Two Novel er1 Alleles Conferring Powdery Mildew (Erysiphe pisi) Resistance Identified in a Worldwide Collection of Pea (Pisum sativum L.) Germplasms

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Abstract: Powdery mildew caused by Erysiphe pisi DC. severely affects pea crops worldwide. The use of resistant cultivars containing the er1 gene is the most effective way to control this disease. The objectives of this study were to reveal er1 alleles contained in 55 E. pisi-resistant pea germplasms and to develop the functional markers of novel alleles. Sequences of 10 homologous PsMLO1 cDNA clones from each germplasm accession were used to determine their er1 alleles. The frame shift mutations and various alternative splicing patterns were observed during transcription of the er1 gene. Two novel er1 alleles, er1-8 and er1-9, were discovered in the germplasm accessions G0004839 and G0004400, respectively, and four known er1 alleles were identified in 53 other accessions. One mutation in G0004839 was characterized by a 3-bp (GTG) deletion of the wild-type PsMLO1 cDNA, resulting in a missing valine at position 447 of the PsMLO1 protein sequence. Another mutation in G0004400 was caused by a 1-bp (T) deletion of the wild-type PsMLO1 cDNA sequence, resulting in a serine to leucine change of the PsMLO1 protein sequence. The er1-8 and er1-9 alleles were verified using resistance inheritance analysis and genetic mapping with respectively derived F2 and F2:3 populations. Finally, co-dominant functional markers specific to er1-8 and er1-9 were developed and validated in populations and pea germplasms. These results improve our understanding of E. pisi resistance in pea germplasms worldwide and provide powerful tools for marker-assisted selection in pea breeding.

Keywords: Erysiphe pisi; er1-8; er1-9; KASPar marker; pea

1. Introduction

Pea (Pisum sativum L.) is a widely distributed legume crop, which frequently suffers from various stresses, including abiotic and biotic factors in the season of growth [1,2]. Powdery mildew, induced by Erysiphe pisi DC., severely reduces the yield and quality of pea crops worldwide [3–5]. Severe E. pisi infections of peas can lead to yield losses of up to 80% in regions which are suitable for disease development [5,6]. The use of resistant cultivars carrying the E. pisi-resistant gene er1 has been considered to be the most effective and environmentally friendly way to prevent this disease to date [6,7].

Formerly, E. pisi infection was the only known cause of pea powdery mildew. However, since 2005, two other Erysiphe species, Erysiphe trifolii and Erysiphe baemleri, have been reported to also infect peas and induce the same powdery mildew symptoms as E. pisi in some regions [8–10]. Previous
studies of pea powdery mildew have primarily focused on breeding peas resistant to *E. pisi*. Their results have indicated that resistance to *E. pisi* is controlled by two single recessive genes (*er1* and *er2*) and one dominant gene (*Er3*) [11–14]. The *er1*, *er2*, and *Er3* genes have been mapped using linked markers [15–27]. The genes *er1* and *er2* map to pea linkage groups (LGs) VI and III, respectively [17,28]. *Er3*, which was isolated from wild pea (*Pisum fulvum*), was initially mapped on an uncertain pea LG, but it was more recently assigned to pea LG IV [29].

As *er1* confers high resistance or complete immunity to *E. pisi* in most pea germplasms, it is currently the most widely used gene in pea production [30]. In contrast, *er2* is only found in a few pea germplasms resistant to *E. pisi* [30]. Moreover, the efficacy of *E. pisi* resistance conferred by *er2* varies with leaf development stage and plant location [12,30–32]. *Er3* was known from wild pea (*P. fulvum*), and there have not been extensive studies conducted to date [13,33].

Gene *er1* confers stable, durable, and broadly effective resistance to *E. pisi*. This gene inhibits the incursion of *E. pisi* into pea epidermal cells [32]. Recent studies have shown that the *er1*-resistant phenotype is caused by loss-of-function mutations in the pea MLO (Mildew Resistance Locus O) homolog (*PsMLO1*). The MLO gene family has been identified in both dicots (e.g., *Arabidopsis thaliana* and tomato: *Solanum lycopersicum*) and monocots (e.g., barley: *Hordeum vulgare*) [14,34–39].

To date, nine *er1* alleles resistant to *E. pisi* have been identified in *E. pisi*-resistant pea germplasms: *er1*-1 (also known as *er1mut1*) [14,21,25,40,41], *er1*-2 [14,24,25], *er1*-3 [14], *er1*-4 [14], *er1*-5 [38], *er1*-6 [27], *er1*-7 [26], *er1*-10 (also known as *er1mut2*) [21,40,42], and *er1*-11 [42,43]. Each *er1* allele corresponds to a different *PsMLO1* mutation site and pattern. Among the nine *er1* alleles identified, only *er1*-1 and *er1*-2 are commonly applied in pea breeding programs [14,38]. Several studies have attempted to design functional markers of *er1* alleles to allow for the rapid selection of pea germplasms resistant to *E. pisi* [24,26,27,38,42–44].

The yield and quality of the Chinese pea crop are severely damaged by powdery mildew [2], with the disease affecting up to 100% of pea plants in some regions of China [4]. Several studies have focused on the identification of Chinese pea germplasms resistant to *E. pisi* [41,44–49]. In the Chinese pea cultivars X9002 and Xucai 1, *E. pisi* resistance is conferred by the *er1*-2 allele [24,25,47], while in some Chinese pea landraces from Yunnan Province, *E. pisi* resistance is conferred by the *er1*-6 allele [27,48]. *E. pisi* resistance in the Indian pea cultivar DDR11 is conferred by the *er1*-7 allele [26]. Thus, natural resistance to *E. pisi* conferred by the *er1* gene has been observed in pea germplasms worldwide, providing a rich source of genetic material that can be used to improve the *E. pisi* resistance of Chinese pea cultivars [41,46,48,50]. Allelic diversity of this locus in the cultivated pea has been well characterized; however, relatively few studies have investigated and characterized *E. pisi*-resistant pea germplasms in an international collection. Thus, this study aimed to identify and characterize the *E. pisi*-resistant alleles at the *er1* locus in a worldwide collection of pea germplasms resistant to *E. pisi*. Additionally, any novel *er1* alleles were genetically mapped, and functional markers specific to these novel *er1* alleles were developed to improve marker-assisted selection in pea breeding programs.

2. Results

2.1. Phenotypic Evaluation

Fifty-five *E. pisi*-immune or -resistant pea germplasm accessions from 13 countries were re-evaluated for their resistance to the *E. pisi* isolate EPYN. At 10 days post-inoculation, the *E. pisi* disease severity of all susceptible controls (Bawan 6 and Longwan 1) were rated as score 4. In contrast, the 55 *E. pisi*-resistant germplasm accessions appeared to be either immune (symptom-free; disease severity 0) or resistant (slight infection; disease severity 1–2) to *E. pisi* isolate EPYN. Of the 55 resistant germplasm accessions, 46 were classified as immune and nine as resistant to *E. pisi* (Table 1). To provide comprehensive information for the resistance of a worldwide collection of 86 pea germplasms to *E. pisi*, the phenotypes of 31 resistant pea germplasms carrying known *er1* alleles are also shown in Table 1.
Table 1. Information about phenotype and the resistance gene at the er1 locus of the 86 *Erysiphe pisi*-resistant and the two *E. pisi*-susceptible controls (two controls are bolded).

| No. | Accession No./Germplasm Name | Origin | Phenotype | er1 Allele | Reference |
|-----|-----------------------------|--------|-----------|------------|-----------|
| 1   | G0004389 Afghanistan        | I      | er1-8     | This study |           |
| 2   | G0004382 Australia          | I      | er1-9     | This study |           |
| 3   | G0004400 Australia          | I      | er1-2     | This study |           |
| 4   | G0004417 Australia          | I      | er1-2     | This study |           |
| 5   | G0004434 Australia          | I      | er1-2     | This study |           |
| 6   | G0004448 Australia          | I      | er1-2     | This study |           |
| 7   | G0004450 Australia          | I      | er1-2     | This study |           |
| 8   | G0002102 Canada             | I      | er1-6     | This study |           |
| 9   | G0006514 Canada             | R      | er1-2     | This study |           |
| 10  | G0006515 Canada             | R      | er1-2     | This study |           |
| 11  | G0006516 Canada             | I      | er1-2     | This study |           |
| 12  | G0006519 Canada             | I      | er1-2     | This study |           |
| 13  | G0003925 Canada             | I      | er1-1     | [41]       |
| 14  | Cooper                      | Canada | I         | er1-1     | [41]      |
| 15  | G0005576 China, Chongqing   | I      | er1-2     | [27]       |
| 16  | G0008273 China, Gansu       | I      | er1-2     | [24]       |
| 17  | 20012 China, Gansu          | I      | er1-2     | This study |           |
| 18  | Jia2                        | China, Gansu | I      | er1-2     | This study |           |
| 19  | Texuan11                    | China, Gansu | I      | er1-2     | This study |           |
| 20  | Hehuaun66                   | China, Gansu | R      | er1-3     | This study |           |
| 21  | Longwan 1 China, Gansu      | S      | er1       | [51]       |
| 22  | PI91630                     | China, Guangdong | I      | er1-4     | [14]      |
| 23  | Xucai                       | China, Hebei | I      | er1-2     | [25]      |
| 24  | G0003694 China, Hebei       | I      | er1-6     | [27]       |
| 25  | Basan 6 China, Hebei        | S      | er1       | [24]       |
| 26  | L0314                       | China, Yunnan | I      | er1-1     | [51]      |
| 27  | L1332                       | China, Yunnan | I      | er1-2     | [51]      |
| 28  | G0001747 China, Yunnan      | I      | er1-2     | This study |           |
| 29  | G0001752 China, Yunnan      | R      | er1-6     | This study |           |
| 30  | G0001763 China, Yunnan      | I      | er1-6     | This study |           |
| 31  | G0001764 China, Yunnan      | I      | er1-6     | This study |           |
| 32  | G0001767 China, Yunnan      | I      | er1-6     | This study |           |
| 33  | G0001768 China, Yunnan      | I      | er1-6     | This study |           |
| 34  | G0001777 China, Yunnan      | I      | er1-6     | This study |           |
| 35  | G0001778 China, Yunnan      | I      | er1-6     | This study |           |
| 36  | G0001779 China, Yunnan      | I      | er1-6     | This study |           |
| 37  | G0001780 China, Yunnan      | I      | er1-6     | This study |           |
| 38  | G0001781 China, Yunnan      | I      | er1-6     | This study |           |
| 39  | G0001782 China, Yunnan      | R      | er1-6     | This study |           |
| 40  | G0001783 China, Yunnan      | I      | er1-6     | This study |           |
| 41  | G0001784 China, Yunnan      | I      | er1-6     | This study |           |
| 42  | G0001785 China, Yunnan      | I      | er1-6     | This study |           |
| 43  | G0001786 China, Yunnan      | I      | er1-6     | This study |           |
| 44  | G0001787 China, Yunnan      | R      | er1-6     | This study |           |
| 45  | G0001788 China, Yunnan      | R      | er1-6     | This study |           |
| 46  | G0001789 China, Yunnan      | R      | er1-6     | This study |           |
| 47  | G0001790 China, Yunnan      | R      | er1-6     | This study |           |
| 48  | G0001791 China, Yunnan      | R      | er1-6     | This study |           |
| 49  | Yunwan4                     | China, Yunnan | R      | er1-3     | This study |           |
| 50  | Yunwan18                    | China, Yunnan | R      | er1-2     | This study |           |
| 51  | Yunwan35                    | China, Yunnan | I      | er1-2     | This study |           |
| 52  | Yunwan37                    | China, Yunnan | I      | er1-6     | This study |           |
| 53  | L2157                       | China, Yunnan | I      | er1-2     | This study |           |
| 54  | G0002848 Denmark            | I      | er1-2     | This study |           |
| 55  | G0002971 England            | I      | er1-2     | This study |           |
| 56  | G0002859 Germany            | I      | er1-2     | This study |           |
| 57  | G0002860 Germany            | I      | er1-2     | This study |           |
| 58  | G0002883 Germany            | I      | er1-2     | This study |           |
| 59  | G0003895 ICRISAT            | I      | er1-7     | [26]       |
| 60  | G0003897 ICRISAT            | I      | er1-2     | This study |           |
| 61  | G0003899 ICRISAT            | I      | er1-7     | [26]       |
| 62  | G0003901 ICRISAT            | I      | er1-2     | This study |           |
| 63  | G0003911 ICRISAT            | I      | er1-2     | This study |           |
| 64  | G0003961 India              | I      | er1-2     | This study |           |
| 65  | G0003967 India              | I      | er1-7     | [26]       |
| 66  | G0003958 India              | I      | er1-7     | [26]       |
| 67  | G0006285 Japan              | R      | er1-2     | This study |           |
| 68  | G0004332 Mexico             | R      | er1-2     | This study |           |
| 69  | G0004394 Nepal              | R      | er1-7     | [26]       |
| 70  | G0002890 Unknown country    | I      | er1-2     | This study |           |
Table 1. Cont.

| No. | Accession No./Germplasm Name | Origin       | Phenotype | er1 Allele | Reference |
|-----|-------------------------------|--------------|-----------|------------|-----------|
| 71  | G0003931                      | Unknown country | I         | er1-7      | [26]      |
| 72  | G0003935                      | Unknown country | I         | er1-2      | This study |
| 73  | G0003936                      | Unknown country | I         | er1-7      | [26]      |
| 74  | G0003942                      | Unknown country | I         | er1-1      | This study |
| 75  | G0003943                      | Unknown country | I         | er1-1      | This study |
| 76  | G0002128                      | USA          | I         | er1-2      | This study |
| 77  | G0002129                      | USA          | I         | er1-2      | This study |
| 78  | G0002131                      | USA          | I         | er1-2      | This study |
| 79  | G0002132                      | USA          | I         | er1-2      | This study |
| 80  | G0002134                      | USA          | I         | er1-2      | This study |
| 81  | G0002137                      | USA          | I         | er1-2      | This study |
| 82  | G0002183                      | USA          | I         | er1-2      | This study |
| 83  | G0002235                      | USA          | I         | er1-6      | This study |
| 84  | G0002250                      | USA          | I         | er1-2      | This study |
| 85  | G0002602                      | USA          | I         | er1-2      | This study |
| 86  | G0002608                      | USA          | I         | er1-2      | This study |
| 87  | G0002847                      | USA          | I         | er1-2      | This study |
| 88  | G0002960                      | USA          | I         | er1-2      | This study |

"R", "I", and "S" stand for resistant, immune, and susceptible, respectively.

2.2. PsMLO1 Sequence Analysis

The PsMLO1 cDNA sequence of Bawan 6 and Longwan 1, the susceptible controls, was consistent with that of the wild-type PsMLO1 cDNA (Table 1). Among the 55 resistant pea germplasms with previously unknown er1 alleles, er1-1 was identified in seven germplasm accessions, er1-2 in 37, er1-6 in seven, and er1-7 in two (Tables 1 and 2).

Table 2. The distribution and numbers of pea germplasm accessions carrying er1 alleles.

| Country        | No. of Pea Germplasm Accessions Contained er1 Alleles |
|---------------|------------------------------------------------------|
|               | er1-1 | er1-2 | er1-3 | er1-4 | er1-5 | er1-6 | er1-7 | er1-8 | er1-9 | Total |
| USA           |   -   |  12   |   -   |   -   |   1   |   -   |   -   |   -   |   -   |   13   |
| Canada        |   -   |   4   |   -   |   -   |   -   |   1   |   -   |   -   |   -   |   5    |
| Germany       |   -   |   3   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   3    |
| ICRISAT       |   -   |   3   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   3    |
| India         |   -   |   1   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   1    |
| Australia     |   1   |   4   |   -   |   -   |   -   |   -   |   -   |   1   |   -   |   6    |
| England       |   -   |   1   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   1    |
| Denmark       |   -   |   1   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   1    |
| Nepal         |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   0    |
| Japan         |   -   |   1   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   1    |
| Afghanistan   |   -   |   -   |   -   |   -   |   -   |   -   |   1   |   -   |   -   |   1    |
| Mexico        |   1   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   1    |
| China         |   3   |   5   |   -   |   -   |   5   |   2   |   -   |   -   |   -   |   15   |
| Unknown country | 2   |   2   |   -   |   -   |   -   |   -   |   -   |   -   |   -   |   4    |
| Total         |   7   |   37  |   -   |   -   |   7   |   2   |   1   |   1   |   55   |

"-" indicates there was no pea germplasm containing this er1 allele.

Two novel er1 alleles were discovered in the two remaining germplasms: G0004389 (from Afghanistan) and G0004400 (from Australia). A novel mutation pattern was found in the G0004389 cDNA fragment homologous to PsMLO1: a 3-bp deletion (GTG) corresponding to positions 1339–1341 in exon 15 (the final exon) of the PsMLO1 cDNA sequence. This deletion caused the loss of the amino acid valine at position 447 of the PsMLO1 protein sequence, probably resulting in a functional change (Figure 1A). This mutation differed from all known er1 alleles, indicating that the E. pisi resistance of G0004389 was controlled by a novel allele of er1. This novel allele was designated er1-8, following the accepted nomenclature [14, 26, 27, 42, 44, 51]. In pea germplasm G0004400, a 1-bp deletion (T) was identified in a previously unreported position homologous to position 928 in exon 10 of the PsMLO1 cDNA sequence. This deletion caused a substitution of the amino acid serine with leucine at position...
310 of the PsMLO1 protein sequence (Figure 1B). This change caused the early termination of protein translation, probably also resulting in a functional change of PsMLO1 (Figure 1B). Thus, E. pisi resistance in G0004400 was also controlled by a novel er1 allele, herein designated er1-9.

Interestingly, frame shift mutations, where small fragments are deleted or inserted, were identified in the cloned sequences of several pea germplasms. The fragments homologous to the wild-type PsMLO1 cDNA in seven pea germplasms (G0002602, G0006515, G0002883, G0004448, G0002848, G0003935, and G0005117) had 5-bp deletions (GTTAG) at positions 700–704 of wild-type PsMLO1 cDNA, while three pea germplasm accessions (G0002883, G0002971, and L0368) had another 5-bp deletion (TAGGG) at positions 1235–1239 of the wild-type PsMLO1 cDNA. In accession G0006514, there was a 4-bp deletion (GGAG) at positions 181–184 of the wild-type PsMLO1 cDNA. In four pea accessions (G0002847, G0004434, G0003974, and Texuan 11) and two pea accessions (G0002235 and G0002848), there were a 16-bp deletion (CTCATCTTCCTCCAGG) at positions 776–791 and a 16-bp insertion (AATTTTTCTGTTTCAG) at position 1171 of the wild-type PsMLO1 cDNA, respectively. In germplasm accession Jia 2, there was a 7-bp insertion (TAATAAG) at position 921 of the wild-type
PsMLO1 cDNA. It was probable that these indels resulted from aberrant splicing events during transcription. Each frame shift mutation was observed in only one or two of ten cloned PsMLO1 cDNA sequences per germplasm accession.

Various alternative splicing patterns, including intron retention and exon skipping, were also observed in multiple PsMLO1 sequences cloned from the 55 resistant pea germplasm accessions. The eight introns retained were 1, 2, 4, 6, 7, 9, 12, and 13, and the three exons skipped were 4, 10, and 11 of the wild-type PsMLO1. Each intron retention and exon skipping event were discovered in only one or two of ten cloned PsMLO1 cDNA sequences.

2.3. Genetic Analysis and Mapping of er1-8 and er1-9

As expected, the two resistant pea parents, G0004389 and G0004400, were immune to E. pisi infection (disease severity 0), while the two susceptible parents (Bawan 6 and WSU 28) were heavily infected (disease severity 4) (Figure 2). The segregation patterns of E. pisi resistance in the F1, F2, and F2:3 populations derived from the crosses WSU 28 × G0004389 and Bawan 6 × G0004400 are presented in Table S1.

![Figure 2](image_url)

**Figure 2.** Phenotypic evaluation of the Erysiphe pisi-resistant pea germplasms G0004389 and G0004400, as well as the E. pisi-susceptible cultivars WSU 28 and Bawan 6, after inoculation with E. pisi isolate EPYN. (A) G0004389 and E. pisi-susceptible cultivar WSU 28. (B) G0004400 and E. pisi-susceptible cultivar Bawan 6.

Six F1 plants produced from the cross WSU 28 × G0004389 were susceptible to E. pisi (Table S1). One of the six plants generated 120 F2 and F2:3 offspring through self-pollination. Of these 120 F2 plants, 30 were resistant (R) to E. pisi, and 90 were susceptible (S) to E. pisi-. This indicates that the segregation ratio (resistance:susceptibility) in the F2 population was exactly 1:3 ($\chi^2 = 0.01; P = 0.92$), indicating recessive heredity of a single gene. Moreover, a segregation ratio of 30 (homozygous resistant): 63 (segregating): 27 (homozygous susceptible) in the F2:3 population fitted well with the genetic model of 1:2:1 ratio ($\chi^2 = 0.48, P = 0.79$) (Table S1), confirming that the E. pisi resistance in G0004389 was controlled by a single recessive gene.

The cross of Bawan 6 × G0004400 generated five F1 plants which showed E. pisi-susceptibility (Table S1). One of five F1 plants generated 119 F2 offspring. 32 of 119 were resistant, and 87 of 119 were susceptible to E. pisi. The segregation ratio in the F2 population of resistance to susceptibility fitted a genetic model ratio of 1:3 ($\chi^2 = 0.14; P = 0.71$), also indicating recessive heredity of a single gene. Moreover, a segregation ratio of 32 (homozygous resistant): 64 (segregating): 23 (homozygous...
was then used to identify the genotypes of the 120 F\textsubscript{2} plants derived from WSU 28 × G0004389 and G0004400, indicating that these markers were likely linked to the \textit{E. pisi} resistance gene. Thus, the five and the seven parental polymorphic markers were used to confirm the genotypes of each F\textsubscript{2} plant derived from WSU 28 × G0004389 and Bawan 6 × G0004400, respectively. This genetic linkage analysis suggested that three markers (c5DNAmet, AA200, and AA224) and six markers (AD160, PSMPSAD51, ScOPD10-650, ScOPX04-880, ScOPE16-1600, and AD59) were linked to the resistance gene \textit{er1} in G0004389 and G0004400, respectively (Figure 3). Our results also indicated that the resistance genes in both germplasm accessions were located in the \textit{er1} region. In G0004389, the linkage map indicated that the markers (c5DNAmet and AA200) were mapped on both sides of the target gene with 9.6 cM and 3.5 cM genetic distances, respectively (Figure 3A). In G0004400, two other markers (PSMPSAD51 and ScOPX04-880) were located on both sides of the target gene with 12.2 cM and 4.2 cM genetic distances, respectively (Figure 3B). Our linkage and genetic map analyses confirmed that \textit{er1-8} and \textit{er1-9} controlled \textit{E. pisi} resistance in G0004389 and G0004400, respectively (Figure 3).

![Figure 3](image-url). Genetic linkage maps constructed using the \textit{er1}-linked markers and the functional markers for \textit{er1-8} and \textit{er1-9}, based on the F\textsubscript{2} populations derived from (A) WSU 28 × G0004389 and (B) Bawan 6 × G0004400. Map distances and loci order were determined with MAPMAKER v3.0 (Lander et al. 1993). Estimated genetic distances between loci are shown to the left of the maps in centiMorgans (cM).

### 2.4. Development of Functional Markers for \textit{er1-8} and \textit{er1-9}

The indel marker, InDel-\textit{er1-8} flanking the 3-bp deletion in \textit{er1-8}, amplified 231-bp and 228-bp fragments in the parents WSU 28 and G0004389, respectively. The amplicons were clearly polymorphic between the contrasting parents, as visualized on an 8% polyacrylamide gel (Figure S1A). InDel-\textit{er1-8} was then used to identify the genotypes of the 120 F\textsubscript{2} plants derived from WSU 28 × G0004389. Three distinct electrophoretic bands corresponding to the homozygous resistant (R), homozygous susceptible (S), and heterozygous (H) genotypes were observed (Figure S1A). Each F\textsubscript{2} genotype corresponded to a phenotype of the 120 F\textsubscript{2,3} families. A chi-squared ($\chi^2$) test showed that the segregation ratio of InDel-\textit{er1-8} in the F\textsubscript{2,3} population derived from WSU 28 × G0004389 fit a 1:2:1 ($\chi^2 = 0.48; P = 0.79$). All results suggested that the marker InDel-\textit{er1-8} co-segregated with gene \textit{er1-8}, indicating a co-dominant marker.
In the Kompetitive allele-specific PCR (KASPar) assay, KASPar-er1-8 and KASPar-er1-9 successfully distinguished the contrasting parents (WSU 28 and G0004389, Bawan 6 and G0004400) into two different clusters corresponding to the FAM-labeled and HEX-labeled groups, respectively (Figure S2). When KASPar-er1-8 and KASPar-er1-9 were used to analyze the 120 and 119 F2 progeny derived from WSU 28 × G0004389 and Bawan 6 × G0004400, the KASPar markers clearly separated the F2 progeny into three clusters corresponding to three genotypes: homozygous resistant, homozygous susceptible, and heterozygous (Figure S2). In the F2 population derived from WSU 28 × G0004389, 30 plants were identified as homozygous resistant, 63 were heterozygous, and 27 were homozygous susceptible. In the F2 population derived from Bawan 6 × G0004400, 32 plants were homozygous resistant, 64 were heterozygous, and 23 were homozygous susceptible. These results were completely consistent with the phenotypes of both F2:3 populations, suggesting that KASPar-er1-8 and KASPar-er1-9 co-segregated with er1-8 and er1-9, respectively. A chi-squared ($\chi^2$) test showed that both segregation ratios of KASPar-er1-8 and KASPar-er1-9 in respective F2 populations fit 1:2:1 (KASPar-er1-8; $\chi^2 = 0.48$, $P = 0.79$; KASPar-er1-9; $\chi^2 = 2.51$; $P = 0.29$), indicating co-dominant markers.

2.5. Validation and Application of Functional Markers

Of the 169 germplasm accessions selected and tested for their phenotypic resistance to E. pisi isolate EPYN (Table S2), 19 were phenotypically immune to E. pisi, 22 were resistant, and 128 were susceptible (Table S2).

Among the 169 germplasms genotyped with InDel-er1-8, the 228-bp fragment corresponding to er1-8 was only amplified in G0004389 (Figure S1B). In all of the other tested germplasm accessions, a 231-bp fragment was consistently amplified by InDel-er1-8, indicating that no accessions besides G0004389 carried er1-8 (Figure S1B; Table S1).

When the 169 germplasm accessions were genotyped with KASPar-er1-8, two distinct clusters were recovered, with one gene (er1-8) corresponding to G0004389 and the other (non-er1-8) to the other germplasms, respectively. Similarly, when the germplasms were genotyped with KASPar-er1-9, two distinct clusters were recovered, corresponding to G0004400 and all of the other germplasms, respectively (Figure S2; Table S1). Thus, markers KASPar-er1-8 and KASPar-er1-9 effectively identified pea germplasms carrying the er1-8 and er1-9 alleles, respectively. Our results also showed that none of the other 169 pea germplasm accessions carried the er1-8 or er1-9 alleles.

3. Discussion

Powdery mildew induced by E. pisi DC. is a major disease on pea and causes considerable yield losses worldwide. The resistance gene er1 is the most widely deployed gene controlling powdery mildew in pea cultivars worldwide. Furthermore, er1 allelic diversity has been widely reported in pea [14,21,25–27,38,40–44,51].

To date, more than 40 MLO mutant alleles have been described in the monocotyledonous plant barley [52]. It is predicted that additional er1 alleles resulting from natural mutations would be present among pea germplasms from around the world. As expected, we not only encountered the four known er1 alleles (er1-1, er1-2, er1-6, and er1-7) across the 53 E. pisi-resistant pea germplasms, but we also discovered two novel er1 alleles: er1-8 in germplasm G0004389 from Afghanistan and er1-9 in germplasm G0004400 from Australia (Table 1).

Among the nine known er1 alleles, er1-1 and er1-2 are most commonly used in pea breeding programs because they confer stable resistance to E. pisi [14,25,38,51]. Our results indicated that these two alleles were common in the tested pea germplasm accessions resistance to E. pisi. The er1-1 allele was found in seven accessions (12.73%), and er1-2 was found in 37 accessions (67.27%) (Table 2). Among the 86 E. pisi-resistant pea accessions, er1-1 and er1-2 were identified in 10 (11.62%) and 42 (48.84%) accessions, respectively (Table 1). Previously, er1-1 has been identified in four E. pisi-resistant pea cultivars (Stratagem, Franklin, Dorian, Nadir, X9002, Xucai 1,
and G0005576 [14,24,25,27,38]. Here, more E. pisi-resistant germplasm accessions carrying the er1-1 and er1-2 alleles were identified.

At the genomic level, seven alleles (er1-1/er1mut1, er1-3, er1-4, er1-5, er1-6, er1-9, and er1-10/er1mut2) are the result of point mutations in the exons of wild-type PsMLO1. Four alleles result from single base substitutions in wild-type PsMLO1 cDNA: in er1-1, a C→G at position 680 (exon 6); in er1-5, a G→A at position 570 (exon 5); in er1-6, a T→C at position 1121 (exon 11); and in er1-10, a G→A at position 939 (exon 10) (Figure S3) [14,27,38,40]. Three alleles result from single base deletions in wild-type PsMLO1 cDNA, including ΔG at position 862 (exon 8) in er1-3; ΔA at position 91 (exon 1) in er1-4; and AT at position 928 (exon 10) in er1-9 identified in this study [14]. Two alleles result from small fragment deletions in wild-type PsMLO1 cDNA, including a 10-bp deletion of positions 111–120 (exon 1) in er1-7 [26]; and a 3-bp deletion of positions 1339–1341 (exon 15) in er1-8. To date, only the er1-11 mutation is known to have resulted from an intron mutation in PsMLO1 (a 2-bp insertion in intron 14) [42,43], and only er1-2 results from a large indel of unknown size in wild-type PsMLO1 cDNA [14,24,27].

Previous studies have indicated that the er1-2 allele produces three distinct PsMLO1 transcripts [14,25,27,51]. Interestingly, this study observed that the er1-2 carried by the pea germplasm accession G0002860 produced four distinct PsMLO1 transcripts. One of these transcripts was characterized by a 129-bp deletion, corresponding to the deletion of exon 13 (68 bp) and exon 14 (61 bp) from wild-type PsMLO1 cDNA, indicating alternative splicing of exon skipping. Previously, two transcripts of er1-2 were observed to have large insertions (155-bp and 220-bp) based on comparisons with the transcripts of wild-type PsMLO1 cDNA [14,24,25,27,51]. Here, we discovered that the 155-bp “insertion” in er1-2 resulted from a 192-bp insertion at position 1263 and a 37-bp deletion of positions 1263–1299 in exon 14 of wild-type PsMLO1, while the 220-bp “insertion” resulted from a 257-bp insertion at position 1263 and a 37-bp deletion of positions 1263–1299 in exon 14 of wild-type PsMLO1. Another alternative transcript of er1-2, an 87-bp “insertion”, was observed and resulted from a 192-bp insertion and a 37-bp deletion in exon 14 and a 68-bp deletion corresponding to exon 13 of wild-type PsMLO1. Our blast analysis indicated that the 192- and 257-bp insertions had 95% sequence identity with a five-part repetition in the pea genomic BAC sequence (GenBank accession number CU655882). These insertions were also highly similar (~85–87% identity) to a portion of the giant Ogre retrotransposon in the pea genome (GenBank accession numbers AY299395, AY299398, AY299397, and AY299394).

Based on 10 cloned sequences, several pea germplasms had frame shift mutations with small fragment indels (4-bp, 5-bp, or 16-bp) in one or two cloned PsMLO1 cDNA sequences. Previously, a 5-bp (GTAG) insertion was identified in G0001763 and G0003831; 11-bp (GTAGAATAAG) and 13-bp (GTAATCTTATTAG) deletions were identified in G0003831 and G0001778; and a 16-bp (CTCATTTCTCAGG) deletion was detected in G0001778 [27]. These small fragment indels in the PsMLO1 cDNAs were assumed to have resulted from aberrant splicing events during transcription [27].

Alternative splicing in euakaryotes is a pervasive molecular mechanism that significantly increases transcriptome and proteome complexity [53]. Four main types of alternative splicing are known: exon skipping, alternative 5’ splice sites, alternative 3’ splice sites, and intron retention [54]. Exon skipping is common in humans, while intron retention is common in plants [55]. Alternative splicing is involved in many physiological processes, including response to biotic and abiotic stressors [56]. In the pea germplasms, three types of alternative splicing, intron retention, exon skipping, and alternative 5’ splice site selection, were observed in this study. Interestingly, pea germplasms carrying identical er1 alleles varied in their resistance to E. pisi, from immune (disease severity of 0) to merely resistant (disease severity of 1–2) (Table 1). Alternative splicing in response to biotic stress may affect the expression of regulatory genes. Thus, it is speculated that the alternative splicing of er1 alleles might affect the expression of the E. pisi resistance genes er1. In addition, the different levels of resistance to E. pisi might result from other related gene regulation. It is possible that multiple molecular processes and pathways contribute to MLo-based E. pisi resistance in peas.
Several functional markers specific to the previously recognized er1 alleles have already been developed to facilitate marker-assisted breeding of pea cultivars resistant to *E. pisi* [14,24,26,27,40,42–44]. Pavan et al. [38] developed a functional cleaved amplified polymorphic sequence (CAPS) marker for er1-5, while Pavan et al. [44] developed functional markers for the five er1 alleles, er1-1 through er1-5. Santo et al. [40] developed functional markers for er1mut1 and er1mut2, and Wang et al. [24] developed a dominant marker for er1-2. Sudheesh et al. [43] developed a functional marker for er1-11, while Sun et al. [26,27] developed co-dominant functional markers for er1-6 and er1-7. More recently, Ma et al. [42] developed eight KASPar markers for eight known er1 alleles, excluding er1-2.

This study discovered two novel er1 alleles resulting from novel mutations of wild-type *PsMLO1* cDNA: er1-8 was generated by a 3-bp deletion in exon 15, and er1-9 was generated by a 1-bp deletion in exon 10. The co-dominant functional markers specific to er1-8 (InDel-er1-8 and KASPar-er1-8) and to er1-9 (KASPar-er1-9) were developed. These markers were validated in genetic populations and in pea germplasms. Our results are vital for future studies of powdery mildew resistance and for the development of *E. pisi*-resistant pea cultivars. The novel er1 alleles and the corresponding co-dominant functional markers developed herein could constitute efficient and powerful tools for the breeding of *E. pisi*-resistant peas.

4. Materials and Methods

4.1. Plant Material and *E. pisi* Isolate

Previously, 86 pea germplasms had been found to be *E. pisi*-resistant in screenings of over 1000 pea accessions in a worldwide collection [27,48,50]. And, 31 of 86 resistant pea germplasms had been previously identified the *E. pisi*-resistant er1 allele [24–27,48,51]. In this study, the remaining 55 of the 86 *E. pisi*-resistant pea germplasms from the United States of America, Canada, Germany, India, Australia, Columbia, England, Denmark, Nepal, Japan, Afghanistan, and Mexico, as well as data from the International Crop Research Institute for Semi-arid Tropics (ICRISAT) and conserved in the China National Genebank (http://www.cgris.net/), were used as research materials to reveal their *E. pisi*-resistant genes at er1 locus (Table 1). The Chinese pea cultivars Bawan 6 and Longwan 1, which carry the *E. pisi*-susceptible gene Er1, were used as susceptible controls [24,51]. The Chinese pea cultivars Xucai 1, carrying er1-2, and YI (JI1591), carrying er1-4, were used as *E. pisi*-resistant controls [14,25].

The *E. pisi* isolate EPYN from Yunnan Province of China was used as the inoculum [26,27,41,48,50,51]. The EPYN isolate was maintained through continuous re-inoculation of seedlings of the pea cultivar Longwan 1 under controlled conditions. The inoculated plants were incubated in a growth chamber to prevent contamination with other isolates [25].

4.2. Phenotypic Evaluation

Twenty seeds were planted from each of the 55 *E. pisi*-resistant pea germplasm accessions, from the susceptible controls Bawan 6 and Longwan 1, and from the resistant controls Xucai 1 and YI [27]. The seedlings were thinned to 15 per pot before the phenotypic evaluation. Three replications were planted. Seeded pots were placed in a greenhouse maintained at 18 to 26 °C. At the same time, the *E. pisi* inoculum was prepared by inoculating the 10-day-old seedlings of the susceptible pea cultivar Longwan 1, which were incubated in a growth chamber at 20 ± 1 °C with a 12-h photoperiod. Two weeks later, the 14-day-old seedlings of 55 germplasm accessions and controls were inoculated by gently shaking off conidia of the Longwan 1 plants. Inoculated plants were incubated in a growth chamber at 20 ± 1 °C with a 12-h photoperiod. Ten days later, disease severity was rated based on a scale (0–4 scale) [27]. Plants with a score of 0 were considered *E. pisi*-immune, while those with scores of 1 and 2, 3 and 4 were considered as *E. pisi*-resistant and *E. pisi*-susceptible, respectively. For those identified as immune or resistant to *E. pisi*, repeated identification was performed.
4.3. RNA Extraction and PsMLO1 Sequence Analysis

The extraction of total RNA and synthesis of cDNA from the 55 pea germplasms and controls were completed according to our previous studies [25–27]. To identify the resistance alleles at the er1 loci, the full-length cDNAs of the PsMLO1 homologs were amplified using the primers specific for PsMLO1 [14]. The PCR cycling conditions were as follows: 95 °C for 5 min; then 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The purified amplicons were cloned with a pEasy-T5 vector (TransGen Biotech, Beijing, China). The sequencing reactions of 10 clones per germplasm (including controls) were performed by the Shanghai Shenggong Biological Engineering Co., Ltd. (Shanghai, China). The resulting sequences were aligned with wild-type PsMLO1 of pea (NCBI accession number: FJ463618.1) using DNAMAN v6.0 (Lynnon Biosoft, Quebec, Canada).

4.4. Genetic Analysis of Pea Germplasms Carrying Novel Alleles

To confirm the resistance genes, er1-8 and er1-9, G0004389 and G0004400 were crossed with the E. pisi-susceptible cultivars WSU 28 and Bawan 6, respectively, to generate genetic populations. The derived F1, F2, and F2:3 populations from both crosses (WSU 28 × G0004389 and Bawan 6 × G0004400) were used to evaluate the E. pisi resistance and genetic analysis of G0004389 and G0004400. The four parents and the derived F1 and F2 populations were planted in a propagation greenhouse to generate F2 and F2:3 family seeds, respectively.

Plants of the F2 populations at the fourth or fifth leaf stage were inoculated with the E. pisi isolate EPYN using the detached leaf method [25–27,57]. After inoculation, the treated leaves were placed in a growth chamber at 20 °C with a 14-h photoperiod. The four parents (WSU 28, G0004389, Bawan 6, and G0004400) were also inoculated as controls. Ten days after inoculation, disease severity was rated based on a scale of 0–4 as described above. Plants with scores of 0–2 and 3–4 were classified as resistant and susceptible, respectively [25–27,31,58]. Those plants identified as E. pisi-resistant were tested again to confirm their resistance.

Twenty-five seeds were selected randomly from each of the 120 F2:3 families derived from WSU 28 × G0004389, and from each of the 119 F2:3 families derived from Bawan 6 × G0004400. These seeds were planted and cultivated together with their parents, following previously published protocols [25–27]. Disease severity was scored 10 days after inoculation using the 0–4 scale, as described above for the phenotypic identification of the pea germplasms. The F2:3 families with scores of 0–2 and 3–4 were classified as homozygous resistant and homozygous susceptible, respectively. Families with scores of 0–2 and 3–4 were considered segregated to E. pisi resistance [27,31,58]. The families identified as homozygous resistant or resistance segregated were subjected to repeated testing.

A chi-squared (χ²) analysis was used to evaluate the goodness-of-fit to Mendelian segregation ratio of the F2 and F2:3 phenotypes derived from WSU 28 × G0004389 and Bawan 6 × G0004400.

4.5. Genetic Mapping of the Resistance Alleles er1-8 and er1-9

The Genomic DNA was isolated from the leaves of the F2 populations and of their parents using the cetyltrimethylammonium bromide (CTAB) extraction method [59]. The DNA solution was diluted and stored at −20 °C until use.

To map the novel er1 alleles er1-8 and er1-9, the 10 known er1-linked markers on the pea LG VI, including four sequence-characterized amplified region (SCAR) markers [ScOPD10-650 [17], ScOPE16-1600 [18], ScOP018-1200 [18], and ScOPX04-880 [23]; five simple sequence repeat (SSR) markers (PSMPSSD51, PSMPSA5, PSMPSSD60, i.e., AD60, PSMPSSA374e, and PSMPSSA369); a gene marker [Cytosine-5, DNA-methyltransferase (c5DNAmet)] [20,24–27,48,60]; and 10 additional molecular markers on the pea LG VI (AD160, AC74, AC10_1, AA224, AA200, AD159, AD59, AB71, AA335, and AB86], were used to screen for polymorphisms between the crossed parents (i.e., WSU 28 and G0004389; Bawan 6 and G0004400) [61]. The parental polymorphic markers were then used for
genetic linkage analysis based on the genotype of each F$_2$ plant. PCR amplification of each marker was conducted in a total volume of 20 µL according to the previous descriptions [25–27]. PCR reactions were performed in a thermal cycler (Biometra, Göttingen, Germany) [25–27]. The PCR products were separated on 6% polyacrylamide gels.

The segregation data of the polymorphic markers in the F$_2$ populations were evaluated for goodness-of-fit to Mendelian segregation patterns with a chi-squared ($\chi^2$) test. Genetic linkage analyses were completed using MAPMAKER/EXP version 3.0b. A logarithm of odds (LOD) score $> 3.0$ and a distance $< 50$ cM were used as the thresholds to determine the linkage groups [62]. Genetic distances were determined using the Kosambi mapping function [63]. The genetic linkage map was constructed using the Microsoft Excel macro MapDraw [64].

4.6. Development of Functional Markers for er1-8 and er1-9

Primers flanking the mutation site (GTG/—) were designed based on the PsMLO1 gene sequence (GenBank accession number KC466597), using Primer Premier v5.0, to develop an insertion/deletion (indel) functional marker specific to allele er1-8, InDel-er1-8 (Table 3). The marker InDel-er1-8 was used to determine the genotypes of the 120 F$_2$ offspring derived from WSU 28 × G0004389. PCR amplification was performed as described above on a thermal cycler with the following cycling program: 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and 72 °C for 7 min. PCR products were separated on 8% polyacrylamide gels.

Table 3. Sequence information for the indel and Kompetitive allele-specific PCR (KASPar) markers specific to er1-8, and for the KASPar marker specific to er1-9.

| Markers     | Primers       | Sequence Information (5'-3') | Annealing Tm |
|-------------|---------------|-----------------------------|--------------|
| InDel-er1-8 | Forward       | GTTTTGACTGATATGACAGATGGGA   | 55 °C        |
|             | Reverse       | GTTTAGACTGATATGACAGATGGGA   |              |
| KASPar-er1-8| Forward-TGG   | TGCAACACGGCTTAAGAACTGG      | 65–57 °C touchdown |
|             | Forward       | GAGCAACACGGCTTAAGAARYA      |              |
|             | Common reverse| TGGTTGATATCGTTGATCCATC       |              |
| KASPar-er1-9| Forward-T     | TTTTGTATATGGGACGGTTGTAAT   | 65–57 °C touchdown |
|             | Forward       | TTTTGTATATGGGACGGTTGTAAT   |              |
|             | Common reverse| CAAAATGTAGATTATGCTAATAGTG   |              |

Based on allele er1-8 indels (a 3-bp deletion) and er1-9 SNPs (1-bp deletion) in PsMLO1, the forward primers and the common reverse primers specific to er1-8 (KASPar-er1-8) and er1-9 (KASPar-er1-9) were designed for Kompetitive allele-specific PCR (KASPar) markers by LGC KBioscience (KBioscience, Hoddesdon, UK), respectively. In brief, two KASPar markers (KASPar-er1-8 and KASPar-er1-9) were used to detect parental polymorphisms (WSU 28 × G0004389, and Bawan 6 × G0004400), and then used to analyze the genotypes of the F$_2$ offspring (WSU 28 × G0004389: 120 F$_2$ individuals; Bawan 6 × G0004400: 119 F$_2$ individuals).

KASPar markers were amplified with a Douglas Scientific Array Tape Platform (China Golden Marker, Beijing, Biotech Co., Ltd.) in a 0.8 µL Array Tape reaction volume with 10 ng dry DNA, 0.8 µL 2 × KASP master mix, and 0.011 µL primer mix (KBioscience, Hoddesdon, UK). A Nexar Liquid handling instrument was used to add the PCR solution to the Array Tape (Douglas Scientific). PCRs were performed on a Soellex PCR Thermal Cycler with the following conditions: initial denaturation at 94 °C for 15 min; followed by 10 cycles of denaturation at 94 °C for 20 s, and 65 °C for 60 s at an annealing temperature that decreased by 0.8 °C per cycle; and then 26 cycles of denaturation at 94 °C for 20 s and 57 °C for 60 s; and a final cooling to 4 °C. A fluorescent end-point reading was completed with the Araya fluorescence detection system (part of the Douglas Scientific Array Tape Platform). Genotypes and clusters were visualized with Kraken (http://ccb.jhu.edu/software/kraken/MANUAL.html).
4.7. Validation and Application of Functional Markers

To test the efficacy of the novel functional markers specific to er1-8 (InDel-er1-8 and KASPar-er1-8) and er1-9 (KASPar-er1-9), 169 pea germplasm accessions were tested for (a) their phenotypic resistance to E. pisi isolate EPYN and (b) whether they carried the er1 alleles er1-8 or er1-9 (Table S2). The four parent cultivars (WSU 28, G0004389, Bawan 6, and G0004400) were used as contrasting controls, and seven cultivars, including Tara (er1-1) [41], Xucai 1 (er1-2) [25], JI210 (er1-3) [14], Yi (er1-4) [14], G0001778 (er1-6) [27], DDR11 (er1-7) [26], and Gl2480 (er2) [28], were used as positive controls (Table S2).

DNA was extracted from the 169 selected pea germplasm accessions and the 11 controls (four parents and seven resistant cultivars with known er1 alleles) using the CTAB method (Shure et al. 1983). PCR amplifications of the indel and KASPar markers were performed as described above (in the section “Development of functional er1-8 and er1-9 markers”).

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/20/5071/s1.

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Abbreviations

SSR Simple sequence repeat  
SNP Single nucleotide polymorphism  
InDel Insertion/deletion  
KASPar Kompetitive allele-specific PCR

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