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# Genetische und molekulare Charakterisierung züchtungsrelevanter Merkmale der Blauen Süßlupine (*Lupinus angustifolius* L.)

Dissertationen aus dem Julius Kühn-Institut

Julius Kühn-Institut
Bundesforschungsinstitut für Kulturpflanzen

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Institut für Pflanzenbau und Pflanzenzüchtung I

- Professur für Pflanzenzüchtung -

# Genetische und molekulare Charakterisierung züchtungsrelevanter Merkmale der Blauen Süßlupine (*Lupinus angustifolius* L.)

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I

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Kristin Fischer, Regine Dieterich, Matthew N. Nelson, Lars G. Kamphuis, Karam B. Singh, Björn Rotter, Nicolas Krezdorn, Peter Winter, Peter Wehling, Brigitte Ruge-Wehling

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

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II

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Kristin Fischer, Eicke Rudloff, Steffen R. Roux, Regine Dieterich, Peter Wehling, Wolfgang Friedt, Brigitte Ruge-Wehling

Generating genetic variation in plant architecture for breeding of narrow-leafed lupin (*Lupinus angustifolius* L.) by EMS mutagenesis

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# 1 Einleitung

## 1.1 Die Süßlupine – eine fast vergessene Proteinressource

Es sind über 300 Lupinenarten (*Lupinus* sp.) in der Familie der *Fabaceae* beschrieben, die aufgrund ihrer geographischen Herkunfts- und Verbreitungsgebiete grob in Altwelt- und Neuweltlupinen unterschieden werden. Heutzutage landwirtschaftlich genutzte Altweltlupinen sind die Blaue Lupine (*L. angustifolius*), Gelbe Lupine (*L. luteus*), Weiße Lupine (*L. albus*) *sowie L. cosentinii*, deren Ursprung im mediterranen Europa liegt. Herkunft der Neuweltlupinen ist Südamerika, wobei allein *L. mutabilis*, die Andenlupine, zur landwirtschaftlichen Kulturart domestiziert worden ist.

Die ersten Versuche, Lupinen als Nutzpflanzen in Deutschland zu etablieren, gehen auf die Initiative von Friedrich dem Großen im 18. Jahrhundert zurück (Fischer und v. Sengbusch 1935). Bereits zu dieser Zeit wollte man Lupinen, die aufgrund einer Symbiose mit Knöllchenbakterien an den Wurzeln in der Lage sind, Stickstoff aus der Luft zu fixieren, aus ökonomischen Gründen als natürliche Stickstofflieferanten und zur Verbesserung der Bodenfruchtbarkeit einsetzen. Die Bemühungen schlugen damals jedoch fehl, da die aus Italien eingeführten Formen der Weißen Lupine aufgrund des gemäßigten Klimas in Deutschland und der dadurch verzögerten Reife für die Körnernutzung nicht geeignet waren (Hondelmann 1996).

Mitte des 19. Jahrhunderts gelang es dem Landwirt Joachim Borchardt, Gelbe Lupinen für die Gründüngung anzubauen und auch erfolgreich zu vermehren (Hondelmann 1984). Gleichzeitig erfreute sich auch die Blaue Lupine mit einer Anbaufläche von rund 400.000 ha (Hondelmann 1984) immer größerer Beliebtheit in Deutschland. Die Nachfrage nach Lupinen als Pflanze zur Gründüngung ging jedoch zu Beginn des 20. Jahrhunderts mit der Entwicklung des Haber-Bosch-Verfahrens und der daraufhin einsetzenden industriellen Produktion von kostengünstigem, mineralischem Stickstoffdünger erneut drastisch zurück.

Die Erschließung alternativer Verwendungen der Lupine, wie z.B. in der Tierfutter- und Lebensmittelindustrie scheiterte zunächst an dem hohen Alkaloidgehalt in den Lupinen-Samen. Vorwiegend enthält der Samen Alkaloide, die strukturell von dem

Amin Chinolizidin abgeleitet werden (Wink 1985, Lee *et al*. 2007, Frick *et al*. 2017) und einen Gehalt von ca. 0,25 %–2,5 % aufweisen.

Erst die Selektion von alkaloidarmen Samen (Gehalt bis 0,1 %) bei der Blauen und Gelben Lupine durch Reinhold von Sengbusch in den Jahren 1927–1931 eröffnete die Nutzung dieser Körnerleguminosen als Eiweißpflanzen (v. Sengbusch 1931). Aufgrund der daraufhin einsetzenden Züchtung Alkaloid-armer Lupinensorten stieg die Anbaufläche in Deutschland erneut an.

Ein massives Auftreten der Brennfleckenkrankheit, auch genannt Anthraknose, verursacht durch den Pilz *Colletotrichum lupini*, führte in den 1990er Jahren indes zu einem kompletten Zusammenbruch des Anbaus der Weißen Lupine. Infolgedessen ging der Anbau aller Lupinenarten bis auf weniger als 1.000 ha im Jahr 1992 in Deutschland (Schätzung der FAOSTAT 2017) zurück, woraufhin auch die Züchtungsaktivitäten stark eingeschränkt wurden.

Aufgrund ihrer im Vergleich zu Weißer und Gelber Lupine höheren Toleranz gegenüber der Brennfleckenkrankheit, verursacht durch *Colletotrichum lupini* (Römer 1998), wird im Nordosten von Deutschland seit Beginn des 21. Jahrhunderts bevorzugt die Blaue Süßlupine mit wieder steigender Anbaufläche genutzt. Mit einem Proteingehalt der Samen von 30–35 % und Kornerträgen von 20–35 dt/ha (Schachler *et al*. 2016) ermöglicht diese Lupinenart den landwirtschaftlichen Betrieben Proteinflächenerträge von 6–12 dt/ha. Die ausgewogene Aminosäurezusammensetzung der Blauen Lupine begründet die Anbauwürdigkeit und ermöglicht eine breite Eignung im Futter- und Lebensmittelbereich. Die Blaue Lupine ist gut angepasst an das feuchte Kontinentalklima und diluviale Standorte, insbesondere an die Löß- und Verwitterungsstandorte Nord-Ostdeutschlands, die sich durch hohe Sand- und Schluffanteile und einen niedrigen pH-Wert auszeichnen.

Die Anbaufläche der Blauen Lupine in Deutschland lag im Jahr 2017 bei 28.900 ha (DESTATIS 2017). Global betrachtet kommt der Anbaufläche von Lupinen als Proteinquelle eine größere Bedeutung zu als in Deutschland. Die Region mit der derzeit größten Anbaufläche liegt in Australien, im Bundesstaat *Western Australia*. Hier wurden in 2016 ca. 485.000 ha der Blauen Lupine und

35.000 ha der Weißen Lupine angebaut (mündl. Mitteilung Jon Clements, DAFWA, Perth). Weltweit betrug die Lupinenanbaufläche in 2014 rund 622.400 ha, davon 62 % in Australien, 32 % in Europa und der Rest in Amerika und Afrika, während in Asien praktisch keine Lupinen angebaut wurden (FAOSTAT 2017).

Infolge geänderter Verbraucheransprüche bezüglich der Nutztierhaltung und -fütterung wird hierzulande angestrebt, den Einsatz von importiertem und zum Teil gentechnisch verändertem Sojaextraktionsschrot zu reduzieren. Beispielsweise kann die Proteinversorgung von Milchkühen erfolgreich über die Zufütterung hofeigener Lupinen in der Ration gedeckt werden (Roth-Maier *et al*. 2004). Vorteilhaft sind dabei der geringe Stärkegehalt sowie stickstofffreie Extraktstoffe und Galactane, die die Verdaulichkeit im Pansen fördern und damit gesundheitlichen Beeinträchtigungen des Verdauungstrakts bei Milchtieren vorbeugen (Pries *et al*. 2005). Auch in der Aquakultur und Fischfütterung ist der Einsatz von Lupinenmehl als Ersatz für das immer kostenintensiver werdende Fischmehl eine nachhaltige Alternative. Fütterungsversuche der Forschungsgruppe um Slater *et al* am Alfred-Wegener-Institut in Bremerhaven zeigen sowohl eine gute Akzeptanz des Lupinenmehls bei den Versuchstieren als auch eine gute Futterverwertung (Slater *et al*. 2016).

Zudem steigt auch die Nachfrage nach veganen Lebensmitteln mit wertvollem und hohem Proteinanteil. Eine Arbeitsgruppe des Fraunhofer Instituts für Verfahrenstechnik und Verpackung in Freising, entwickelte ein Verfahren, mit dem das Protein der Lupine hochrein isoliert und als biofunktionelle Lebensmittelzutat verwendet werden kann (Wäsche *et al*. 2001). Im Rahmen eines vom Bundesministerium für Bildung und Forschung geförderten Drittmittelprojekts (PlantsProFood, 2011–2014) konnte dieses Verfahren hinsichtlich unterschiedlicher Einsatzmöglichkeiten des Proteins optimiert werden (Sussmann *et al*. 2013). Dieses etablierte Verfahren ermöglicht derzeit die Isolation von ausreichend mit sensorisch positiven Eigenschaften ausgestatteten Proteins aus dem Lupinenkorn, um damit Milchersatzprodukte herzustellen. Diese auf pflanzlichem Protein basierenden Lebensmittel stellen eine Alternative zum tierischen Eiweiß für die Humanernährung dar und sind gerade auch unter Gesundheitsaspekten – zum Beispiel aufgrund des positiven Einflusses auf chronische Volkskrankheiten, wie Hypercholesterinämie,

Hypertensie oder Hyperglykämie – wertvoll (Arnoldi *et al*. 2015; Villarino *et al*. 2016; Lima-Cabello *et al*. 2017).

Neben den genannten positiven Eigenschaften als Rohstoff erbringt die Lupine im praktischen Anbau umfassende Ökosystemleistungen die sie zu einem wertvollen Fruchtfolgeglied machen, wie z.B. Stickstofffixierung, Verbesserung der Bodenfruchtbarkeit, hoher Vorfruchtwert, Regionalität der Erzeugung sowie Erhöhung der Agrobiodiversität.

Aufgrund eines vergleichsweise niedrigen Ertragsniveaus, Einschränkungen in der Ertragsstabilität, vermehrten Auftretens von Krankheiten (u.a. Anthraknose, *Botrytis*-Befall) und Schädlingen (u.a. Blattrandkäfer, Blattläuse als Virusvektoren) und zum Teil fehlender oder mangelhafter Anbautechnik stellt die Lupine jedoch eine besondere Herausforderung für den praktischen Anbau und damit auch für die Züchtung dar.

## 1.2 Domestikation der Blauen Süßlupine

Mit der Selektion alkaloidarmer Samen und der damit verbundenen Möglichkeit ihrer Nutzung im Futter- und Lebensmittelbereich wurde die Basis für die Züchtung von Süßlupinen geschaffen. Voraussetzung dafür war die Entwicklung spezieller Nachweismethoden, die erst eine gezielte Selektion von süßen Linien bis hin zur Sorte ermöglichte (v. Sengbusch und Zimmermann 1937). Neben der Züchtung Alkaloid-armer Sorten lag ein weiterer Forschungsschwerpunkt auf der Verbesserung agronomischer Merkmale der Lupinen als Voraussetzung der Anbauwürdigkeit.

Zusätzliche Zuchtziele waren Platzfestigkeit, Frühzeitigkeit und Standfestigkeit sowie das Merkmal Weichschaligkeit. Krankheiten spielten anfangs keine wesentliche Rolle im Lupinen-Anbau. Dies änderte sich jedoch in den 1990er Jahren entscheidend mit dem Auftreten der Brennfleckenkrankheit (siehe Kapitel 1.3.1). Als eine essenzielle Voraussetzung für eine erfolgreiche Selektion auf züchterisch relevante Merkmale wurde die genetische Analyse der Merkmalsausprägung als Basis der Züchtung zunehmend als wichtig erkannt. Auf der Basis so aufgeklärter Vererbungsmodi kam es

schließlich zur Entwicklung von molekularen Selektionsmarkern, die verstärkt in modernen Zuchtprogrammen eine Rolle spielen (vgl. Han *et al*. 1996, Gupta *et al*. 1999).

Im Folgenden ist der Kenntnisstand für die wichtigsten, züchterisch relevanten Merkmale der Süßlupine zusammengefasst.

Für die Alkaloidarmut sind bis heute drei Gene bekannt: *iucundus*, *esculentus* (Hackbarth und v. Sengbusch 1934) und *depressus* (Schwarze and Hackbarth 1957), sowie eine induzierte Mutation in einem vierten Gen, *tantulus* (Zachow 1967). Alle vier Gene werden monogen-rezessiv vererbt. Für das Gen *iucundus* stehen bereits Selektionsmarker zur Verfügung (Li *et al*. 2011) und werden in einem am Julius Kühn-Institut aktuell bearbeiteten Projekt zur Selektion Alkaloid-haltiger bzw. Alkaloid-armer Kreuzungspartner eingesetzt (unveröffentlicht).

Das ebenfalls rezessiv vererbte Gen *mollis* verleiht den Samen der Lupinen ihre Weichschaligkeit (v. Sengbusch 1938), wodurch die Wasserdurchlässigkeit verbessert und das Keimverhalten positiv beeinflusst wird. Der Wildtyp, d.h. Hartschaligkeit, bedingt Dormanz, welche die Keimfähigkeit stark verzögert (Boersma *et al*. 2007).

Eine verlustarme Ernte der reifen Lupinensamen setzt voraus, dass die Hülsen nicht vorzeitig platzen; Platzfestigkeit ist somit eine wichtige Komponente der Ertragssicherheit. Sie wird über die rezessiven Gene *lentus* und *tardus* (v. Sengbusch & Zimmermann 1937; v. Sengbusch 1938) reguliert, beide Gene sind für sich selbst wirksam, verursachen aber gemeinsam einen additiven Effekt und somit eine bessere Platzfestigkeit (Gladstones 1967). Molekulare Selektionsmarker (Boersma *et al*. 2009; Li *et al*. 2010; Li *et al*. 2012) erlauben eine Marker-gestützte Selektion auf betreffende Genvarianten im Züchtungsprozess.

Gladstones & Hill (1969) berichteten erstmals von einem dominanten Gen (*Ku*), welches das Vernalisationsbedürfnis der Pflanzen aufhebt. Die hierdurch bedingte, frühzeitige Blüte erlaubt eine bessere Eingliederung der Lupine in die Fruchtfolge. Auch dieses Merkmal kann über molekulare Selektionsmarker im Züchtungsprozess verfolgt werden (Brien *et al*. 2000, Nelson et al. 2017).

Die oben beschriebenen züchterischen Erfolge betreffen einfach vererbte Merkmale, die zu den typischen Domestikationsmerkmalen der Lupinen zählen. Die weitere züchterische Bearbeitung ist vor allem auch auf quantitative Eigenschaften wie Kornertrag und Ertragsstabilität ausgerichtet; solche Merkmale sind in der Regel polygenisch vererbt. Die Selektion ertragsrelevanter Genotypen setzt eine hinreichend breite genetische Basis im Zuchtmaterial voraus. Süßlupinen gelten jedoch als ein Musterbeispiel für eine extreme Einengung der genetischen Variabilität von Zuchtmaterial als Ergebnis der Selektion auf essenzielle Nutzmerkmale (hier Alkaloidarmut). Aktuelles Zuchtmaterial der Blauen Süßlupine geht im Wesentlichen auf nur drei durch v. Sengbusch selektierte Alkaloid-arme Samen zurück. Mit der damit einhergehenden deutlichen Unterschreitung einer kritischen Populationsgröße war ein ausgeprägter genetischer Flaschenhals ('*genetic bottle neck*') verbunden, der bis heute die weitere züchterische Verbesserung von Ertragseigenschaften der Blauen Süßlupine erschwert.

## 1.3 Aktuelle Züchtungsfortschritte bei der Blauen Süßlupine

Neue genetische Variabilität kann auf verschiedenen Wegen erzielt werden, die zu züchterisch unterschiedlich nutzbaren Ergebnissen führen und insofern im Kontext mit den jeweiligen Zuchtzielen zu bewerten sind.

Soll der genetisch eingeengte Hintergrund von Zuchtmaterial mit neuer genetischer Variabilität angereichert werden, ist die Einkreuzung genetisch weiter entfernter pflanzengenetischer Ressourcen erforderlich, wie es beispielsweise bei der Selektion auf Anthraknose-Resistenz erfolgreich durchgeführt wurde.

Liegt hingegen der Schwerpunkt auf der Selektion einfach vererbter, rezessiver Defektmutanten, wie etwa Alkaloidarmut oder spezieller Wuchstyp vor einem züchterisch bereits adaptierten genetischen Hintergrund, erscheint nach wie vor die Mutagenese als Methode der Wahl.

### 1.3.1 Erhöhung der Anthraknose-Resistenz

Anthraknose, hervorgerufen durch das Pilzpathogen *Colletotrichum lupini* (Nirenberg *et al*. 2002, Sweetingham und Thomas 2004), ist nach wie vor weltweit

die bedeutendste Krankheit im Lupinenanbau (Talhinas *et al*. 2016). Erstmalig wurde von der samenübertragbaren Krankheit, charakterisiert durch seine typischen Symptome wie das Eindrehen und Abknicken der Stängel sowie durch auffällig orangefarbene Konidienlager (Feiler und Nirenberg 2004), im Jahre 1939 in den USA berichtet (Weimer 1943). Zu Beginn der 1990er Jahre traten erste Fälle in Europa (Gondran *et al*. 1994, Frencel 1998) sowie in Australien (Sweetingham *et al*. 1995) auf. Der Anthraknosebefall, führte dabei zu partiellen bis vollständigen Ernteausfällen.

Aufgrund der großen wirtschaftlichen Bedeutung dieser Krankheit erfolgt seitdem sowohl in der Züchtungsforschung als auch in der praktischen Züchtung eine Prüfung und Selektion anhand sonst bislang nicht weiter charakterisierter genetischer Ressourcen auf Anthraknose-Resistenz.

Durch die australische Arbeitsgruppe um Yang *et al*. wurde das Resistenzgen *Lanr1* (Yang *et al*. 2004) in der Sorte 'Tanjil' identifiziert. Der Resistenzlocus *Lanr1* ist auf der Kopplungsgruppe NLL-11 lokalisiert (Nelson *et al*. 2010; Yang *et al*. 2012, Kamphuis *et al*. 2015). Mit Hilfe der veröffentlichten Sequenzdaten des Lupinengenoms konnte der Locus durch zwei flankierende Marker begrenzt werden. Dieser Bereich enthält fünf kosegregierende Marker sowie 41 vorhergesagte Gene. Unter diesen befindet sich ein Gen, welches der NLR-Genfamilie zugeordnet werden kann und somit ein potentieller Kandidat für das Resistenzgen ist (Hane *et al*. 2017). Die kosegregierenden Selektionsmarker können in aktuellen Züchtungsprogrammen vermehrt zur Selektion von Anthraknose-resistenten Stämmen eingesetzt werden.

Ein weiteres Gen, welches Resistenz gegen *C*. *lupini* bedingt, wurde in dem Zuchtstamm Bo7212 gefunden. Die Charakterisierung dieses Zuchtstammes erfolgte in Resistenztests am Julius Kühn-Institut in Groß Lüsewitz im Gewächshaus und im Freiland (Ruge-Wehling *et al*. 2009). In den Freilandtests konnte die im Gewächshaus ermittelte Symptomfreiheit des Zuchtstammes Bo7212, hervorgegangen aus einen russischen Akzession, über mehrere Jahre bestätigt werden. Darüber hinaus konnte mit Hilfe von genetischen Analysen nachgewiesen werden, dass der Resistenzlocus, bezeichnet als *LanrBo*, monogen-dominant wirkt (Fischer *et al*., 2015, siehe Kapitel 2).

Mit Hilfe der zur Verfügung stehenden genetischen Karten und Ankermarker konnte der Locus *LanrBo* auf Kopplungsgruppe NLL-11 von *L. angustifolius* lokalisiert werden. Weitere SSR-Marker aus dem Modellgenom *Lotus japonicus*, AFLP-Marker (Vos *et al*. 1995) sowie Transkriptom-basierte SNP-Marker, wiesen eine Kopplung mit dem Resistenzlocus auf. In der Nähe von *LanrBo* sind zwei flankierende Marker identifiziert worden, die den Locus umschließen und für Züchtungszwecke als Selektionswerkzeug eingesetzt werden können (Fischer *et al*. 2015, siehe Kapitel 2).

### 1.3.2 Identifikation ertragreicher Wuchstypen

Ethylmethansulfonat (EMS) ist ein chemisches Mutagen, welches bereits seit Jahrzehnten angewendet wird, um in unterschiedlichen Kulturpflanzenarten genetische Variabilität für die züchterische Nutzung zu generieren (Nichterlein *et al*. 1989, Büchsenschütz-Nothdurft *et al*. 1998, Kodym and Afza 2003; Sikora *et al*. 2011; Serrat *et al*. 2014). Bei der Sojabohne sind beispielsweise mutagene Linien mit erhöhter Nodulierungsfähigkeit selektiert worden, die eine deutlich erhöhte Kapazität zur Stickstofffixierung aufweisen als die ursprüngliche Linie (Carroll *et al*. 1986). Ein weiteres Beispiel ist die Entwicklung einer früh blühenden Sommerraps-Mutante (Thurling and Depittayanan 1992), deren Frühreife die Eingliederung in die Fruchtfolge erleichtert und einen verbesserten Ertrag ermöglicht.

In der vorliegenden Arbeit sind Ergebnisse eines Experiments dargestellt, in dem zur Erweiterung der genetischen Variabilität 40.000 Samen der endständigen Blaue- Lupine Sorte 'Boruta' mit EMS mutagen behandelt wurden (Rudloff 2008).

M2-Pflanzen mit abweichender Pflanzenarchitektur im Feldanbau wurden vermehrt und die daraus resultierenden M3-Linien im Feld geprüft (Rudloff 2011). Dabei wurden primär solche Wuchstypen berücksichtigt, die im Vergleich zu der Ausgangssorte einen abweichenden Verzweigungstyp zeigten und so eine Verbesserung des Ertragspotentials erwarten ließen. Ein anschließender Ertragskomponenten-Feldversuch führte zur Identifikation von Linien mit signifikanten Verbesserungen in einzelnen Ertragskomponenten im Vergleich zur Ausgangssorte Boruta (Fischer *et al*. 2017, siehe Kapitel 2).

Zur Untersuchung der Frage, ob die erzeugten neuen Phänotypen tatsächlich auf eine rezessive Mutation zurückgehen, wurden genetische Analysen durchgeführt. Die dafür analysierten F2-Populationen gehen auf Rückkreuzungen zwischen Pflanzen des neuen Wuchstyps und der Ausgangssorte Boruta zurück. Die in der Arbeit gezeigten Spaltungsverhältnisse belegen eindeutig die zu erwartende monogen-rezessive Vererbung (Fischer *et al*. 2017, siehe Kapitel 2). Der einfache Vererbungsmodus ermöglicht es, diese Wuchstypen in zukünftigen Züchtungsprogrammen effektiv einzusetzen.

# 2 Kumulativer Teil der Dissertation

I

Kristin Fischer, Regine Dieterich, Matthew N. Nelson, Lars G. Kamphuis, Karam B. Singh, Björn Rotter, Nicolas Krezdorn, Peter Winter, Peter Wehling, Brigitte Ruge-Wehling

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

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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

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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** $\chi^2$ 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 | $\chi^2$ (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

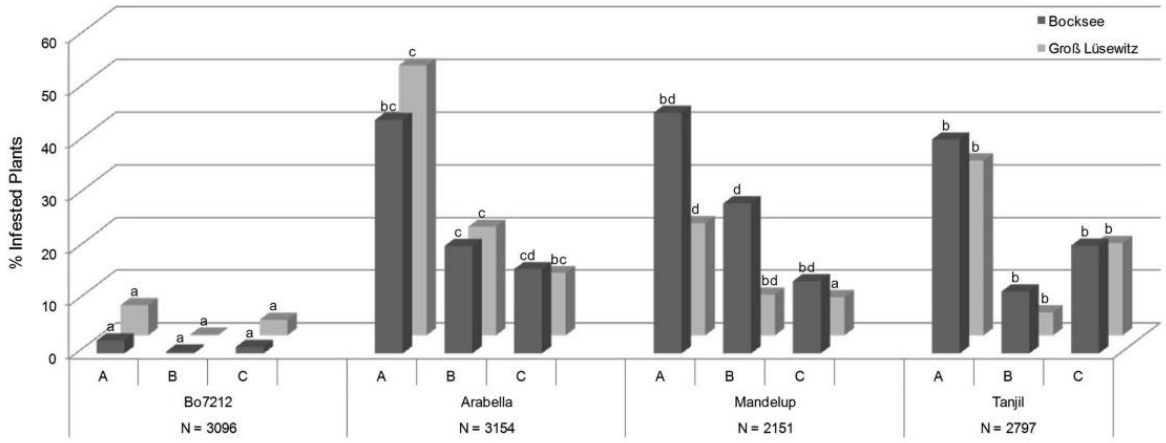


**Fig. 3** Infestation of three *L. angustifolius* cultivars and breeding line Bo7212 under field conditions in northeast Germany. Percentage of *A* of infested plants, *B* infested plants without pod formation, *C* plants with symptoms restricted to pods. Mean percentages per test location over 3 years 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

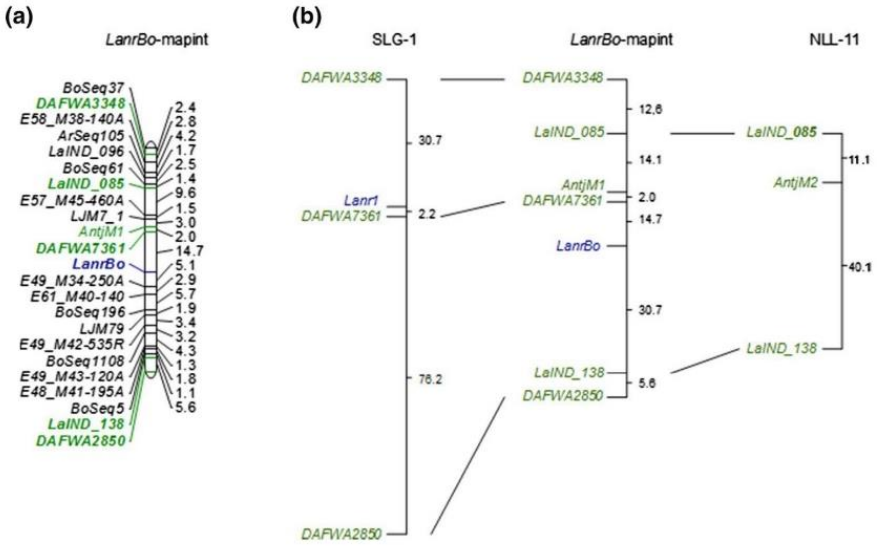


**Fig. 4** **a** Integrated genetic map (*LanrBo*-mapint) of resistance locus *LanrBo*. Markers common in *LanrBo*-mapint and any of the two other maps in Fig. 4b are shown in *bold*. **b** Schematic depiction of anchor-marker positions and distances in *LanrBo*-mapint relative to those reported for 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 $r = r_1 r_2 / (1 - r_1 - r_2 + 2 r_1 r_2)$, 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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II

Kristin Fischer, Eicke Rudloff, Steffen R. Roux, Regine Dieterich, Peter Wehling, Wolfgang Friedt, Brigitte Ruge-Wehling

## Generating genetic variation in plant architecture for breeding of narrow-leafed lupin (*Lupinus angustifolius* L.) by EMS mutagenesis

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ORIGINAL ARTICLE

WILEY Plant Breeding

# Generating genetic variation in narrow-leafed lupin (*Lupinus angustifolius* L.) for plant architecture by ethyl methanesulfonate mutagenesis

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## Abstract

Narrow-leafed lupin (NLL) is a source of protein and fibre with exceptional functional properties useful for a variety of purposes. In adapted NLL germplasm selected for low alkaloid contents, the genetic base is narrow, thus limiting fast breeding progress. In an EMS-based mutagenesis programme, we have treated 40,000 seeds of cv. 'Boruta' and obtained a variety of phenotypic mutant lines expressing novel growth types with altered plant height and/or branching. In 3-year field trials at two locations, three of these lines exhibited significantly improved grain yield components compared to the cv. 'Boruta'. Growth types of these promising lines were shown to be inherited in a monogenic recessive manner and should, thus, be easily fixable by breeding.

KEYWORDS
ethyl methanesulfonate mutagenesis, genetic analysis, genetic variability, *Lupinus angustifolius*, narrow-leafed lupin, yield components

## 1 | INTRODUCTION

Narrow-leafed lupin (*Lupinus angustifolius* L., NLL), a member of the *Fabaceae* family, is used as a grain legume crop mainly for feeding purposes. Recently, the food sector has taken an increased interest in NLL owing to the health benefits of its grain ingredients in human nutrition (Arnoldi, Boschin, Zanoni, & Lammi, 2015; Lucas et al., 2015; Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2016).

Further, the NLL has a number of agro-ecological benefits (Foyer et al., 2016). In contrast to many other crops, the NLL is reasonably yielding even on sandy soils with a low pH value. It also improves the soil structure by mobilizing soil-bound phosphorus and fixing atmospheric nitrogen, thus providing nutrients to the succeeding crop(s). Despite its value in crop rotations, NLL so far is an underutilized crop due to its low grain yield stability. To improve yield stability and yield potential of NLL, intense breeding efforts are required. However, genetic variability as a precondition for this purpose is restricted in recent NLL breeding material due to preceding intensive selection for low alkaloid content after the initial report of von Sengbusch (1930).

As a result, there is a need for broadening the genetic variation in NLL as a prerequisite for a sustainable breeding progress (Allard & Hansche, 1964; Berger et al., 2013; Duc et al., 2014). The main avenue to accomplish this is prebreeding for introducing exotic accessions into existing breeders' germplasms. A different, more limited option is mutagenic treatment of adapted breeding lines or elite cultivars in order to induce a range of novel phenotypes, some of which might be of potential value for further breeding progress.

Ethyl methanesulfonate, introducing single point mutations randomly in the genome, has long been successfully used to produce variants in many different plant species (Kodym & Afza, 2003) as it

**Abbreviations:** cv., cultivar; df, degrees of freedom; EI, ethylenimine; EMS, ethyl methanesulfonate; FS, fertile side shoots; GL, Groß Lüsewitz; GZ, Gülzow; LSD, least significant difference; M lines, mutant lines; NLL, narrow-leafed lupin; NMH, N-nitroso-N-methyl urea; PH, plant height; PN, number of pods; SD, standard deviation; SN, number of seeds; SP, seeds per pod; SW, seed weight.

  

is known as an effective and powerful mutagen for breeding purposes (Serrat et al., 2014; Sikora, Chawade, Larsson, Olsson, & Olsson, 2011). It has also been used in leguminous crops such as chickpea (Wani, Kozgar, Khan, Ahanger, & Ahmad, 2014), soybean (Tsuda et al., 2015) or lentil (Rana & Solanki, 2015). In the FAO/IAEA Mutant Variety Database (Maluszynski, 2001), a total of 106 varieties based on the EMS mutagenesis are recorded, including 43 leguminous cultivars. As an example, the white lupin (*L. albus*) cv. 'Drujba,' selected and bred for early maturity and high yield, was released already in 1984.

With regard to the NLL, two mutant cultivars were bred in Poland (cv. 'Bar,' year of registration: 1991) and Australia (cv. 'Chittick': 1982). These cultivars resulted from mutagenic treatment by NMH (N-nitroso-N-methyl urea) and EI (ethylenimine), respectively, and have been selected for uniform ripening.

The present paper reports on the results obtained with EMS-induced mutants showing deviating growth types. Yield components of the selected mutant lines were assessed, and the inheritance of the deviant growth types was analysed.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and mutagenic treatment

A total of 40,000 seeds ($M_0$ generation) of cv. 'Boruta' (determined growth type with restricted branching) were soaked in tap water at 4°C for 24 hrs. Subsequently, the swollen seeds were transferred to a 1.5% EMS (Sigma-Aldrich) aqueous solution, a concentration which in preliminary tests generated satisfactory phenotypic variability while still allowing for sufficiently high seed germination rates (Rudloff & Wehling, 2008). Seeds were shaken for 4 hrs at room temperature on a platform shaker. The seeds were then rinsed two times in 5% sodium thiosulphate followed by soaking in tap water for 24 hrs. The treated seeds ($M_1$ generation) were dried back at room temperature before sowing in field plots with a plot driller at a seed density of 83 seeds/$m^2$. The mature $M_1$ plants were harvested separately to obtain $M_2$ seeds. $M_2$ plants were grown in field plots, selected for deviating branching types, selfed and harvested. Phenotypic stability and genetic homogeneity of the selected M lines were assessed in the following two generations, yielding phenotypically and genetically stable $M_4$ lines (Rudloff, Eickmeyer, & Wehling, 2008) that were used for subsequent tests and breeding.

$F_2$ families segregating with mutant and wild-type growth types were generated by reciprocal crosses of mutant lines M431, M1424b, M280, M348, M945b, M991, M190, M116, M411, and M21 to a wild-type parent. $F_1$ offspring plants were raised and self-pollinated. For crossings, flowers of mutant lines were emasculated and hand-pollinated with pollen of cv. 'Boruta' or the GenBank Accession No. 22662 (donated by The Australian Lupin Collection, Lupin Breeding, Department of Agriculture, Western Australia, origin: Israel, hereinafter L115). Due to the wide crosses with L115, a higher rate of polymorphisms has been expected regarding further marker analysis for future development of selection tools. $F_3$ seeds harvested from $F_2$ individual plants were used for progeny tests to verify phenotypes and determine the underlying genotypes of plants in segregating $F_2$ families.

### 2.2 | Phenotyping

Mutant lines were grown in rows with 20 plants per row (plant distance 15 cm, row distance 10 cm). The cv. 'Boruta' was simultaneously grown as a wild-type control. Phenotyping took place in the developmental stages from BBCH 60 through BBCH 80. Growth types were defined according to the scheme presented in Figure 1.

The field trials were carried out at two locations in Mecklenburg-Western Pomerania, Germany: (i) Groß Lüsewitz (GL), 54°07′N, 12°33′E, sandy loam soil, average annual precipitation 692 mm, mean annual temperature 8.3°C, (ii) Gülzow: (GZ), 53°82′N, 12°07′E, sandy soil, average annual precipitation 569 mm, mean annual temperature 8.6°C.

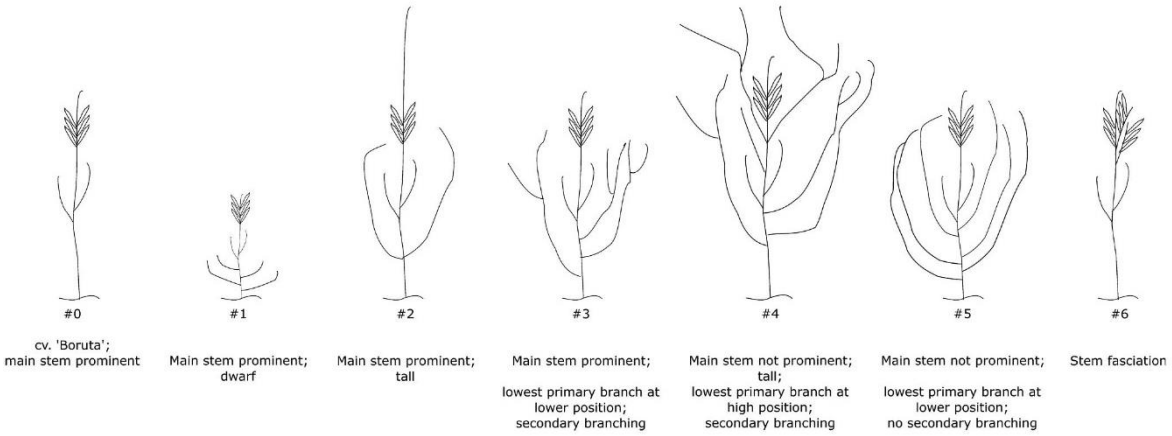


**FIGURE 1** Growth types observed among mutant lines (according to the Lupin Descriptor of the International Board for Plant Genetic Resources, 1981, modified)

The following traits were assessed in three successive years (2012–2014): Plant height (*PH*, cm), number of fertile side shoots per plant (*FS*), number of pods per plant (*PN*), number of seeds per plant (*SN*), seed weight per plant (*SW*, g) and seed number per pod (*SP*).

### 2.3 | Assessing yield components

For assessment of the *PH* and yield components *FS*, *PN*, *GN*, *SW*, and *SP*, mutant lines and wild-type cultivar "Boruta" were grown in experimental plots of 8 $m^2$ at GL and 13.5 $m^2$ at GZ, respectively. The trials were conducted as a randomized block design with two replications and a seed density of 50 seeds/$m^2$. Pre-emergence herbicides were applied according to good farmers' practice, and insecticide treatment was carried out as required. Traits were assessed on 20 manually harvested plants per plot.

### 2.4 | Statistical analysis

Statistical analysis was performed using the R software package (R Core Team, 2015). ANOVA was conducted, and least significant differences ($LSD_{5\%}$) between lines were calculated using the R package "predict means" (Dongwen, Siva, & John, 2014). To estimate heritability ($h^2$), genotypic ($V_G$), genotype by environment ($V_{GLY}$) and residual ($V_F$) variance components were estimated and broad-sense heritability was calculated according to

$$h^2 = \frac{V_G}{V_G + \frac{V_{GLY}}{N_{LY}} + \frac{V_F}{N_{LYR}}}$$

where $N_{LY}$ is the amount of environments (two locations by 3 years, $N = 6$) and $N_{LYR}$ displays the amount of environments and replications (two locations by 3 years with two replications, $N = 12$).

## 3 | RESULTS

### 3.1 | Novel growth types

From approximately 40,000 $M_0$ seeds of cv. 'Boruta,' a total of 27,300 fertile $M_1$ plants were obtained, corresponding to a germination rate of nearly 70%. The $M_2$ seeds were harvested as selfed families from individual $M_1$ plants, and $M_2$ families were repeatedly field-phenotyped in the years 2007 to 2010. About 10,000 $M_2$ families expressing phenotypes deviating from the wild-type "Boruta" were propagated to $M_3$ seeds. Finally, 2,320 $M_3$ lines were found to be phenotypically stable.

Novel growth types comprised, for instance, altered dimensions (length/width) of leaflets, variation in the anthocyanin and/or chlorophyll content, flower colour, fasciation of the stem, plant height or growth type. Among these growth types, lines were found to express markedly different plant heights and/or branching compared to the determinate growth type of cv. 'Boruta' with restricted branching (Figure 1, growth type #0). Growth types #1 and #2 mainly differed from "Boruta" concerning their plant height. Growth type #3 was particularly characterized by strong branching. Then again, growth type #4 showed apical secondary branching and was taller compared to cv. 'Boruta,' while growth type #5 was devoid of secondary branching and had a plant height similar to 'Boruta' (Figure 2). Additionally, branching started at a higher position of the stem in growth type #4 as compared to #5. Another growth type (#6) developed a fasciated stem accompanied by a pronounced lodging resistance.

**FIGURE 2** Typical shoots of the wild-type cv. 'Boruta' (a), as compared to phenotype #4 (b), and phenotype #5 (c). Numbering of phenotypes as in Figure 1

A total of twelve mutant lines falling into either one of the phenotypic classes #1 to #6 were selected for further examination and propagated to the $M_4$ generation (Table 1).

### 3.2 | Inheritance of different growth types

Ten of the mutant lines listed in Table 1 were used as crossing parents to generate twelve $F_2$ families segregating for individuals expressing either the growth of the wild-type parent or the respective mutant growth type. As could have been expected, the segregation ratios were consistent with a monogenic recessive inheritance of the respective mutant growth type (Table 2).

Individuals of $F_2$ families from the crosses M1424b × 'Boruta' and M280 × L115 were progeny-tested by raising 20 $F_3$ offspring plants per $F_2$ individual each and assessing their growth types. While $F_2$ mutant growth type gave phenotypically homogeneous $F_3$ mutant offspring, approximately one-third of the $F_2$ phenotypes resembling the wild type resulted in homogeneous wild-type progeny while two-thirds gave segregating $F_3$ offspring, thus indicating wt+/wt+ and wt+/wt− genotypes underlying the heterozygous and heterozygous dominant phenotype classes, respectively, in $F_2$. For instance, the 71 $F_3$ families derived from M1424b × 'Boruta' were found to comprise 21 wild-type families, 35 segregating and 15 mutant families, consistent with a genotypic 1:2:1 segregation ratio underlying the two phenotypic classes in $F_2$. The same conclusion could be drawn for the cross M280 × L115 where 88 $F_3$ families were found to segregate into 22 wild types, 41 segregating and 25 mutant families.

**TABLE 1** Growth types of selected mutant lines

| | Mutant lines | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Growth type [a] | M116 | M1424b | M190 | M21 | M280 | M348 | M411 | M431 | M945 | M991 | M1725 | M1743 |
| 1 | | | | x | | x | | | x | x | | |
| 2 | | | | | | | | x | | | | |
| 3 | x | | x | | | | | | | | | |
| 4 | | x | | | | | | | | | x | x |
| 5 | | | | | x | | | | | | | |
| 6 | | | | | | | x | | | | | |

[a]Growth types numbered according to Figure 1.

**TABLE 2** Genetic segregation of mutant vs. wild-type growth types in $F_2$ families

| | Nos. of segregants in $F_2$ | | | |
|---|---|---|---|---|
| $F_1$ cross | Total | Mutant | Wild type | $\chi^2$ (1:3) |
| M431 × 'Boruta' | 75 | 15 | 60 | 1.00 n.s. |
| M1424b × 'Boruta' | 71 | 15 | 56 | 1.03 n.s. |
| M280 × 'Boruta' | 87 | 25 | 62 | 0.65 n.s. |
| M348 × 'Boruta' | 66 | 17 | 49 | 0.02 n.s. |
| M945b × 'Boruta' | 38 | 10 | 28 | 0.04 n.s. |
| 'Boruta' × M945b | 31 | 6 | 25 | 0.53 n.s. |
| M991 × 'Boruta' | 39 | 10 | 29 | 0.01 n.s. |
| M190 × L115 | 96 | 27 | 69 | 0.50 n.s. |
| M116 × L115 | 105 | 27 | 78 | 0.03 n.s. |
| M411 × L115 | 127 | 34 | 93 | 0.21 n.s. |
| M280 × L115 | 101 | 29 | 72 | 0.74 n.s. |
| M21 × L115 | 109 | 19 | 90 | 3.33 n.s. |

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

### 3.3 | Plant height and seed yield components

Eight mutant lines belonging to the phenotypic classes #2, 3, 4, 5 or 6 were examined for *PH* and yield components. Traits were assessed in a multi-environmental field trial (3 years, two locations) with two replicated plots per entry. The cv. 'Boruta' was used as check. Twenty plants per plot were assessed for the *PH* and yield components *PN*, *GN*, *SW*, *SP*, and *FS*. Consequently, the mean values in Table 3 are based on 240 plants per entry.

For all traits, heritability was calculated. High estimates ($h^2 > 0.80$) were obtained for *PH* and *SW*. *FS*, *PN*, and *SP* showed moderate heritabilities whereas *GN* displayed the lowest heritability (Table 3).

For all traits measured, significant differences were observed between cv. 'Boruta' and mutant lines. For five of the six traits, two or more of the mutant lines proved to be superior to the original cv. 'Boruta' (Table 3). Four of the eight mutant lines proved to be outstanding regarding *PN*, *GN* or *SW* (Table 3, Figure 3).

While cv. 'Boruta' tends to develop smaller side shoots depending on the local conditions such as water availability or soil quality, the mutant lines M116, M1424b, and M1725 showed a significant increase in the number of fertile side shoots per plant. Concerning the trait number of pods per plant, the mutant lines M116, M1424b, M190, and M280 showed significant increases compared to "Boruta." In particular, the mutant M280 developed almost twice as many pods per plant than cv. 'Boruta' (Table 3, Figure 3a). In addition, lines M116, M1424b, and M280 showed significant increases regarding the number of seeds per plant. As an outstanding example,

| | Plant height cm | No. of fertile side shoots/plant | No. of pods/plant | No. of seeds/plant | No. of grains per pod/plant | Seed weight g/plant |
|---|---|---|---|---|---|---|
| 'Boruta' | 63.1 | 11.0 | 23.4 | 93.0 | 3.7 | 13.8 |
| M116 | 60.4 | 12.1* | 34.2* | 110.6* | 3.2 | 15.2* |
| M1424b | 56.4 | 12.7* | 35.9* | 138.4* | 3.5 | 22.2* |
| M1725 | 66.6* | 11.8* | 23.1 | 75.7 | 3.4 | 11.2 |
| M1743 | 59.8 | 10.7 | 24.4 | 91.7 | 3.6 | 13.6 |
| M190 | 61.5 | 11.3 | 28.3* | 93.9 | 3.4 | 12.6 |
| M280 | 49.4 | 11.0 | 45.6* | 112.7* | 2.7 | 13.8 |
| M411 | 65.5* | 8.0 | 20.6 | 53.5 | 3.1 | 7.2 |
| M431 | 78.8* | 10.6 | 20.8 | 66.3 | 3.3 | 10.2 |
| $LSD_{5\%}$ | 1.7 | 0.5 | 2.4 | 8.0 | 0.12 | 1.2 |
| $h^2$ | 0.83 | 0.52 | 0.67 | 0.34 | 0.61 | 0.85 |

**TABLE 3** Yield components of the NLL mutants tested (mean values of 3 years and two locations, least significant differences, and heritability $h^2$). Lines that are significantly superior to cv. 'Boruta' are indicated with an asterisk, $P = .05$

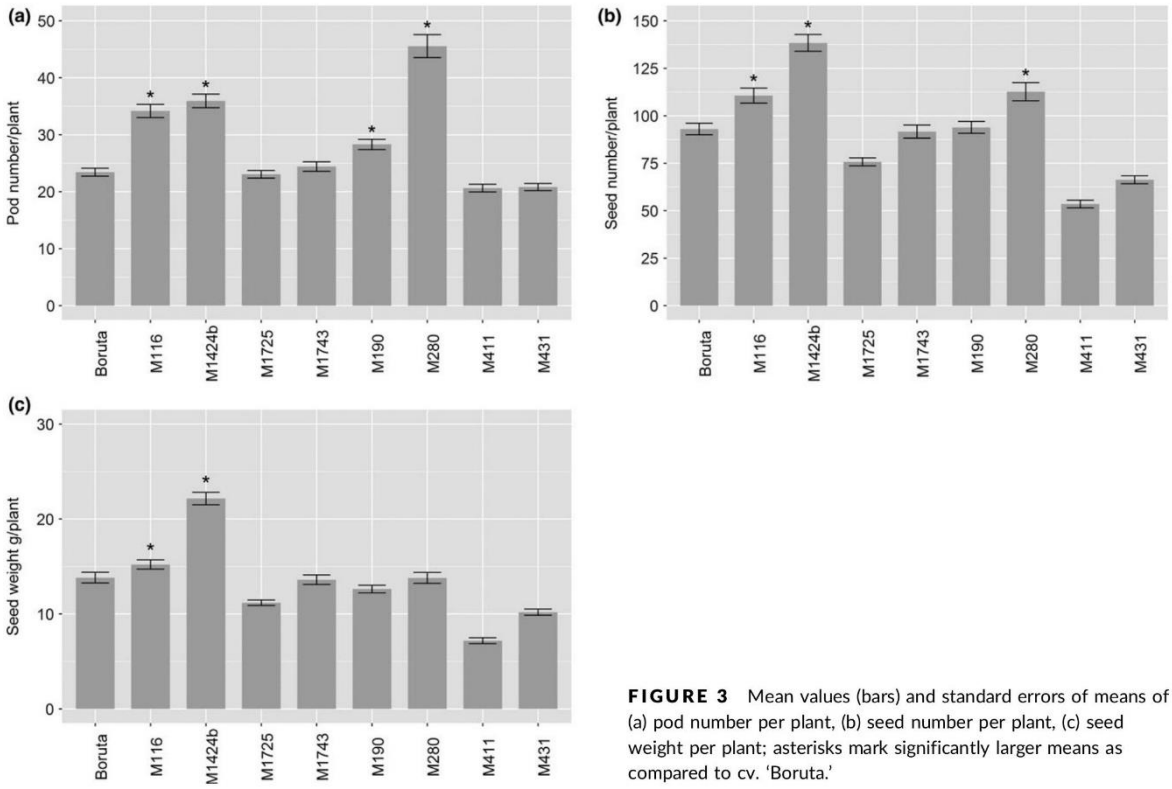


**FIGURE 3** Mean values (bars) and standard errors of means of (a) pod number per plant, (b) seed number per plant, (c) seed weight per plant; asterisks mark significantly larger means as compared to cv. 'Boruta.'

the line M1424b yielded almost 50% more seeds than cv. 'Boruta' (Table 3, Figure 3b).

The seed weight per plant was found to be increased in two lines, namely M116 and M1424b. The best line M1424b reached a 61% increase for the seed weight per plant compared to cv. 'Boruta' (Table 3, Figure 3c).

## 4 | DISCUSSION

Narrow-leafed lupin is characterized by a relatively high seed protein content and favourable protein composition within the grain legume species (Foyer et al., 2016; Lucas et al., 2015). On the other hand, NLL is also known for its unstable grain yield in different environments (locations, years). However, this relative instability has to be regarded in view of the fact that NLL is often grown on the poorest, usually sandy soils, for example, in north-eastern regions of Germany. Crops grown under such conditions are particularly sensitive to weather conditions and inappropriate farming practice. Grown on better soils, the NLL can exploit its genetic yield potential and is able to produce protein yields comparable or superior to other legume species including soybean raised under similar conditions. Nevertheless, there is no doubt that substantial breeding progress will be needed to further develop NLL as a stable high-yielding crop competitive under agricultural farm conditions. As a self-pollinating species, the genetic variation in NLL is comparatively limited due to the genetic bottleneck resulting from rigorous selection for sweetness necessary to make NLL basically suitable as a profitable feed and food crop (Berger, Buirchell, Luckett, & Nelson, 2011; Berger et al., 2013; Duc et al., 2014; von Sengbusch, 1930).

To introduce novel genetic variability into current NLL germplasm, we used the approach of EMS-based mutagenesis. The commercial cultivar 'Boruta' was chosen for mutagenesis as it is well adapted to agricultural conditions in Germany. Our results indicate that NLL is easily accessible to the EMS treatment, although this classical approach is quite lengthy (Figure 4) and requires a broad-based starting material in terms of the seed amount to be treated. In our hands, EMS mutagenesis resulted in a plethora of phenotypic variation, which may be used for selecting offspring expressing specific trait variants of potential interest. It should be noted, though, that EMS-based mutagenesis cannot substitute for the introduction of plant genetic resources if existing genetic bottlenecks are to be widened in a given breeder`s germplasm in order to ensure future breeding progress.

Three M lines, that is, M116, M1424b, and M280, were identified which proved to be superior to the original cv. 'Boruta' regarding a number of yield components. For instance, line M1424b showed a highly heritable seed weight per plant that was 60%

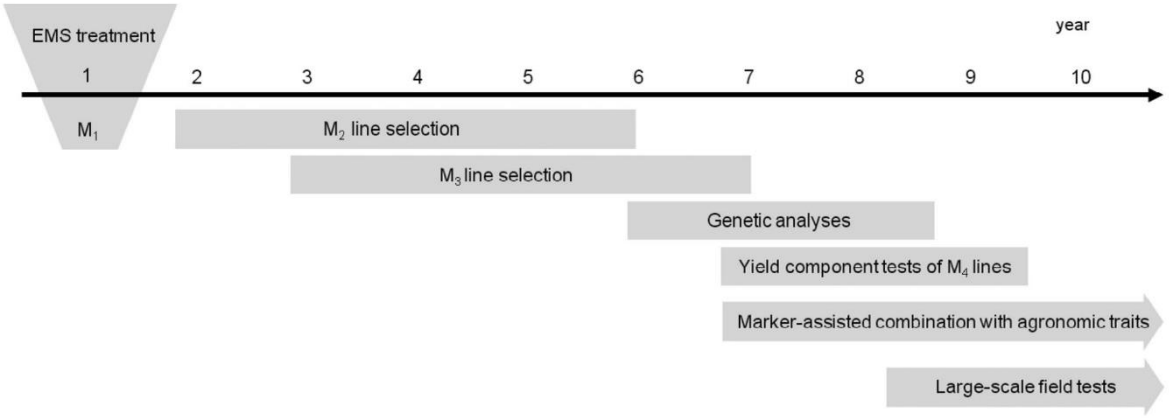


**FIGURE 4** Time scheme of mutant line development and evaluation

higher compared to cv. 'Boruta'. The three lines expressed the growth types #3, #4 or #5, respectively, and are characterized by pronounced branching (Figure 1). These plant types show an early pod set in the higher (i.e., younger) plant parts, potentially leading to a more uniform seed maturation which would facilitate mechanical combine harvesting. Other mutant lines expressing interesting traits such as dwarfism, that is growth type #1 (mutants M21, M945, M348, M991), or stem fasciation (#6, line M411) were not among the best lines regarding yield potential and seed yield.

The growth types of mutant lines M116, M1424b, and M280 were shown to be simply inherited, each by a single recessive gene. Thus, these growth types can be easily traced and fixed, for example, in backcross programmes with elite cultivars showing a restricted branching, aiming at the transfer into high-yielding germplasm by eliminating background mutations expected after EMS-mediated mutagenesis.

In contrast to recent genome-editing approaches (Cardi & Neal Stewart, 2016; Mahfouz, Cardi, & Neal Stewart, 2016) like TALEN (Cermak et al., 2011) or the CRISPR–Cas system (Mao et al., 2013; Schiml, Fauser, & Puchta, 2017), EMS-mediated mutagenesis is an undirected approach. Recent progress in NLL genome sequencing (Gao et al., 2011; Kamphuis et al., 2015; Hane et al., 2017) is expected to widen future possibilities for site-directed mutagenesis also in this species. Despite these powerful novel approaches, EMS treatment remains an option as long as physiologically complex and polygenic traits such as seed yield are addressed which are not yet fully understood in terms of the genes involved, their sequence, function, as well as regulation. In the present work, seed yield components could be improved by selecting novel growth types randomly induced *via* EMS mutagenesis.

The specific value of the NLL mutant lines selected in this prebreeding project remains to be demonstrated. To this end, after seed multiplication, the respective lines will be seed-multiplied and subjected to large-scale field tests (Figure 4) in multiple environments to assess seed yield on an area basis rather than on a single plant basis. Furthermore, protein content and composition, as well as alkaloid levels, will have to be analysed in detail for those lines which prove to be promising in the field trials.

In prior research, selection markers for resistance to anthracnose have been developed including the loci *LanrBo* (Ruge-Wehling, Dieterich, Thiele, Eickmeyer, & Wehling, 2009; Fischer et al., 2015) and *Lanr1* (Yang, Boersma, You, Buirchell, & Sweetingham, 2004; Kamphuis et al., 2015). These molecular tools are now available to introduce known resistance genes into novel prebreeding lines like those identified in the present work. What remains to be done is to develop molecular selection markers for promising EMS-induced growth types identified in the present study. In a first attempt, we applied a transcriptomic sequencing approach (unpublished) to compare the wild-type (cv. 'Boruta') and mutant growth types developed thereof. However, this NIL-based gene-tagging approach was not successful, possibly due to the presence of frequent additional EMS-induced mutations elsewhere in the mutants' genome.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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# 3 Diskussion und Zusammenfassung

Im Rahmen der Eiweißpflanzenstrategie, die 2012 vom Bundesministerium für Ernährung und Landwirtschaft verabschiedet wurde, soll der Leguminosenanbau insbesondere in Deutschland forciert und gestärkt werden. Der damit verbundene Nutzen für die lokale Landwirtschaft durch die vielfältigen Ökosystemleistungen sollte Argument genug sein, Soja-Importe aus Süd- und Mittelamerika auf ein Mindestmaß zu reduzieren und somit, besonders in der Tierfütterung, auf heimisches Protein zu setzen und die sogenannte „Fernfütterung“ durch Soja möglichst gering zu halten. Dazu kommt, dass 80 % der importierten Sojaprodukte gentechnisch verändert sind, was mit der Tierfütterung in der ökologischen Landwirtschaft und auch mit den Zielen der Lebensmittelwirtschaft in Deutschland nicht im Einklang steht. Zunehmend werden in beiden Sektoren gentechnik-freie Produkte gefordert. Sowohl “einheimisch“ als auch „gentechnik-frei“ sind neben anderen Ökosystemleistungen Attribute, die die Blaue Lupine für einen Leguminosenanbau, im Nordosten Deutschlands als Alternative zu importiertem Soja attraktiv machen.

Dennoch kann die Wirtschaftlichkeit des Lupinenanbaus und der Körnerproduktion aktuell nicht mit sogenannten *Cash Crops* wie beispielsweise Weizen oder Mais mithalten. Gründe dafür sind hauptsächlich eine unzureichende Ertragsstabilität und eine geringe Ertragsleistung, die in den letzten Jahren aufgrund zu geringer Züchtungsaktivitäten nahezu konstant unbefriedigend geblieben sind.

## 3.1 Anthraknose-Resistenz für eine höhere Ertragsstabilität

Die Ertragsstabilität im Lupinenanbau wird maßgeblich durch die Pilzkrankheit Anthraknose (Erreger *Colletotrichum lupini*), beeinträchtigt. Die samenübertragbare Krankheit kann in ihrer Ausprägung teilweise eingedämmt werden, indem die Samen langfristig gelagert werden. Die Lebensfähigkeit des Pilzes wird mit Erhöhung der Lagertemperatur reduziert, allerdings ist dies auch mit Einbußen der Keimfähigkeit verbunden (Talhinhas *et al*., 2016) und zudem in der praktischen Landwirtschaft nahezu nicht umsetzbar. Im Gegensatz zum Ökolandbau ist der Einsatz von Pestiziden in der konventionellen Landwirtschaft zwar möglich, jedoch aufgrund der mangelnden Mittelverfügbarkeit nur bedingt praktikabel, da Pflanzenschutzmittelzulassungen sowohl zeit- als auch kostenintensiv sind und sich

daher für sogenannte „kleine Kulturen“ selten lohnen. Ökologisch als auch ökonomisch sinnvoller ist es demzufolge, gezielt nach Resistenzen gegen das jeweilige Pathogen zu forschen und diese näher zu charakterisieren. In der vorliegenden Arbeit konnte ein funktionaler Resistenzlocus, *LanrBo*, genetisch und molekularbiologisch charakterisiert werden, der Widerstandsfähigkeit gegen *Colletotrichum lupini* vermittelt (Fischer *et al*. 2015, Kapitel 2).

Mit Hilfe der neuen genomischen Ressourcen – insbesondere aufgrund der Verfügbarkeit der Genomsequenz von *L. angustifolius* (Yang *et al*. 2013, Hane *et al*. 2017) – sind mittlerweile enger gekoppelte Marker für *LanrBo* identifiziert worden. Auf Grundlage der kartierten Ankermarker (Fischer *et al*. 2015) wurde auf dem Chromosom NLL-11 eine Region von 6,6 Mbp bis 10,8 Mbp definiert, in der *LanrBo* lokalisiert ist. Um für diesen Bereich spezifische Marker zu entwickeln, wurden SNP-Sequenzen genutzt, die aus einem MACE-Experiment hervorgegangen sind (*Massive Analysis of cDNA ends*, Kahl *et al*. 2012). Darin wurden differentiell – d.h. anfällig versus resistent – exprimierte Sequenzen generiert und auf Sequenzunterschiede hin untersucht. Diese wurden anschließend mithilfe eines BLAST-Algorithmus‘ gegen das Lupinen-Genom abgeglichen. Für die Markerentwicklung und Kopplungsanalyse wurden ausschließlich SNPs verwendet, die im eingangs definierten Bereich vorab lokalisiert werden konnten. Dabei erfolgte die Identifizierung von Markern für *LanrBo*, die in einem Abstand von ~ 1 cM kartieren und somit verlässlich für eine markergestützte Selektion auf Anthraknose-Resistenz in der Züchtung eingesetzt werden können (unveröffentlicht). Die für den BLAST verwendeten Sequenzinformationen stehen unter ‚Lupin Genome Portal‘ (http://www.lupinexpress.org/) zur Verfügung.

Die molekularen Selektionsmarker machen es jetzt möglich, *LanrBo* in Kreuzungsprogrammen gezielt zu verfolgen, und für die sichere und effektive Selektion resistenter Nachkommen zu nutzen.

Der Nachweis, dass die beiden auf der Kopplungsgruppe NLL-11 lokalisierten Resistenzgene *Lanr1* und *LanrBo* tatsächlich zwei unterschiedliche Genorte repräsentieren, wurde zum einen anhand der genetischen Abstände der Ankermarker zu den Resistenzloci erbracht (Fischer *et al*. 2015). Aktuelle Analysen ergaben einen genetischen Abstand des Selektionsmarkers von *Lanr1* zu dem Resistenzlocus

*LanrBo* von ~ 30 cM. Andererseits wurden Alleliekreuzungen beider Resistenzträger durchgeführt und in der F2-Nachkommenschaft eine genetische Aufspaltung von resistent zu anfällig im Verhältnis von 15:1 beobachtet. Die Markerallele der Loci wiesen eine 9:3:3:1-Aufspaltung auf; *Lanr1* und *LanrBo* sind somit nicht eng gekoppelt (unveröffentlicht).

Der Nachweis über Alleliekreuzungen, dass es sich bei den nunmehr zwei bekannten monogenisch dominanten Resistenzloci *Lanr1* (Yang *et al*. 2004) und *LanrBo* (Fischer *et al*. 2015) um zwei nicht gekoppelte Loci handelt, sowie die Verfügbarkeit molekularer Selektionswerkzeuge macht eine Pyramidisierung dieser Loci sinnvoll. Somit können die unterschiedlichen Resistenzlevel (Fischer *et al*. 2015) der beiden Ressourcen kombiniert werden um somit möglichst eine stabile Resistenz gegen das Pathogen zu schaffen.

## 3.2 Neue Wuchstypen als Basis für eine hohe Ertragsleistung

Die nach wie vor geringe Ertragsleistung der Süßlupine ist Folge eines genetischen Flaschenhalses (Berger *et al.* 2011), verursacht durch die praktizierte, erfolgreiche Selektion auf Alkaloidarmut. In dieser Arbeit ist im Rahmen eines Mutageneseprogramms ein Set an vielversprechenden Mutantenlinien untersucht worden. Diese Linien zeigten ein diverses phänotypisches Spektrum hinsichtlich des Verzweigungsgrades (Fischer *et al*. 2017, siehe Kapitel 2). Hinsichtlich einzelner Ertragskomponenten konnten signifikante Veränderungen im Vergleich zur Ausgangssorte nachgewiesen werden. Somit konnte gezeigt werden, dass über die Selektion aberranter Phänotypen indirekt auch auf Ertragsmerkmale selektiert wurde. Der postulierte monogenisch-rezessive Vererbungsmodus erlaubt es, die angestrebten Phänotypen im Züchtungsprozess weiter zu verfolgen und verbesserte, ertragreiche Sorten zu züchten.

Im Gegensatz zu aktuellen *Genome-Editing*-Methoden - wie beispielsweise dem Einsatz von Zink-Finger-Nukleasen (*ZNF*), Transkription-like-effector-Nukleasen (*TALEN*) oder dem *CRISPR/Cas9*-System (Songstad *et al*. 2017) - ist die Induktion von Mutationen über EMS nicht zielgerichtet, stellt aber im weiteren Sinne eine gewisse Form des *Genome Editings* durch das Einführen von Mutationen in ein Genom dar. Einfach einsetzbare Screening-Methoden sind dabei notwendig, um erwünschte

Mutationen in großen Populationen zu detektieren. Dazu gehören zum Beispiel die Bonitur von Merkmalen wie Pflanzenlänge oder neue Verzweigungstypen, welche möglicherweise eine indirekte Selektion auf quantitative Merkmale wie den potenziellen Ertrag zulassen. Die Anwendung neuer *Genome Editing*–Methoden erfordert die Kenntnis der DNA-Sequenz des zu verändernden Gens. Das ist durch die Veröffentlichung der genomischen Sequenz von *L. angustifolius* nunmehr möglich geworden (Yang *et al*. 2013, Hane *et al*. 2017). Quantitative Merkmale wie der Ertrag werden jedoch nicht nur von einem Gen kontrolliert, sondern durch einen Komplex sich gegenseitig beeinflussender Faktoren. Die Anwendung der chemischen Mutagenese ist daher gerade bei komplexen Merkmalen nach wie vor, wenn auch zeitaufwändiger, die Methode der Wahl.

In umfassenden Leistungsprüfungen im Feld wurden auf der Basis der Ergebnisse aus dem Ertragskomponententest Linien auf Kornertrag, Platzfestigkeit sowie die Qualitätsmerkmale Protein- und Alkaloidgehalt untersucht. In bisher sechs Umwelten (2 Jahre, 3 Standorte) konnte das verbesserte Ertragspotential in Form von signifikant erhöhten Flächenkornerträgen sowie höheren Proteinerträgen bestätigt werden (Fischer *et al*. 2016).

Linien mit signifikant höheren Erträgen wurden mit anderen Linien gekreuzt, welche die Anthraknoseresistenzgene *LanrBo* und *Lanr1* besitzen. Mit Hilfe von Selektionsmarkern für diese Resistenzgene konnten bereits ertragreiche, neue Wuchstypen selektiert werden, die zusätzlich eine Anthraknoseresistenz tragen (unveröffentlicht). Dieses *Prebreeding*-Material mit höherer Ertragsleistung und besserer Ertragsstabilität bildet eine wertvolle Basis für die Sortenzüchtung.

Zusammenfassend kann demzufolge festgestellt werden, dass diese Arbeit verschiedene hochrelevante Ergebnisse gezeigt hat:

i) Der enge genetische Flaschenhals bei der Süßlupine wurde durch die Nutzung genetischer Ressourcen erweitert und damit konnte ein positiver Effekt auf die Ertragsstabilität erzielt werden.
ii) Mit Blick auf die Ertragsleistung konnte die genetische Variabilität mit der chemischen Mutagenese erfolgreich erhöht werden.

Diese beiden Effekte bringen ertragsstabile Linien hervor, d.h. einerseits Anthraknose-resistente sowie andererseits potenziell ertragsstarke, neue verzweigte Wuchstypen. Die Möglichkeit der Kombination beider Merkmale in gezielten Kreuzungsprogrammen unter Nutzung neuer, molekularer Selektionswerkzeuge ist eine wichtige und erfolgversprechende Basis für die Züchtung neuer, leistungsstarker Sorten der Blauen Lupine. Somit liefert diese Arbeit einen Impuls für die Züchtung auf eine verbesserte Stabilität und Leistung der Lupine für gemäßigte Klimate und neue Verwendungen.

# 4 Summary

Narrow-leafed lupin is considered to be a legume with great future potential because of its high and valuable protein content. Cultivated lupins appear in agricultural production in diverse forms and for very different purposes: grain production and forage in crop rotation, green manure, and as a relatively new area the production of healthy food products. Therefore novel cultivars for these particular applications are needed. Successful breeding of varieties requires genetic variability with a broad spectrum of agronomic traits. But genetic variation of narrow-leafed lupin, a self-pollinator, is limited due to the bottleneck of former selection for sweetness.

These days overriding goals in lupin breeding are yield stability and yield increase.

Yield stability may be achieved by breeding for anthracnose resistance as it is the most severe disease in lupin cultivation worldwide. Therefore, a screening for anthracnose resistance of a set of plant genetic resources of NLL identified the breeding line Bo7212 as being highly resistant to anthracnose (*C. lupini*). Segregation analysis of experimental mapping populations indicated that the resistance of Bo7212 is inherited by one single dominant locus. Published molecular anchor markers as well as further sequence information obtained from the model genome *Lotus japonicus*, RNASeq data and genome sequence information of NLL allowed us (i) to locate the locus *LanrBo* on linkage group NLL-11 and (ii) to identify closely linked markers that allow for precise marker-assisted selection for *LanrBo* in narrow-leafed lupin breeding programs.

In an EMS-based mutagenesis program 40,000 seeds of cv. 'Boruta' were treated and a variety of phenotypic mutant lines expressing novel growth types with altered plant height and branching were obtained. These lines promise a potential yield increase. In field trials at two locations for three years, three of a subset of these lines exhibited significantly improved grain yield components compared to the wild type cv. 'Boruta', indicating a successful yield increase by indirect selection for novel branching behaviour. Growth types of these promising lines were shown to be inherited in a monogenic recessive manner and should, hence, be easily fixable in breeding programs.

To conclude, this work demonstrates (i) the successful exploitation of plant genetic resources regarding yield stability and (ii) the increase of genetic variability by chemical

mutagenesis. This may result in anthracnose-resistant lines and high-yielding growth types. The combination of both factors in targeted breeding programs by using novel molecular selection tools is a promising and efficient basis for the development of powerful lupin varieties.

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# 6 Abkürzungsverzeichnis

| | |
|---|---|
| AFLP | *Amplified fragment length polymorphism* |
| BLAST | *Basis local alignment search tool* |
| cM | centi-Morgan |
| DAFWA | *Department for Agriculture and Food Western Australia* |
| DESTATIS | Statistisches Bundesamt |
| dt | Dezitonne |
| EMS | Ethylmethansulfonat |
| FAOSTAT | *Food and Agriculture Organization Corporate Statistical Database* |
| ha | Hektar |
| MACE | *Massive analysis of cDNA ends* |
| Mbp | Megabasenpaare |
| NLL | *Narrow-leafed lupin* |
| NLR | *NOD-like receptor* |
| SNP | *Single nucleotide polymorphism* |
| SSR | *Simple Sequence Repeats* |
| TALEN | *transcription-like-effector*-Nukleasen |
| ZNF | Zink-Finger-Nukleasen |

# 7 Anhang

Ergänzendes Material aus:

I

Kristin Fischer, Regine Dieterich, Matthew N. Nelson, Lars G. Kamphuis, Karam B. Singh, Björn Rotter, Nicolas Krezdorn, Peter Winter, Peter Wehling, Brigitte Ruge-Wehling

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

*Theoretical and Applied Genetics* (2015), 128: 2121 – 2130

DOI: 10.1007/s00122-015-2572-3

Supplemental Table S1 Genebank accessions tested in this study

| Designation | Origin | Donor |
|---|---|---|
| 28079 | Australia | DAFWA, Perth |
| 28502 | Australia | DAFWA, Perth |
| PI283633 | Germany | Washington State University, Pullman, Washington |
| 27052 | Belarus | DAFWA, Perth |
| 27053 | Belarus | DAFWA, Perth |
| 27054 | Belarus | DAFWA, Perth |
| 27061 | Belarus | Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben |
| 27479 | Belarus | DAFWA, Perth |
| 28471 | Belarus | Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben |
| 28481 | Belarus | DAFWA, Perth |
| PI180708 | Germany | Washington State University, Pullman, Washington |
| PI237721 | Germany | Washington State University, Pullman, Washington |
| PI283633 | Germany | Washington State University, Pullman, Washington |
| PI289162 | Hungary | Washington State University, Pullman, Washington |
| PI289163 | Hungary | Washington State University, Pullman, Washington |
| LUP102/73 | Israel | Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben |
| 27254 | Morocco | DAFWA, Perth |
| 27447 | Poland | DAFWA, Perth |
| 28806 | Poland | Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben |
| PI274811 | Poland | Washington State University, Pullman, Washington |
| PI274812 | Poland | Washington State University, Pullman, Washington |
| PI274814 | Poland | Washington State University, Pullman, Washington |
| PI274817 | Poland | Washington State University, Pullman, Washington |
| LUP106/73 | Portugal | Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben |
| LUP141/80 | Russia | Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben |
| 27479 | Russia | DAFWA, Perth |
| PI255472 | Slovenia | Washington State University, Pullman, Washington |

Supplemental Table S1 Genebank accessions tested in this study (continued)

| Designation | Origin | Donor |
|---|---|---|
| PI283636 | South Africa | Washington State University, Pullman, Washington |
| PI300023 | South Africa | Washington State University, Pullman, Washington |
| PI308615 | South Africa | Washington State University, Pullman, Washington |
| PI308616 | South Africa | Washington State University, Pullman, Washington |
| PI308619 | South Africa | Washington State University, Pullman, Washington |
| BGE023639 | Spain | The National Institute for Agricultural Research and Experimentation, Madrid |
| BGE007278 | Spain | The National Institute for Agricultural Research and Experimentation, Madrid |
| BGE001174 | Spain | The National Institute for Agricultural Research and Experimentation, Madrid |
| LUP165/79 | Spain | Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben |
| 27436 | Syria | DAFWA, Perth |
| BGE001626 | Spain | The National Institute for Agricultural Research and Experimentation, Madrid |
| LUP155/80 | Frankreich | Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben |
| LUP154/80 | Belarus | Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben |
| BGE0165526 | Spain | The National Institute for Agricultural Research and Experimentation, Madrid |
| PI255472 | Yugoslavia | Washington State University, Pullman, Washington |
| PI255473 | Yugoslavia | Washington State University, Pullman, Washington |

Supplemental Table S2 Percentage of 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 (sd, standard deviation)

| **Entry** | | **Bocksee** | | **Groß Lüsewitz** | |
|---|---|---|---|---|---|
| **Number of plants** | | **% infested plants** | **sd** | **% infested plants** | **sd** |
| Bo7212 | A | 2.5 | 1.2 | 5.7 | 3.0 |
| (N = 3096) | B | 0.3 | 2.3 | 0.1 | 0.9 |
| | C | 1.2 | 3.2 | 3.0 | 3.3 |
| Arabella | A | 44.3 | 1.1 | 51.2 | 2.0 |
| (N = 3154) | B | 20.5 | 9.6 | 20.6 | 6.1 |
| | C | 16.0 | 6.0 | 11.9 | 6.8 |
| Mandelup | A | 45.7 | 4.0 | 21.3 | 7.0 |
| (N = 2151) | B | 28.5 | 6.2 | 7.7 | 2.4 |
| | C | 13.7 | 5.7 | 7.2 | 3.8 |
| Tanjil | A | 40.6 | 0.4 | 33.2 | 0.3 |
| (N = 2797) | B | 11.7 | 7.2 | 4.4 | 4.5 |
| | C | 20.5 | 10.3 | 17.6 | 4.4 |

Supplemental Table S3 $F_3$ progeny testing of $F_2$ phenotypes

| $F_2$ parent plant | | $F_3$ offspring No. of plants | | | |
|---|---|---|---|---|---|
| **Plant designator** | **Phenotype** | **Total** | **Non-infested** | **Infested** | **$\chi^2$(3:1)** |
| 1013/4-5 | non-infested | 11 | 9 | 2 | 0.273 n.s. |
| 1013/4-13 | non-infested | 12 | 12 | - | - |
| 1013/4-15 | non-infested | 12 | 12 | - | - |
| 1013/4-31 | non-infested | 12 | 9 | 3 | 0.000 n.s. |
| 1013/4-32 | non-infested | 11 | 8 | 3 | 0.031 n.s. |
| 1013/4-37 | non-infested | 10 | 7 | 3 | 0.133 n.s. |
| 1013/4-38 | non-infested | 12 | 12 | - | - |
| 1013/4-45 | non-infested | 12 | 10 | 2 | 0.444 n.s. |
| 1013/4-88 | non-infested | 11 | 9 | 2 | 0.273 n.s. |
| 1013/4-90 | non-infested | 11 | 7 | 4 | 0.757 n.s. |
| 1013/4-98 | non-infested | 8 | 0 | 8 | - |
| 1013/4-123 | non-infested | 12 | 12 | - | - |

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

Supplemental Table S4 Molecular markers used for establishing the *LanrBo*-mapint map

| Marker name | cM position | Source | Assay method | F primer (5'–3') | R Primer (5'–3') | GenBank accession number |
|---|---|---|---|---|---|---|
| *BoSeq37* | 0.0 | RNASeq data, this paper | HRM[1] | GCATGAAGAAGGTGCTCTCC | CATGGGATACCCCTTGAACTT | KP760854 |
| *DAFWA3348* | 2.4 | Yang et al. 2013 | HRM[1] | - | - | |
| *E58_M38-140A* | 5.2 | - | AFLP[2] | | | |
| *ArSeq105* | 9.4 | RNASeq data, this paper | HRM[1] | TTCCTATTATCTGACCCTTGAGAA | TGGTCCAGATATCAATACTAAGGG | KP760858 |
| *LaIND_096* | 11.1 | Kamphuis et al. 2014 | Multi Plex Ready PCR[3] | - | - | |
| *BoSeq61* | 13.6 | RNASeq data, this paper | HRM[1] | GAACGCCACTTTGTCAAACA | ATGGATATTTTCAATAGCAATTTGG | KP760855 |
| *LaIND_085* | 15.0 | Kamphuis et al. 2014 | Multi Plex Ready PCR[3] | - | - | |
| *E57_M45-460A* | 24.6 | - | AFLP[2] | - | - | |
| *LJM7_1* | 26.1 | *Lotus japonicus* EST, this paper | Length polymorphism | CTCACCAACCCCTTCTACCA | CAGCCTTCACCACCGTTATT | |
| *AntjM1* | 29.1 | Yang et al. 2004 | Length polymorphism | - | - | |
| *DAFWA7361* | 31.1 | Yang et al. 2013 | HRM[1] | - | - | |
| *LanrBo* | 45.8 | - | - | - | - | |
| *E49_M34-250A* | 50.9 | - | AFLP[2] | - | - | |

[1]High-Resolution Melt Analysis, [2]According to Vos et al. 1995, [3]According to Hayden et al. 2008

Supplemental Table S4 Molecular markers used for establishing the LanrBo-mapint map (continued)

| Marker name | cM position | Source | Assay method | F primer (5'–3') | R Primer (5'–3') | GenBank accession number |
|---|---|---|---|---|---|---|
| *E61_M40-140* | 53.8 | - | AFLP[2] | - | - | |
| *BoSeq196* | 59.5 | RNASeq data, this paper | HRM[1] | CACCCAACATTCCAGCTCTG | CTTCAGCCAAGGAAAAGAAGTC | KP760856 |
| *LJM79* | 61.4 | *Lotus japonicus* EST, this paper | Length polymorphism | GTTCCCATCACCAACAACCT | GGAACGGAAAAATCCATGTG | |
| *E49_M42-535R* | 64.8 | - | AFLP[2] | | | |
| *BoSeq1108* | 68.0 | *RNASeq data, this paper* | HRM[1] | CAAGAAGAAACAGGACAGGACC | TGTGGTGATGGAAGGAAGAGA | KP760857 |
| *E49_M43-120A* | 72.3 | - | AFLP[2] | - | - | |
| *E48_M41-195A* | 73.6 | - | AFLP[2] | - | - | |
| *BoSeq5* | 75.4 | RNASeq data, this paper | HRM[1] | TGCACTTTCTAACCGTTCCA | AAGCTACCGTGGATGCATTT | KP760856 |
| *LaIND_138* | 76.5 | Kamphuis et al. 2014 | Multi Plex Ready PCR[3] | - | - | |
| *DAFWA2850* | 82.1 | Yang et al. 2013 | HRM[1] | - | - | |

[1]High-Resolution Melt Analysis, [2]According to Vos et al. 1995, [3]According to Hayden et al. 2008

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# Erklärung

Erklärung gemäß der Promotionsordnung des Fachbereichs 09 §17(2) (07. Juli 2004)

„Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe.

Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht.

Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Oktober 2017, Kristin Fischer


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