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Originalarbeit

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# Mapping of the *Rpv* Resistance Gene against Downy Mildew in Pea (*Pisum sativum* L.)

Kartierung des Resistenzgens *Rpv* gegen den Falschen Mehltau bei der Erbse (*Pisum sativum* L.)



## Abstract

Downy mildew causes severe yield and quality losses in pea (*Pisum sativum* L.). Therefore, the development of downy mildew resistant varieties is of high priority for pea breeders. Within this study in total 335 $F_3$ families from a cross of the highly susceptible green pea variety `Topaz´ with the resistant breeding line `Gen. 27´ were tested for resistance behaviour against *Peronospora viciae* f. sp. *pisi* to determine the genotype of the corresponding $F_2$ parental plants. The ratio 94:161:80 for homozygous resistant, heterozygous resistant and homozygous susceptible $F_2$ plants was not significantly different from 1:2:1, expected for the effect of a single dominant resistance gene. The resistance gene *Rpv* was mapped to linkage group 1 of the pea genetic map. This linkage group was saturated by molecular markers available from literature. The marker AD147 was identified as nearest proximal flanking marker with 4.4 cM distance, and in distal position marker AB28 with 18.8 cM distance. Further marker saturation using the syntenic relationship of *P. sativum* and *Medicago truncatula* was not effective. The usefulness of the identified markers for marker assisted selection has been confirmed in independent pea breeding material and the results of this study should lay the basis for future fine mapping studies.

**Key words:** *Pisum sativum* L., resistance gene, *Rpv*, downy mildew, *Peronospora viciae*, genetic linkage map, flanking markers

## Zusammenfassung

Die Entwicklung von Sorten, welche eine Resistenz gegen den Erreger des Falschen Mehltaus (*Peronospora viciae* f. sp. *pisi*) aufweisen, hat für Erbsenzüchter einen hohen Stellenwert, da dieser Pilz deutliche Ertragsminderungen und Qualitätseinbußen verursachen kann. In der vorliegenden Arbeit wurde eine spaltende Nachkommenschaft von 335 $F_2$-Nachkommen einer Initialkreuzung zwischen der anfälligen Hochleistunssorte `Topaz´ und der resistenten Zuchtlinie `Gen. 27´ erstellt. Jeweils 10 $F_3$-Nachkommen dieser 335 $F_2$-Pflanzen wurden in der Klimakammer auf Resistenz gegen den Falschen Mehltau getestet um auf den Genotyp der ursprünglichen $F_2$-Pflanze schließen zu können. Das Verhältnis von 94 homozygot resistenten, 161 heterozygot resistenten und 80 homozygot anfälligen $F_2$-Pflanzen weicht nicht signifikant von einer 1:2:1-Spaltung ab, die für das Vorliegen eines dominanten Resistenzgens erwartet wird. Das

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Resistenzgen *Rpv* wurde auf Kopplungsgruppe 1 der Erbse kartiert. Diese Kopplungsgruppe wurde anschließend mit weiteren molekularen Markern, die in der Literatur beschrieben wurden, gesättigt. Der Marker AD147 wurde dabei als proximaler Marker mit der geringsten Distanz (4,4 cM) bestimmt, während in distaler Position AB28 eine Distanz von 18,8 cM aufwies. Durch Nutzung der Synthenie zum *Medicago truncatula* Chromosom 5 konnten keine enger gekoppelten Marker ermittelt werden. Der Nutzen der identifizierten Marker in unabhängigem Zuchtmaterial konnte jedoch demonstriert und auch die Basis für eine zukünftige Feinkartierung des Resistenzgens gelegt werden.

**Stichwörter:** *Pisum sativum* L., Resistenzgen, *Rpv*, Falscher Mehltau, *Peronospora viciae*, Kopplungskarte, flankierende Marker

## Introduction

The genus *Pisum* belongs to the Fabaceae family, which is the third largest family in the plant kingdom and is second only to the Poaceae as the most important plant family for human consumption (YOUNG et al., 2003; GEPTS et al., 2005; WEEDEN, 2007; CANNON et al., 2009). Peas (*Pisum sativum* L.) serve as a protein-rich food with a high content of important minerals and vitamins and were cultivated already 8,000 years B.C. in the Near East (ZOHARY and HOPF, 2000). While dry peas are primarily used as animal feed, immature green peas are harvested for human consumption as fresh market peas, or as freezing or canning product. In 2013, a total of 6.72 million ha of dry peas were cultivated, compared with 2.30 million ha of green peas, of which China and India alone grew 1.30 and 0.42 million ha, respectively (http://faostat3.fao.org).

Pea is affected by a number of biotic stresses (HAGEDORN, 1991), among them downy mildew, which is responsible for substantial yield losses especially upon systemic infection (STEGMARK, 1994). The disease occurs under cool and moist climatic conditions and is caused by the pathogen *Peronospora viciae* (Berk.) Caspary f. sp. *pisi* (DIXON, 1981; BIDDLE, 2001). Its oospores are the main source for systemic infections (STEGMARK, 1994) and are able to survive in the soil for up to 10 years (VAN DER GAAG and FRINKING, 1997). Systemic infections lead to severe stunting of the pea seedlings and can cause floral sterility or even early death of the plants. Under conditions of high humidity also local infections of leaves and pod infections can occur. These symptoms are caused by conidia spread by water droplets or wind, which land on the surface of the plants and result in the typical grayish-white mycelium on the lower leaf surface or in yellow lesions on the pods. Together with systemic infections, pod infections cause the highest economic damage since they negatively affect pea quality (TAYLOR et al., 1990; STEGMARK, 1994; CHANG et al., 2013). Good agricultural practice and the application of fungicides are recommended to avoid infection with downy mildew (CHANG et al., 2013). However, for economic reasons and due to the growing demand for environment-friendly agriculture, the best solution is to provide resistant cultivars. Marker assisted selection (MAS) can greatly support resistance breeding efforts by selecting genotypes carrying the optimal combination of favorable alleles and avoid time consuming resistance screenings.

According to TAYLOR (1986), a single dominant resistance gene found in an Afghan pea line (JI 85) was first described by MATTHEWS and DOW (1973), who denominated it *Rpv*. TAYLOR (1986) mapped *Rpv* between the morphological markers *D* and *af* on *P. sativum* linkage group 1 (LG1). Additionally, two recessive genes (*rpv-1* and *rpv-2*) were reported in pea line JI 411 (MATTHEWS and DOW, 1971; LEWIS and MATTHEWS, 1985) and one single recessive resistance gene in line JI 314 (MATTHEWS and DOW, 1972). Also partially resistant lines were reported (STEGMARK, 1991a).

For breeding line `Gen. 27´ a high resistance against downy mildew was observed under field conditions (Irina WEIL, personal communication). However, nothing was known about the genetics underlying this resistance. Therefore, the aims of this study were: i) to clarify the mode of resistance, ii) to map the resistance gene(s) against downy mildew in pea line `Gen. 27´ and iii) to identify molecular markers which can be used for marker assisted selection in the breeding process.

## Material and Methods

### *Plant material*

The $F_2$ mapping population was developed by crossing `Gen. 27´ with the variety `Topaz´, susceptible to *P. viceae* f. sp. *pisi*. Both genotypes were kindly provided by van Waveren Saaten GmbH. The $F_2$ population comprised 335 plants derived from seven individual $F_1$ plants. The $F_2$ plants were self pollinated to produce $F_3$ families.

### *Resistance test*

The resistance tests were performed on $F_3$ progenies. In order to distinguish between homozygous resistant and heterozygous resistant $F_2$ plants, ten $F_3$ plants per $F_2$ plant were tested for resistance/susceptibility against *P. viciae* f. sp. *pisi*. The ten seeds were sown in two $8 \times 8$ cm pots (five seeds per pot) and grown for two weeks under greenhouse conditions until reaching the two-leaf to four-leaf stage.

Plants showing fresh field infections with downy mildew served as source for the inoculum. Mycelium was collected from infected pea leaves and suspended in 30 ml distilled water. The solution was homogenized continuously by mixing and kept on ice at all times. Immediately before inoculation, the viability of conidia and inoculum density was determined by staining with Phloxine B and counting with a Fuchs-Rosenthal counting chamber. A minimum percentage of viable conidia of 10% and a concentration of $10^4$ conidia/ml were necessary for successful infection.

The inoculation protocol with this conidia suspension was adapted from a standard protocol (Stegmark, 1991a; Stegmark, 1991b) and carried out as follows: For inoculation, the pots were placed on trays in a climate chamber (Fig. 1a) under controlled conditions with a photoperiod of 16 h light, 15/10°C (day/night) and a continuous humidity of at least 75–80%. Apical buds between the last emerging leaves were inoculated with 20 µl well mixed mycelium suspension (Fig. 1b). Right after inoculation the trays were covered with plastic bags, which were removed 48 hours later. Seven days after inoculation, the trays were placed under foil cages and regularly sprayed with water to create optimal conditions for sporulation (approx. 100% humidity; Fig. 1c). Symptoms of downy mildew were observed between 10 and 14 days after inoculation (Fig. 1d).

*Scoring of disease symptoms and segregation analysis*

The disease symptoms were scored two days after appearance of first symptoms. Since no previous knowledge existed about the mode of inheritance of the resistance, a scoring interval from 0–9 was chosen allowing a fine graduation of the symptoms (0–3 being resistant, 4–6 being medium resistant, and 7–9 being susceptible).

A $\chi^2$ test comparing the observed with the expected distribution was conducted to test the hypothesis of the existence of a single dominant resistance gene.

*DNA extraction and used molecular markers*

DNA was extracted from 100 mg of young leaf material using the DNeasy Plant Mini Kit (Qiagen). The DNA concentration was determined spectrophotometrically and adjusted to 8 ng/µl.

A total of 354 published molecular markers were tested for polymorphisms between `Topaz´ and `Gen. 27´. For 207 SSR markers (Burstin et al., 2001; Gutierrez et al., 2005; Loridon et al., 2005; DeCaire et al., 2012) an universal fluorescent labeling strategy was applied (Oetting et al., 1995). The unlabeled forward primer, extended at the 5′ end by a 19 bp M13 sequence, was combined with the original reverse primer and the fluorescently labeled M13 universal primer. The PCR reactions were conducted in 6 µl reaction volumes containing 8 ng DNA, 0.07 µM M13 tailed forward primer, 0.27 µM reverse primer, 0.2 µM IRD700 or IRD800 labeled M13 primer, 5x reaction buffer (including $MgCl_2$ and dNTPs) and 0.2 units of MyTaq™ (Bioline). The PCR reactions were performed using a touchdown protocol using a 0.5°C decrement for the first cycles and annealing temperatures according to the requirements of the primers. The PCR products were analyzed on a LI-COR 4300 DNA Analyzer and bands were scored manually.

82 gene based markers (Gilpin et al., 1997; Schneider et al., 1999; Brauner et al., 2002; Hecht et al., 2005; Konovalov et al., 2005; Aubert et al., 2006; Jing et al., 2007; Zhukov et al., 2007; Bordat et al., 2011; Jain et al., 2013) were either already described as CAPS-markers or converted into this marker class by testing a range of restriction endonucleases (*Alu*I, *Eco*RI, *Hae*III, *Hinf*I, *Mse*I, *Rsa*I, *Taq*I). Alternatively, the PCR products were sequenced and later analyzed with the program SNP2CAPS (Thiel et al., 2004) for finding an appropriate restriction enzyme.

Furthermore, 21 INDEL-marker (Brauner et al., 2002; Aubert et al., 2006; Zhukov et al., 2007; Bordat et al., 2011) and 44 RBIP marker (Flavell et al., 1998; Jing et al., 2005) were tested.

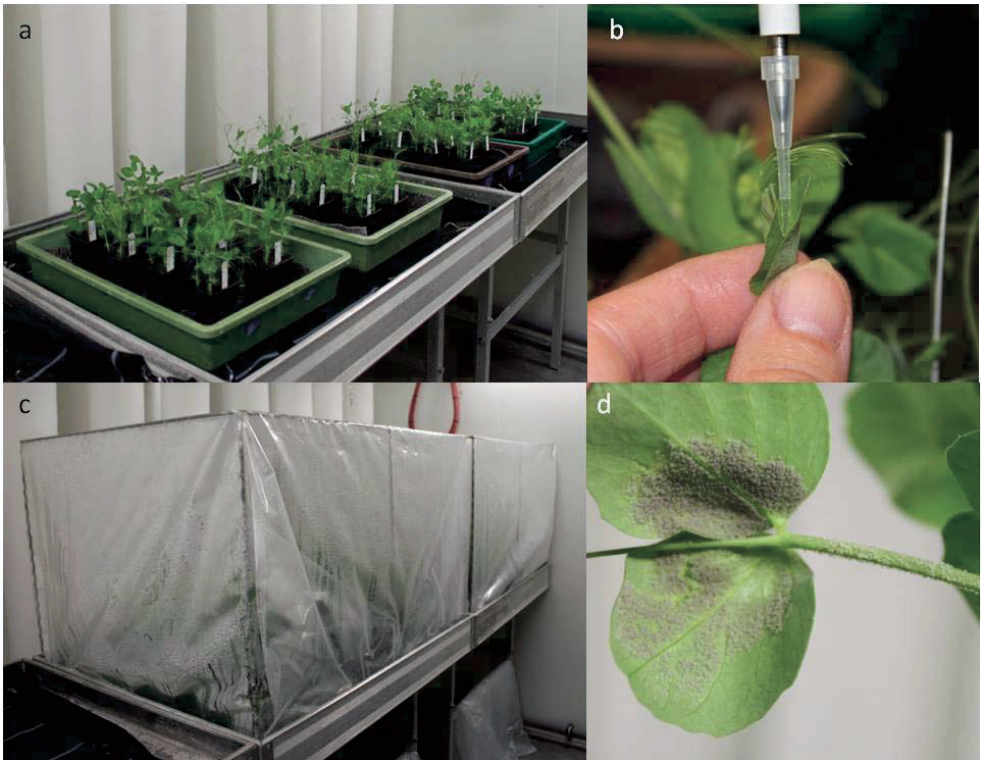


**Fig. 1.** Method of inoculation with downy mildew. a: Plants for resistance test in the climatic chamber, b: Mechanical inoculation with 20 µl mycelium suspension, c: Usage of foil cages for optimal conditions for sporulation, d: Symptoms of downy mildew 10 days after inoculation.

A homology search using the syntenic relationship between Pisum and Medicago was carried out based on *Medicago* assembly 4.0 (TANG et al., 2014, http://jcvi.org/medicago/index.php, 11.03.2014).

*Data scoring, linkage analysis and map construction*
The phenotypic and molecular data were formatted as required for JoinMap® version 4.1 (VAN OOIJEN, 2006). Linked loci were grouped using a LOD threshold from 2.0 to 10.0 in steps of 1.0 and recombination frequency lower than 0.4. The jump threshold was set to 5.0 and the third mapping round was carried out. The recombination frequencies were converted to mapping distances (in cM) using the Kosambi function.

## Results

*Inheritance of resistance*
For 94 of the 335 $F_2$ plants a homozygous resistant phenotype was deduced from the resistance behaviour of their $F_3$ progenies. 161 $F_2$ plants were heterozygous and for 80 progenies a homozygous susceptible genotype was concluded. The ratio 94:161:80 is not significantly different from the ratio of 1:2:1 ($\chi^2$ = 1.44), expected for the effect of a single dominant resistance gene.

*Genetic mapping and marker saturation*
Markers known from literature were tested for polymorphisms between the parental lines `Topaz´ and `Gen. 27´. Seventy nine polymorphic markers were used for constructing a preliminary genetic linkage map of *P. sativum*. Due to linkage to the markers AA155 and AD147 the resistance gene was mapped on LG1 (Fig. 2a). Based on this result the focus for further marker saturation was put on this linkage group. Additional markers known from literature to be located on LG1 or with unknown chromosomal location (Table 1) were used to screen for polymorphisms between the parental lines and, if polymorphic, used to saturate the map around *Rpv*. In that way the SSR marker PSSRAATTGA001 (DECAIRE et al., 2012) was mapped to LG1, which to our knowledge is the first description of its position. Furthermore, the RAPD-markers OPR13_600 and OPR13_2000 and the two genes *Enod7* (KOZIK et al., 1996) and *Enod40* (SCHNEIDER et al., 1999) were mapped to the *Rpv* region on LG1. The general marker order in the `Gen. 27´ × `Topaz´ map correlates well with other published pea genetic maps (Table 1, LORIDON et al., 2005; AUBERT et al., 2006; BORDAT et al., 2011).

Further marker saturation was intended by using the syntenic relationship of pea to *M. truncatula* (Mt) (KAL et al., 2004). This revealed that the homologous chromosomal region of the *Rpv*-locus in *Medicago* is located on Mt Chromosome 5. Twenty seven new primer pairs were developed for *Medicago* genes, which were located in the syntenic region and which had previously been described in the context of resistances or fungicidal effects. Although there was synteny on the macro level between the *Rpv* region of *Pisum* and *Medicago*, we observed that on the

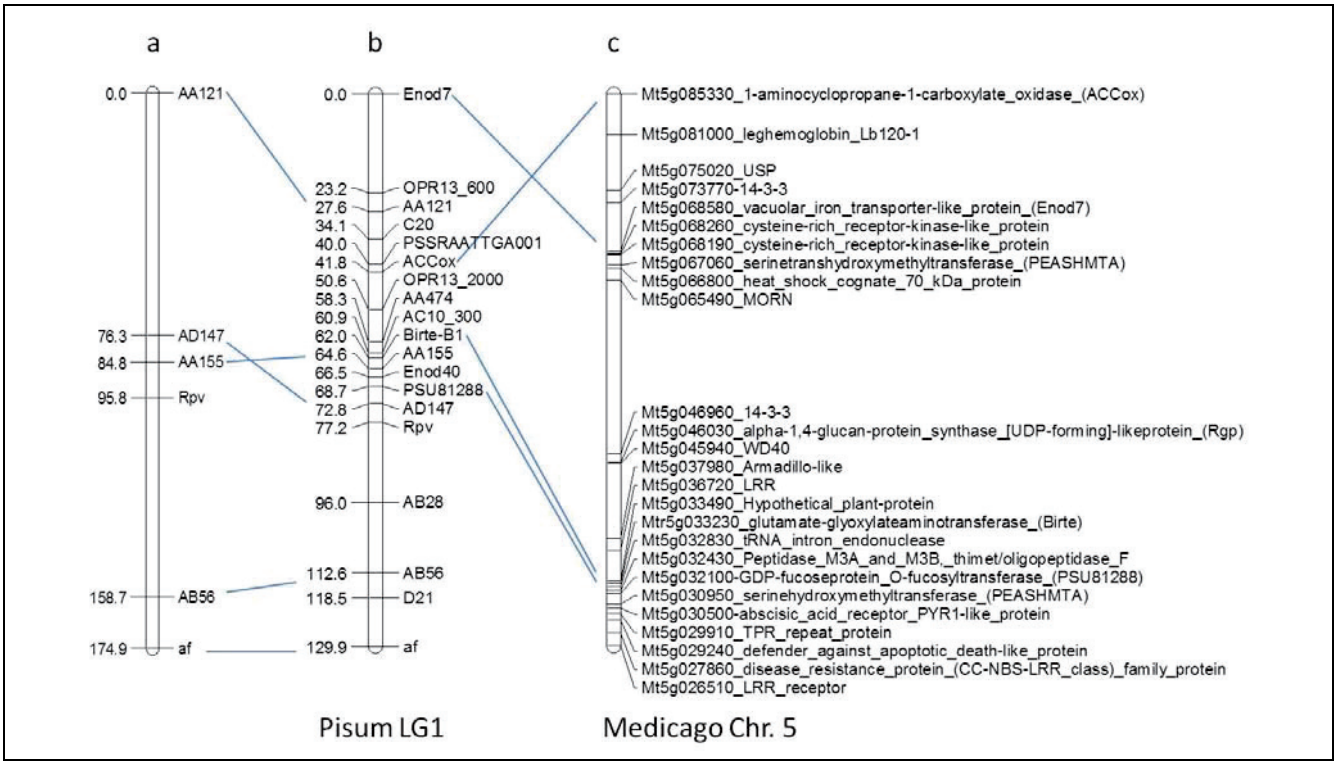


**Fig. 2.** Genetic map of the *Rpv* region on *Pisum* LG1 based on the `Gen. 27´ × `Topaz´ mapping population; a) rough genetic map after first mapping; b) genetic map of the *Rpv* region after marker saturation; c) syntenic region on *Medicago truncatula* chromosome 5 based on *Medicago* assembly 4.0 (http://jcvi.org/medicago/index.php, 11.03.2014).

**Table 1. Overview of markers mapped on LG 1 and their position in published pea genetic maps**

| Marker | Position [cM] (Bordat et al., 2011) | Position [cM] (Loridon et al., 2005) | Position [cM] this paper | Primer | Primer sequence | Marker type | Ref. |
|---|---|---|---|---|---|---|---|
| Enod7 | – | – | 0.0 | Enod7_2F<br>Enod7_3R | TGTGGCAATTGAGCCTTATG<br>CATGGTCCCATCTTTATTTTACA | CAPS | 1) |
| OPR13_600 | – | – | 23.2 | OPR13 | GGACGACAAG | RAPD | 2) |
| AA121 | 16.3 | 10.2 | 27.6 | AA121_F<br>AA121_R | TCCATACCTTAGTGTTAAA<br>ACTAATAAGGTAAACATGTG | SSR | 3) |
| C20 | 22.5 | 26.0 | 34.1 | C20_F<br>C20_R | GAGTTCTCCGTAATAGAAGGCT<br>CACTCTGTTCTGCTTCATCATC | CAPS | 3) |
| PSSRAATT-GA001 | – | – | 40.0 | PSSRAATTGA001_F<br>PSSRAATTGA001_R | TGGACAAGAGCCCATGATAA<br>CCAATACCCATTTCTCAACTCA | SSR | 4) |
| ACCox | 30.3 | – | 41.8 | ACCox_F<br>ACCox_R<br>ACCox_2F<br>ACCox_2R | GAATCATGGCATACCTCATGACC<br>GAGAAGGATGATCCCGCCAG<br>CAGGGTTGTAGAATGAGGCTATGG<br>GCTTGGAGAGACTCAATGGTGAGG | Gene<br>CAPS | 5)<br>3) |
| OPR13_2000 | 43.9 | 55.7 | 50.6 | OPR13 | GGACGACAAG | RAPD | 2) |
| AA474 | 56.9 | 59.7 | 58.3 | AA474_F<br>AA474_R | GCCCACACAAGTGGTTCTATAAAT<br>ATTAGTCGTTTTTCTGAAACATCAAAG | SSR | 3) |
| AC10 | – | 93.2 | 60.9 | AC10_F<br>AC10_R | TAAAGATGAGCCACGAACAGGC<br>AACGCGGCGATTAGGGATC | SSR | 3) |
| Birte-B1 | – | 72.4* | 62.0 | Birte-B1_F<br>Birte-B1_R | CCCATTGATTCTCGTCTCAAGAC<br>TCACGAGGGTGTGATAGTAACTCA | CAPS | 6) |
| AA155 | 69.6 | – | 64.6 | AA155_F<br>AA155_R | CATTTGAATAGTTGCAATTTCA<br>TATTTCTCCACCAGAGTTAGGT | SSR | 3) |
| Enod40 | – | – | 66.5 | Enod40_F<br>Enod40_R | CCTCTAAACCAATCCATTAT<br>TAACAGTTACCTACCTTAC | CAPS | 7) |
| PSU81288 | 73.7 | 77.0 | 68.7 | PSU81288_F<br>PSU81288_R | CGCCATGGAGCTTAGCTTCC<br>CGAGTAGATAGAAGAAGATGC | SSR | 8) |
| AD147 | 72.5 | 78.3 | 72.8 | AD147_F<br>AD147_R | AGCCCAAGTTTCTTCTGAATCC<br>AAATTCGCAGAGCGTTTGTTAC | SSR | 3) |
| AB28 | 102.0 | 113.9 | 96.0 | AB28_F<br>AB28_R | CCTGAGTCATCACATAGGAGAT<br>GCAGAAGTATTTGACTTGATGGAA | SSR | 3) |
| AB56 | 115.9 | 130.0 | 112.6 | AB56_F<br>AB56_R | GTGCAGAAGCATTTGAGCAGTT<br>TGAATGGACCAAAATGAAGGAG | SSR | 3) |
| D21 | 119.8 | 134.9 | 118.5 | D21_F<br>D21_R | TATTCTCCTCCAAAATTTCCTT<br>GTCAAAATTAGCCAAATTCCTC | SSR | 3) |
| af | – | – | 129.9 | | | mor-phol. | |

1) EST information from Kozik et al. (1996)
2) this paper
3) Loridon et al. (2005)
4) DeCaire et al. (2012)
5) Bordat et al. (2011)
6) Flavell et al. (1998); Jing et al. (2005)
7) Schneider et al. (1999b)
8) Burstin et al. (2001)
* According to JI281xJI399 map from Noel Ellis (cmap.comparative-legumes.org)

micro level the syntenic relationship was not completely linear (Fig. 2b + 2c). However, four new primer pairs from three *Medicago* genes were successfully developed and allele-specifically sequenced on the parental pea lines. None of the sequences showed polymorphisms between `Gen. 27´ and `Topaz´.

## Discussion

This paper describes the molecular mapping of a resistance gene in pea breeding line `Gen. 27´ against downy mildew using genetic resources available in pea and the synthenic relationship to *M. truncatula*.

Downy mildew is caused by *P. viciae* and yield losses of up to 70% were reported (CHANG et al., 2013). Oospores of the fungus are known to survive in soil for up to ten years (VAN DER GAAG and FRINKING, 1997). Infections with downy mildew can be prevented by either using chemicals in combination with cultural practices or by growing resistant cultivars (CHANG et al., 2013). The application of marker assisted selection could speed up resistance breeding and lead to a quicker introduction of high yielding resistant pea cultivars. This in turn would reduce the need to use agrochemicals to fight against downy mildew and would contribute to a more environment-friendly agriculture.

In earlier publications it was assumed that the resistance against downy mildew may be inherited by one dominant and two recessive genes (ALI et al., 1994; DAVIDSON et al., 2004). Because of this information the mapping project was started using a QTL-mapping approach. As it became clear that the resistance was inherited by one single dominant gene on linkage group 1 the mapping procedure was switched to a fine mapping approach. MATTHEWS and DOW (1973) first described and named the dominant resistance gene *Rpv* which was later mapped by TAYLOR (1986) to linkage group 1 between morphological markers *D* and *af*. The resistance gene in breeding line `Gen. 27´ is also located between those two markers and is inherited in a dominant fashion. As a result, there is well-founded reason to postulate that `Gen. 27´ carries the dominant resistance gene *Rpv*. Further fine mapping of *Rpv* using published pea markers from linkage group 1 or markers with unknown position in the pea genome was limited at the time of the experiments by the lack of sufficient known markers on linkage group 1 and by the lack of polymorphism between `Topaz´ and `Gen. 27´. The approach to exploit the syntenic relationship of pea and *M. truncatula* did yield functioning PCR primers in pea, however the amplified sequences showed no polymorphisms which could be used for marker development. This lack of polymorphisms between both parental genotypes was a general problem encountered in this study as only 24.6% of the tested pea markers were polymorphic. Due to this limitation the position of *Rpv* could not be refined further. Nevertheless, SSR marker AD147 mapping only 4.4 cM proximal and marker AB28 with 18.8 cM distance on the distal side could be identified as the closest flanking markers to *Rpv*. Both SSR markers are well suited for MAS in breeding programs. Even though they are not tightly linked to *Rpv*, if used in combination, they can increase the reliability for selection of downy mildew resistant plants. The applicability of both markers in pea breeding programs was confirmed successfully in independent breeding material.

With the establishment of the syntenic relationship to *M. truncatula* and the finer positioning of *Rpv* on the pea genetic map the present work lays the foundation for the future identification of this resistance gene. It should be possible to identify candidate genes for *Rpv* in a relatively short period of time by taking advantage of additional pea markers (SUN et al., 2014), a newly published high-resolution consensus genetic map as well as state-of- the art genomic tools like the recently described 13.2K SNP array (TAYEH et al., 2016) and molecular methods like genotyping by sequencing, exome capture or whole genome shotgun sequencing in combination with bulked segregant analysis (ELSHIRE et al., 2011; MASCHER et al., 2013; BOLGER et al., 2014; HIMMELBACH et al., 2014; WENDLER et al., 2014: RIES et al., 2016). Additionally, findings about factors involved in resistance mechanisms against downy mildew in other plant species, for example *A. thaliana*, should be exploited (PARKER et al., 1996; SINAPIDOU et al., 2004; VAN DAMME et al., 2009; TEN HOVE et al., 2011; ASAI et al., 2014).

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