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LpBRI1 polymorphism association with flag leaf architecture in perennial ryegrass

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Abstract

In order to evaluate association between sequence polymorphism in candidate genes and phenotypic traits in perennial ryegrass (*Lolium perenne* L.), mapping population, consisting of 96 genotypes, was assessed for traits related to seed yield with four replications over two years. The traits flag leaf length and width, inflorescence length, spikelet number, seed weight per plant, seed weight per inflorescence, 1000 seed weight and heading date revealed heritability ranging from 0.14 to 0.84 and considerable amount of variation. Marker-trait associations were studied between sequence polymorphism of five candidate genes *LpIAA1*, *LpRUB1*, *LpBRI1*, *LpSHOOT1* and *LpTB1* with putative function in plant architecture and phenotypic traits. Thirteen marker-trait associations were identified in total. Relation between 3 bp INDEL polymorphism in 3'UTR region of *LpBRI1* gene and flag leaf width was confirmed in two consecutive years of field experiment. The possible regulatory role of the identified INDEL is discussed.

Key words: *Lolium perenne*, marker-trait association, plant architecture, seed yield.

Introduction

Perennial ryegrass is one of the most valuable temperate crops widely used for turf or amenity purposes due to its rapid establishment rate and excellent tolerance to traffic (Wilkins, 1991), while superior grazing tolerance, high digestibility and adequate seed production make it the most dominant grass species for forage (Quesenberry, Casler, 2001). It is a self-incompatible, wind-pollinated species that is propagated by seed, thus the economics of seed production has been increasingly important for the commercial success of a new cultivar and plant breeders need selection criteria to ensure a high and stable seed production of new cultivars (Elgersma, 1990 b). Therefore, high and stable seed yield is a key factor for producing a sufficient amount of forage for effective livestock feeding, but in contrast to cereals and edible legumes, the disparate breeding goals for both agronomic yield and seed productions make the seed production of forage grasses complicated. Breeding for improved vegetative traits like leafiness, tillering capacity and persistency of forage species lead to decrease of their ability to produce seeds, but some studies of different forage grass species in various environmental conditions showed promising results. Combining high seed yield with high dry matter production should be possible in grass species by breaking the negative association between hay and seed yields, when considering that breeding for higher seed yield would negatively affect the forage yield and quality (Marshall, Wilkins, 2003). Later studies of smooth bromegrass, narrow-leaved meadow grass and orchardgrass supported the hypothesis by showing a strong positive correlation between seed yield and dry matter yield (Parsa et al., 2012).

Quantitative trait locus (QTL) mapping, identification of markers and candidate genes associated with seed yield components, and the use of comparative genomics in cereal species have revealed several key components which may facilitate development of markers for marker-assisted breeding for the improvement of seed yield (Humphreys et al., 2010). Genes with putative impact on seed yield components have been identified in monocots (Doebley et al., 1995). Moreover, sequence homology to dicotyledonous model crop species *Arabidopsis thaliana* and *Glycine max* has been used to tag gene orthologous to *IAA1*, *RUB1*, *BRI1*, *SHOOT1* and *TB1* with putative functions in plant architecture, axillary tiller formation and hormone response in perennial ryegrass (Brazauskas, Pašakinskienė, 2007).

The objective of this study was to evaluate a perennial ryegrass association mapping population for variation in phenotypic traits related to seed yield, and identify associations between these traits and sequence variants within the *LpIAA1*, *LpRUB1*, *LpBRI1*, *LpSHOOT1* and *LpTB1* genes.

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Materials and methods

A panel of 213 perennial ryegrass (*Lolium perenne* L.) genotypes was established in the field in 2013 as described earlier (Statkevičiūtė et al., 2015). The panel consisted of 91 cultivars, 121 natural ecotypes and one colchicine induced mutant (Pašakinskienė, 2005). A sub-panel consisting of 96 genotypes (44 cultivars, 51 ecotypes and colchicine induced mutant genotype) was randomly selected for sequencing of candidate genes as well as phenotyping evaluation. Flag leaf length, flag leaf width, inflorescence length, spikelet number, seed weight per inflorescence, seed weight per plant, thousand seed weight and heading date were assessed in 2013 and 2014. Flag leaf length and width were measured at full emergence of inflorescences. Three leaves of each plant were measured and the stems were marked. The inflorescence of the marked stems were collected at full ripening stage and dried for the inflorescence length, spikelet number and seed weight per inflorescence determination. Each plant was cut at full ripening stage and dried to constant weight for seed weight per plant and thousand seed weight measurement. The generative and vegetative stems were separated and weighed. The seeds of generative stems were threshed and weighed. A thousand seed weight was calculated with a seed counter Contador (Pfeuffer GmbH, Germany). Pearson correlation coefficients among phenotypic traits and variation coefficients of phenotypic traits among genotypes were calculated with software *STATISTICA 7* (StatSoft Inc., USA). Broad sense heritabilities were estimated with software *PLABSTAT* (Utz, 2011) by the formula $H^2 = \sigma^2_g / (\sigma^2/re + \sigma^2_{ge}/e + \sigma^2_g)$, where *r* is the number of replicates, *e* – the number of environments.

Genomic DNA was isolated from 100 mg of fresh leaf material from each accession according to the modified protocol (Lassner et al., 1989). Gene fragments of *LpIAA1*, *LpBRI1*, *LpRUB1* and *LpSHOOT1* were amplified with primers described in Brazauskas et al. (2010), except for *LpTB1*, where sequences of forward and reverse primers were 5'-TGATCTGCTCCTGCTAGTCCT-3' and 5'-TGCAGATTAGAATCCACGCAAGA-3', respectively. Amplified fragments were cloned and transformed into *Escherichia coli* strain as described in Statkevičiūtė et al. (2015). Five bacterial colonies were isolated for each perennial ryegrass accession. Isolated colonies were propagated on LB (lysogeny broth) medium supplemented with ampicillin. Plasmid extraction was carried out using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Lithuania) according to the manufacturer's protocol. The fragments were sequenced by GATC Biotech (GATC Biotech AG, Germany). Each fragment was sequenced from both strands using forward and reverse pJET 1.2 sequencing primers. Sequence chromatograms were assembled into contigs using Chromas Pro v.1.7.5 (Technelysium Pty Ltd., Australia). Obtained sequences were aligned using software *MEGA6* (Tamura et al., 2013).

Association analysis between single nucleotide polymorphisms (SNPs) and phenotypic traits was performed using a mixed linear model (MLM) implemented in the software *Tassel v.4.3* (Bradbury et al., 2007). SNPs with minor allele frequency of less than 5% were excluded from further analysis. Population structure and relative kinship estimations were based on AFLP markers as described in Aleliūnas et al. (2015). Specific primers, flanking 3bp INDEL polymorphism in *LpBRI1* gene, were used to identify allelic variation for this INDEL in broader perennial ryegrass population, consisting of 213 genotypes. Sequences of forward and reverse primers were 5'-GACTGGGAGGCGGTACAAG-3' and 5'-CGACCTCACATGGAAACAGGAG-3', respectively. The amplified product was 120 (deletion) or 123 (insertion) bp long. PCR products were separated on 6.5% poly-acrylamide gels in DNA Analyzer 4300 (Licor). Fragments were scored using software *SAGA Generation 2* (Licor). The phenotypic means of marker genotype classes were compared using post-hoc Unequal N HSD test implemented in software *STATISTICA 7*.

Results

Phenotypic variation. Phenotypic data was collected over two consecutive years – 2013 and 2014, characterized by contrasting meteorological conditions. The spring of 2013 was late; therefore the first panel of genotypes could be transferred from the greenhouse to the field only in the second half of April. The autumn was warm and the vegetation continued until the end of December, thus the plants in the second field test group had better conditions to establish and develop roots. Different growth conditions determined that the values of phenotypic traits in 2014 were higher compared with those in 2013 (Table 1). Low variation between genotypes was established according to heading date (CV% 1.48 and 2.72) in both years and for spikelet number (CV% 9.83) in 2014. Moderate variation was determined for flag leaf length, flag leaf width, inflorescence length, spikelet number and thousand seed weight in 2013 (CV% 10.74–19.18) and flag leaf width, inflorescence length and thousand seed weight in 2014 (CV% 10.19–15.04). High polymorphism between genotypes was established

Table 1. Variation and heritability of phenotypic traits in 96 perennial ryegrass genotypes

Trait	2013		2014		Heritability
	mean ± SD	CV%	mean ± SD	CV%	
Flag leaf length cm	9.60 ± 2.81	19.18	13.41 ± 3.02	20.08	0.70
Flag leaf width cm	0.43 ± 0.09	12.91	0.46 ± 0.07	13.11	0.71
Inflorescence length cm	14.47 ± 3.19	10.74	20.33 ± 3.63	10.19	0.84
Spikelet number	18.14 ± 3.16	12.49	22.57 ± 3.66	9.83	0.70
Seed weight per inflorescence g	0.14 ± 0.07	52.97	0.19 ± 0.07	28.15	0.63
Seed weight per plant g	1.94 ± 1.29	59.00	20.03 ± 10.26	40.00	0.14
Thousand seed weight g	2.03 ± 0.37	14.43	1.95 ± 0.37	15.04	0.68
Heading date	21 May ± 4.30	1.48	3 June ± 8.06	2.72	0.77

SD – standard deviation, CV% – coefficient of variation

for other traits. Strong (0.730) positive correlation ($p < 0.01$) was determined between flag leaf width and flag leaf length in 2013 and moderate (0.559) in 2014. The seed weight per inflorescence moderately correlated with the spikelet number in both years and with the flag leaf length in 2014 (Table 2).

Table 2. The relationship between phenotypic traits of perennial ryegrass genotypes (upper triangle – correlation coefficients in 2014, lower triangle – in 2013)

	Flag leaf length	Flag leaf width	Inflorescence length	Spikelet number	Seed weight per inflorescence	Seed weight per plant	Thousand seed weight	Heading date
Flag leaf length	–	0.56**	0.68**	0.50**	0.64**	0.53**	0.07	0.15
Flag leaf width	0.73**	–	0.52**	0.23*	0.60**	0.39**	0.31**	−0.09
Inflorescence length	0.78**	0.62**	–	0.60**	0.66**	0.44**	0.08	0.17
Spikelet number	0.50**	0.47**	0.54**	–	0.52**	0.40**	−0.33**	0.28**
Seed weight per inflorescence	0.36**	0.35**	0.45**	0.49**	–	0.57**	0.12	0.03
Seed weight per plant	−0.03	−0.11	0.15	0.06	0.24*	–	0.03	−0.21*
Thousand seed weight	0.20	0.28**	0.33**	−0.05	0.09	0.22*	–	−0.50**
Heading date	0.38	0.25*	0.01	0.21*	−0.08	−0.44	−0.45	–

* – $P \leq 0.05$, ** – $P \leq 0.01$

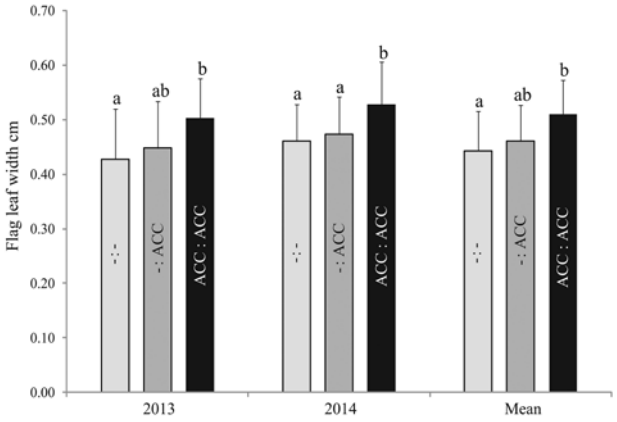
Marker-trait associations. Associations were estimated between twelve phenotypic traits and 270 polymorphic sites in five genes in total. The analysis yielded twelve marker-trait associations with $p < 0.01$ and one with $P < 0.001$ (Table 3). One single nucleotide polymorphism (SNP) in noncoding region was associated ($P = 0.009$) with inflorescence length in 2014 in *LpIAA1* gene. The only non-synonymous substitution associated with phenotypic trait was detected in *LpTB1*, where SNP causing amino acid change from asparagine to tyrosine was associated with seed weight per plant in 2014 ($P = 0.007$). The marker-trait association with the lowest *P* value (0.00055) was detected in *LpBRI1*. The 3 bp long INDEL at the position 1155–1157 of *LpBRI1* was associated with flag leaf width in 2014.

Table 3. Associations between phenotypic traits and sequence polymorphism in 5 perennial ryegrass genotypes

Locus	Trait	*P*	Allele	Effect	n
LpIAA1 SNP-278 (N)	inflorescence length 2014	0.0092	C:A	2.41	31
			C:C	−0.17	40
			A:A	0	21
LpBRI1 SNP-964 (N)	flag leaf width 2013	0.00176	A:A	0.10	9
			T:A	0.04	28
			T:T	0	54
	flag leaf width 2014	0.0014	A:A	0.09	10
			T:A	0.02	27
			T:T	0	58
	seed weight per inflorescence 2013	0.00478	A:A	0.08	9
			T:A	0.03	27
			T:T	0	55
	spikelet number 2013	0.00773	A:A	3.34	9
			T:A	−0.35	27
			T:T	0	55
	inflorescence length 2013	0.00852	A:A	3.72	9
			T:A	0.59	27
			T:T	0	55
LpBRI1 INDEL 1155-1157 (N)	flag leaf width 2013	0.00326	-:ACC	−0.06	25
			-:-	−0.1	58
			ACC:ACC	0	8
	flag leaf width 2014	0.00055	-:ACC	−0.09	24
			-:-	−0.1	61
			ACC:ACC	0	8
	seed weight per inflorescence 2013	0.00246	-:ACC	−0.05	25
			-:-	−0.09	58
			ACC:ACC	0	8
	spikelet number 2013	0.00442	-:ACC	−4.17	25
			-:-	−3.66	58
			ACC:ACC	0	8
	inflorescence length 2013	0.00974	-:ACC	−0.29	25
			-:-	−0.38	58
			ACC:ACC	0	8
LpSHOOT1 SNP-602 (Cs)	seed weight per plant 2014	0.00331	C:C	−0.12	78
			T:T	−0.17	3
			C:T	0	10
LpTB1 SNP-371 (Cm)	seed weight per plant 2014	0.00724	G:T	0.057	20
			T:T	0.049	25
			G:G	0	29

n – number of genotypes in allelic group; N – non-coding sequence, Cs – coding sequence, synonymous substitution, Cm – coding sequence, missense mutation

Analysis of variance (*ANOVA*) revealed that the flag leaves of plants harbouring insertion were wider ($P < 0.05$) compared to genotypes with deletion (Fig.). The same INDEL was also associated with seed weight per inflorescence ($P = 0.002$), spikelet number ($P = 0.004$) and inflorescence length ($P = 0.01$) in 2013. SNP 190 bp upstream from the INDEL was associated with the same phenotypic traits. A synonymous substitution in *LpSHOOT1* gene was associated with seed weight per plant ($P = 0.003$) in 2014. The association between flag leaf width and polymorphism in *LpBRI1* gene was the only one confirmed in both 2013 and 2014 in an association mapping panel of 96 genotypes. The sizes of DEL/DEL, DEL/IN and IN/IN genotypic groups were as follows: 61, 25 and 8 (*LpBRI1* sequence of two genotypes did not amplify).



Note. The INDEL position in the alignment was 1155–1157; values with the same letter are not significantly different ($p < 0.05$); the error bars represent standard deviation of the group mean.

Figure. Marker-trait association and variant alleles of *LpBRI1* gene

A total of 122 genotypes homozygous for deletion, 77 heterozygous genotypes and 14 genotypes homozygous for insertion were identified in a larger set of 213 genotypes. All IN/IN genotypes were natural ecotypes, collected in Lithuania and Ukraine.

Discussion

Association mapping panel consisting of 44 cultivars of both turf and forage type and 52 natural ecotypes used in this study revealed high variability for phenotypic traits as could be expected for genotypes of such a diverse origin (Jonavičienė et al., 2014). High heritabilities were observed for the studied seed yield traits (Table 1), even though previous study of Casler et al. (1996) stated that the seed yield appears to be a moderately heritable trait. The panicle length, known to be governed by genetic factors with minimal environmental influence, has shown highest heritability, thus selection for this trait would be effective in future breeding programs as it is likely to be controlled by additive genes (Byrne et al., 2009).

The number of spikelets per inflorescence was in accordance with the typical number reported by Humphreys et al. (2010). Moreover, this trait was highly heritable as it was reported previously by Byrne et al. (2009). Although the number of spikelets and florets per inflorescence may vary to some extent from season to season and between cultivars, this component did not have large effect on seed yield in perennial ryegrass (Elgersma, 1990 a). The seed yield improvement is more likely to be attained through increasing the number of seeds per spikelet and improved floret site utilization (Humphreys et al., 2010). A positive correlation between spike length and the number of spikelets per spike found in this study confirms previous report of Casler et al. (1996) and could be considered when breeding for improved seed yield.

Besides other numerous environmental factors, such as nitrogen availability, water availability and disease incidence, affecting fertility of the grasses, the timing of flowering plays one of the most important roles in forming the seed yield. Yet the interrelationships between heading date and seed traits were not identified in this study. High heritability of heading date, observed among the genotypes of our collection (Table 1) was reported in some earlier studies (Wedderburn et al., 1992) suggesting that achieving desired earliness in newly bred cultivars should be relatively straightforward.

Seed yield was shown to be mostly influenced by seed yield per inflorescence by Studer et al. (2008), yet in this study the correlation between these traits was moderate (in 2014) or low (in 2013). The different experiment conditions could explain contrasting results as this study was carried out in natural conditions while the experiment by Studer et al. (2008) was performed in a greenhouse enabling reduction of the influence of environment. The contradictory outcomes of the experiments confirm seed yield being a complex trait, affected by agricultural practices and environmental factors, including biotic and/or abiotic stresses. The reduction of green flag leaf area due to disease infection was shown to have a negative effect on grain yield and mean grain weight in wheat (Brathal et al., 2003). Genetic determination of flag leaf architecture and its relation with plant productivity have been studied in major crops (Chen et al., 2010; Tian et al., 2011; Wang et al., 2012) as well as in perennial ryegrass (Studer et al., 2008). The positive effect of flag leaf size of meadow fescue on seed yield was demonstrated by Fang et al. (2004). Yamada et al. (2004) reported about a QTL for flag leaf width identified on linkage group (LG) 3 in rice, whereas in a later study on perennial ryegrass by Studer et al. (2008) the QTL determining flag leaf width was identified on LG 5 and explained 21.7% of phenotypic variation. In this study, flag leaf was associated with INDEL in 3'UTR region of *LpBRI1* gene, located on LG 3 in perennial ryegrass. The gene codes for cell surface receptor kinase brassinosteroid-insensitive 1 (BRI1) (reviewed in Fariduddin et al., 2014) and brassinosteroids (BR), plant-originated steroidal lactones, are well known for promoting growth (Choe, 2006), increasing the leaf number and area (Bhat et al., 2011) as well as having a favourable effect on the yield of plants (Janeczko et al., 2010). The INDEL polymorphism identified in this study cannot alter the activity of the protein through change in amino acid composition as it is allocated in non-coding region. However, the importance of the non-coding intragenic sequences in regulation of gene expression in plants and other eukaryotes are undoubted (reviewed in Rose, 2008) considering the impact of 3-UTR's as well. Long 3-UTRs or the presence of introns in the 3-UTR can subject mRNAs to nonsense-mediated mRNR decay in plants (Kertesz et al., 2006). Down-regulation of *BRI1* expression has been demonstrated to have paramount

effect on plant phenotypic performance. *Brachypodium distachyon* BRI1-RNAi mutants with decreased *BRI1* gene expression level showed decreased plant height with compact stature, narrow and short leaf, short internode, decreased BR response, and modulated expression level of BR-related genes (Feng et al., 2015). Barley uzu1.a mutant with altered brassinosteroid signalling due to amino acid exchange in *HvBRI1* gene was shown to have altered leaf architecture, besides other typical BR-insensitive phenotypic traits (Dockter et al., 2014), and similar effect was observed for maize plants also exhibited shorter leaf blades and sheathes after knock-down of the expression of *BRI1* homologs (Kir et al., 2015). Even though most researches primarily stress the influence of genes involved in brassinosteroid signalling on plant height, the impact on leaf architecture is also apparent. The positive effect on flag leaf width was observed for the group of Lithuanian and Ukrainian genotypes which harboured 3 bp insertion in the non-coding sequence of *LpBRI1*. The genotypes had significantly wider flag leaves compared to genotypes with deletion, and the leaves of heterozygous genotypes also tended to be wider. Narrower leaves were observed in a group of genotypes, representing turf type cultivars of the collection. Whilst longer and wider leaves are favourable trait when breeding for higher yield and indirectly for higher seed yield, plants with narrow leaves have wide application in turf for sport fields or in the areas for recreation. The group of narrow-leaved genotypes consisted of sixteen genotypes having the deletion and six genotypes being heterozygous in the locus associated with flag width. There seems to be a strong bias towards deletion in this non-coding sequence at the 3'UTR end of putative *LpBRI1* sequence in acquired genotype collection, yet there is not enough information about *BRI1* gene structure and function in perennial ryegrass for a firm conclusion about the role of identified polymorphism. We propose that the 3 bp insertion might play a role in up-regulating *LpBRI1* expression in homozygous plants based on the results of this study.

Conclusions

1. The association between 3 bp INDEL polymorphism in 3'UTR region of *LpBRI1* gene and flag leaf width was confirmed in perennial ryegrass (*Lolium perenne* L.) mapping panel.

2. The genotypes which harboured deletion in the identified locus had significantly wider flag leaves compared to genotypes with deletion.

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Daugiametės svidrės *LpBRI1* geno sekos polimorfizmo ir lapų architektūros sąsajų analizė

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Santrauka

Sėklų derlių lemiantys požymiai buvo tirti 96 genotipų daugiametės svidrės populiacijoje; tyrimai atlikti keturiais pakartojimais dvejus metus. Buvo nustatyta didelė viršūninio lapo ilgio ir pločio, žiedyno ilgio, varpučių skaičiaus, vieno augalo sėklų masės, vieno žiedyno sėklų masės, 1000 sėklų masės ir plaukėjimo variacija. Šių požymių paveldimumas svyravo nuo 0,14 iki 0,84. Atlikus tirtų požymių ir kandidatinių genų *LpIAA1*, *LpRUB1*, *LpBRI1*, *LpSHOOT1* bei *LpTB1* sekų polimorfizmo sąsajų analizes buvo identifikuotos sąsajos tarp fenotipinių sėklų derliaus požymių ir šiuose genuose esančių trylikos polimorfiškų lokusų. Dvejų metų lauko eksperimento duomenimis nustatyta, kad *LpBRI1* geno 3'UTR regione esanti 3 bp iškrita (INDEL) lemia viršūninio lapo plotį; taip pat aptarta ir galima šio geno įtaka reguliaciniams procesams.

Reikšminiai žodžiai: asociacinė analizė, augalo architektūra, *Lolium perenne*, sėklų derlius.