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; Lyngkjær 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 F1 and F2 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...
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 (Büsches 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).

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; Büsches 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 (Kleinholds 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

![Fig. 1](image)

**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.

**Table 1** Novel mlo mutant alleles.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mother variety</th>
<th>Mutagen</th>
<th>Mutational event at Mlo*</th>
<th>Effect on amino acid level</th>
</tr>
</thead>
<tbody>
<tr>
<td>mlo-31</td>
<td>Ursula (six-rowed)</td>
<td>Sodium azide</td>
<td>ΔG326</td>
<td>Frame shift after Gly326</td>
</tr>
<tr>
<td>mlo-32</td>
<td>Prudentia (two-rowed)</td>
<td>Sodium azide</td>
<td>G103→T</td>
<td>Stop after Met34</td>
</tr>
<tr>
<td>mlo-33</td>
<td>Ursula (six-rowed)</td>
<td>Sodium azide</td>
<td>G916→A</td>
<td>Ala916→Thr</td>
</tr>
</tbody>
</table>

*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.

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
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 HaeIII 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 HaeIII 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 HaeIII 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 HaeIII 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 Brilliant 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 Haell 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′-CGATGGAGGCCACTCTCAAGG-3′; forward primer). Aliquots (10 µL) of the amplification products were subjected to Haell 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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**REFERENCES**


