Mol Breeding (2013) 32:723–727
DOI 10.1007/s11032-013-9889-x

SHORT COMMUNICATION

The ENU-induced powdery mildew resistant mutant pea (*Pisum sativum* L.) lines S(*er1mut1*) and F(*er1mut2*) harbour early stop codons in the *PsMLO1* gene

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Received: 16 January 2013 / Accepted: 16 May 2013 / Published online: 15 June 2013

Abstract Two pea (*Pisum sativum* L.) powdery mildew-resistant mutant lines, S(*er1mut1*) and F(*er1mut2*), were previously obtained by experimental chemical mutagenesis with ethylnitrosourea. Identification and subsequent analysis of the genomic sequence of the *PsMLO1* gene revealed one single nucleotide mutation in each mutant line that leads to either a transversion or a transition, respectively, resulting in premature stop codons that drastically truncate the protein product of this gene in these two mutant lines. These results confirm the previous findings that *PsMLO1* is the powdery mildew resistance gene *er1*. Only one additional mutation (transition) was observed in the S(*er1mut1*), downstream of the protein-truncating stop codon. Mutations were not identified in the intron regions of the gene. Specific molecular markers (cleaved amplified polymorphic sequences and sequence-tagged sites) were generated for the protein-truncating mutations, and these provide breeders with very efficient tools for marker-assisted selection when either of the two mutated lines are used in plant breeding programmes.

Keywords *PsMLO1* · Powdery mildew resistance · *Pisum* · CAPS · Marker assisted selection · Ethylnitrosourea

Electronic supplementary material The online version of this article (doi:10.1007/s11032-013-9889-x) contains supplementary material, which is available to authorized users.

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In an earlier publication we reported the first experimentally induced powdery mildew-resistant lines of pea (*Pisum sativum* L.), which were obtained by chemical mutagenesis with ethylnitrosourea (ENU) (Pereira et al. 2001). The cross between the mutant lines, S(*er1mut1*) and F(*er1mut2*), and the respective original cultivars, Solara and Frilene, confirmed the recessive nature of the induced mutations, while the complementation crosses between the two mutant lines revealed that the two mutations affected the same locus. Additional complementation tests with line E835 carrying the powdery mildew resistance gene *er1* from cv. Mexique 4 demonstrated that both mutations were located at this resistance locus (Pereira and Leitão 2010). Additional evidence, though indirect, that the two mutations are located at the *er1* locus was also provided by their mapping to pea chromosome 6 (Pereira et al. 2010) where this locus had been previously located to by other research groups (Timmerman et al. 1994; Weeden et al. 1998).

To date, only a second recessive powdery mildew resistance locus, namely, *er2* has been identified in a number of *Pisum sativum* lines—SVP 951 and SVP 952 (Heringa et al. 1969) and JI 2480 (Ali et al. 1994; Tiwari et al. 1997). This locus was recently mapped to chromosome 3 (Katoch et al. 2010). However, in contrast to *er1*, which confers strong broad resistance to the pathogen, *er2* provides only leaf resistance which depends on plant age and temperature (cf. Ghafoor and McPhee 2012).

Humphry et al. (2011) recently found the *er1* locus to be a MLO homologous gene, *PsMLO1*, the loss of function of which leads to the powdery mildew-resistant phenotype. This conclusion was drawn based on the presence of severe mutations on *PsMLO1* shown by multiple *er1er1* genotypes, such as Mexique 4 (Ser to stop codon), Stratagem (insert of unknown size), JI210 (frameshift) and Ji1951 (frameshift), and by the partial single cell complementation of the er1 phenotype by the transient expression of functional *PsMLO1* on detached leaves of resistant genotypes. These results were almost simultaneously confirmed by Pavan et al. (2011), who showed that the powdery mildew resistance of a mutant line selected after diethyl sulphate treatments was associated to a G/A transition that truncates the PsMLO1 protein at the second N-proximal intracellular loop. In this last study, three other powdery mildew-resistant cultivars were also shown to produce aberrant transcripts of the *PsMLO1* gene, and a cleaved amplified polymorphic sequence (CAPS) marker was developed for the identification of the induced resistance allele of the mutated RO13/02 line.

In the study reported here we present the genomic sequence of the *PsMLO1* gene and show that ENU-induced mutant lines S(*er1mut1*) and F(*er1mut2*) harbour point mutations in coding sequences that, in both cases, drastically truncate the protein product of this gene. Two types of molecular markers, CAPs and sequence-tagged sites (STS), are also reported which will be useful in marker-assisted selection (MAS) by breeders that use our mutated lines for breeding purposes.

Plants of cvs. Solara and Frilene, which are powdery mildew mutant lines S(*er1mut1*) and F(*er1mut2*), respectively, and F_2 plants resulting from the crosses S(*er1mut1*) × cv. Frilene and F(*er1mut2*) × Solara were cultivated in greenhouses. Total genomic DNA was extracted from leaves using a common phenol/chloroform method, as previously described (Pereira et al. 2010). Primers to retrieve the genomic sequence of *PsMLO1* were designed firstly based on the published expressed sequence (www.ncbi.nlm.nih.gov/nuccore/FJ463618.1) and subsequently based on the successively unveiled genomic sequence.

The full genomic sequence of the *PsMLO1* gene (www.ncbi.nlm.nih.gov; GenBank: KC466597.1) was obtained, in the various genotypes, resulting in the assembly of partially overlapping PCR-amplified fragments. Particularly difficult to unveil was the sequence of intron 5 due the presence of a dinucleotide (TA) microsatellite. The PsMLO gene spans over 4,729 nucleotides in cv. Frilene and 4,708 nucleotides in cv. Solara, with the same coding region of 1,722 nucleotides spread throughout 15 exons, of which ten are <100 bp. The differences in size between the *PsMLO1* sequence in cvs. Solara and Frilene are due to differences in the dinucleotide microsatellite in intron 5 which in the first cultivar is 36 nucleotides long (TA_{18}) and in the second cultivar spans over 58 nucleotides (TA_{29}). An additional nucleotide (T) is also present in intron 14 of cv. Solara.

The coding sequences of the mutant lines and respective original cultivars differ uniquely by very small point mutations. However, although minor at the nucleotide level, these point mutations have drastic consequences on *PsMLO1* protein function.

Only two induced single nucleotide polymorphisms (SNPs) were identified in the S(*er1mut1*) line: (1) a C/G transversion in exon 6 that results in a premature stop codon (Table 1) interrupting translation from the second N-proximal intracellular domain between transmembrane domains 3 and 4 onwards; (2) a G/A transition in exon 11 which alters codon GAT (aspartic acid) into AAT (asparagine). However, this second mutation is assumed to have no practical effect on the function of the gene or on the powdery mildew resistance as the translation is interrupted upstream at exon six.

In the second mutant line F(*er1mut2*) only one single, induced SNP was identified: a G/A transition that results in a premature stop codon in exon 10 (Table 1), terminating protein synthesis at the middle of the fifth transmembrane domain (TM5).

In order to create the conditions for efficient MAS for the presence of the powdery mildew resistance alleles we tested two analytical procedures. The first method is based on the analysis of a CAPS marker.

Table 1 Amplified fragments for cleaved amplified polymorphic sequence discrimination between powdery mildew resistance mutated alleles and wild alleles for susceptibility

Cv. Solara (wild)

GATCCTGAGAGGTTTAGGTTTGCAAGGGACACAACATTTGGAAGAAGGCACTTGAGCATGT
GGGCTCAGTCACCTATTTTGTTATGGATTGTAAGGGAACTTTTGTTACATAAAATTAATCATACACATTAATTAA
ATGATTAAGATAAACACAAAATTAATTATACTATTAGTGACACATATTATGGTTGAATCAGGTTAGCTTCTTCAGACAA
TTCTTTGGATCTATCAGTAGAGTTG

S(*er1mut1*)

GATCCTGAGAGGTTTAGGTTTGCAAGGGACACAACATTTGGAAGAAGGCACTTGAGCATGT
GGGCTCAGTGACCTATTTTGTTATGGATTGTAAGGGAACTTTTGTTACATAAAATTAATCATACACATTAATTAA
ATGATTAAGATAAACACAAAATTAATTATACTATTAGTGACACATATTATGGTTGAATCAGGTTAGCTTCTTCAGACAA
TTCTTTGGATCTATCAGTAGAGTTG

Cv. Frilene (wild)

TATATGGGCAGGGTGGTATTCTTATTATTGGCTTCCATTTCTTCCACTAATTGTAAGCATAATCTACAT
TTTGTTTCTTAATTAAGGGTTGAAATAAATAACAAATAATAAGTTCTAATTTAAAAATTACAGGTAATCTTATTAGTTGGT
GCTAAGTTACAAATGATCATAACAAAAATGGGATTAAGGATTCAAGACAGAGGAGAAGTAA
TCAAGGGTGCACCT

F(*er1mut2*)

TATATGGGCAGGGTGGTATTCTTATTATTGACTTCCATTTCTTCCACTAATTGTAAGCATAATCTACATTT
TGTTTCTTAATTAAGGGTTGAAATAAATAACAAATAATAAGTTCTAATTTAAAAATTACAGGTAATCTTATTAGTTGGTG
CTAAGTTACAAATGATCATAACAAAAATGGGATTAAGGATTCAAGACAGAGGAGAAGTAAT
CAAGGGTGCACCT

Arrowheads indicate restriction sites, primers and restriction recognition sequences are underlined, mutated nucleotides are highlighted gray and intron sequences are in the relatively smaller letters

The second method is based on differential (positive) PCR amplification of the mutated alleles versus the lack of amplification of the wild-type (WT; susceptible) allele.

The presence of the induced C/G transversion in the mutated line S(*er1mut1*) eliminates a restriction site recognized in the WT by the restriction enzyme *Hph*I. In the WT allele this restriction enzyme cuts an amplified fragment of 240 bp into two fragments (178 and 62 bp), allowing easy discrimination between homozygous susceptible (WT allele), heterozygous susceptible (carrier) and homozygous resistant plants by simple agarose gel electrophoresis (Table 1; Fig. 1).

In a similar way the presence of the induced G/A transition in the mutated line F(*er1mut2*) eliminates a *CviKI*-1 restriction site. This enzyme cuts a 225-bp fragment amplified in the WT allele into two pieces (31 + 194 bp), allowing the above-mentioned three genotypes to be clearly discriminated (Table 1; Fig. 1).

Nonetheless, since the use of restriction enzymes can be hampered by loss of enzyme activity, incomplete restriction, among other factors, an alternative differential analysis more affordable and easier to perform can be achieved by simple and direct agarose gel electrophoresis analysis of a PCR-amplified product. However, this simpler procedure does not allow plants homozygous for the presence of the powdery mildew-resistant allele to be distinguished from heterozygous (carrier) plants.

The primer pairs used to produce the 341- and 372-bp-long fragments (Fig. 2) that identify the mutated alleles S(*er1mut1*) and F(*er1mut2*), respectively, are displayed in Table 2. In each pair, one of the primers was designed to amplify the mutant allele starting exactly from the respective mutated nucleotide and to contain an intentional mismatch at the fourth nucleotide at the 3′ terminus (Monteros et al. 1998; Xiao-Yong et al. 2003). This mismatching, while not affecting the amplification of the resistance allele when combined with the mismatch between the last nucleotide of the primer and the non-mutated nucleotide of the WT sequence, completely prevents the amplification of a PCR product in the WT (susceptible) allele.

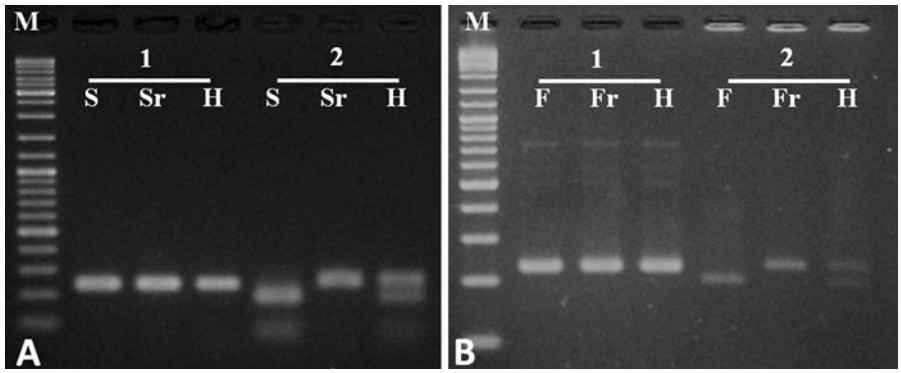

Fig. 1 Cleaved amplified polymorphic sequence (CAPS) discrimination between the *PsMLO1* alleles of powdery mildew-resistant lines and the respective original susceptible cultivars. **a** S(*er1mut1*) versus the wild-type (WT) (cv. Solara) allele. Agarose gel electrophoresis: *lanes 1* Amplified DNA fragment surrounding the C/G transversion, *lanes 2 Hph*I digestion of amplified fragments of cv. Solara (*S*), S(*er1mut1*) (*Sr*) and heterozygous (*H*) [S(*er1mut1*) × (cv. Frilene)] F_1 plants. **b** F(*er1mut2*) versus WT (cv. Frilene) allele. Agarose gel electrophoresis *lanes 1* Amplified DNA fragment surrounding the G/A transition, *2 CviKI*-1 digestion of amplified fragments of cv. Frilene (*F*), F(*er1mut2*) (*Fr*) and heterozygous (*H*) [F(*er1mut2*) × (cv. Solara)] F_1 plant. *M* Molecular-weight marker

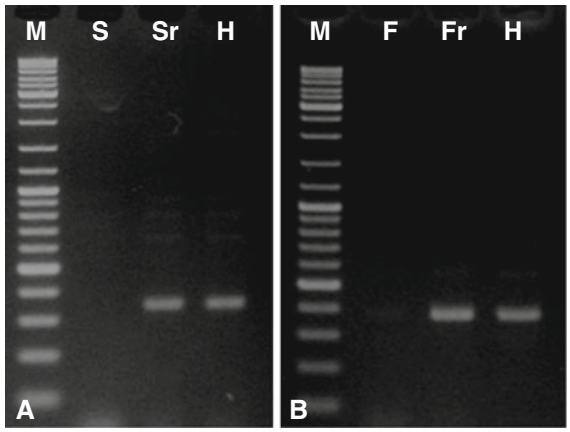

Fig. 2 Discrimination, by direct amplification, of the mutated *PsMLO1* alleles induced in cvs. Solara and Frilene, respectively. **a** (*left to right*) cv. Solara (*S*), S(*er1mut1*) (*Sr*) and heterozygous (*H*) [S(*er1mut1*) × (cv. Frilene)] F_1 plant. **b** (*left to right*) cv. Frilene (*F*), F(*er1mut2*) (*Fr*) and heterozygous (*H*) [F(*er1mut2*) × (cv. Solara)] F_1 plant. *M* Molecular-weight marker

Two of the three mutations in the *PsMLO1* gene induced by ENU were a G/A transition, which is the expected outcome of the alkylation of guanine at the oxygen 6 position and one of the most common pre-mutagenic DNA lesions induced by this alkylating agent. However, the third mutation was a C/G transversion, a much less frequently induced mutation by this mutagen (Leitão 2012).

The drastic protein-truncating effects of the induced point mutations (SNPs) on the PsMLO1 protein in the two ENU-induced powdery mildew-resistant lines provides additional evidence for the observed resistance resulting from the loss of function of this protein (Humphry et al. 2011; Pavan et al. 2011). Nevertheless, the molecular mechanism that leads to resistance still waits future elucidation and constitutes a specific exciting modern research topic in the field of the plant–pathogen relationships.

Both mutant lines are presently available at the John Innes Institute and can also be requested from our laboratory (www.lggi.ualg.pt). The genomic sequence of the *PsMLO1* gene was uploaded to the NCBI public genomic database (www.ncbi.nlm.nih.gov) and can also be found, with additional information, in the Electronic Supplementary Material accompanying this publication. The amplification of the fragments for CAPS analysis and of the specific STS markers was performed in a 25-µl final volume of reaction mixture constituted by 0.16 mM of each dNTP, 0.2 µM of each primer, 15 ng of genomic DNA, 1 U of Dream Taq™ DNA polymerase and 1× Dream Taq™ Green Buffer (Thermo Fisher Scientific, Waltham, MA), using an initial denaturation step of 1.5 min at 94 °C, followed by 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 60 °C) and extension (1 min at 72 °C), with a final extension step at 72 °C for 10 min. The digestion with restriction enzymes *Hph*I (New England Biolabs, Ipswich, MA) and *CviKI*-1 (New England Biolabs) were performed according to the instructions of the suppliers, except for the digestion

Table 2 Primers for direct PCR identification of the powdery mildew resistance alleles

S(*er1mut1*)	
Forward	5′-AGCATGTGGGCTCCGT**G**-3′
Reverse	5′-CATCATGTCCTGGAGGAAG-3′
F(*er1mut2*)	
Forward	5′-ATCTTCCTCCAGGACATG-3′
Reverse	5′-AATTAGTGGAAGAAATGG**T**AG**T-**3′

Additional information is provided in the Electronic Supplementary Material

Underlined nucleotides are intentionally mismatching

with *CviKI*-1 which was performed for only 15 min at 37 °C.

The selection based on molecular markers genetically linked to the phenotype of interest enables an enormous amount of time, material and human resources to be saved. This highly estimated aspect of MAS is even more evident when the trait of interest is recessive, which requires homozygosity for clear phenotypic expression. Nevertheless, the efficiency of the methodology can be increased if the markers are based on molecular features of the gene of interest itself. Here we provide two types of markers for the induced resistant genes: (1) CAPS markers for discriminating between heterozygous and homozygous plants for the resistance genes and (2) STS markers, which are easier to analyse but which do not allow heterozygous and homozygous plants to be discriminated. Either of these two types of markers will allow breeders to advance rapidly through successive plant generations without requiring either the presence of the pathogen and the conditions for the infectious process to occur and the expression and analysis of the plant–pathogen interaction phenotype, until advanced generations are reached.

Acknowledgments This work was funded by the Research Contract 15653/R0 granted by IAEA/FAO Vienna.

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