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IDENTIFICATION OF THE *Lanr1* GENE OF RESISTANCE TO ANTHRACNOSE OF NARROW-LEAFED LUPINE (*Lupinus angustifolius* L.) USING DNA-MARKERS AnSeq3 AND AnSeq4

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Abstract

Anthracnose is one of the fungal diseases of the narrow-leafed lupine (*Lupinus angustifolius* L.) caused by *Colletotrichum lupini*. Resistance to anthracnose is not absolute in character, as the plants with high resistance can be affected by the pathogen but in less extent than those non-resistant. Recent suggestions of the total number of genes involved in control of anthracnose tolerance are discrepant. Current approach in breeding anthracnose-tolerant lupine is based on combination of non-allele genes of resistance in a single genotype. Specific DNA markers are being developed which are linked to the genes of resistance and can be used for rapid and effective selection of resistant plants, but prior to their application the efficacy of DNA marker should be specifically tested with the breeding material of interest to avoid false positive responces. The AnSeq3 and AnSeq4 DAN markers flanking *Lanr1* gene at 0.9 cM distance are considered the closest to it (H. Yang et al., 2012). In this article we report the possibility of using DNA markers AnSeq3 and AnSeq4, the single nucleotide polymorphisms (SNPs), in selecting forms resistant to anthracnose among varieties of narrow-leafed lupine. A total of 50 Russian, Belarusian, Polish and Australian varieties and samples were tested to detect the allele DNA markers of susceptibility or resistance to anthracnose. DNA was individually isolated from seeds in three replicates. Polymerase chain reaction (PCR) was used to detect alleles of DNA markers linked to the *Lanr1* gene. The list of tested plants, PCR mix composition and protocol are specified. PCR enzymes and reagents of the SibEnzyme company (Russia) were used. The praimers for AnSeq3 and AnSeq4 markers were site-specific and synthesized by the Syntol company (Russia). Polyacrylamide gel electrophoresis was used for visualization of the allele markers. The Australian varieties resistant and susceptible to anthracnose were used as a control for AnSeq3 and AnSeq4 alleles. DNA fragments of 92 and 87 base pairs corresponding to the markers AnSeq3 and AnSeq4, respectively, were obtained for all 50 breeding samples included in the study. For 13 Russian and 10 Polish varieties the marker alleles of susceptibility to anthracnose were detected. For BGB-6 Belarusian sample the resistance alleles were identified by AnSeq3 and AnSeq4 markers, and for Myrtan variety only the AnSeq3-specific pattern was shown. The rest of 21 Belarusian samples possessed the alleles of susceptibility to anthracnose. Earlier by means of other DNA markers, AntjM1 and AntjM2, we showed the absence of alleles linked to *Lanr1* in currently registered varieties originated from All-Russian Research Institute of Lupine. Thus, the DNA markers AnSeq3 and AnSeq4 linked to gene *Lanr1* may be useful in breeding Russian and Belarusian anthracnose-resistant lupine varieties.

Keywords: *Lupinus angustifolius* L., anthracnose, polymerase chain reaction (PCR), DNA markers AnSeq3, AnSeq4.

Anthracnose is an infectious disease of many agricultural crops, including the blue lupine, *Lupinus angustifolius* L. This disease of lupine plants is caused by the fungus *Colletotrichum lupini* [1]. In the years of anthracnose epiphytoty development, significant reduction of yields is observed for the varieties susceptible to the pathogen. It is not recommended to use heavily affected lupine crops for seeding, grain forage and ensiling purposes [2]. It is reported that it

was the blue lupine where anthracnose was found for the first time in the USSR; it was damaged to the greatest extent as compared to the other species [3]. However, due to many years of breeding for resistance to anthracnose, some present-day varieties of *L. angustifolius* L. are tolerant to the disease. At the same time, it should be noted that this resistance is not absolute, but relative, because plant specimens with high resistance can be affected by the pathogen, but in less extent than those considered to be non-resistant.

Among researchers, yet there is no consensus about the total number of genes associated with resistance to anthracnose [4]. American varieties of the blue lupine, Rancher and Frost, and Australian varieties Marri, Illyarrie, Yandee and Danja carry dominant anthracnose resistance gene *An* [5]. Australian variety Kalya has dominant gene *Lanr2* providing medium resistance to anthracnose [6]. High anthracnose resistance of Australian varieties Wonga and Tanjil is controlled by dominant gene *Lanr1* [7]. Based on the results of hybridological analysis, the genotype of Belarusian varieties Mirtan and Pershatsvet, which exhibit resistance to anthracnose at the level of Wonga and Tanjil, contains blocks of three nonallelic dominant genes *Rcl1, Rcl2,* and *Rcl3*.

It should be emphasized that, at present, breeders from various countries carry out studies for creation of more anthracnose-resistant varieties by combining nonallelic genes of resistance to the disease in a single genotype [4]. DNA markers linked to disease-resistance genes are developed in order to rapidly and efficiently select anthracnose-resistant plants. Thus, Australian researchers have proposed DNA markers AntjM1 [7], AntjM2 [8], AnSeq3, and AnSeq4 [9] linked to gene *Lanr1*. With help of primers for the corresponding anthracnose resistance markers of the blue lupine, it is possible to determine if gene *Lanr1* is present in the specimens under study using the polymerase chain reaction (PCR) with subsequent analysis of DNA fragments.

Screening of breeding material with use of DNA markers makes it possible to identify anthracnose-resistant plants and recommend them for inclusion into the selection process. At the same time, according to the results obtained by Australian researches, DNA markers may be used in selection only after preliminary analysis of their efficiency for particular breeding specimens [10]. Earlier, in studies of anthracnose resistance controlled by gene *Lanr1*, we applied markers AntjM1 and AntjM2 for DNA analysis of 14 Russian and Belarusian varieties of the blue lupine [11]. At this point, DNA markers AnSeq3 and AnSeq4 seem to be the closest to *Lanr1*; they flank the gene at a distance of 0.9 cM [9].

The purpose of this work was to investigate a capability of using DNA markers AnSeq3 and AnSeq4 linked to gene *Lanr1* in breeding of Russian and Belarusian specimens of the blue lupine. In this regard, we have assessed the presence of anthracnose resistance gene *Lanr1* in varieties and specimens of various selection origin using the two mentioned molecular markers.

Technoque. The test material was represented by 50 Russian, Belarusian, Polish and Australian varieties and specimens of the blue lupine (*Lupinus angustifolius* L.).

DNA was individually isolated from seeds in three replicates by a previously used method [11]. PCR was carried out in a Tertsik amplifier (DNA-Technology, Russia). 10 rl reaction mixture was composed of genomic DNA (10-20 ng), forward and reverse primers (0.3 μM), Taq polymerase E 338 (0.25 U), $MgCl_2$ (1.5 mM), dNTPs (0.15 mM), B321 AS buffer (1 μl). Enzymes and reagents of SibEnzyme (Russia) were used. Amplification conditions were es follows: initial denaturation for 1 min at 95 °C; then 30 cycles including denaturation for 30 s at 95 °C, annealing for 20 s at 51 °C for primers in case of marker AnSeq3 and at 53 °C for primers in case of AnSeq4, elongation for 10 s at 72 °C; final elongation for 2 min at 72 °C. The primers used for markers An-

Seq3 and AnSeq4 are site-specific [9] and have been synthesized by Sintol (Russia). DNA fragments were separated in 11-13 % polyacrylamide gels using a VE-20 vertical electrophoresis chamber (Helicon, Russia). PCR products were separated within 5-6 h at 270-300 V. The gel was placed into ethidium bromide solution (0.5 mg/l) for 20-30 min. After staining, it was visualized using a Gel-DocXR gel scanner (Bio-Rad, USA). The control for marker allele length determination comprised PCR products with DNA of the plants representing Australian varieties Tanjil, Wonga, Kalya.

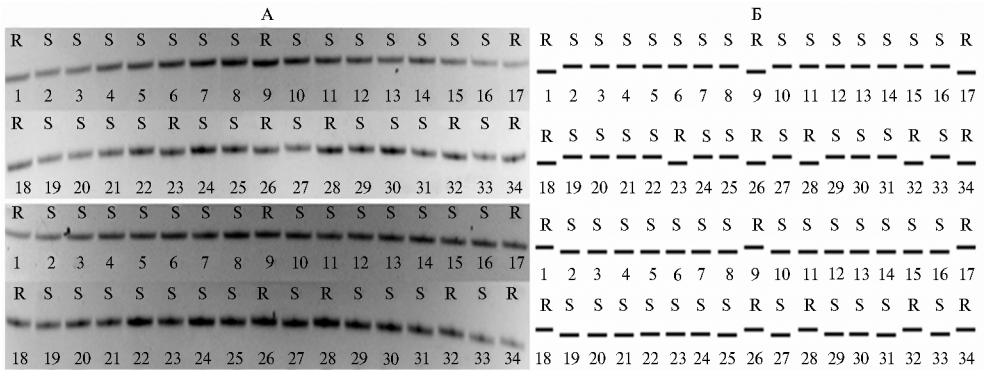
Results. We have investigated 50 blue lupine specimens of various origin (see table).

Blue lupine specimens included in the study for identifying the alleles of DNA markers AnSeq3 and AnSeq4 of anthracnose resistance gene *Lanr1*

Provided by Russia	Provided by Belarus
Russian varieties and specimens (total 13): Raduzhny, Siderat 38, Kristall, Snezhet, Belozerny 110, Smena, Nadezhda, Bryansky 123, Vityaz, Uzkolistny 53-02, Vektor, Belozerny 121, SN 78-07	Belarusian varieties and specimens (total 23): Privabny, Pershatsvet, Mitan, Gelena, Mirtan, Khvalko, Yan, BGB-6, Gerkules, Zhodinsky, Ranny, BGB-3, Mirtan 312, MK-VDS-Beis, PEL-MRL, Ramonok, 4B-129, BGB-1, BGB-2, BGB-4, Vada 40, Vasilek, Divny
Polish varieties and specimens (total 10): Baron, Boruta, Boyar, Graf, Zevs, Kalif, Neptun, Regent, Tsezar, Elf	
Australian varieties and specimens (total 4): Tanjil, Wonga, Kalya, W2248	

N o t e: The Russian and Belarusian breeding material was provided by All-Russian Research Institute of Lupine (Bryansk) and Scientific and Practical Center for Agriculture of NAS of Belarus (Zhodino), respectively.

DNA fragments of 92 and 87 base pairs corresponding to markers AnSeq3 and AnSeq4, respectively, were obtained for all 50 breeding specimens included in the study. The alleles of susceptibility (S) and resistance (R) to anthracnose have been determined in the course of comparison of the marker alleles identified for Australian control varieties and other breeding specimens. Alleles of AnSeq3 and AnSeq4 are single nucleotide polymorphisms (SNP) with the same length for each marker. In electrophoretic separation in polyacrylamide gel, the fragments corresponding to the marker alleles of resistance for AnSeq3, due to presence of one C→T base substitution, were positioned below the amplicons of the alleles of susceptibility, whereas inverted distribution was observed for AnSeq4 due to a single G→A substitution in the base sequence [7]. Thus, in case of varieties Tanjil and Wonga (positive controls of resistance for gene *Lanr1*), DNA fragments for the alleles of AnSeq3 and AnSeq4 located, respectively, below and above the amplified ones for variety Kalya (negative control of resistance for gene *Lanr1*). The PCR products obtained for DNA of each of the blue lupine variety specimens included in the study were reproduced in all three replicates. The marker alleles of AnSeq3 and AnSeq4 for different Russian, Belarusian, Polish and Australian varieties and specimens are shown in the figure below.



Results of electrophoretic separation of PCR products obtained for alleles of DNA markers AnSeq3

(1-34, top) **and AnSeq4** (1-34, bottom) **of susceptibility and resistance to anthracnose in the studied varieties and breeding specimens of various origin:** A — photo documentation, B — schematic representation; 1, 17, 18, 34 — Tanjil, 2 — Raduzhny, 3 — Siderat 38, 4 — Kristall, 5 — Snezhet, 6 — Belozerny 110, 7 — Smena, 8 — Nadezhda, 9, 26 — Wonga, 10, 27 — Kalya, 11 — Bryansky 123, 12 — Vityaz, 13 — Uzkolistny 53-02, 14 — Vektor, 15 — Belozerny 121, 16 — SN 78-07, 19 — Privabny, 20 — Pershatsvet, 21 — Mitan, 22 — Gelena, 23 — Mirtan, 24 — Khvalko, 25 — Yan, 28 — BGB-6, 29 — Gerkules, 30 — Zhodinsky, 31 — Ranny, 32 — W 2248, 33 — BGB-3; S and R — marker alleles of susceptibility and resistance, respectively, linked to gene *Lanr1* (12 % polyacrylamide gel; please refer to the body of the article for the description of the varieties and breeding specimens).

For the Russian specimens, the PCR analysis has not resulted in identification of R-alleles of DNA markers AnSeq3 and AnSeq4 linked to gene *Lanr1* which controls the anthracnose resistance of Australian varieties Wonga and Tanjil. Earlier, using other DNA markers, in particular, AntjM1 and AntjM2 [11], we also obtained the information that the varieties of All-Russian Research Institute of Lupine which are currently included in the State Register of Selection Achievements of the Russian Federation do not contain resistant marker R-alleles linked to gene *Lanr1*. It is known that the genetic material that provided a basis for anthracnose resistance of Australian varieties Wonga and Tanjil was not used in breeding of the Russian varieties studied herein, which was confirmed by the results of the molecular genetic analysis of gene *Lanr1* DNA marker alleles. As with the Russian specimens, the Polish varieties were matched by anthracnose susceptibility marker alleles for gene *Lanr1*. With regard to phenotypic manifestations for the Polish varieties included in this study, there was no pronounced anthracnose resistance similar to that of Australian varieties Tanjil and Wonga, which is consistent with the data on markers AnSeq3 and AnSeq4.

Anthracnose susceptibility alleles were characteristic for most of the Belarusian specimens. Resistance alleles for two markers, AnSeq3 and AnSeq4, were only found in component BGB-6 of the blue lupine biological gene bank. This breeding specimen is closely related to varieties Wonga and Tanjil and also demonstrates high anthracnose resistance in field and laboratory trials [4].

For variety Mirtan, resistance marker alleles were only identified for AnSeq3. In a previous study of this variety with markers AntjM1 and AntjM2, we found susceptible alleles, and primers for marker AntjM2 allowed us to amplify the allele that was not identified for the other specimens, which characterizes the distinctiveness of the mentioned variety [11]. The variation of the anthracnose resistance/susceptibility alleles for markers An-Seq3 and AnSeq4 can be attributable to differences in the genetic make-up of Belarusian variety Mirtan and Australian variety Tanjil. In spite of the fact that Mirtan demonstrates relatively high anthracnose resistance, it was bred without the use of the Australian material resistant to the disease. Thus, it seems possible that, in case of variety Mirtan, marker AnSeq3 gives «false-positive» results as compared to marker AnSeq4, and the resistance allele identified is not associated with gene *Lanr1*.

In addition to variety Mirtan and specimen BGB-6, relatively high anthracnose resistance has been observed for variety Pershatsvet due to the presence of the block of several nonallelic genes *Rcl* in plant genotypes. It seems that the anthracnose resistance genes specific for this variety are not allelic to *Lanr1*, which is consistent with the results of these studies where anthracnose resistance for marker alleles linked to gene *Lanr1* was not observed for variety Pershatsvet. It should be noted that susceptibility alleles have been identified in the other Belarusian varieties of the blue lupine, which were not closely related to Australian varieties Wonga and Tanjil. R-alleles of markers AnSeq3 and AnSeq4 have also been found in Australian breeding line Walan 2248. This fact reflects the results of the anthracnose resistance breeding program implemented in Australia as part of creation of blue lupine varieties.

In general, it turned out that DNA markers AnSeq3 and AnSeq4 allows quite efficient identification of anthracnose resistant and susceptible genotypes by gene *Lanr1*. Thus, for 50 varieties and breeding specimens of the blue lupine, discrepant information about the alleles of the studied markers has been obtained only in case of variety Mirtan. These results have confirmed the need for preliminary analysis of DNA marker applicability in large-scale crossings because the use of «false-positive» marker alleles may lead to errors in selection of anthracnose resistant plants.

The stably high resistance of varieties Tanjil and Wonga carrying gene *Lanr1* has been confirmed in various test conditions [4, 5, 12]. Therefore, inclusion of gene *Lanr1* in the genotypes of new varieties obtained after crossing of Belarusian or Russian breeding material and anthracnose-resistant Australian specimens can improve total resistance to this disease. Appropriate DNA markers can be applied for individual, high-precision and rapid selection of the genotypes containing gene *Lanr1*.

So, the study of blue lupine (*Lupinus angustifolius* L.) specimens of various origin has not revealed anthracnose resistance alleles for markers AnSeq3 and AnSeq4 linked to gene *Lanr1* in case of Russian and Polish varieties. For Belarusian breeding specimen BGB-6, resistance alleles have been identified for both markers AnSeq3 and AnSeq4 studied, for variety Mirtan they were found only for marker AnSeq3. The controls for corresponding alleles of AnSeq3 and AnSeq4 were represented by the Australian breeding material resistant and susceptible to anthracnose. The undertaken studies have demonstrated that the use of DNA markers AnSeq3 and AnSeq4 linked to gene *Lanr1* may be useful in breeding of the Russian and Belarusian varieties carrying anthracnose resistance gene *Lanr1*. At the same time, in case of large-scale crossings, it is necessary to preliminarily assess if the selected DNA markers are applicable or not because the use of «false-positive» marker alleles may lead to errors in selection of anthracnose resistant plants.

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