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ORIGINAL ARTICLE

Characterization and mapping of *LanrBo*: a locus conferring anthracnose resistance in narrow-leafed lupin (*Lupinus angustifolius* L.)

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Abstract

Key message **A novel and highly effective source of anthracnose resistance in narrow-leafed lupin was identified. Resistance was shown to be governed by a single dominant locus. Molecular markers have been developed, which can be used for selecting resistant genotypes in lupin breeding.**

Abstract A screening for anthracnose resistance of a set of plant genetic resources of narrow-leafed lupin (*Lupinus angustifolius* L.) identified the breeding line Bo7212 as being highly resistant to anthracnose (*Colletotrichum lupini*). Segregation analysis indicated that the resistance of Bo7212 is inherited by a single dominant locus. The corresponding resistance gene was given the designation *LanrBo*. Previously published molecular anchor markers allowed us to locate *LanrBo* on linkage group NLL-11 of narrow-leafed lupin. Using information from RNAseq data obtained with inoculated resistant vs. susceptible lupin entries as well as EST-sequence information from the model genome *Lotus japonicus*, additional SNP and EST markers linked to *LanrBo* were derived. A bracket of two *LanrBo*-flanking markers allows for precise marker-assisted selection of the novel resistance gene in narrow-leafed lupin breeding programs.

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Introduction

Recent developments in the EU Common Agricultural Policy, as well as national policies, to open up ecosystem services provided by domestic legumes, have called attention in so far underutilized grain legumes in European agriculture.

Among these, sweet narrow-leafed lupin (*Lupinus angustifolius* L.) offers a highly valuable protein source for both feed and food purposes. Moreover lupin cultivation provides benefits for sustainable agriculture as they are able to mobilize soil phosphorous and fix atmospheric nitrogen; hence they offer attractive options to provide a more flexible crop rotation (Lambers et al. 2013).

Present acreage of sweet lupins in Germany amounts to 21,400 ha (DESTATIS 2014), which although still quite low, represents a 23 % increase as compared to 2013. Reflecting the lupin production worldwide in 2013, 58.4 % took place in Australia and Oceania, followed by Europe with 32 % with the remaining 9.6 % lupin production happened in Africa and America. Being the most important lupin producer worldwide, acreage of lupins in Australia and Oceania amounts to 450,200 ha (FAOSTAT 2014).

In Germany, narrow-leafed lupin largely displaced white and yellow lupins (*L. albus* L., *L. luteus* L.) in the mid-nineties of the last century because of its somewhat higher

tolerance to the fungus *Colletotrichum lupini*, the causal agent of anthracnose (Wolko et al. 2011). However, most if not all narrow-leafed lupin cultivars adapted to agricultural conditions in Central Europe lack strong resistance to the fungus. Being a seed-transmitted disease (Gondran et al. 1994), anthracnose continues to pose a latent threat to the cultivation of narrow-leafed lupin, not only in Germany but also worldwide (Paulitz 1995; Reed et al. 1996; Sweetingham et al. 1995), thereby emphasizing the necessity of resistant cultivars.

In Australia, after dealing with a disease spread in the mid-1990s, the anthracnose-resistant cvs. 'Mandelup' and 'Tanjil' were released, thereby exemplifying that genes for anthracnose resistance exist in *L. angustifolius* and that resistance breeding provides an option to fight the disease (Yang et al. 2004, 2008). Resistance in cv. 'Tanjil' is inherited by a single dominant gene named *Lanr1* (Yang et al. 2004), which is located on linkage group NLL-11 (Nelson et al. 2010). This resistance gene can be tracked in breeding programs by use of closely linked co-dominant molecular markers (Yang et al. 2012; You et al. 2005).

When grown under local German growing conditions, cvs. 'Tanjil' and 'Mandelup' prove to be less susceptible than standard cultivars; however they still become infested by the pathogen to considerable extents (Ruge-Wehling et al. 2009; this paper). Thus, provision of additional genes for anthracnose resistance appears desirable to lupin breeders.

In the present study, we performed a screening of cultivars, breeding lines and genebank accessions and assessed their susceptibility to anthracnose in the greenhouse and under field conditions at diverse locations. We report on a novel anthracnose-resistance gene, *LanrBo*, and the development of molecular markers using existing and novel resources, which may be used to select for this gene in breeding programs.

Materials and methods

Material

L. angustifolius *accessions*

A set of 13 *L. angustifolius* cultivars ('Arabella', 'Bolivio', 'Bora', 'Bordako', 'Boregine', 'Boruta', 'Borweta', 'Haagena', 'Haags Blaue', 'Mandelup', 'Polonez', 'Tanjil', and 'Vitabor'), 15 breeding lines from the seed breeding company Saatzucht Steinach GmbH & Co KG, Bocksee, Germany, and 43 genebank accessions (cf. Supplement, Table S1) were tested with respect to their susceptibility to anthracnose in an initial greenhouse testing.

Field testing included breeding lines Bo7212, Metel1 and Bo5333, cvs. 'Tanjil' and 'Mandelup' which are known to be resistant to anthracnose under Australian growing conditions, as well as the German cultivar 'Arabella' which was used as a susceptible standard.

F_2 *mapping populations*

Flowers of plants from the susceptible cvs. 'Arabella' and 'Probor' and genebank accession PI308616, respectively, were emasculated and hand-pollinated with pollen from breeding line Bo7212. This yielded up to five pods per plant with 1–5 seeds/pod. Seeds were germinated and molecular markers used to identify true F_1 hybrid plants, the latter of which were selfed to the F_2 generation (Ruge-Wehling et al. 2009).

Altogether, five F_2 families, each segregating with susceptible and resistant individuals, were used for genetic analysis and mapping. Three families stemmed from different cv. 'Arabella' seed-parent plants and two from a cv. 'Probor' and PI308616 seed parent, respectively (Table 1). Each of the three 'Arabella' F_2 families originated in 4–5 seeds, respectively, of a single F_1 pod.

Table 1 χ^2 analysis of segregations in F_2 with infested and non-infested individuals

F_2	Seed parent	Pollen parent	N individuals	N non-infested	N infested	χ^2 (3:1)
1013/4	Arabella	Bo7212	104	76	28	0.205 n.s.
1014/1	Arabella	Bo7212	133	100	33	0.003 n.s.
1015/2	Arabella	Bo7212	131	97	34	0.064 n.s.
		Total	368	273	95	0.131 n.s.
1104/1	Probor	Bo7212	101	72	29	0.744 n.s.
1110/2	PI308616	Bo7212	51	38	13	0.006 n.s.

n.s. non-significant at $\alpha = 0.05$

F3 progeny testing

In the case of F_2 family 1013/4, randomly selected plants were selfed to F_3 and 10–12 plants per F_3 progeny subjected to a greenhouse anthracnose testing to check the inheritance of phenotypes ("infested" vs. "non-infested") in the segregating F_2 families and to distinguish homozygous from heterozygous genotypes among resistant F_2 individuals.

C. lupini *strains*

The *C. lupini* var. *setosum* strains BBA70400, BBA70397, BBA70358, and BBA70385, which have been collected and isolated from different lupin species (Nirenberg et al. 2002), were kindly provided by N. I. Nirenberg from the former Federal Biological Research Centre for Agriculture and Forestry (BBA), Germany. Storage and propagation of *C. lupini* were performed according to Nirenberg et al. (2002).

Testing for susceptibility to anthracnose

Greenhouse testing

Twelve to fifteen plants per entry were grown in the greenhouse and inoculated following the procedure by Yang et al. (2004). Briefly, 8-week-old plants were prepared by removing their lateral shoots and flowers. The remaining shoot including the main inflorescence was sprayed with a conidial suspension (500,000 conidia/ml, strain BBA70385). The inoculated plants were incubated in the dark for 16 h at 18 °C. Following incubation, the plants were held in the greenhouse at long-day conditions (16 h/8 h) and 18 °C until phenotyping.

Field testing

Field testing was done over six environments, namely, two locations (Groß Lüsewitz and Bocksee, in the northeast of Germany) and three years (2007, 2009 and 2010; except for cv. 'Mandelup' which was not included in 2007). Unlike the greenhouse testing, field testing left the plants intact, with lateral shoots and flowers. Plot design was a randomized block with two replications per location and year. Plots were 2 × 1.5 m (L × W) and made up of six rows with 20 cm spacing. The seed rate was calculated with 90 g/m^2. Seeds of the susceptible cv. 'Arabella' were inoculated with a mixture of four strains of *C. lupini* (see above) and seeded as infection rows with 15 seeds per row between every single row of each entry to obtain a high and permanent infection pressure. Infection rows were sown 2 weeks after sowing the experimental entries.

Fig. 1 Phenotypes of plant response after inoculation with *C. lupini* in the greenhouse. **a** Plant of the "infested" phenotype, with twisted and bended stem and conidia bearings; **b** plant representing the "non-infested" phenotype

Phenotyping

In the greenhouse testing procedure, 12–15 plants per entry were phenotyped 10–14 days past inoculation. Phenotypes were defined with regard to the expression of the typical anthracnose symptoms, namely, twisting and bending of the stem and lesions at the stems and pods (Fig. 1). Plants expressing these symptoms were given the phenotype "infested", without any further differentiation in symptom severity. A second group of plants, which stayed free of symptoms, was assigned to the "non-infested" phenotype.

In the field tests, incidence of the "infested" phenotype was recorded at three dates, namely, at the six-leaf stage (BBCH30), time of flowering (BBCH60), as well as at the early stage of pod formation (BBCH70). The percentage of infested plants was calculated by relating the number of infested plants per plot to the total count of plants established in a given plot. Plants were assigned to the "infested" (Fig. 2a) and "non-infested" (Fig. 2b) phenotype, respectively, as described for the greenhouse testing procedure, with no further gradation of symptom severity. However, a distinction was made between plants, which became infested at an early stage and as a consequence, failed to form pods and those which developed symptoms exclusively on their pods.

Fig. 2 Field-testing plots. **a** Infested plants of cv. 'Arabella', **b** non-infested breeding line Bo7212

Statistical analysis

Statistical analysis of field-testing data was accomplished by an analysis of variances (ANOVA) and a LSD test as a post hoc analysis with a significance level of $p = 0.05$. Thestatistics software PLABSTAT (Utz 2001) was used.

Molecular marker analysis

DNA isolation

Genomic DNA was isolated following a slightly modified protocol after Stein et al. (2001). DNA was dissolved in TE buffer, quantified via photometric approach (NanoQuant, Tecan, Austria) and diluted to a working concentration of 10 ng/µl.

Anchor markers

The genetic reference map published previously by Nelson et al. (2010) provided primer information for potential anchor markers. 140 STS primer pairs were tested for polymorphism between cv. 'Arabella' and breeding line Bo7212. Sequence information on additional markers was taken from Yang et al. (2012, 2013).

For PCR, 50–100 ng of template DNA was used in a solution containing 1× reaction buffer (Promega), 0.8 mM dNTP mix, 0.5 µM of each primer, 1.5 mM $MgCl_2$ and 0.3 U *Taq* DNA polymerase (Promega). PCR was conducted by a touchdown protocol: after an initial denaturing step for 2 min at 95 °C, the cycling started for 1 min at 95 °C, followed by an annealing step for 30 s at 60 °C and an extension for 1 min at 72 °C. The annealing temperature decreased by 1 °C during the first 10 cycles. Annealing temperature was adjusted according to the respective markers. Amplification products were separated either on 2.5 % agarose gels followed by ethidium bromide staining or on 10 % PAGE followed by silver staining (Budowle et al. 1991).

Screening for polymorphism of 239 indel and simple-sequence repeat (SSR) markers (Kamphuis et al. 2014) was performed by the multiplex-ready PCR method (Hayden et al. 2008) as described in Gao et al. (2011). Amplification products were analyzed on an AB3730 DNA Analyzer (Applied Biosystems). Allele scoring was carried out using the GeneMarker software (Version 1.91, SoftGenetics, LLC).

Simple-sequence repeat (SSR) markers based on sequence information from Lotus japonicus

EST sequences from *L. japonicus* were transferred from the NCBI database (http://www.ncbi.nlm.nih.gov/nucest/) to SSRIT [Simple Sequence Repeat Identification Tool, Temnykh et al. (2001)] to search for SSR motifs of various lengths. 100 primer pairs were designed using the Primer3 software (Untergasser et al. 2012) and designated as *LJM* with consecutive numbering. PCR amplification was carried out by using 50–100 ng template DNA in a 1× buffer solution containing 0.8 mM dNTP mix, 0.5 µM of each primer pair, 4.5 mM $MgCl_2$ and 0.3 U *Taq* DNA polymerase and performed by a touchdown protocol as described for anchor markers.

Amplified fragment-length polymorphism (AFLP) markers

AFLP analysis was performed according to Vos et al. (1995). In detail, DNA samples were digested with *Eco*RI as a non-frequently cutting endonuclease and *Mse*I as a frequent cutter, and ligated with the appropriate double-strand adaptors. A total of 256 primer combinations were screened using parent DNA samples. All PCR reactions were conducted on a peqSTAR thermal cycler (PEQLAB Biotechnologie GmbH). The PCR products were run on 6.5 % denaturing polyacrylamide gels and fractionated on a 4300 DNA Analyzer (LI-COR Biosciences).

RNAseq-based SNP markers

SNP markers were developed by high-throughput sequencing of the transcriptomes (RNAseq) of susceptible cv. 'Arabella' and resistant breeding line Bo7212. To allow for the identification of resistance-related transcripts, both parents were inoculated with a *C. lupini* conidial suspension at BBCH 60 (first flowers start opening). Leaf material was collected 4, 8, 24, and 48 h post inoculation, snap-frozen in liquid nitrogen and assigned to a 'susceptible' and 'resistant' bulk, respectively, for RNA isolation.

After grinding the leaf material in liquid nitrogen RNA was extracted using silica bead-columns (RNA extraction kit, Invitek). The poly-adenylated mRNA was captured using oligo-dT-coupled magnetic beads (Dynabeads mRNA purification kit, Ambion) according to the manufacturer's description.

The poly-adenylated RNA was then fragmented using buffer containing Zn^{2+}, and Illumina sequencing-adapters were ligated with the fragment-ends via RNA ligase according to an internal protocol of GenXPro GmbH, Frankfurt/M., Germany. The adapter-fragment combinations were transcribed into cDNA amplified by PCR using 12 cycles and sequenced on an Illumina Hiseq 2000 machine to generate 2 × 100 bp reads.

The raw data was cleansed of adapter sequences using the software TagDust (Lassmann et al. 2009). All RNAseq datasets were combined to create a reference assembly using the software Trinity (Grabherr et al. 2011). The reads of the individual libraries were mapped to the reference library and single nucleotide variants (SNVs, or SNPs) were identified using the software SNVMix (Goya et al. 2010). Sequences 100 bps up- and downstream of the SNPs were determined and utilized to generate PCR primers, using default settings with Primer 3 (Untergasser et al. 2012). The SNP containing contigs were annotated by BLASTX to the Swiss-Prot database (Boeckmann et al. 2003).

Detection of SNPs as genetic markers was performed by high-resolution melt analysis (HRM). PCR was carried out in 20 µl volume containing 40 ng template DNA, 1× buffer (Promega), 2.5 mM $MgCl_2$, 0.8 mM dNTP mix, 0.5 µM of each primer, 1× EvaGreen Dye (Biotium, Inc.) and 0.3 U *Taq* DNA polymerase (Promega). A touchdown PCR protocol was conducted with a temperature gradient from 60 to 50 °C. The melting curve analysis was conducted by ramping from 65 °C to 95 °C with a 0.1 °C increase per capture. Primer pairs derived from sequences specific for either Bo7212 or cv. 'Arabella' were termed with the prefix *BoSeq* and *ArSeq*, respectively.

The sequence data have been deposited in GenBank and are accessible through accession numbers KP760854—KP760858 (cf. Supplement, Table S4).

Genetic mapping

Mapping of the resistance present in breeding line Bo7212 was performed using the software package JoinMap4.1 (Van Ooijen 2011). Loci were grouped with LODs ranging from 3.0 to 4.0. Locus ordering was performed with the regression mapping algorithm with default parameters. The Kosambi mapping function was used to estimate genetic distances.

The JoinMap4.1 function 'Combine Groups for Map Integration' was used to carry out an integrated-map calculation based on mapping populations 1013/4, 1014/1 and 1015/2.

Genetic maps were displayed and edited in MapChart 2.2 (Voorrips 2002).

Results

Identification of anthracnose resistance source

In the greenhouse testing, plants fell into either of two phenotypic classes, namely, plants which displayed the typical anthracnose symptoms of twisting and bending of the main stem (Fig. 1a) and those which stayed free of these symptoms (Fig. 1b). Consequently, the phenotypes were designated "infested" and "non-infested", respectively.

Among the 13 cultivars assessed in the greenhouse test, plants of all the 11 European cultivars expressed the "infested" phenotype. In contrast, the Australian cvs. 'Tanjil' and 'Mandelup' stayed free of symptoms. All the tested genebank accessions as well as 12 of 15 breeding lines fell into the "infested" phenotypic class. Three breeding lines (Metel1, Bo7212, and Bo5333) remained free of symptoms (not shown).

Breeding lines Bo7212, Bo5333, and Metel1, as well as cvs. 'Tanjil' and 'Mandelup' were tested for their susceptibility to anthracnose under field conditions at two test sites over 2–3 years. Figure 3 illustrates the results by showing the percentages of three classes of diseased plants, namely, plants which (A) became infested by the fungus, (B) became infested and failed to develop pods, (C) became infested and developed symptoms exclusively on the pods. Class C is relevant since symptom expression on a pod often is attended by the presence of contaminated seeds within the pod. Supplementary Table S2 presents the percentages underlying Fig. 3.

The high infection pressure in these field trials is documented by the fact that in the case of the susceptible standard (cv. 'Arabella'), the percentage of diseased plants averaged 44.3 and 51.2 % at the test sites of Bocksee and Groß Lüsewitz, respectively (Fig. 3, class A). As a consequence of infestation, 20.5 and 20.6 %, respectively, of all plants of cv. 'Arabella' tested at the two sites did not develop pods (Fig. 3, class B).

Among the three breeding lines tested, Metel1 and Bo5333 could not resist the fungus in the field testing; hence they were regarded as susceptible (not shown). In contrast, of the 3096 plants of breeding line Bo7212 tested over 3 years, only 2.5 and 5.7 % became infested and as few as 0.3 and 0.1 % ceased pod formation at the Bocksee and Groß Lüsewitz trial sites, respectively (Fig. 3, classes

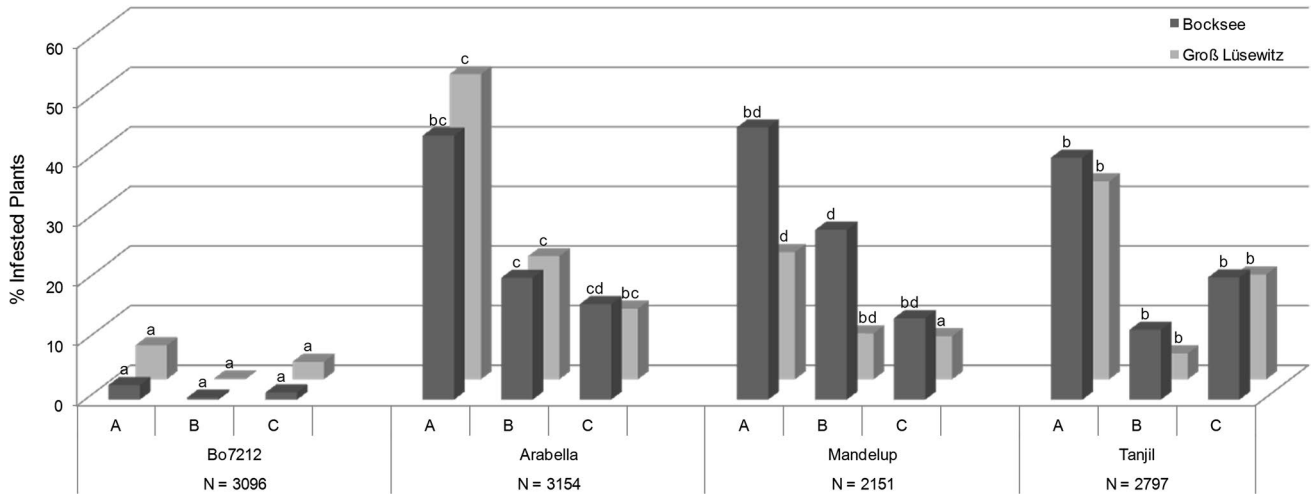

Fig. 3 Infestation of three *L. angustifolius* cultivars and breeding line Bo7212 under field conditions in northeast Germany. Percentage of *A* of infested plants, *B* infested plants without pod formation, *C* plants with symptoms restricted to pods. Mean percentages per test location over 3 years and 2 replications/year are shown. Significant differences according to the LSD test ($\alpha = 0.05$) between entries are indicated by different *lower-case letters*

A and B). At the two sites, 1.2 and 3.0 %, respectively, of the tested plants developed symptoms on their pods (class C in Fig. 3 and Suppl. Table S2).

The two cvs. 'Tanjil' and 'Mandelup' were somewhat outstanding, in that although they expressed the "non-infested" phenotype in the greenhouse test they were found to show up quite high percentages of infested plants under field conditions. Namely, 40.6 and 33.2 % of the tested plants from cv. 'Tanjil' and 45.7 and 21.3 % of 'Mandelup' plants became diseased at the two test locations, respectively. Thus, infestation rates of these cultivars were somewhat lower than for cv. 'Arabella'; however they were significantly above the infestation rate of breeding line Bo7212. Likewise, the rates of plants among the two cultivars, which failed to develop pods upon infection were significantly higher as compared to Bo7212. Strikingly, the two cultivars also displayed high rates of pod infestation (class C) at the two test sites, with 20.5 and 17.6 % for cv. 'Tanjil', and 13.7 and 7.2 % for cv. 'Mandelup'.

To conclude, breeding line Bo7212 turned out to be the only entry, which resisted the fungus both under the controlled conditions in the greenhouse test and under field conditions in hitherto six environments. Bo7212 appears, thus, as a potential source of highly effective resistance to *C. lupini*, provided that its resistance has a clear-cut genetic basis trackable in a breeding programme.

Genetic analysis of the resistance of Bo7212

F_2 families derived from five independent crosses of Bo7212 and three susceptible accessions were phenotyped for their anthracnose reaction under greenhouse conditions (Table 1). All the five F_2 families segregated with non-infested and infested offspring. Proportions of these two phenotypes were consistent with a 3:1 ratio in each case.

In addition, 12 F_2 plants taken from family 1013/4 were selfed and 10–12 F_3 offspring, respectively, subjected to a greenhouse testing. Of 12 non-infested F_2 parents, 4 gave rise to non-infested and 7 to segregating F_3 progeny. One F_2 plant, 1013/4-98, which had been phenotyped as "non-infested" gave homogeneous-infested F_3 progeny, thus indicating a case of mis-phenotyping (cf. Supplement, Table S3). As a result, sampling of F_2 individuals via F_3 progeny tests generally confirmed the F_2 phenotypes and corroborated their monogenic mode of inheritance observed in F_2, with one phenotype being conditioned by the homozygous-resistant and heterozygous-resistant genotypes, and the other by a homozygous-susceptible genotype.

To conclude, F_2 and F_3 segregation analyses suggest that the "non-infested" phenotype observed for breeding line Bo7212 has a clear-cut genetic basis, with a single dominant resistance factor effective in various genetic backgrounds. Taking into account the report on anthracnose-resistance gene *Lanr1* by Yang et al. (2004), the resistance factor described in the present report is named *LanrBo*.

Genetic mapping of LanrBo

Mapping of *LanrBo* was based on F_2 families 1013/4 ($N = 104$), 1014/1 ($N = 133$), and 1015/2 ($N = 131$). A set of 201 molecular markers were used, which proved to be polymorphic in these F_2 families. The marker set comprised 92 anchor markers (Kamphuis et al. 2014; Nelson et al. 2010; Yang et al. 2013), 17 SSR markers based on

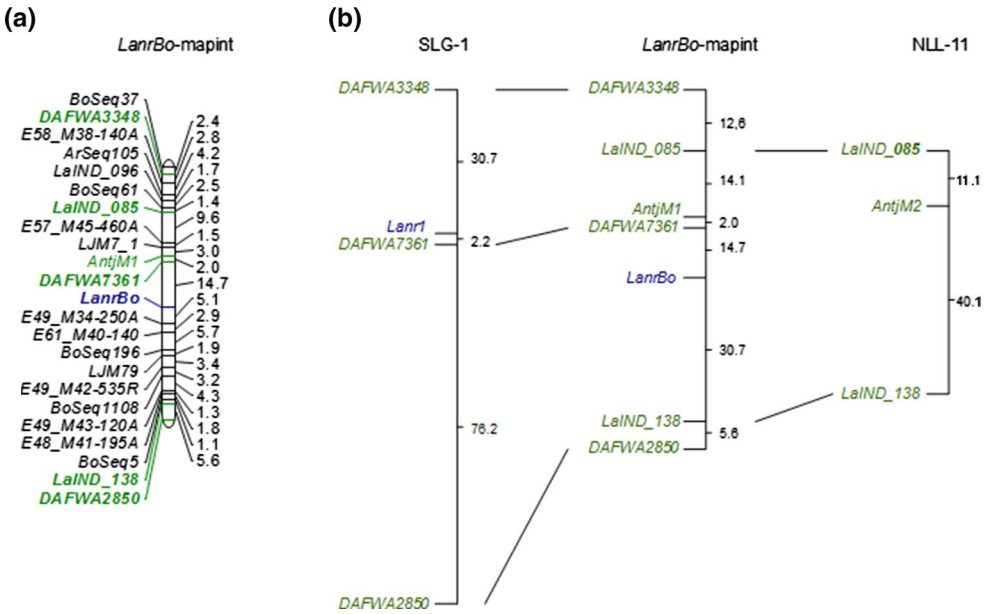

Fig. 4 **a** Integrated genetic map (*LanrBo*-mapint) of resistance locus *LanrBo*. Markers common in *LanrBo*-mapint and any of the two other maps in Fig. 4b are shown in *bold*. **b** Schematic depiction of anchor-marker positions and distances in *LanrBo*-mapint relative to those reported for linkage groups SLG-1 (Yang et al. 2013) and NLL-11 (Kamphuis et al. 2014) *Anchor markers* used in the present study are *highlighted in green*; *distances* are given in cM

Lotus japonicus sequences (*LJM*), a set of amplified fragment-length polymorphisms (AFLP) and 14 *ArSeq* and 78 *Boseq* sites. Of these, 32 markers proved to be linked to *LanrBo* in mapping family 1014/1 ($N = 133$) whereas less markers were scorable in the remaining two families (not shown). To improve mapping precision an integrated map (*LanrBo*-mapint) was calculated based on the three family-wise maps. In total, *LanrBo*-mapint comprises the *LanrBo* locus together with 22 marker loci and covers 82.0 cM (Fig. 4a). Of the 22 markers, 15 were developed in the present study, namely, 7 AFLP markers, 2 *LJM* markers and 6 RNAseq markers (cf. Supplement Table S4). These novel markers could be anchored to two other published maps using three polymorphic markers from map NLL-11 (Kamphuis et al. 2014) and three markers from map SLG-1 (Yang et al. 2013) (Fig. 4b). Within the *LanrBo* linkage group, the resistance locus is delimited to a genetic interval of 19.8 cM by the flanking markers *DAFWA7361* 14.7 cM proximal and *E49_M34-250A* 5.1 cM distal of *LanrBo*.

Discussion

Testing susceptibility to anthracnose

To characterize plants from accessions, cultivars and breeding lines with regard to their susceptibility to anthracnose, we applied two test schemes in a consecutive manner, namely, a greenhouse test, which was followed by field testing. The two test schemes differed from each other in respect to the mode of inoculation. The mapping populations used in this study were only tested under greenhouse conditions.

The greenhouse test encompassed manual ablation of 2nd order stems of the plants prior to inoculation. The protocol was adopted from Yang et al. (2004) who successfully used the test to identify the first anthracnose-resistance gene, *Lanr1*, in narrow-leafed lupin. Clearly, such a harsh treatment opens an artificial portal of entry of the pathogen in each tested plant and is not representative for the situation met under field conditions. To conclude, the greenhouse test appears to be more suited for detecting broad, qualitative differences in the susceptibility of entries rather than more subtle, quantitative graduations in disease severity. Using this test, entries and individual plants thereof could be grouped to either of two phenotypes, namely, plants developing the typical symptoms like twisting and bending of the stem ("infested") or plants not showing these symptoms at all ("non-infested"). As could perhaps be anticipated from the rigorous treatment of plants in the course of the greenhouse testing scheme, quantitative differences in symptom expression (twisting and bending) among the first group of plants were negligible. We decided to use the greenhouse test for testing breeding line Bo7212 in an initial approach, after we had obtained first indications that Bo7212 might carry a qualitatively effective resistance. The rationale behind this was that plants withstanding the fungus in the rigorous greenhouse test were expected to prove resistant also under field conditions. This expectation was confirmed by the observation that breeding line Bo7212 withstood the infection pressure in the field trial. A low percentage of plants of line Bo7212, though, became diseased in the field trial. We assume some resting genetic inhomogeneity of the breeding line as a possible reason for this observation. The expectation was, however, contradicted by the field testing in the case of cvs. 'Tanjil' and 'Mandelup' which proved at

best moderately susceptible to the infection pressure under field conditions (see below).

Breeding line Bo7212 as a promising source of anthracnose resistance

Bo7212 expressed an effective, qualitative resistance to *C. lupini* under Central European growing conditions. The resistance was effective against various *C. lupini* strains and proved stable over 3 years and two sites.

Notably, in the field experiments Bo7212 performed quite differently from the cvs. 'Tanjil' and 'Mandelup', which are considered to be resistant to anthracnose under Australian growing conditions. Compared to these cultivars, Bo7212 appeared distinctly more resistant to the infection pressure under the field conditions effective in our study.

There are various conceivable reasons for the difference in the levels of resistance observed between Bo7212 and the resistant Australian cultivars.

One reason may lie in different genetic backgrounds present in these germplasms. As a consequence, differing physiological adaptation to the specific environmental conditions of our study might have favoured the expression of the resistance gene in one genetic background (Bo7212) and compromised the expression of the same gene in the others (cvs. 'Tanjil' and 'Mandelup'). However, the fact that in our study the resistance drawn from Bo7212 was equally effective in three different genetic backgrounds (cv. 'Arabella', cv. 'Probor', accn. PI308616) contradicts the assumption of a pronounced influence exerted by the genetic background. Furthermore, in our study plant development of cv. 'Tanjil' was comparable to that of Bo7212, making it less probable that its higher infestation by anthracnose would have been caused by some inadequate adaptation of this Australian cultivar to Central European growth conditions. A strong argument against environment x genetic background interactions as a cause of the differences in resistance levels as shown in Fig. 3 is that Bo7212 proved to be more resistant than cv. 'Tanjil' also under Australian growing conditions (person. commun. B. Buirchell, Department of Agriculture and Food, Perth, Western Australia).

Another reason, which we deem more probable may be the presence of differing anthracnose-resistance genes in Bo7212 on the one hand and in the cvs. 'Tanjil' and 'Mandelup' on the other. The resistance present in cvs. 'Tanjil' and 'Mandelup' was shown to be dominantly inherited (Yang et al. 2004, 2008). Likewise, the resistance effective in Bo7212 is inherited by a single dominant factor, *LanrBo*, as shown in the present study. The progenitors of Bo7212 trace back to genetic resources from Russia while the two Australian cultivars have other origins, probably from Spain (Cowling and Gladstones 2000). Thus, it appears reasonable to assume that different resistance alleles, if not loci, are effective in Bo7212 as compared to cvs. 'Tanjil' and 'Mandelup'.

Molecular markers and map position of LanrBo

For developing molecular markers, which could serve in mapping *LanrBo* in the lupin genome we made use of genomic resources available in narrow-leafed lupin, namely, several dense genetic maps (Boersma et al. 2005; Kroc et al. 2014; Nelson et al. 2010; Yang et al. 2013), a draft genome sequence (Yang et al. 2013) as well as extensive transcriptome datasets (Kamphuis et al. 2014). We found, however, that molecular markers described for Australian breeding material were difficult to transfer and that generally, marker polymorphism in the *LanrBo* mapping family was low, thus obstructing the construction of a saturated linkage map for *LanrBo*. Limited polymorphism in our plant material may be traced back to the genetic bottleneck introduced to European breeding materials by the intense selection for sweetness (v. Sengbusch 1930).

Among the molecular markers we used for mapping *LanrBo* (Fig. 4a), markers *DAFWA3348*, *DAFWA7361*, and *DAFWA2850* had previously been mapped to linkage group SLG-1 by Yang et al. (2013) (Fig. 4b). Likewise, the markers *LaIND_085* and *LaIND_138* included in *LanrBo*-mapint were assigned by Kamphuis et al. (2014) to a linkage group named NLL-11 by Nelson et al. (2010) (Fig. 4b). Linkage group SLG-1 comprises the resistance locus *Lanr1* (Yang et al. 2013; Fig. 4b). To conclude, SLG-1, NLL-11, and *LanrBo*-mapint are anchored by five markers and, thus, represent the same genomic region, with resistance genes *Lanr1* and *LanrBo* falling into this region (Fig. 4).

With regard to the resistance factors *Lanr1* in SLG-1 and *LanrBo* in *LanrBo*-mapint, marker positions point to these being distinct loci. For instance, while the marker *DAFWA7361* was mapped 2.2 cM distal of *Lanr1* the same marker appears to be located 14.7 cM proximal of *LanrBo*. Another marker, *DAFWA2850*, mapped 76.2 cM distal of *Lanr1* while it maps only half the distance relative to *LanrBo*. Likewise, marker *AntjM1* was reported to map 3.5 cM apart from *Lanr1* (Yang et al. 2004; not shown in Fig. 4) while the same marker showed up 16.7 cM apart from *LanrBo* in the present study.

As shown in Fig. 4b, the orientation of anchor markers in *LanrBo*-mapint is the same as in SLG-1 and NLL-11, respectively. Likewise, marker distances are in comparable ranges.

Distances between individual anchor markers and *LanrBo* on the one hand and *Lanr1* on the other appear to be quite different, leaving open the question whether the two resistance genes are allelic or are distinct resistance genes that belong to a R gene cluster as is commonly observed

for plant R genes, particularly those undergoing diversifying selection (Michelmore and Meyers 1998). While the phenotyping for anthracnose resistance is robust, mis-phenotyping may have occurred to some extent, which could have lead to inflated marker-trait distances. For instance, while marker–marker distances were low in most cases, the closest markers flanking *LanrBo* had distances of 14.7 and 5.1 cM, respectively, relative to *LanrBo* (Fig. 4). For more conclusive data on the allelic states of the two resistance genes, progeny testing of crosses between lines homozygous for *Lanr1* and *LanrBo* will have to be performed.

Of the two flanking markers most tightly linked to LanrBo, *DAFWA7361* is inherited in a co-dominant fashion and can easily be assayed by high-resolution melt techniques. The other marker, *E49_M34-250A*, is a dominant-recessive AFLP, which possibly may be converted into an easy reproducible agarose-based marker assay by fragment isolation, sequencing and re-amplification. While each of the two markers is positioned at some distance from *LanrBo*, they allow for precise selection decisions when taken together as a single selection criterion. According to Weber and Wricke (1994) the recombination r between a target locus and two flanking markers is $r = r_1 r_2/(1 - r_1 - r_2 + 2r_1 r_2)$, where r_1 and r_2 are the recombination fractions between the target gene and either of the two flanking markers. Given $r_1 = 0.143$ for *LanrBo–DAFWA7361* and $r_2 = 0.051$ in the case of *LanrBo–E49_M34-250A*, the combined recombination r is 0.0089, which seems sufficiently low to use the marker bracket for marker-assisted selection of *LanrBo* carriers in a breeding programme.

Author contribution statement KF contributed to the experimental process, carried out greenhouse tests, molecular analysis, and preparation of plant material for RNASeq experiment. RD provided breeding lines. MNN, LGK and KBS performed screening for polymorphism of SSRs and InDels of mapping population parents via MultiPlex Ready PCR. Genotyping of SSRs and InDels was performed by KF. BR, NK and PW performed RNASeq and bioinformatics analyses. BRW designed the study, carried out greenhouse tests, field tests and supervised further experiments. KF, BRW and PW formulated the manuscript. All authors critically proof read the manuscript and provided valuable inputs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants or animal performed by any of the authors.

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