André Filipe Dias Almeida Cardoso

Establishment of a broad-spectrum marker for er1/PsMLO1 powdery mildew resistance in pea (*Pisum sativum* L.)

(Dissertation for Mestrado Integrado em Engenharia Biológica)

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Accomplished under the guidance of Prof. Dr. José M. Leitão

Universidade do Algarve

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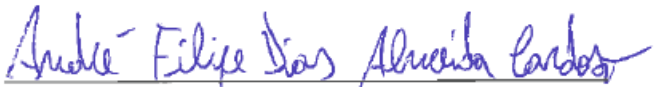
2018

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.



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Resumo

O oídio, causado pelo fungo ascomycete *Erysiphe pisi* Syd, é uma das doenças mais importantes que afectam a produção de ervilha (*Pisum sativum* L.) em Portugal e em países por todo o mundo (Sousa, 1999). Esta doença prospera em climas húmidos temperados causando perdas até 50% nas colheitas de ervilha, uma leguminosa muito importante e vastamente cultivada na Europa, sendo a quarta mais cultivada em todo o mundo (Rubiales et al., 2009; Warkentin et al., 1996).

A resistência a esta doença tem sido estudada desde 1925 (Hammarlund, 1925). No final da Segunda Guerra Mundial, Harland (1948) identificou uma nova fonte natural desta resistência numa variedade do Peru designada "Huancabamba" e através de cruzamentos do material resistente com a linha susceptível "First of all", observou que a geração F1 era susceptível e que na geração F2 se observava uma segregação mendeliana 3:1 (susceptível:resistente), concluindo que esta resistência era determinada por um único locus (*er*). Esta conclusão foi corroborada nas décadas seguintes por investigadores como Heringa (1969) e Sanexa et al. (1975), que efectuaram cruzamentos entre linhas resistentes (T10) e linhas susceptíveis (T163) e observaram que a resistência ao oídio era atribuída a uma condição monogénica e recessiva homozigótica.

A resistência ao oídio (Powdery Mildew Resistance - PMR) em *Pisum* foi identificada em germoplasma originário de locais de todo o mundo. Sabe-se na actualidade que a grande maioria das PMRs identificadas em ervilheira são determinadas pelo locus *er1* (Harland 1948; Sharma 2003; Liu et al., 2003; Sun et al., 2015). A resistência atribuída por este locus, é de amplo espectro e caracterizada pela falha do patogénio em penetrar e infectar as células da epiderme da planta (Fondevilla et al., 2006).

Um segundo locus monogénico recessivo para resistência ao oídio (*er2*) foi identificado em poucas linhas de ervilheira. Esta resistência invulgar, em que os indivíduos demonstram alta resistência na folhagem, enquanto o caule e os botões florais são infectados, foi mapeada posteriormente no grupo de ligamento III de ervilheira (Katoch et al., 2010).

Um locus de resistência ao oídio de hereditariedade dominante *Er3*, foi identificado em *Pisum fulvum*, um parente selvagem de ervilheira (Fondevilla et al., 2007). A resistência conferida pelo *Er3* é de largo espectro e pode ser introgredida em *Pisum sativum* L. *via* cruzamentos inter-específicos (Fondevilla et al., 2007, 2008).

Através mutagénese experimental química usando o agente alquilante Etilnitrosoureia (ENU), Leitão et al. (1998) induziram os primeiros mutantes resistentes ao oídio em *Pisum sativum* nas cultivares Solara e Frilene. Através do cruzamento destes mutantes com as respectivas cultivares originais susceptíveis, Pereira e Leitão (2010), verificaram: que estas PMRs induzidas eram recessivas e monogénicas. Através de cruzamentos de complementação entre os mutantes resistentes e uma linha resistente (E835) contendo o gene de resistência ao oídio *er1* (proveniente da linha Mexique 4), foi possível observar que ambas as mutações afectavam o mesmo locus e que este locus era o *er1*.

Pouco depois, Pavan et al. (2011) induziram, por mutagénese experimental química com sulfato de dietilo, um novo mutante resistente ao oídio, também monogénico e recessivo, e afectando o locus *er1*.

Marx (1974) observou que o locus *er1* se encontrava próximo do locus para o tegumento da semente *Gty* (Gritty). Dirlewanger et al. (1994) identificaram pela primeira vez um marcador de DNA (RFLP p236) a 9.8 (± 5.9) centimorgans (cM) do locus *er1*. Quase simultaneamente, usando a análise por "Bulk segregant analysis" (BSA), Timmerman et al. (1994) identificaram um marcador RAPD ($OPD10_{650}$) muito próximo (2.0 cM) do *er1* que mapearam no grupo de ligamento VI. A construção posterior de um mapa genético permitiu mapear definitivamente o locus *er1* no grupo de ligamento VI (Weeden et al., 1998).

Com os objectivos de isolar o locus *er1* e identificar marcadores úteis para selecção assistida por marcadores (marker assisted selection - MAS) de indivíduos resistentes ao oídio em programas de melhoramento, múltiplos grupos de investigadores continuaram a busca por marcadores próximos ao locus *er1*.

Tiwari et al., (1998) identificaram três RAPD loci fortemente ligados ao *er1*: $OPE\text{-}16_{1600}$(4 ± 2 cM), $OPL\text{-}06_{1900}$ (2 ± 2 cM) and $OPO\text{-}18_{1200}$ (r.f. = 0.0), onde dois destes foram convertidos a marcadores SCAR e a ligação completa do marcador *Sc-OPO*$_{1200}$ ao locus *er1*foi confirmada.

Janila e Sharma (2004) identificaram dois marcadores RAPD, $OPO02_{1400}$ (4.5 cM) $OPU17_{1000}$ (10.3 cM), ligados ao locus *er1*.

Ek et al. (2005) identificaram 5 marcadores SSR, em que o mais próximo (*PSMPSAD60*) está localizado a 10.4 cM do locus *er1*.

No nosso laboratório, Pereira et al. (2010) tentaram identificar marcadores moleculares ligados a um dos alelos de resistência induzidos usando duas abordagens: 1) "near isogenic lines" – NILs, e 2) "Bulk Segregant Analysis", onde incluiram todos os marcadores moleculares com ligação ao *er1* identificados previamente.

Na análise NILs, foram usados um grande número de marcadores: 2800 RAPD, 280 ISSR, 3300 AFLP e só um marcador RAPD ($OPL13_{990}$) demonstrou polimorfismo entre a cultivar Frilene e a linha mutante F(*er1mut2*), que foi convertido em SCAR dominante mas observou-se que não tinha ligação à mutação PMR induzida.

A utilização de um elevado número de primers RAPD na análise "Bulk Segregant Analysis" (200 convecionais e 189 não convencionais) permitiu a identificação de 6 marcadores ligados ao locus mutado *er1mut2*. A identificação de marcadores adicionais, permitiu a construção de um grupo de ligamento com 16 marcadores moleculares e o locus *er1*.

Presentemente, apesar da sequência do *er1* já ser conhecida, a busca por marcadores de DNA ligados a este gene continua, pois é importante identificar novos marcadores polimórficos ligados a novas fontes de resistência ao oídio, ou quando marcadores já publicados não são polimorficos entre as linhas progenitoras utilizadas num programa específico de melhoramento (Srivastava et al., 2012; Sudheesh et al., 2014; Javid et al., 2015; Sun et al., 2015, 2016).

"Mildew resistance Locus O" (MLO) é uma família de proteínas inter-membranares que ocorrem no reino vegetal e foram inicialmente descritas no contexto da resistência ao oídio, onde as perdas de função devido a mutações naturais ou induzidas na cevada (*Hordeum vulgare* L.) conferiram resistência de largo espectro contra *Erysiphe graminis* DC f.sp. *hordei*, o fungo causador do oídio nesta espécie vegetal.

De forma semelhante, foi demonstrado em *Arabidopsis thaliana* e no tomate (*Solanum lycopersicum*) mutações que causam a perda de função em loci MLO, que conferiram resistência ao oídio (Büschges et al., 1997; Consonni et al., 2006; Bai et al., 2008). A resistência nestas três espécies foi observado ter características muito

semelhantes à resistência conferida pelo locus *er1* – monogénica, hereditariamente recessiva, de largo espectro, efectiva sob condições de campo e impedindo a invasão das células da epiderme por parte do patogénio. Assim, foi colocada a hipótese da resistência conferida pelo locus *er1* ser também conferida pela perda de função MLO (Bai et al., 2008).

Poucos anos depois, Humpry et al. (2011), com base na sequência de genes ortólogos do locus MLO (*A. thaliana* – *AtMLO2*, tomate – *SlMLO1*, *Medicago truncatula* – *MtMLO1*) amplificaram o cDNA de um locus *MLO* em *Pisum*.

Através de expressão génica transiente, este grupo demonstrou que este locus de *Pisum* (*PsMLO1*) coincide com o locus *er1* e publica a sequência expressa deste gene. Quase em simultâneo, Pavan et al. (2011) chegaram à mesma conclusão.

Pouco tempo após a publicação da sequência expressa do gene *PsMLO1*, Santo et al. (2013) obtiveram a sequência genómica completa deste gene no nosso laboratório. No entanto, a sequenciação do quinto intrão foi particularmente difícil, devido à presença de um motivo microsatélite (TA).

O locus *PsMLO1* tem 4,729 nucleótidos na *cv*. Frilene e 4,708 na *cv*. Solara, ambos com uma região codificante de 1,722 nucleótidos organizados em 15 exões. A diferença de tamanho da sequência deste gene deve-se a diferenças no microsatélite do intrão 5: 36 nucleótidos (TA_{18}) na cultivar Solara e 58 nucleótidos (TA_{29}) na cultivar Frilene. Em adição, na cultivar Solara este gene tem um nucleótido adicional (T) no intrão 14, sendo a diferença total entre estes genótipos de 21 nucleótidos (Santo et al., 2013).

Os métodos estabelecidos até à data para identificar marcadores moleculares adequados para selecção assistida por marcadores (MAS) usando novos alelos de resistência, requerem tempo e são propensos a falhar em certas condições.

Este trabalho teve como objectivo criar um procedimento simples e acessível, que permita a identificação e distinção dos alelos de ambos os progenitores e a análise da segregação entre a descendência, necessitando de um mínimo de trabalho laboratorial e investimento de tempo e que seja funcional para um amplo leque de diferentes alelos.

Para este efeito, foram testados primers que flanqueiam a sequência do microsatelite localizado no intrão 5 do *PsMLO1* em 12 cultivares e 2 linhas de ervilheira. Os produtos de amplificação foram analisados em electroforese em gel de agarose (2 a 4 %) e em poliacrilamida (10%).

A sequência do microsatelite apresentou tamanho diferente em cada um dos genótipos, permitindo a discriminação clara entre as diferentes cultivares e linhas testadas. Apesar da sequência que rodeia o microsatelite não ser adequada ao desenho de primers, três das combinações de primers deram bons resultados.

Marcadores SSR baseados neste microsatelite podem ser usados para seleção assistida por marcadores (MAS) em programas de melhoramento de resistência ao oídio em ervilheira, para um grande número de alelos do locus *er1*/*PsMLO1*, sem necessidade prévia de sequenciação, identificação exacta das mutações e deselvolvimento de marcadores específicos.

Palavras-chave: resistência ao oídio, *er1*, *Pisum*, *PsMLO1*, seleção assistida por marcadores, marcador SSR

Title: "Establishment of a broad-spectrum marker for er1/PsMLO1 powdery mildew resistance in pea (*Pisum sativum* L.)"

Abstract

Powdery mildew caused by the biotrophic ascomycete fungus *Erysiphe pisi* Syd. is one the most devastating diseases of (*Pisum sativum* L.) with a considerable impact in seed production. So far, the most efficient genetic resistance to this disease identified is conferred by the naturally occurring or experimentally induced by chemical mutagenesis recessive state of the locus *er1*. Identified over 6 decades ago and genetically mapped to the *Pisum sativum* Linkage Group VI over 20 years ago, this gene was recently identified as a homolog of the barley (*Hordeum sativum* L.) powdery mildew resistance gene MLO, and renamed as PsMLO1. The broad spectrum resistance conferred by the *er1/PsMLO1* locus was found to be a consequence of the loss of function of the encoded PsMLO1 protein. After the publication of the expressed sequence of this gene by another research group, we published the genomic sequence of this gene, which harbors a relatively long (TA) microsatellite sequence (SSR) in the fifth intron. SSR markers based on this highly polymorphic microsatellite can be used for marker-assisted selection in multiple pea powdery mildew resistance breeding programs involving the er1/ PsMLO1 resistance, except in the rare circumstances where the progenitor lines are monomorphic for the microsatellite sequence. The use of established SSR markers is an affordable and straightforward approach for identification and discrimination of alleles of progenitors in breeding programs, permiting the easy analysis of their inheritance among progenies.

Key words: Powdery mildew resistance; *Pisum*; *PsMLO1*; *er1*; SSR marker;

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Abbreviations

AFLP – Amplified fragment length polymorphism

APS – Ammonium persulfate

AtMLO– *Arabidopsis thaliana* Mildew resistance Locus O

BSA – Bulked segregant analysis

CAPS – Cleaved amplified polymorphism sequence

cv- cultivated variety

cDNA – Complementary deoxyribonucleic acid

cM – CentiMorgan

DNA – Deoxyribonucleic acid

EDTA –Ethylenediaminetetra-acetic acid

ENU – *N*-ethyl-*N*-nitrosourea

EST – Expressed sequence tag

FAO/IAEA- Food and Agriculture Organisation/International Atomic Energy Agency

INRB –"Instituto Nacional de Recursos Biológicos"

ISSR – Inter simple sequence repeat

LGMG – "Laboratório de Genómica e Melhoramento Genético"

MLO – Mildew resistance Locus O

NIL – near isogenic line

PCR – Polymerase chain reaction

PMR – Powdery mildew resistance

PsMLO – Pisum sativum Mildew resistance Locus O

RACE - rapid amplification of cDNA ends

RAPD – Random amplified polymorphic DNA

r.f. – recombination frequency

RFLP – Restriction fragment length polymorphism

SCAR – Sequence characterized amplified region

SDS – Sodium dodecyl sulphate

SlMLO – *Solanum lycopersicum* Mildew resistance Locus O

SSR – Simple sequence repeat or microsatellite

TBE – tris - borate - EDTA

T-DNA – transfer DNA

TE – Tris-EDTA

TEMED – Tetramethylethylenediamine

TM – transmembrane motiff

Tris –tris(hydroxymethyl)aminomethane

TRV – tobacco rattle virus

1. Introduction

1.1 - Identification of natural sources of powdery mildew resistance

Powdery mildew, caused by the biotrophic ascomycete fungus *Erysiphe pisi* Syd, is one of the most important diseases affecting pea (*Pisum sativum* L.) production in Portugal and in countries all over the world (Sousa., 1999). This disease causes yield losses up to 50% in pea, a very important and widely grown grain legume in Europe and the fourth most grown in the world (Rubiales et al., 2009; Warkentin et al., 1996). Powdery mildew is prevalent in humid and temperate climates, significantly impacting yield and crop quality in field and greenhouse farming (Warkentin et al., 1996).

As early as 1925, Hammarlund (1925) was investigating powdery mildew resistance (PMR) in Sweden and this resistance was thought to be regulated by 4 genes acting additively. Soon after the Second World War, PMR has been reported and identified by Harland (1948) in the pea landrace "Huancabamba", an adapted peruvian type of pea originally brought to the Andes by the Spanish during the sixteenth and subsequent centuries. This resistance was investigated through crosses of this resistant material with the susceptible variety "First of all". The subsequent analysis of the progeny revealed a simple Mendelian recessive segregation leading to the conclusion that the resistance was governed by a single gene (*er*). Heringa (1969) and Saxena et al. (1975) also observed that when a highly susceptible line (T163) was crossed with a highly resistant one (T10) the F1 progeny was found to be completely susceptible and the F2 progeny segregating in a 3 susceptible to 1 resistant ratio. In conclusion, in these three cases, the resistance was attributed to a monogenic recessive homozygous condition.

Natural resistance to powdery mildew in pea (*Pisum sativum* L.) has been identified in germplasm from many different places e.g. Sweden (Hammarlund, 1925), Peru (Harland, 1948), India (Saxena et al., 1975; Sharma 2003), Australia (Liu et al., 2003), Spain (Fondevilla et al., 2006) and even China (Sun et al., 2015), and regardless

of its place of origin all sources of the resistance have shown monogenic recessive inheritance.

Today it is known that the large majority of PMRs identified in pea are determined by the locus *er*1 (Harland 1948; Sharma 2003; Liu et al., 2003; Sun et al., 2015). The resistance conferred by this locus, genetically mapped to the pea linkage group VI (Timmerman et al., 1994; Weeden et al., 1998), is characterized by failure of the pathogen to penetrate and infect the epidermal cells (Fondevilla et al., 2006).

A second recessive locus for powdery mildew resistance, *er*2 was identified in very few lines, *e.g.* SVP 951, SVP 952 and JI2480 (Heringa et al., 1969; Tiwari et al., 1997). However, Heringa et al., (1969) observed that this PMR presents an unusual resistance to the pathogen, as the *er*2 bearing plants demonstrated very high resistance in leaves, while the stem and buds were heavily infected, an observation corroborated by later studies (Tiwari et al., 1997; Marx 1986; our own observations). Furthermore, this resistance, also monogenic, was shown to be partially or totally broken depending on biotic and abiotic stress factors such as leaf age, field versus glasshouse growth conditions and temperature (Heringa et al., 1969; Tiwari et al., 1997; Fondevilla et al., 2006).

Recently, Katoch et al. (2010) investigated the unusual resistance exhibited by the JI2480 line andbythe F2 progeny of the cross of this line with the susceptible cultivar Lincoln, confirmed that this resistance was monogenic and recessive and controlled by the *er2* locus that these authors mapped to the pea linkage group III.

A dominantly inherited powdery mildew resistance locus *Er*3 was identified in *Pisum fulvum*, a pea wild relative (Fondevilla et al., 2007). This species, original from the eastern Mediterranean, is more resistant than *P. sativum* to various pea diseases and insect pests, namely powdery mildew (*Erysiphe pisi*), but possesses undesirable agronomical traits (Fondevilla et al., 2007).

Conferring complete powdery mildew resistance against different *E. pisi* isolates, the *Er3* locus can be introgressed into *Pisum sativum* L. *via* interspecific crossings (Fondevilla et al., 2007, 2008).

1.2 -Experimental induction of powdery mildew resistance

Experimental mutagenesis has an important role in plant breeding *via* induction of new mutants of agronomical interest. So far, thirty-four pea (*Pisum sativum* L.) mutant varieties are listed in the FAO/IAEA database (www.mvd.iaea.org). Some of these varieties (e.g. "Stral-art", in Sweden, 1954) were developed from a mutant induced by x-ray irradiation, while others, like the Russian variety "Orphei" (1989) were developed by mutagenic chemical treatments. A large number of induced mutants are additionally maintained as publically accessed accessions at the John Innes Pisum Collection (www.jic.ac.uk).

In 1998, in our lab, Leitão et al., (1998) induced, in the commercial varieties Solara and Frilene, via experimental chemical mutagenesis using the alkylating mutagenic agent ethylnitrosourea (ENU), the first two powdery mildew resistant mutants in *Pisum sativum* L. (Fig.1).

Figure 1 The first induced powdery mildw resistant mutants in *Pisum sativum* L. Left. Resistant mutant S(*er1mut1*). Right – Resistant mutant F(*er1mut2*). Notice the contrast between the susceptible material, completely white - fully infected with powdery mildew – and the resistant mutants, green and healthy, with no disease symptoms.

Several other mutants, exhibiting other traits of agronomical interest such as higher number of productive nodes, erected plants with higher number of pods, short-

internode plants, among others, were also identified and included in the INIAV pea breeding program developed at the National Plant Breeding Station, in Elvas.

Leitão et al., (1998) observed that the powdery mildew resistant (PMR) mutant induced in the cultivar Solara differentiates from the original cultivar by presenting more basal branches than the original, i.e. a *ramosus* tendency, longer period to flowering, less seeds per pod and smaller seeds, while the PMR mutant induced in the cv. Frilene is very similar phenotypically to the original cultivar. In addition, since only one PMR individual was observed among the M2 families of each cultivar, it was hypothesized that both mutations were recessive.

The crosses between the two induced mutants and their original susceptible cultivars resulted in a completely susceptible F1 population, a result that confirmed the recessiveness of both mutations. The segregation analysis of the PMR among the F2 progenies revealed a very clear 3:1 (susceptible : resistant) mendelian ratio evidencing the monogenic character of the induced PMR mutation (Pereira and Leitão, 2010).

Through the complementation (resistant x resistant) crosses performed between both resistant mutant lines and a resistant line (E835) carrying the PMR gene *er*1 (from Mexique 4), it was clear that both PMR mutations affected the same locus and that this locus was the *er*1 (carried by line E835). The mutant alleles were named *er1mut1* and *er1mut2*, by the order of their induction, and the respective mutant lines: S(*er1mut*) and F(*er1mut2*) (Pereira and Leitão, 2010).

Meanwhile, in Italy, Pavan et al., (2011) induced a new PMR mutant also recessive and monogenic and affecting the locus *er1*, through experimental chemical mutagenesis using a different alkylating agent – diethyl sulfate – on a breeding line derived from the cultivar "Sprinter", obtaining a new resistant line named ROI3/02 (Fig. 2).

Figure 2 Powdery mildew resistance mutant line ROI3/02 induced by Pavan et al. (2011)

1.3 - Genetic mapping of the resistance locus *er*1.

Attempts to locate the locus for PMR in pea have been made, starting as far back as 1948, when Harland (1948) suggested that *er1* was located on chromosome 1, relatively away (35 centimorgans - cM) from the main locus *A* for anthocyanin pigmentation of stem axes, flowers and testa, a result that failed to be later corroborated by other authors (Marx, 1971).

Few years later, Marx (1974) reported that *er1* was located in proximity to: 1) the locus for gritty testa *Gty* (Gritty); 2) the locus for begonia flower color *B*; and 3), the locus for ochraceous colored seed coat (*Och*), indicating that all these other loci must all be located in the same chromosome.

Two decades later, Dirlewanger et al. (1994), identified for the first time a DNA marker, the RFLP locus p236, linked to the locus *er1* at 9.8 (±5.9) centimorgans (cM).

Due to the fact that the *er*1 locus location was still not very clear, using a bulk segregant analysis (BSA) approach, Timmerman et al. (1994) performed a detailed genetic analysis of the powdery mildew resistance trait in two segregating populations identifying a RAPD marker ($OPD10_{650}$) tightly linked (2.0 cM) to the locus *er*1 and concluding that this locus was mapped on linkage group VI, and as previously observed by Marx (1971), the *Gty* gene was identified as the closest morphological marker to the locus *er1*.

Integrating the data of these last referred studies with multiple other linkage studies, a consensus genetic linkage map for *Pisum sativum* L. genome was constructed and the recessive locus for powdery mildew resistance *er*1 was definitively mapped to linkage group VI (Weeden et al., 1998).

Aiming both at the isolation of the locus *er1* and identification of markers useful for Marker Assisted Selection (MAS) in order to facilitate the selection processes in pea PMR breeding programs, multiple research groups have searched for molecular markers closely linked to the *er1* locus.

Tiwari et al., (1998), based on bulk segregant analysis, identified three RAPD loci strongly linked to *er1*: OPE-16_{1600} (4 ± 2 cM), OPL-06_{1900} (2 ± 2 cM) and OPO-

18_{1200} (r.f. = 0.0). Two of these RAPD markers were converted to polymorphic SCAR markers, confirming the complete linkage of the marker *Sc-OPO*$_{1200}$ to the *er1* locus.

Janila and Sharma (2004) identified two RAPD markers $OPO02_{1400}$ (4.5 cM) and $OPU17_{1000}$ (10.3 cM) linked to the *er1* locus.

Ek et al. (2005) tested 315 SSR markers of the genetic map constructed by Loridon et al. (2005) and identified 5 SSR markers linked to the *er1* locus. Nevertheless, the closest marker (*PSMPSAD60*) was located relatively apart of this locus (10.4 cM).

In our laboratory, Pereira et al. (2010) tried to identify new molecular markers linked to the PMR mutated allele *er1mut2* induced in *cv*. Frilene through two different approaches: 1) Near isogenic lines (NILs) analysis; and 2) bulk segregant analysis (BSA), while including in the analysis all identified molecular markers linked to *er1* reported thus far.

The NILs analysis, a large number of markers was used: 2800 RAPD, 280 ISSR, 3300 AFLP, and only one RAPD marker ($OPL13_{990}$) was identified as polymorphic between the *cv*. Frilene and the mutant form F(*er1mut2*). Nevertheless, this marker, converted into a dominant SCAR, was found to be unlinked to the induced PMR mutation.

The use of 200 conventional and 189 unconventional RAPD primers in BSA analysis allowed the identification of 6 markers linked to the PMR *er1mut2* locus. The mapping of additional molecular markers, polymorphic among the segregating (F2) progeny, allowed the construction of a linkage group gathering 16 molecular markers and the *er1* locus (Fig. 3).

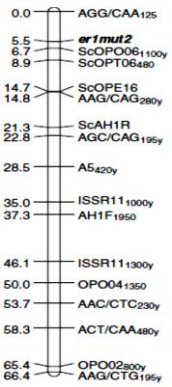

Figure 3 Genetic linkage map containing 16 DNA-markers and the mutated gene *er1mut2* (Pereira et al., 2010)

Presently, in spite that the sequence of the *er1* gene is already deciphered, the search for DNA markers linked to this gene continues, as it is important to identify new polymorphic markers linked to novel source of powdery mildew resistance or when the already published markers are not polymorphic between progenitor lines used in a specific breeding program (Srivastava et al., 2012; Sudheesh et al., 2014; Javid et al., 2015; Sun et al., 2015, 2016).)

1.4 -From the powdery mildew resistance in barley to the molecular identification of the *er*1 (PsMLO1) locus

Barley Mildew resistance Locus O (MLO) is a family of integral membrane proteins that occur in the plant kingdom. These were first described in the context of powdery mildew infection resistance, where losses of function due to natural or induced mutations in barley (*Hordeum vulgare*) conferred broad-spectrum resistance against *Erysiphe graminis* DC f.sp. *hordei*, an obligate biotrophic fungus that is the cause of this widespread plant disease.

The MLO controlled powdery mildew resistance in barley has been reported for more than 60 years, when Freisleben and Lein (1942) induced by X-rays the first powdery mildew resistant mutant, Mutante 66 (M66) in the German *cv*. Haisa. In the following years, many MLO controlled PMR resistant mutants were induced through experimental mutagenesis, until Jørgensen (1976) identified a spontaneously occurring MLO allele in the Ethiopian barley line Grannenlose Zweizeilige - a recessive monogenic locus designated *mlo-11* which conferred broad-range resistance.

Similarly, in the dicots *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*), mutations that caused loss of function in MLO loci were shown to confer powdery mildew resistance (Büschges et al., 1997; Consonni et al., 2006; Bai et al., 2008)

In the model species *Arabidopsis thaliana*, Consonni et al. (2006) investigated that three of its MLO loci (*AtMLO2, AtMLO6 and AtMLO12*) are the functional complements (co-orthologs) of barley MLO, with *AtMLO2* having a major role while

the other two played minor roles, and concluded that full resistance requires loss of function of the three co-orthologs (Fig. 4).

Figure 4 Powdery mildew (*Oidium neolycopersici)* requires AtMLO2 loss of function for pathogenesis on *Arabidopsis thaliana*. Powdery mildew infection in 7-week-old *A. thaliana* wild-type (Col-0) and T-DNA insertion mutants (*Atmlo2*, *Atmlo6*, *Atmlo12* single, double, and triple mutants) (Bai et al., 2008).

In tomato (*Solanum lycopersicum*), the natural allele *ol-2* was identified by Ciccarese et al. (1998), conferring recessively inherited broad-spectrum resistance to powdery mildew (*Oidium neolycopersici*). The resistance allele *ol-2* originated from a wild accession of cherry tomato (*S. lycopersicum* var. *cerasiforme*) LA-1230 collected in 1970 in a home garden in Ecuador. Bai et al. (2008) cloned the tomato MLO gene *SlMLO1* and observed that this powdery mildew resistance is mediated by loss of SlMlo1 function (Fig. 5).

Figure 5 Left. Virus-induced gene silencing of *SlMlo1*. **Right.** Leaves of wild-type tomato *cv.* Moneymaker. (Bai et al., 2008).

This *mlo*-conditioned resistance observed in barley, Arabidopsis and tomato was found to be characteristically very similar to *er1* by Bai et al., (2008) – a monogenic, recessively inherited broad-spectrum resistance, durable under agricultural conditions, with impediment of pathogenesis by preventing epidermal cell invasion. Thus, Bai et al., (2008) hypothesized that *er1* resistance could be conferred by loss of MLO function.

A few years later, Humphry et al. (2011) used a PCR based approach to obtain a *MLO* candidate gene for pea *Er1* plants, due to the fact that BLAST searches did not yield any EST sequence with enough homology to any *MLO* gene. Taking advantage on known sequence information from other plant species, Humphry et al. (2011) performed an alignment of *MLO* coding sequences of suspected orthologs, such as *A. thaliana* (*AtMLO2*), tomato (*SlMLO1*), *Capsicum annuum* (pepper; *CaMLO1*), *Lotus japonicas* (*LjMLO1*) and *Medicago trucatula* (*MtMLO1*). The conserved regions were selected to deduce the oligonucleotide sequences for PCR-based amplification of the respective part of the pea *MLO* cDNA. Reverse transcription – polymerase chain reaction (RT-PCR) was then performed using pea RNA derived from the wild-type cultivar JI 502 as a template. Through BLASTX analysis of the sequenced PCR products, Humphry et al. (2011) observed that the respective cDNA fragments encoded a part of a MLO-like protein. Performing a 5' and 3' rapid amplification of cDNA ends (RACE) with new designed internal oligonucleotide pairs, the full-length sequence information of this cDNA was obtained. The obtained sequences were overlapped and resulted in a cDNA that encodes a protein with a sequence highly related to *AtMLO2, SlMLO1, CaMLO1, LjMLO1*, and through a phylogenetic analysis it was determined to represent a genuine ortholog of these proteins, designated *PsMLO1*.

Four independent lines from distinct geographical origin – JI 210 (India), JI 1559 (Mexique 4, Mexico), JI 1951 (China), JI 2302 (Stratagem, USA) - reported previously by Tiwari et al., (1997) to harbor *er1* resistance were analyzed by Humphrey et al. (2011) and pre-invasive immunity was observed in these lines. The *PsMLO1* cDNA sequences of the four resistant lines were determined through direct sequencing of the full-length RT-PCR products, and polymorphisms were detected: 1) single-nucleotide deletions resulting in frame shifts in lines JI 210 and JI 1951; 2) a nucleotide substitution originating an early stop codon in JI 1559; 3) multiple overlapping sequence traces in JI 2302, that were analyzed and it was concluded to be an insertion of

a large transposable element (transposon). Lastly, to further the claim that *PsMLO1* is *Er1*, Humphrey et al. (2011) performed the complementation of the *er1* resistance by transient gene expression, using a bombardment-mediated transformation of single leaf epidermal cells to test the functionality of *PsMLO1*. It was observed that successfully transformed cells were attacked by powdery mildew while the non-transformed retained resistance against the fungus, corroborating their claim.

Almost simultaneously, Pavan et al. (2011) arrived at the same conclusions. At first, the induced PMR mutant line "ROI3/02", was crossed with the susceptible cultivar "Progress9" and their F_2 progeny segregated in a clear 3:1 Mendelian ratio, confirming that the resistance was recessive and monogenic. In similarity to previous studies (Consonni et al., 2006; Fondevilla et al., 2006), in line ROI3/02 the pathogen (*E. pisi*) could not penetrate the epidermal cells to infect them. DNA from this F_2 (ROI3/02 x Progress9) population and its parental lines was then extracted, and the parental lines were tested with three SCAR markers with known linkage to *er1*: ScOPD-10_{650}, ScOPO-18_{1200} and ScOPO-06_{1100} (Timmerman et al., 1994; Tiwari et al., 1998; Pereira et al., 2010). The markers that presented polymorphisms - ScOPO-18_{1200} and ScOPO-06_{1100} - were then tested on the F_2 progeny and were found to be related to the same locus (*er1*) with a distance of 1.1 cM for the former and 3.1 cM for the latter. Finally, Pavan et al. (2011) performed a cross between the line ROI3/02 and the resistant line Franklin that yielded in an F_1 population where all individuals exhibited the powdery mildew resistance phenotype, providing final proof of the identification of a new mutant at the *er1* locus.

With cDNA from Sprinter and ROI3/02, Pavan et al. (2011) amplified the full-length coding sequence of *PsMLO1* (Fig. 6) and it was found to contain a point mutation: a G/A transition that originated an early stop codon in its sequence, causing a premature termination of translation and consequently a truncated protein. This mutation occurring in the *PsMLO1* sequence of ROI3/02 was observed to result in a cutting site for the restriction enzyme *Sml*I, so Pavan et al. (2011) designed a primer pair that flanked the mutation site and developed a cleaved amplified polymorphic sequence (CAPS) marker GIM-300/*Sml*I. All the resistant individuals from the F_2 population (ROI3/02 x Progress9) were homozygous for the G/A *PsMLO1* transition, while the susceptible phenotypes were either homozygous for the wild-type allele or heterozygous. The same primer pair was used for PCR amplification of cDNA from the

known resistant line Franklin, homozygous for an *er1* allele and from the PMR homozygous lines Dorian and Nadir, whose genotype was unknown at the *er1* locus. The resulting *PsMLO1* transcripts were predicted to be associated with non-functional proteins, providing further proof of the co-segregation of *er1* PMR and *PsMLO1* loss of function.

***Pisum sativum* MLO1 (MLO1) mRNA, complete codons**

(GenBank: FJ463618.1; Panstruga,R. and Reinstaedler,A., 14-NOV-2008)

```
ACCESSION   FJ463618
ORIGIN
     1 aaaacaacaa gtcaaaaaag aaagaaaaaa tggctgaaga gggagttaag gaacgaactt
    61 tggaagaaac accaacttgg gctgttgcag ttgtgtgtct tgtgttgcta gctgtttcaa
   121 tcttaattga acatattatt catgttattg gaaagtggtt gaagaagaga aacaaaaatg
   181 ctctttatga agctttggaa aagatcaaag gagagcttat gctactagga ttcatatcct
   241 tgcttctaac tgtcttccaa gataatattt ctaaaatatg cgtatcacaa aaaattggat
   301 caacttggca tccttgttcc acttcaaaca caaaggccaa ggctaaatct gatgaatcat
   361 tagactataa aaccaacaat gatagaaaac tcttggagta ttttgatcct attcctcgga
   421 gaattcttgc tacaaaagga tatgataaat gttttgataa gggtcaagtt gcattagttt
   481 ctgcatatgg aattcaccaa ctccatatat tcatttttgt gctggcacta tttcatatcc
   541 ttcaatgtat aataacatta actttgggaa gaatcaagat gaggaagtgg aagacttggg
   601 aagatgagac aagaacagtt gaatatcaat tttataatga tcctgagagg tttaggtttg
   661 caagggacac aacatttgga agaaggcact tgagcatgtg ggctcagtca cctattttgt
   721 tatggattgt tagcttcttc agacaattct ttggatctat cagtagagtt gattatatgg
   781 ctcttaggca tggatttatc atggctcatc ttcctccagg acatgatgca caatttgatt
   841 tccaaaagta tataagtaga tcaattgaag aggattttaa agttgttgta ggaataagtc
   901 caactatctg gctcttcaca gtgcttttcc ttcttacaaa tactcatggg tggtattctt
   961 attattggct tccatttctt ccactaattg taatcttatt agttggtgct aagttacaaa
  1021 tgatcataac aaaaatggga ttaaggattc aagacagagg agaagtaatc aagggtgcac
  1081 ctgtggttga gcctggagat caccttttct ggttcaatcg tcctcacctt cttctcttca
  1141 cgattcatct tgttctcttt cagaatgcct ttcaacttgc atttttgct tggagtacat
  1201 atgagttttc cataacctct tgcttccaca aaacaactgc agatagtgtc attagaatca
  1261 ctgtaggggt tgtaatacaa actctatgta gctatgtgac tttgcctctt tatgctctag
  1321 tcacacagat gggatcaacc atgaaaccaa ccattttcaa cgaaagagtg gcaacagcgc
  1381 ttaagaactg gcaccacaca gccaaaaagc aggtaaaaca gagcaaccac tcaaacaaca
  1441 cgacaccgta ttcaagcagg ccatcaaccc caacacatgc catgtctcct gttcacctgc
  1501 tccatagaca cactgctgga aacagcgaca gtctacaaac ttctccggaa aagtctgatt
  1561 ataaaatga acagtgggat attgaaggag aaggaccaac ttccctaaga aacgatcaaa
  1621 cagggcaaca tgagattcaa atagcgggtg tcgagtcatt ttcgtcaacc gaattgccgg
  1681 ttagaattag acatgaaagc acctctggtt caaaagattt ttctttcgag aagcgccact
  1741 tagggagcaa ttagaattgt aggtattgat aaccagttca atgtatacca attaggtaca
  1801 ttcttgcaga taaagataga ggaactcctt ctaagaatgg agtgtaaatt tgttgaggta
  1861 gcagcttgat ttgtggatat aatcataggg tatgaaaatg caagactata ttttgtaaaa
  1921 aaaaaaaa

Submitted (14-NOV-2008) Plant-Microbe Interaction, Max-Planck Institute for
Plant Breeding Research,   Carl-von-Linne-Weg 10, Koeln 50829, Germany
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Figure 6 Expressed sequence of *PsMLO1*, GenBank: FJ463618.1 (Humphry et al., 2011)

The *MLO* genes code for a plant-specific type of integral membrane protein, characterized by seven transmembrane (TM) regions (Fig. 7). The biochemical function of these proteins is unknown. Earlier studies have hypothesized that these proteins could serve as modulators of plant defense or as targets for defense suppression (Panstruga and Schulze-Lefert, 2003).

Later on, Bai et al. (2008) reported that only members of a specific phylogenetic clade are related to powdery mildew resistance/susceptibility. Very recently, Kusch et al. (2016), through phylogenetic analysis, identified evolutionary patterns such as

conserved protein motifs which lead to the hypothesis that MLO proteins are related to transmembrane transport and regulation functions.

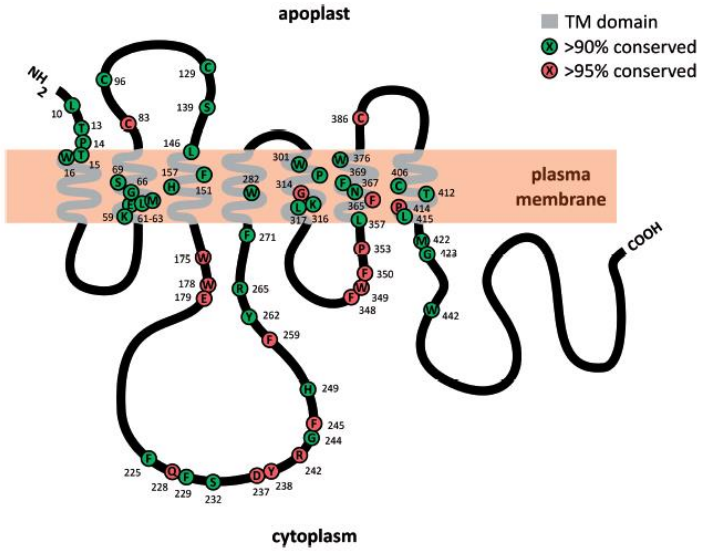


Figure 7 The plant MLO protein. Notice the seven trans-membrane domains of this cell membrane protein (Kusch et al., 2016).

1.5 - Identification of the genomic sequence of the gene PsMLO1

Not long after Humphry et al. (2011) published the expressed sequence of the *PsMLO1* gene, Santo et al. (2013) published the full genomic sequence of this gene.

The work was carried out using genomic DNA from leaves of: 1) plants of *cvs*. Solara and Frilene and their powdery mildew resistant mutant lines S(*er1mut1*) and F(*er1mut2*); 2) F_2 progeny plants of the crosses S(*er1mut1*) × *cv*. Frilene and F(*er1mut2*) × *cv*. Solara.

Using primers initially based on the expressed sequence, then based on the successively uncovered genomic sequence, and assembling the resulting partially overlapping fragments, the full genomic sequence of *PsMLO1* was deciphered (Fig. 8). Nevertheless, the uncovering of the sequence of intron 5 was particularly difficult, a consequence of the presence in this intron of a dinucleotide (TA) microsatellite sequence (Santo et al., 2013).

PsMLO1 was reported to be 4,729 nucleotides long in *cv*. Frilene and 4,708 in *cv*. Solara, both presenting the same coding region of 1,722 nucleotides organized in 15 exons, which led to the observation that this difference in size of the gene sequence between these cultivars is due to the differences in the microsatellite on intron 5. The identified SSR spans over 36 nucleotides (TA_{18}) in *cv*. Solara and 58 nucleotides (TA_{29}) in *cv*. Frilene. Also, the *PsMLO1* sequence has an additional nucleotide (T) in intron 14 of *cv*. Solara, amounting to a total difference of 21 nucleotides between these genotypes (Santo et al., (2013).

In both PMR mutant lines, S(*er1mut*) and F(*er1mut2*), Santo et al. (2013) observed that their sequences differed from the sequences of the respective original cultivars by induced small point mutations.

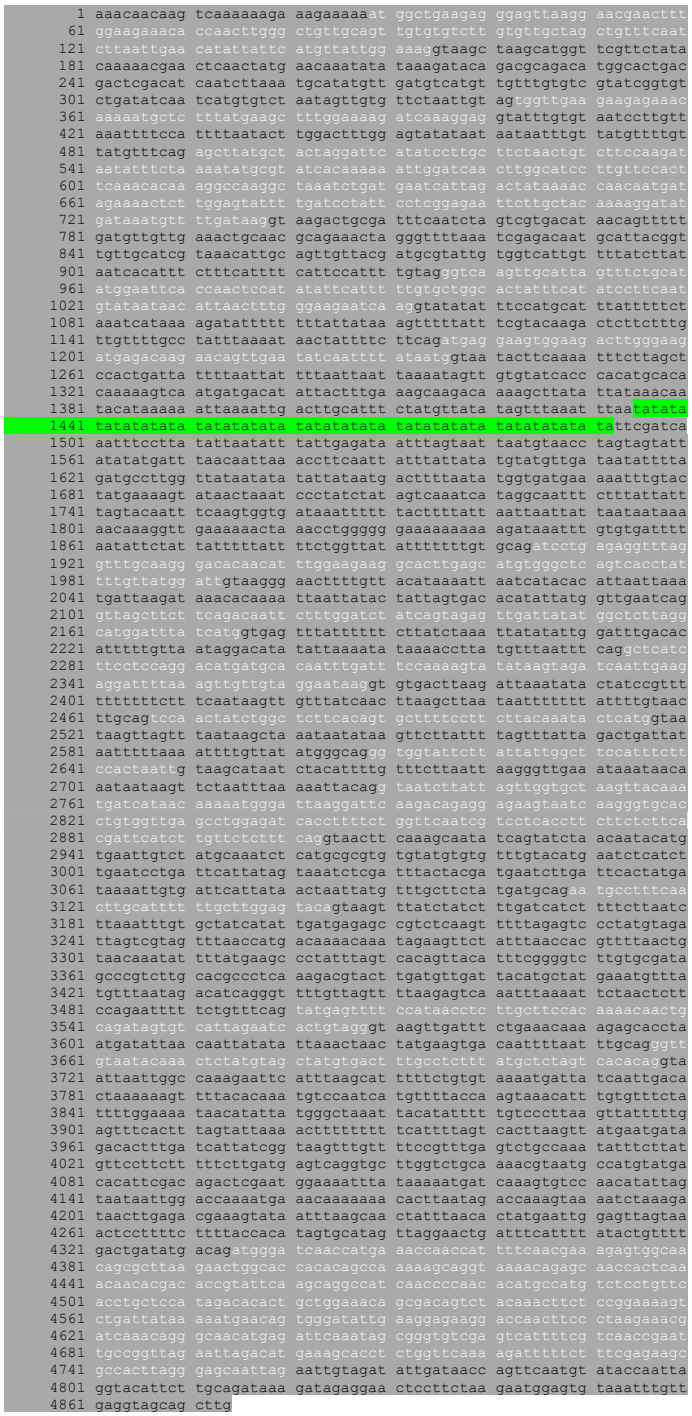
In S(*er1mut*) two SNPs were identified: 1) a C/G transversion in exon 6, resulting in an early stop codon, interrupting the translation between transmembrane domains 3 and 4; 2) a G/A transition in exon 11, which alters codon GAT (aspartic acid) into AAT (asparagine). Nevertheless, this second mutation was assumed to have no practical effect since the translation of the MLO protein was stopped upstream at exon 6.

In the Frilene mutant line F(*er1mut2*), a G/A transition was identified which also resulted in an early stop codon in exon, 10 interrupting the protein synthesis at the middle of the fifth transmembrane domain (TM5).

The presence of these mutations in these lines eliminated restriction sites recognized in the respective wild-types by the enzymes *Hph*I (S(*er1mut*)) and *CviKI*-1 (F(*er1mut2*)). These circunstances allow the creation of CAPS markers that discriminate between homozygous susceptible (wild-type allele), heterozygous susceptible (carrier) and homozygous resistant individuals by simple agarose gel electrophoresis.

Pisum sativum subsp. *sativum* cultivar Frilene MLO1 protein (MLO1) gene, AND complete codons

(GenBank: KC466597.; Leitao, J., Santo, T., Rashkova, M. and Alabaca, C., 10-Jan-2013)



Submitted (10-JAN-2013) FCT, Universidade Do Algarve, Campus de Gambelas, Faro 8005-139, Portugal

Figure 8 *PsMLO1* genomic sequence, *cv*. Frilene. Exons in white, introns in black, microsatellite motif highlighted in green (Santo et al., 2013).

1.6 - Objective of the work: To determine if the microsatellite present in the genomic sequence of PsMLO1 can be used as an efficient marker for powdery mildew resistance

Multiple research groups have been identifying molecular markers linked to *er1* (*PsMLO1*) aiming at the map-based cloning of this gene and for use in marker assisted selection (MAS) in PMR breeding projects.

Although important, the thus far established methods to identify markers suitable for (MAS) using novel alleles are time consuming and are prone to failure in certain conditions. For example, the direct sequencing of the new allele and the identification of suitable markers for it can be hampered when the mutation is created by a large indel or a transposon (Humphry et al., 2011).

Our objective was to create a straightforward and affordable procedure that would enable the identification and distinction of the alleles of both progenitors and the segregation analysis among the progeny, requiring minimal laboratory work and time investment, and functional in a broad-spectrum of different alleles.

Taking into account the fact that the *PsMLO1* gene harbors a microsatellite motif $(TA)_n$ located in the fifth intron (Fig. 9), this microsatellite motif could be transformed into a highly polymorphic genetic marker with maximal genetic linkage to the gene, which could be used for MAS in almost any pea powdery mildew resistance breeding program.

ACTTGGGAAGATGAGACAAGAACAGTTGAATATCAATTTTATAATGGTAATACTTCAAAATTTCTTAGCT
CCACTGATTATTTTAATTATTTTAATTAATTAAAATAGTTGTGTATCACCCACATGCACACAAAAAGTCA
ATGATGACATATTACTTTGAAAGCAAGACAAAAGCTTATATTATAAACAATACATAAAAAATTAAAATTG
ACTTGCATTTCTATGTTATATAGTTTAAATTTAATATATATATATATATATATATATATATATATATA
TATATATATATATATATATATATTCGATCAAATTTCCTTATATTAATATTTATTGAGATAATTTAGTAAT
TAATGTAACCTAGTAGTATTATATATGATTTAACAATTAAACCTTCAATTATTTATTATATGTATGTTGA
TAATATTTTAGATGCCTTGGTTATAATATATATTATAATGACTTTTAATATGGTGATGAAAAATTTGTAC
TATGAAAAGTATAACTAAATCCCTATCTATAGTCAAATCATAGGCAATTTCTTTATTATTTAGTACAATT
TCAAGTGGTGATAAATTTTTTTACTTTTATTAATTAATTATTAATAATAAAAACAAAGGTTGAAAAAACTA
AACCTGGGGGGAAAAAAAAAAAGATAAATTTGTGTGATTTTAATATTCTATTATTTTTATTTTCTGGTTAT
ATTTTTTTGTGCAG***ATCCTGAGAGGTTTAGGTTTGCAAGGGACACAACATTTGGAAGAAGGCACTTGAGC***

Figure 9 Sequence of the fifth intron of the *PsMLO1* locus in *cv*. Frilene. The $(TA)_n$ microsatellite is highlighted in *gray*. The initial and end sequence of the flanking exons are shown in bold and italic.

2 - Materials and Methods

2.1 - Plant Material

In the present work were used 12 *Pisum sativum* L. cultivars: Douce de Provence, Fallon, Frilene, Grisel, Kelvedon Wonder, Lincoln, Rondo, Senador Cambados, Telephone, Television, Progress 9 and Progreta; and 2 lines: JI2480 and S(er1mut1). Seeds were washed with tap water and immediately immersed for 5 minutes in disinfecting solution containing 10% bleach and 0,5% SDS, rinsed with tap water and germinated over moist paper in petri dishes for 48h, at 24ºC in a dark greenhouse.

2.2 - DNA extraction

Roots of *Pisum sativum* L. seedlings were cut, dried with absorbent paper and macerated in a sterilized ceramic mortar with liquid nitrogen until a homogeneous white powder was obtained. The material was transferred to sterilized eppendorf tubes and re-suspended in 900µL of extraction buffer, composed of TrisHCl 50mM, pH 7,5, 10mM EDTA and 1% SDS, and the tubes incubated for 15 minutes at 65ºC in a water bath. A volume of a phenol : clorophorm : isoamylalcohol (25:24:1) was added to the eppendorf tubes, the tubes were softly mixed by hand for 2 minutes and centrifuged at 13000 rpm for 5 minutes at 4ºC. This extraction was repeated twice with clorophorm :isoamylalcohol (24:1) and the aqueous phase was transferred to a new sterile eppendorf tube and the DNA precipitated by the addition of 1 volume of ice-cold isopropanol. Precipitated DNA was centrifuged at 13000 rpm for 10 minutes at 4ºC. The supernatant was decanted carefully, 80% ethanol was added to the pellet and centrifuged again at 13000 rpm for 5 minutes at 4ºC. The ethanol was decanted and the pellet was dried at room temperature for 30 minutes, then dissolved in 1x TE. The sample of DNA was treated with RNaseA (10µg/mL) for 1 hour at 37ºC, and extracted once with clorophorm :isoamylalcohol (24:1). The purified DNA was precipitated nadressupeded in $TE_{0.1}$ as above described, and stored at -20ºC.

2.3 - PCR Amplification

A PCR mix of 30µL was prepared by combining 3µL of 10xNZYtaq™ buffer, 0,48µL of dNTPsNZYMix™ (10mM), 1,5µL of $MgCl_2$, 0,24µL of NZYtaq™ polymerase, 4µL of sample DNA at 5ng/µL, 4µL of PCR primer (10mM)andMiliQ H_2O up to 30µL.

The thermocycler (VWR) was programmed as follows: 1 minute and 30 seconds initial denaturation cycle at 94ºC followed by 35 (or 28) cycles of 30 sec at 94ºC, 1 min at the annealing temperature adequate to the primer pair used (58ºC, 60.5ºC, or 62ºC) and 1 min at 72ºC, ending with a final elongation cycle of 10 min at 72ºC.

2.4 - Gel Electrophoresis – agarose

The amplification products were put through an electrophoresis in 2 to 4% agarose gels, ran at 8V/cm for the appropriate amount of time. Gels were stained with ethidium bromide and photographed under UV trans-ilumination with a digital camera "Kodak EDAS 120".

2.5 - Gel Electrophoresis – acrylamide

Amplified products were analyzed by 10% and 15% polyacrylamide gel electrophoresis. A solution was prepared with stock acrylamide (40%), in 1X TBE (Tris-Borate-EDTA) electrophoresis buffer. After this, in a glass recipient, APS (ammonium persulfate) and TEMED (tetramethylethylenediamine) were added to the solution to catalyze the polymerization.

Gels were run at 220 volts for 1h and 10 min after the marker has left the gel, stained with ethidium bromide and photographed under UV trans-ilumination with a digital camera "Kodak EDAS 120", in similarity to the agarose gels.

3 - Results

Primers were designed closely flanking the microsatellite sequence. However, due to the fact that the genomic sequence surrounding the microsatellite has low suitability for primer designing, multiple primers and primer combinations were tested. Three of the primer combinations worked well and can be used for marker-assisted selection (Table 1).

Table 1 Primer combinations for PsMLO1-SSR amplification

Primer combinations	Primers	Annealing Temperature (ºC)	Expected size[a] (bp)
1	5′ - GACTTGCATTTCTATGTTATATAG – 3′ 5′ - AATATAAGGAAATTTGATCGAATAT - 3′	58	~115
2	5′ - AAATTGACTTGCATTTCTATGTT – 3′ 5′ - TACTACTAGGTTACATTAATTACTA – 3′	60,5	~175
3	5′ - AAATTGACTTGCATTTCTATGTT - 3′ 5′ - AGAAATTGCCTATGATTTGACT - 3′	62	~338

[a] Estimated size of the amplification product in *cv*. Frilene

Even so, the use of the first pair is recommended, for it produces shorter fragments easier to discriminate by agarose or polyacrylamide gel electrophoresis.

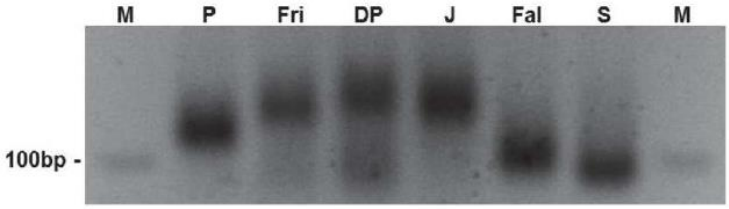


Figure 10 SSR-*PsMLO* marker amplification after 35 cycles (using primer combination 1) in six different pea genomes, analyzed on 3.5% agarose gel. Notice the high polymorphism of the marker. Stutter bands, a common problem of SSR amplifications, are visible. **P** - *cv*. Progreta, **Fri** - *cv*. Frilene, **DP** - *cv*. Douce de Provence, **J** - line JI2480, **Fal** - *cv*. Fallon, **S** - line S(er1mut1), **M** - molecular weight marker.

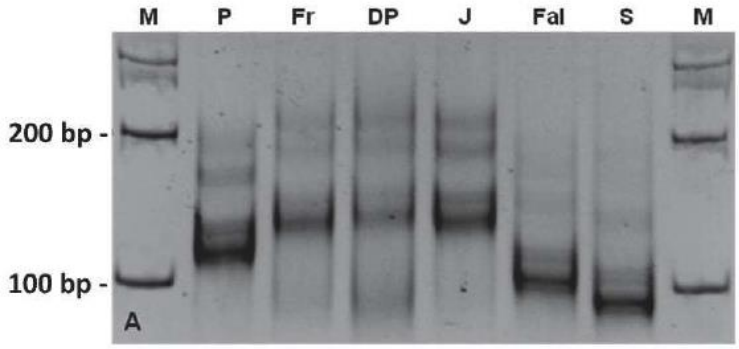


Figure 11 SSR-PsMLO1 marker (primer combination 1) amplified in six pea genomes analyzed on 10% polyacrylamide gels. Amplification after 35 cycles. Notice the high polymorphism of the marker. The smear of bands (stutter bands) and non specific products are clearly visible. **P** - *cv*. Progreta, **Fr** - *cv*. Frilene, **DP** - *cv*. Douce de Provence, **J** - line JI2480, **Fal** - *cv*. Fallon, **S** - line S(er1mut1), **M** - molecular weight marker.

Although the amplification of the SSR marker evidences a clear discrimination between most of the analysed MLO genes, a problem was found with all primer combinations: a systematic appearance of stutter bands which complicate the exact discrimination between alleles of similar size (Fig. 10 and Fig. 11)

Sutter bands are a very common problem of SSR markers, but following the recommendations of Bovo et al. (1999) we diminished the amplification cycles of our PCR from 35 to 28 (while the remainder of the amplification protocol was maintained), significantly reducing the appearance of these bands (Fig. 12 and Fig. 13).

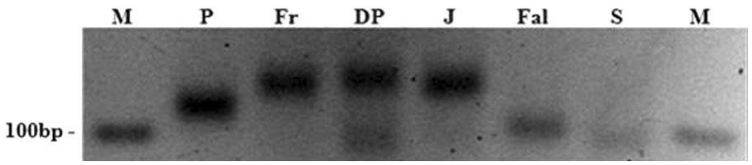


Figure 12 SSR-*PsMLO1* marker (primer combination 1) amplified in six pea genomes and analyzed on 3.5% agarose gel. Amplification after 28 cycles. High polymorphism of the marker is observed and better defined bands in comparison to Figure 10 after lower number of amplification cycles. **P** - *cv*. Progreta, **Fr** - *cv*. Frilene, **DP** - *cv*. Douce de Provence, **J** - line JI2480, **Fal** - *cv*. Fallon, **S** - line S(er1mut1), **M** - molecular weight marker.

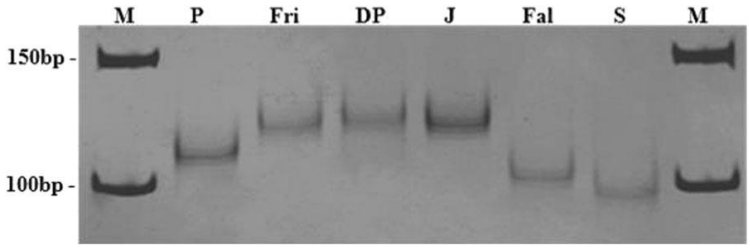


Figure 13 SSR-*PsMLO1* marker (primer combination 1) amplified in six pea genomes analyzed on 10% polyacrylamide gel. Amplification after 28 cycles. High polymorphism of the marker is clearly visible. The smear of bands and non specific products are clearly reduced in comparison to Figure 11 by lowering the number of amplification cycles. **P** - *cv*. Progreta, **Fri** - *cv*. Frilene, **DP** - *cv*. Douce de Provence, **J** - line JI2480, **Fal** - *cv*. Fallon, **S** - line S(er1mut1), **M** - molecular weight marker.

In addition to a large number of tested primer combinations until the three better combinations were selected (Table 1), multiple gel electrophoresis tests were performed to determine the best percentage for easy discrimination of the sizes of the bands of the SSR in the gel, and we concluded that high-percentage (3 to 4%) agarose gel electrophoresis or (10%) TBE – polyacrylamide get electrophoresis worked best. The use of agarose *vs.* polyacrylamide gels will depend mainly on the size difference between the microsatellite sequences that are being discriminated.

The use of close flanking primers generated genomic fragments that contained the microsatellite sequence, polymorphic in size for each of the tested individuals. These differences in size in the SSR sequence were, in most cases, easily discriminated between the different cultivars/lines.

In later analysis of additional genotypes, unexpected problems were found in the amplification of this SSR marker. This problem was further identified as being caused by the utilization of a new brand of Taq-polymerase. For that reason we recommend consultation of the Tm calculator of the DNA polymerase manufacturer, as some brands were not suitable for this work.

4 - Discussion

Microsatellites, or simple sequence repeats (SSR), are tandem repeats of 1 to 6 nucleotides that can be used as a molecular marker. Despite their initial isolation being somewhat costly and time consuming, these bring several advantages in comparison to other molecular marker systems: they are frequent, dispersed throughout the genome of most eukaryotic organisms and generally show high levels of polymorphism. For SSR genotyping assays, only a simple PCR methodology and small amounts of DNA are required. The SSR markers are usually co-dominant, enabling distinction between homozygotes and heterozygotes, and their analysis can be automated, allowing high throughput analysis of large sample populations, which make these markers more suitable for marker assisted selection (MAS) than most of the other available genetic marker systems.

The analysis carried out with this large group of cultivars and lines has showed that the SSR locus present in the fifth intron of the MLO gene is highly polymorphic and in most crosses, the resistant and susceptible progenitor lines have high probability of harboring microsatellite sequences of different size.

This microsatellite sequence can be used to develop SSR markers useful for marker-assisted selection for a very large number of *er1*/*PsMLO1*-resitant loci, without the need for previous sequencing of the mutant allele, the exact identification of the mutation that caused the loss-of-function of the *PsMLO1* allele, and the following developing of specific markers for the specific mutation.

This SSR marker is internal to the gene, therefore absolutely linked to the resistant or susceptible powdery mildew phenotype. It provides a highly accurate and secure assay for identification of progeny plants harboring the recessive powdery mildew resistence allele. Being co-dominant, it permits distinction between homozygous and heterozygous individuals for the *PsMLO1* resistance allele.

Furthermore, this procedure is an affordable and straightforward approach, requiring minimal laboratory conditions, for use in breeding programs, permiting the easy identification and discrimination of alleles of both progenitors and their segregation among the progeny.

There can be cases of very small differences (e.g. 1 or 2 TA-repeats) between the progenitor alleles. In these cases, their discrimination will require the use of higher sensibility assays such as long denaturing polyacrylamide get electrophoresis stained by silver nitrate or revealed by autoradiography (using radioactively labeled primer). Nevertheless, the better alternative option would be the analysis by polyacrylamide capillary electrophoresis of the PCR products generated using a fluorescence-labeled primer.

To facilitate analysis of large amounts of progeny plants, including very young seedlings, the DNA extraction can be carried out according to the protocol described by Elisário et al. (1999) which has been tested and used in our lab for multiple plant species including pea, making the procedure faster, easier, and more affordable.

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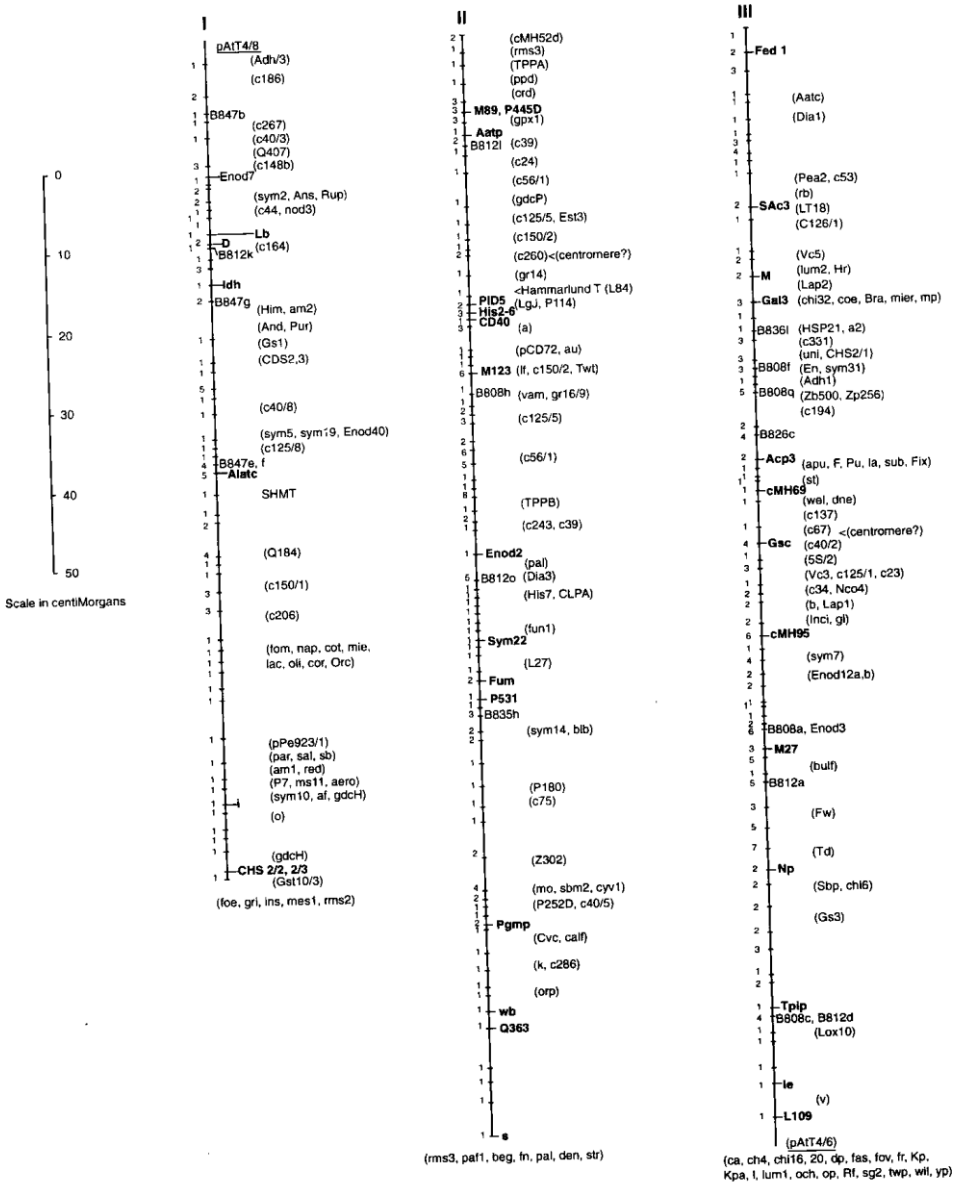
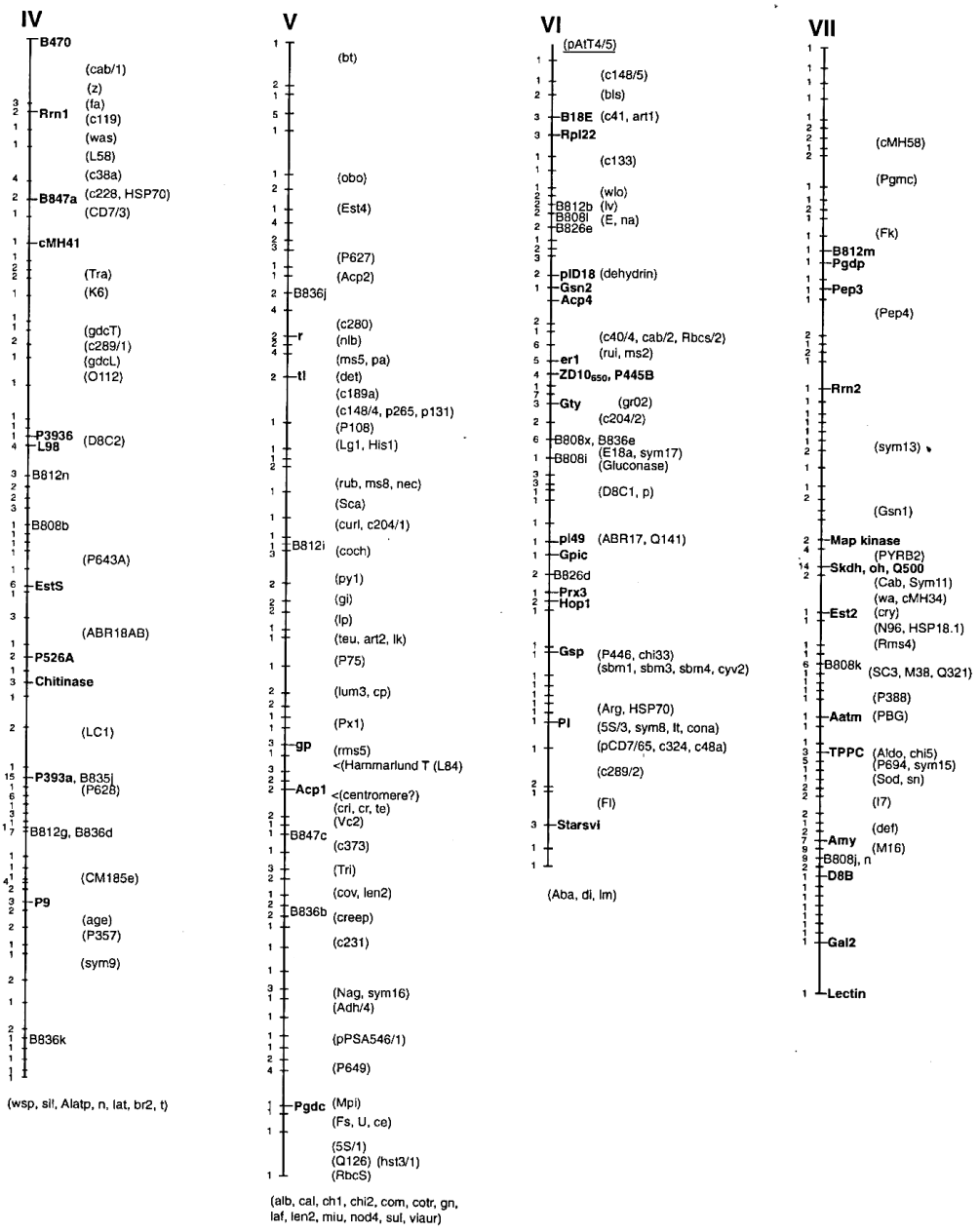
6 - ANEXXES

Annex I

Consensus Linkage map for *Pisum sativum* L. (Weeden et al., 1998)




IV
B470
(cab/1)
(z)
(fa)
Rrn1
(c119)
(was)
(L58)
(c38a)
B847a
(c228, HSP70)
(CD7/3)
cMH41
(Tra)
(K6)
(gdcT)
(c289/1)
(gdcL)
(O112)
P3936
L98
(D8C2)
B812n
B808b
(P643A)
EstS
(ABR18AB)
P526A
Chitinase
(LC1)
P393a, B835
(P628)
B812g, B836d
(CM185e)
P9
(age)
(P357)
(sym9)
B836k
(wsp, sil, Alatp, n, lat, br2, t)
V
(bt)
(obo)
(Est4)
(P627)
(Acp2)
B836j
(c280)
r
(nlb)
(ms5, pa)
tl
(det)
(c189a)
(c148/4, p265, p131)
(P108)
(Lg1, His1)
(rub, ms8, nec)
(Sca)
(curl, c204/1)
B812i
(coch)
(py1)
(gi)
(lp)
(teu, art2, lk)
(P75)
(lum3, cp)
(Px1)
gp
(rms5)
<(Hammarlund T (L84)
Acp1
<(centromere?)
(crl, cr, te)
(Vc2)
B847c
(c373)
(Tri)
(cov, len2)
B836b
(creep)
(c231)
(Nag, sym16)
(Adh/4)
(pPSA546/1)
(P649)
Pgdc
(Mpi)
(Fs, U, ce)
(5S/1)
(Q126)
(hst3/1)
(RbcS)
(alb, cal, ch1, chi2, com, cotr, gn, laf, len2, miu, nod4, sul, viaur)
VI
(pAtT4/5)
(c148/5)
(bls)
B18E
(c41, art1)
Rpl22
(c133)
(wlo)
B812b
B808l
B826e
(lv)
(E, na)
pID18
(dehydrin)
Gsn2
Acp4
(c40/4, cab/2, Rbcs/2)
(rui, ms2)
er1
ZD10650, P445B
Gty
(gr02)
(c204/2)
B808x, B836e
B808i
(E18a, sym17)
(Gluconase)
(D8C1, p)
pI49
(ABR17, Q141)
Gpic
B826d
Prx3
Hop1
Gsp
(P446, chi33)
(sbm1, sbm3, sbm4, cyv2)
(Arg, HSP70)
PI
(5S/3, sym8, lt, cona)
(pCD7/65, c324, c48a)
(c289/2)
(FI)
Starsvi
(Aba, di, lm)
VII
(cMH58)
(Pgmc)
(Fk)
B812m
Pgdp
Pep3
(Pep4)
Rrn2
(sym13)
(Gsn1)
Map kinase
(PYRB2)
Skdh, oh, Q500
(Cab, Sym11)
(wa, cMH34)
Est2
(cry)
(N96, HSP18.1)
(Rms4)
B808k
(SC3, M38, Q321)
(P388)
Aatm
(PBG)
TPPC
(Aldo, chi5)
(P694, sym15)
(Sod, sn)
(I7)
(def)
Amy
(M16)
B808j, n
D8B
Gal2
Lectin

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SHORT COMMUNICATION

A microsatellite sequence in the fifth intron provides a broad-spectrum SSR marker for multiple alleles of the *er1*/*PsMLO1* powdery mildew resistance gene in *Pisum sativum* L.

André Cardoso · Ricardo Pereira · Maria Fonseca · José Leitão

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Abstract Powdery mildew caused by the biotrophic ascomycete fungus *Erysiphe pisi* Syd. is one the most devastating diseases of peas (*Pisum sativum* L.) with enormous impact in seed production. The most efficient genetic resistance to this disease, so far identified, is conferred by the naturally occurring or experimentally induced by chemical mutagenesis recessive state of the locus *er1*. Genetically mapped over 2 decades ago, this gene was recently identified as a homolog of the barley (*Hordeum sativum* L.) powdery mildew resistance gene MLO, and renamed as PsMLO1. The broad wide resistance conferred by the *er1/PsMLO1* locus was found to be a consequence of the loss of function of the encoded PsMLO1 protein. After the publication of the expressed sequence of this gene by another research group, we published the genomic sequences of this gene which harbors a relatively long (TA) microsatellite sequence (SSR) in the fifth intron. SSR markers based on this highly polymorphic microsatellite can be used for marker-assisted selection in multiple pea powdery mildew resistance breeding programs involving the er1/PsMLO1 resistance, except in the rare circumstances where the progenitor lines are monomorphic for the microsatellite sequence.

Keywords Powdery mildew resistance · *Pisum* · *PsMLO1* · *er1* · SSR marker

Introduction

Powdery mildew, elicited by the biotrophic ascomycete fungus *Erysiphe pisi* Syd., is among the most ravaging diseases affecting pea (*Pisum sativum* L.) production (Warkentin et al. 1996).

The so far identified naturally occurring genetic resistances to powdery mildew in pea are monogenic recessive and conferred by two independent loci named *er1* and *er2*, after the causing agent and identification date. A third source of genetic resistance, also monogenic but dominant (Er3), although identified in *P. fulvum* can be introgressed into *P. sativum* by interspecific crossing (Fondevilla et al. 2007, 2008).

Genetically mapped, respectively, to linkage group VI (Timmerman et al. 1994; Weeden et al. 1998) and linkage group II (Katoch et al. 2010), the er1 and er2 loci exhibit very different phenotypic expression. The *er2* locus confers uniquely leaf resistance to powdery mildew which, while impeded to grow on the leaves, can develop intensively on the stem and pods (Katoch et al. 2010; authors' personal observation). The resistance conferred by the *er1* locus is stable towards a broad specter of pathotypes of the pathogen on all plant tissues, particularly on pods which remain free of symptoms even under the most favorable environmental conditions for disease development (Pereira and Leitão 2010)

Electronic supplementary material The online version of this article (doi:10.1007/s11032-017-0685-x) contains supplementary material, which is available to authorized users.

A. Cardoso · R. Pereira · M. Fonseca · J. Leitão (✉)
Laboratory of Genomics and Genetic Improvement, FCT, Universidade do Algarve, 8005-139 Faro, Portugal
e-mail: jleitao@ualg.pt

Fig. 1 Sequence of the fifth intron of the PsMLO1 gene in cv. Frilene. The $(TA)_n$ microsatellite is highlighted in *gray*. The end and initial sequence of the flanking exons are depicted in *bold* and *italic*

ACTTGGGAAGATGAGACAAGAACAGTTGAATATCAATTTTATAATGGTAATACTTCAAAATTTCTTAGCT
CCACTGATTATTTTAATTATTTTAATTAATTAAAATAGTTGTGTATCACCCACATGCACACAAAAAGTCA
ATGATGACATATTACTTTGAAAGCAAGACAAAAGCTTATATTATAAACAATACATAAAAAATTAAAATTG
ACTTGCATTTCTATGTTATATAGTTTAAATTTAATATATATATATATATATATATATATATATATATATA
TATATATATATATATATATATATTCGATCAAATTTCCTTATATTAATATTTATTGAGATAATTTAGTAAT
TAATGTAACCTAGTAGTATTATATATGATTTAACAATTAAACCTTCAATTATTTATTATATGTATGTTGA
TAATATTTTAGATGCCTTGGTTATAATATATATTATAATGACTTTTAATATGGTGATGAAAAATTTGTAC
TATGAAAAGTATAACTAAATCCCTATCTATAGTCAAATCATAGGCAATTTCTTTATTATTTAGTACAATT
TCAAGTGGTGATAAATTTTTTACTTTTATTAATTAATTATTAATAATAAAAACAAAGGTTGAAAAAACTA
AACCTGGGGGGAAAAAAAAAAGATAAATTTGTGTGATTTTAATATTCTATTATTTTTATTTTCTGGTTAT
ATTTTTTTGTGCAG***ATCCTGAGAGGTTTAGGTTTGCAAGGGACACAACATTTGGAAGAAGGCACTTGAGC***

The first two non-naturally occurring powdery mildew resistant mutants were induced in our lab via ethylnitrosourea (ENU) chemical mutagenesis. Complementation crosses between the two experimentally induced mutant lines, and between these lines and the line E835 harboring the powdery mildew resistance gene from cv. Mexique 4, showed that the two mutations affected the same locus, identified as *er1* (Pereira and Leitão 2010).

More recently, Humphry et al. (2011) demonstrated that *er1* is homolog to the barley (*Hordeum sativum* L.) powdery mildew resistance gene MLO1, and as in this monocotyledonous species, the loss of function of the *er1* gene (renamed as *PsMLO1*) was completely associated with the powdery mildew-resistant phenotype in multiple pea lines. To the same conclusion arrived almost simultaneously Pavan et al. (2011) who also determined that the powdery resistance in an induced mutant line and other three resistant genotypes was associated to mutations that truncate the PsMLO1 protein. Later, we found that the ENU mutagenesis has induced one single G/A transition in cv. Frilene and one single C/G transversion in cv. Solara which, in both cases, create stop codons that result in truncate loss of function PsMLO1 proteins (Santo et al. 2013). Simultaneously, based on the published expressed sequence, we deciphered and published the genomic sequence of the PsMLO1 gene (www.ncbi.nlm.nih.gov/nuccore/KC466597.1), which was found to harbor in the fifth intron a polymorphic $(TA)_n$ microsatellite, 36 nucleotides long in cv. Solara (and respective induced powdery mildew resistant line), and 58 nucleotides long in cv. Frilene (and respective induced resistant line) (Santo et al. 2013).

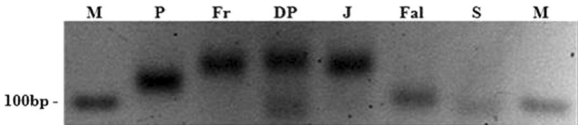

Fig. 2 SSR-*PsMLO1* marker (primer combination 1) amplified in six pea genomes and analyzed on 3.5% agarose gel. Amplification after 28 cycles. Notice the high polymorphism of the marker and better defined bands after lower number of amplification cycles (compare with figures in the Supplemental Electronic Material). *P cv.* Progreta, *Fr cv.* Frilene, *DP cv.* Douce de Provence, *J* line JI2480, *Fal cv.* Fallon, *S* line S(er1mut1), *M* molecular weight marker

During the last 2 decades, multiple research groups (e.g., Dirlewanger et al. 1994, Timmerman et al. 1994, Tiwari et al. 1998, Janila and Sharma 2004, Ek et al. 2005, and Pereira et al. 2010) have been identifying molecular markers linked to the *er1* (*PsMLO1*) gene aiming at the genetic mapping and map-based cloning of the locus, and marker-assisted selection in powdery mildew resistance breeding programs.

Nevertheless, after the publication of the expressed (Humphry et al. 2011) and genomic sequence (Santo et al. 2013) of the gene, the search for DNA-markers linked to the *er1* (*PsMLO1*) gene has continued (Srivastava et al. 2012; Sudheesh et al. 2014; Javid et al. 2015). The identification of new markers is of particular interest when a novel source of powdery mildew resistance is identified or when the progenitor lines used in a specific breeding program are not polymorphic regarding the already published markers (Sun et al. 2015, 2016).

Tightly linked markers to a specific *er1*/*PsMLO1* resistance allele can be generated identifying the mutation by sequencing the PsMLO1 allele, and establishing the specific marker based on the identified mutation. Specific markers can be established designing primers that amplify specifically and discriminatively the mutant allele (Santo et al. 2013) or, in those cases in which the mutation eliminates or creates a restriction enzyme recognition site, by the establishment of a specific internal CAPS (cleaved amplified polymorphism) marker (cf. Pavan et al. 2011; Santo et al. 2013).

Nevertheless, the above described approaches are time consuming and can be unsuccessful: e.g. when

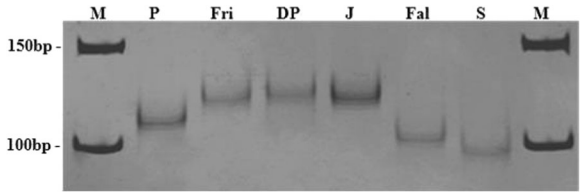

Fig. 3 SSR-*PsMLO1* marker (primer combination 1) amplified in six pea genomes analyzed on 10% polyacrylamide gels. Amplification after 28 cycles. Notice the high polymorphism of the marker. The smear of bands and non-specific products are clearly reduced by lowering the number of amplification cycles (compare with figures in the Supplemental Electronic Material). *P cv.* Progreta, *Fr cv.* Frilene, *DP cv.* Douce de Provence, *J* line JI2480, *Fal cv.* Fallon, *S* line S(*er1mut1*), *M* molecular weight marker

the mutation consists in the insertion of large (e.g. transposable) elements into the PsMLO1 gene (cf. Humphry et al. 2011).

Internal to the gene, the $(TA)_n$ microsatellite marker in the fifth intron of PsMLO1 offers the maximal genetic linkage to this gene, which can only be broken by a very unlikely to occur recombination event.

This SSR marker is suitable for use with a large diversity of genotypes in marker-assisted selection (MAS) in pea powdery mildew resistance breeding programs. Herein, we show that, in multiple cases, the polymorphisms between alleles can be analyzed by common agarose and polyacrylamide gel electrophoresis.

Results and discussion

In our experiments, we have used 12 cultivars: Douce de Provence, Fallon, Frilene, Grisel, Kelvedon Wonder, Lincoln, Rondo, Senador Cambados, Telephone, Television, Progress 9 and Progreta, and 2 lines: JI2480 and S(er1mut1)

The analyses performed with these cultivars and lines showed that this microsatellite locus (Fig. 1) is highly polymorphic and in most crosses (and backcrosses), the resistant and susceptible progenitor lines have high probability of harboring microsatellite sequences of different size (Figs. 2 and 3; Figs. SEM1–SEM4)

SSR markers based on this microsatellite sequence can be used in marker-assisted selection (MAS) for a very large number of *er1/PsMLO1*-resistant loci, without requiring the previous sequencing of the mutant allele, the exact identification of the mutation that caused the loss of function of *PsMLO1*, and the subsequent generation of specific markers.

Internal to the gene and, consequently, absolutely linked to the resistant or susceptible powdery mildew phenotype, this co-dominant SSR marker provides a highly accurate and secure assay for identification of progeny plants harboring the recessive powdery mildew resistance allele and for discrimination between homozygous and heterozygous carriers.

The more affordable, straightforward, and easy procedure for identification and discrimination of the alleles of both progenitors, for analysis of their inheritance among the progeny and requiring a minimum of laboratory conditions, will consist of the use of close flanking primers to generate a genomic fragment containing the microsatellite sequence, that can be discriminated by high-percentage (3–4%) agarose gel electrophoresis or (10%) TBE-polyacrylamide gel electrophoresis. The use of agarose vs. polyacrylamide gels will depend mainly on the size difference between the microsatellite sequences of both progenitors (Figs. 2 and 3).

Since the genomic sequence surrounding the microsatellite is low suitable for primer designing, multiple primers and primer combinations were tested. Three primer combinations worked well (Table 1) and can be used for marker-assisted selection. Nevertheless, we recommend the use of the first primer combination since

Table 1 Primer combinations for PsMLO1_SSR amplification

Primer combination	Primers	Annealing temperature (°C)	Expected size[a] (bp)
1	5′ - GACTTGCATTTCTATGTTATATAG - 3′ 5′ - AATATAAGGAAATTTGATCGAATAT - 3′	58	~115
2	5′ - AAATTGACTTGCATTTCTATGTT - 3 5′ - TACTACTAGGTTACATTAATTACTA – 3″	60,5	~175
3	5′ - AAATTGACTTGCATTTCTATGTT - 3′ 5′ - AGAAATTGCCTATGATTTGACT - 3′	62	~338

[a] Estimated size of the amplification product in cv. Frilene

the produced shorter PCR products are easier to discriminate by common agarose or polyacrylamide gel electrophoresis (Figs. 2 and 3). We also recommend the consultation of the Tm calculator of the DNA polymerase manufacturer as some brands could not be suitable for the work. Nevertheless, a problem subsisted with all primer combinations: the systematic appearance of stutter bands which could complicate the discrimination between alleles of relatively similar size (Figs. SEM1–SEM4). This common problem of SSR markers was significantly diminished reducing the number of amplification cycles from 35 to 28 as recommended by Bovo et al. (1999) (Figs. 2 and 3). The remaining aspects of the amplification protocol were maintained: (i) initial cycle at 94 °C for 90 s; (ii) *N* (28 or 35) cycles of 94 °C for 30 s; (58, 60.5, or 62—depending on the used primer pair) °C for 1 min; 72 °C for 1 min; and (iii) final elongation cycle at 72 °C for 10 min (Figs. 2 and 3)

In case of very small size differences (1 or 2 TA repeats) between the parent alleles, their discrimination will require the use of long denaturing polyacrylamide gel electrophoresis stained by silver nitrate or revealed by autoradiography (using a radioactively labeled primer) or, alternatively, the analysis by polyacrylamide capillary electrophoresis of the PCR products generated using a fluorescence-labeled primer.

The analysis of large amounts of progeny plants, including very young seedlings, can be more affordable, faster, and easier to carry out, using the DNA extraction protocol described by Elisiário et al. (1999) which has been tested and used in our lab for multiple plant species including pea.

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