, brewed under licence in Europe, have greatly increased in popularity. In contrast to Europe, powdery mildew is not a serious pathogen in Midwest USA, the main malting barley-growing region of the United States. Therefore, breeding of six-rowed barley has not focused on powdery mildew resistance in the past. An effort was recently started to identify broad-spectrum powdery mildew-resistant *mlo* mutants (Jørgensen, 1992; Lyngkjaer *et al.*, 2000) in typical North American malting varieties. These could subsequently serve as a genetic resource for agriculture or breeding programmes to obtain powdery mildew-resistant varieties optimized for European production (Molina-Cano *et al.*, 2003). Three potential *mlo* mutants in two North American malting barley cultivars, the two-rowed Prudentia and the six-rowed Ursula, were described. These mutants were designated PRU1 (derived from the parental cultivar Prudentia) and URS1 and URS2 (derived from the parental cultivar Ursula), respectively (Molina-Cano *et al.*, 2003). Although genetic analysis unambiguously identified PRU1 and URS1 as *mlo* mutants (designated as *mlo*-32 and *mlo*-31), the results for URS2 were less clear. Test crosses of URS2 with Alexis (*mlo* genotype) or Rupal (*Mlo* genotype) and subsequent powdery mildew inoculation experiments in the resulting F_1 and F_2 generations provided contradictory results. In addition, targeted inoculation experiments with various powdery mildew isolates indicated partial resistance for URS2, whereas under field conditions URS2 was as resistant as the fully immune URS1 mutant (Molina-Cano *et al.*, 2003).

Barley *Mlo* encodes a plasma membrane-localized protein with seven transmembrane domains (Devoto *et al.*, 1999) that is considered to modulate SNARE protein-dependent and vesicle transport-associated processes at the cell periphery (reviewed in Panstruga, 2005; Schulze-Lefert, 2004). We previously described the molecular analysis of a range of induced barley *mlo* mutants (Büschges *et al.*, 1997; Piffanelli *et al.*, 2002) as well as one mutant allele (*mlo*-11) that arose spontaneously (Jørgensen, 1992; Piffanelli *et al.*, 2004). Whereas the majority of chemical or radiation-induced mutants comprise single amino acid substitutions or small deletions in the heptahelical protein, the natural *mlo*-11 allele is characterized by presence of a complex repeat array that inserted upstream of an intact *Mlo* wild-type copy. The concatemeric repeat units, consisting of *Mlo* 5′ regulatory sequences and part of the *Mlo* coding sequence, presumably interfere with transcription of the downstream *Mlo* wild-type copy (Piffanelli *et al.*, 2004). Surprisingly, the majority of MLO mutant protein variants carrying single amino acid substitutions exhibit reduced *in planta* stability. These protein variants are substrates

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of a conserved post-translational and post-insertional quality control mechanism reminiscent of endoplasmic reticulum-associated protein degradation (ERAD) in yeast and animals (Müller *et al.*, 2005).

RESULTS AND DISCUSSION

We re-examined powdery mildew infection phenotypes of cultivars Prudentia and Ursula as well as the derived sodium azide-induced *mlo* mutant candidates PRU1 (*mlo*-32), URS1 (*mlo*-31) and URS2, respectively, under controlled growth chamber conditions (Fig. 1). Specimens were inoculated with a high density of conidiospores of *Blumeria graminis* f. sp. *hordei* (*Bgh*) isolate K1 that is virulent on the respective parental lines, Prudentia and Ursula. The susceptible cultivar Ingrid and the near isogenic line BC Ingrid *mlo*-5 carrying an *mlo* null mutant allele (Büschges *et al.*, 1997) were included as controls. We found that the PRU1 candidate *mlo* mutant was macroscopically equally resistant against isolate K1 as the BC Ingrid *mlo*-5 null mutant line (Fig. 1). In contrast, candidate *mlo* mutants URS1 and URS2 both showed some fungal sporulation under equal experimental conditions (Fig. 1). As the extent of residual sporulation was similar for both candidate mutants and URS1 was subsequently found to represent an *mlo* null mutant (see below), we conclude that the apparently reduced resistance might be the consequence of the genetic background of the lines possibly containing a natural variant of a modifier gene of *mlo* resistance such as *Ror1* or *Ror2* (Freialdenhoven *et al.*, 1996). Alternatively, the slightly susceptible phenotype could be caused by the combination of the fungal isolate and the growth conditions employed in this experiment. All three *mlo* candidate mutants exhibited premature leaf senescence and necrotic leaf spotting (Molina-Cano *et al.*, 2003; and data not shown), a typical pleiotropic effect of barley *mlo* loss-of-function mutants (Piffanelli *et al.*, 2002; Schwarzbach, 1976).

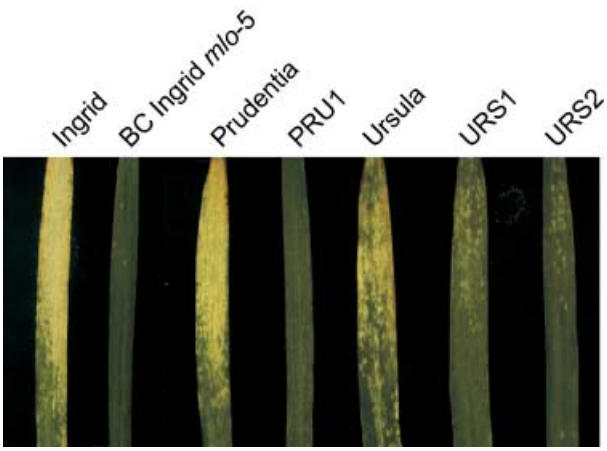

Fig. 1 Powdery mildew infection phenotypes of candidate *mlo* mutants PRU1, URS1 and URS2. Seven-day-old first leaves of barley seedlings were inoculated with conidiospores of *Bgh* isolate K1. The photograph was taken 6 days post inoculation.

To determine potential molecular defects at *Mlo*, we amplified by RT-PCR the *Mlo* coding sequence from cultivars Prudentia and Ursula as well as from the sodium azide-induced *mlo* mutant candidates PRU1, URS1 and URS2. Subsequently, we determined the nucleotide sequence of the RT-PCR products by direct DNA sequencing of the amplicons. This revealed few cultivar-specific alterations (data not shown) as well as mutant-specific changes of the *Mlo* coding sequence as compared with the previously published *Mlo* reference sequence of barley variety Ingrid (GenBank accession number Z83834; Büschges *et al.*, 1997). In PRU1 (*mlo*-32), guanine 103 is replaced by a thymine, resulting (in combination with a cultivar-specific A105G alteration) in the creation of a premature stop codon (TAG; Table 1). This stop codon is predicted to terminate translation of the PRU1 *Mlo* cDNA after 34 (instead of 533) amino acids. In URS1 (*mlo*-31), one of four consecutive guanine nucleotides in position 826–829 (in the genomic DNA sequence at nucleotides 1764–1767) is missing, resulting in a frame shift within the *Mlo* cDNA after glycine 276 and an early stop codon 13 triplets downstream (Table 1). Finally, in URS2, the guanine at position 916 (in the genomic DNA sequence at position 1966) is replaced by an adenine, resulting in a change of the respective codon triplet from GCC (encoding alanine) to ACC (encoding threonine; Table 1).

Sodium azide has been described as a powerful mutagen primarily causing base substitution mutants (Kleinhofs *et al.*, 1974; Sideris and Argyrakis, 1974). In a sodium azide-based mutational analysis of the barley *Ant18* gene, primarily transitions (86%) and to a lesser extent also transversions (14%) were found (Olsen *et al.*, 1993). Among the 21 analysed *Ant18* mutants, no deletion was detected. In this study, however, we identified one G→T transversion, one G→A transition, and a 1-nt deletion.

We previously established transient gene expression in leaf epidermal cells mediated by particle bombardment of detached

Table 1 Novel *mlo* mutant alleles.

Allele	Mother variety	Mutagen	Mutational event at Mlo*	Effect on amino acid level
mlo-31	Ursula (six-rowed)	Sodium azide	ΔG^{826}	Frame shift after Gly^{276}
mlo-32	Prudentia (two-rowed)	Sodium azide	$G^{103}\rightarrow T$	Stop after Met^{34}
mlo-33	Ursula (six-rowed)	Sodium azide	$G^{916}\rightarrow A$	$Ala^{306}\rightarrow Thr$

*Numbers of nucleotides are given relative to the translational start site in the *Mlo* cDNA (GenBank accession number Z83834).

barley leaves as a rapid functional assay for *Mlo* function (Panstruga, 2004; Shirasu *et al.*, 1999). As mutants PRU1 and URS1 revealed obvious lesions in the *Mlo* coding sequence resulting in dramatic changes such as an early stop codon (PRU1) or a frame shift (URS1), we did not further consider these mutants for functional analysis by transient gene expression. By contrast, the URS2 mutant candidate encodes a single amino acid replacement (A306T) in the *Mlo* coding region, rendering this exchange the potential cause of powdery mildew resistance in URS2 plants. However, because this particular mutant had revealed contradictory results in previous complementation studies (Molina *et al.*, 2003), it was equally conceivable that the detected lesion was a second-site event of the mutagenesis procedure without direct impact on *Mlo* function. To address this topic by experimentation, we engineered the URS2 A306T mutation in the *Mlo* coding sequence of expression plasmid pUbi-Mlo-nos and tested the mutant derivative by transient gene expression and subsequent powdery mildew challenge in the barley *mlo*-5 null mutant background (see Experimental procedures). We found that the A306T derivative exhibited ~12% penetration efficiency of wild-type *Mlo* (absolute penetration efficiencies were 8 ± 5% and 65 ± 1%, respectively; Fig. 2A), suggesting severely impaired *Mlo* functionality. The residual activity of the A306T variant is probably the result of non-physiological *Mlo* expression levels upon transient gene expression, a phenomenon also observed with a range of further *mlo* mutant variants (R. Panstruga, unpublished data). Alternatively, the remaining activity could be the consequence of residual functionality of this single amino acid substitution variant. In conclusion, the transient gene expression assay corroborates that the A306T amino acid substitution is the cause of the lack of *Mlo* function in URS2. We therefore designate the URS2 *mlo* mutant allele as *mlo*-33 (Table 1).

We previously reported that the majority of *Mlo* mutant variants characterized by single amino acid substitutions are subject to ERAD-like quality control. Aberrant MLO variants recognized by this quality control mechanism exhibit reduced *in planta* half-lives and are eliminated via a proteasome-dependent degradation route. As a consequence of this tight quality control process, such mutants are characterized by reduced steady-state MLO accumulation levels (Müller *et al.*, 2005). To test whether the *mlo* mutant encoded by barley line URS2 also represents an ERAD substrate, we used the previously established dual luciferase assay to assess *in planta* accumulation of the A306T MLO variant (Müller *et al.*, 2005). Wild-type MLO and the highly unstable MLO-1 mutant variant served as positive and negative control, respectively. We found that the A306T protein variant accumulated to steady-state levels comparable with wild-type MLO (~95% relative to MLO; Fig. 2B), suggesting that the barley *mlo*-33 mutant encodes a stable protein variant. Stable but non-functional MLO variants are assumed to be defective in protein–protein interactions of MLO with essential interaction partners.

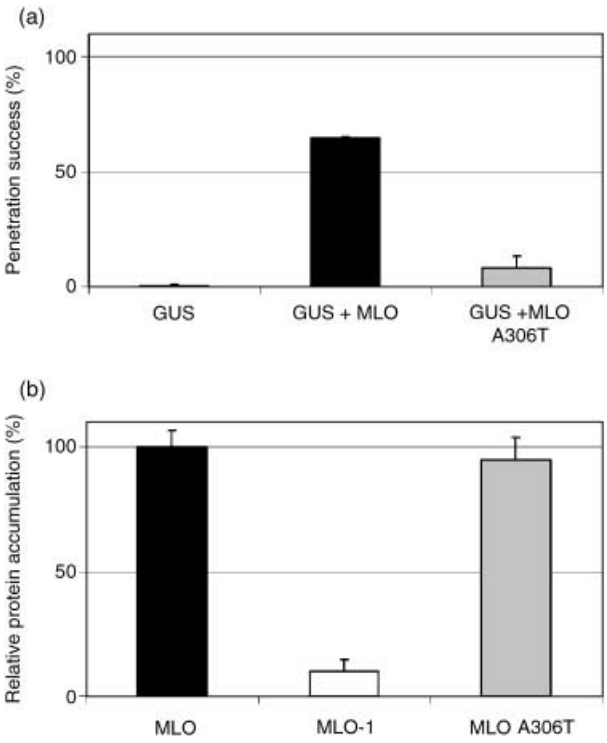

Fig. 2 Mlo A306T encodes an MLO variant with impaired functionality that is stable *in planta*. (a) Detached barley leaves (*mlo*-5 genotype) were either transformed with a β-glucuronidase (GUS) reporter construct alone or co-transformed with the reporter plasmid plus either a construct encoding wild-type MLO or the MLO A306T variant. After inoculation with powdery mildew conidiospores and staining for GUS activity, transformed cells were analysed for fungal penetration success as described in the Experimental procedures. The bars represent the mean ± standard deviation of three independent experiments. (b) Arabidopsis protoplasts were transformed with dual luciferase reporter constructs carrying either the *Mlo* wild-type gene, the *mlo*-1 mutant variant encoding the highly unstable MLO W162R version, or the *Mlo* A306T variant. Luciferase assays were performed as described in the Experimental procedures and relative *in planta* protein accumulation (wild-type MLO set as 100%) was calculated from these values. The bars represent the mean ± standard deviation of four independent experiments.

Thus, the current collection of stable, non-functional *mlo* mutant variants (MLO-10, MLO-27, MLO-29 and MLO-33; Müller *et al.*, 2005; and this study) will be instrumental in determining the *in vivo* relevance of MLO candidate interactors.

It is intriguing that three of the four reported stable MLO mutant variants carry amino acid replacements in the third cytoplasmic loop of the heptahelical protein (MLO-27, MLO-29 and MLO-33; Müller *et al.*, 2005; and this study), whereas the four described single amino acid replacements in the second cytoplasmic loop all result in protein variants with reduced stability (Müller *et al.*, 2005). Future experimentation will be necessary to unravel whether the clustering of stable MLO mutant variants represented by amino acid replacements in cytoplasmic loop 3 represents a

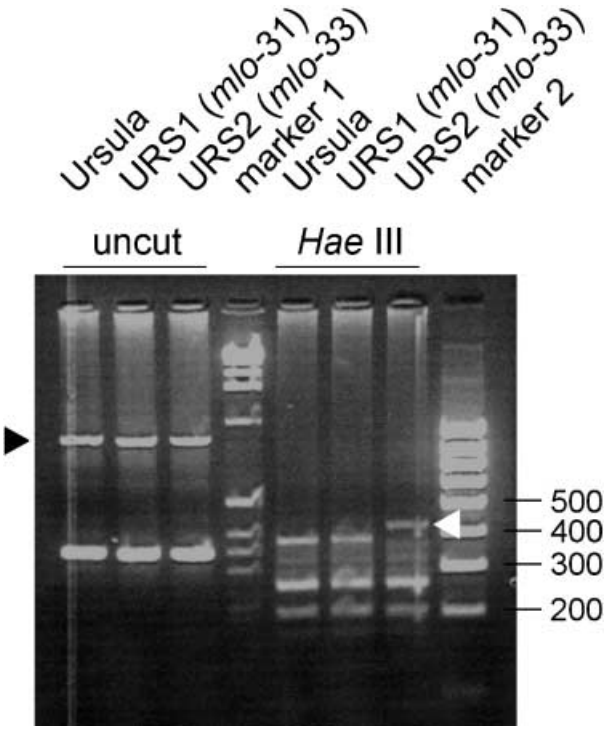

Fig. 3 A CAPS marker for detection of the *mlo*-33 mutant allele. A region of the *Mlo* gene covering the lesion site in URS2 (*mlo*-33) was amplified by PCR using either genomic DNA of Ursula wild-type plants or URS1 (*mlo*-31) or URS2 (*mlo*-33) mutant plants as template (for details, see Experimental procedures). The ~870-bp amplicons were either subjected immediately to agarose gel electrophoresis ('uncut'; the black triangle indicates the specific amplification product while the ~350-bp amplicons represent unspecific PCR by-products) or digested with restriction enzyme *Hae*III before separation by gel electrophoresis. The white triangle on the right indicates the 416-bp product characteristic of *mlo*-33. Marker 1, 1-kb DNA ladder (Invitrogen); Marker 2, Hyper ladder IV (Bioline, London, UK). Numbers on the right indicate band sizes (in bp) of the Hyper ladder IV marker lane.

random accumulation or whether this might reflect a locally reduced stringency of the quality control mechanism that monitors MLO integrity. The latter would be compatible with the concept of the 'distributed degron', a term that describes the phenomenon that dispersed structural features of a protein are decisive for its potential recognition as an ERAD substrate (Gardner and Hampton, 1999).

We inspected the nucleotide sequences of *Mlo* wild-type plants and mutants *mlo*-31, *mlo*-32 and *mlo*-33 at the sites of the respective lesions for altered restriction enzyme recognition sites. We found that the nucleotide exchange in *mlo*-33 eliminates a *Hae*III restriction site present in the wild-type sequence but we could not discover any restriction enzyme recognition sites specific for the positions of the lesions in *mlo*-31 and *mlo*-32. We exploited the differential presence of the *Hae*III restriction site in *mlo*-33 for development of a diagnostic cleaved amplified polymorphic sequence (CAPS) marker (Konieczny and Ausubel, 1993). We designed primers Mlo31 and Mlo34 (see Experimental procedures) suitable for amplifying a region covering the mutant site from genomic DNA of the parental variety Ursula as well as of genomic DNA of the mutants URS1 (*mlo*-31) and URS2 (*mlo*-33). PCR amplicons were subjected to digestion with *Hae*III and fragments resolved by agarose gel electrophoresis. Whereas cleaved PCR products derived from template DNA of Ursula and URS1 resulted only in fragments < 400 bp, the cleaved amplicon from URS2 resulted in a restriction fragment > 400 bp (Fig. 3). The latter is the predicted consequence of absence of the *Hae*III restriction site in the *mlo*-33 allele. This CAPS marker can be used as a tool for the rapid and convenient detection of the *mlo*-33 mutant allele in marker-assisted breeding programmes (Thomas, 2003). Collectively, the set of *mlo* mutants in North American two- and six-rowed barley cultivars represents a valuable extension of the currently available collection of characterized European *mlo* mutants for both basic science and agriculture.

EXPERIMENTAL PROCEDURES

Plant and fungal material

Barley mutants PRU1, URS1 and URS2 as well as parental lines Prudentia and Ursula were previously described (Molina-Cano *et al.*, 2003). Barley lines Ingrid (*Mlo* wild-type) and back-cross Ingrid *mlo*-5 (an *mlo* null mutant, Büschges *et al.*, 1997) were used as control lines. Powdery mildew inoculations were performed with *Blumeria graminis* f. sp. *hordei* isolate K1 (Zhou *et al.*, 2001).

DNA sequencing of *mlo* mutants

Total RNA was isolated from wild-type plants Prudentia and Ursula as well as from mutants PRU1, URS1 and URS2, respectively, using the Trizol reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen). Double-stranded full-size *Mlo* cDNA was amplified by PCR using oligonucleotides ADUP7 and ADDOWN6 (Devoto *et al.*, 1999). Internal *Mlo*-specific oligonucleotides were used for DNA sequencing of the PCR amplicons. Upon detection of specific polymorphisms between wild-type and mutant plants, reverse transcription (RT)-PCR and DNA sequencing were repeated in an independent biological experiment.

Transient gene expression analysis

Ballistic transformation of detached barley leaves was carried out as previously described (Elliott *et al.*, 2005; Schweizer *et al.*, 1999). Bombarded specimens were inoculated with high densities of powdery mildew (*Bgh* isolate K1) conidiospores and GUS staining performed 48 h post inoculation (Schweizer *et al.*, 1999). Epiphytic

fungal structures were stained by Coomassie Brillant Blue. Leaf epidermal cells attacked by the appressorial germ tube of powdery mildew sporelings were microscopically evaluated for presence or absence of haustoria. Penetration success was calculated as the number of transformed cells that exhibit one or multiple haustoria in relation to the total number of transformed cells attacked by powdery mildew sporelings.

Dual luciferase assays

Plasmid K93, a derivative of binary vector pAMPAT-MCS (GenBank accession number AY436765) carries two expression cassettes: one consisting of a doubled cauliflower mosaic virus 35S promoter, an in-frame fusion of *Mlo* and Renilla luciferase cDNAs and 35S terminator, the second comprising 35S promoter, firefly luciferase and 35S terminator. Derivatives of this plasmid expressing *Mlo* variants (MLO-1, MLO-33) as translational fusions with Renilla luciferase were generated by placement of suitable restriction fragments. Protoplast preparations of *Arabidopsis thaliana* cell lines, protoplast transfections and dual luciferase reporter assays (Promega, Madison, WI) were carried out as described previously (Müller *et al.*, 2005). Renilla luciferase activity was set in relation to firefly luciferase activity and the ratio obtained with wild-type *Mlo* defined as 100%.

Development of a CAPS marker for *mlo*-33

DNA sequences flanking the lesion sites in *mlo*-31, *mlo*-32 and *mlo*-33 were inspected electronically for altered presence/absence of restriction sites compared with the respective wild-type sequences. A *Hae*III restriction site (at nucleotide 917 in *Mlo* cDNA, 1967 in *Mlo* genomic DNA) present in wild-type *Mlo* and absent in *mlo*-33 was exploited for CAPS marker analysis (Konieczny and Ausubel, 1993). Fragments (~870 bp) covering the differentially present restriction site were amplified by PCR (50 cycles, 55 °C annealing temperature; 1.5-min extension) from genomic template DNA of either cultivar Ursula or mutants URS1 (*mlo*-31) and URS2 (*mlo*-33) using oligonucleotides Mlo31 (5′-CACCACCTTCATGATGCTCAG-3′; reverse primer) and Mlo34 (5′-CGATGGAGGACGACTTCAAGG-3′; forward primer). Aliquots (10 μL) of the amplification products were subjected to *Hae*III digest (total volume 15 μL) without prior purification. Products of the restriction reaction and equal amounts of untreated PCR products were resolved by gel electrophoresis on 3% agarose gels. Expected fragment sizes were 377, 216, 176 and 61 bp (Ursula and URS1) as well as 416, 216, 176 and 61 bp (URS2), respectively.

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