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ORIGINAL PAPER

Characterization of a QTL affecting spike morphology on the long arm of chromosome 3H in barley (*Hordeum vulgare* L.) based on near isogenic lines and a NIL-derived population

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Abstract Traits related to spike morphology (SM), including grain density (GD), spike length (SL) and awn length (AL), are of central importance in cereal improvement. A recent study based on a two-row landrace of barley, TX9425, detected QTL controlling all of the three traits in a similar region on the long arm of chromosome 3H. To further characterize this chromosomal region, 12 pairs of near isogenic lines (NILs) for GD were generated from two populations between TX9425 and two different commercial cultivars. A population consisting of 1,028 lines segregating primarily for the target region was also developed using materials generated during the production of these NILs. Results from the analysis of the NILs and the NIL-derived population showed that these three traits were likely controlled by a single-locus which was mapped to a 2.84 cM interval between two SSR markers, GBM1495 and HVM33. Across the 12 pairs of NILs, the presence of the 3HL locus increased GD by 53.4 %, reduced SL and AL by 38.8 % and 62.7 %, respectively. In the NIL-derived population, the presence of the 3HL locus increased GD by 64.6 %, reduced SL and AL by 33.7 % and 62.6 %, respectively. An interesting question arising from this research is why some loci such as the one reported here affect several SM-related traits while others appear to affect one of these traits only. The NILs and the NIL-derived population generated in this study will help answer such questions by providing the germplasm to enable cloning and comparative analysis of the genes responsible for these SM-related traits.

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Introduction

Spike length (SL) and grain density (GD) are two of the most important traits of spike morphology (SM) in barley. They not only affect barley grain yield potential, but also affect the yield of malt extract (Wang et al. 2010). Loci controlling SL have been reported on five of the seven barley chromosomes

including 2H (Kjær et al. 1995; Hori et al. 2003; Sameri et al. 2006; Wang et al. 2010), 3H (Sameri et al. 2006; Wang et al. 2010), 4H (Baghizadeh et al. 2007), 5H (Hori et al. 2003) and 7H (Sameri et al. 2006). Loci affecting GD have been detected on three of the seven barley chromosomes: 2H (Wang et al. 2010), 3H (Wang et al. 2010; Shahinnia et al. 2012) and 7H (Shahinnia et al. 2012). The 7H locus controlling GD was recently mapped to a 0.37 cM interval near the centromere (Shahinnia et al. 2012).

The presence of awns is another characteristic of spike morphology with known effects on yield potential in barley. The main physiologic functions of awns are photosynthesis and transpiration (Grundbacher 1963). Even though its significance in transpiration is debatable, as an assimilatory organ awns may contribute as much as 10 % toward grain yield (Kjack and Witters 1974). Short awned cultivars are rare in western countries but they are not uncommon in eastern Asia (Takahashi 1987). Three QTL conferring awn length (AL) have been reported as located on chromosomes 2H, 3H and 7H (Sameri et al. 2006; Wang et al. 2010).

Relationships between those loci on the same chromosomes seem to vary between genotypes. QTL controlling SL, GD and AL in some genotypes were located at similar chromosomal regions (Sameri et al. 2006; Wang et al. 2010). However, close linkages between QTL controlling SL and GD seem clear in other genotypes (Zhang 2000). The likelihood that these SM-related traits can be controlled by different genes is also supported by the existences of genotypes with dense spikes and short awns (Wang et al. 2010) as well as genotypes with dense spikes and long awns (Shahinnia et al. 2012).

With the use of a doubled haploid population, QTL controlling GD, SL and AL were detected at a similar chromosomal region on the long arm of chromosome 3H from a two-row barley landrace, TX9425 (Wang et al. 2010). As QTL mapping using segregating populations has limited resolution (Paterson et al. 1988), the QTL mapping results could not tell if these traits are controlled by closely linked loci or by a single locus with pleiotropic effects. Clarifying the relationship between loci controlling these traits is important for their effective manipulation in a breeding program. Working toward such an objective, several sets of NILs for GD and a large population segregating primarily for the 3HL chromosomal region were generated and analyzed. The results based on the analysis of these three SM-related traits in these genetic stocks are reported in this paper.

Materials and methods

Development and assessment of near isogenic lines (NILs) for the targeted 3HL locus

The heterogeneous inbred family (HIF) method (Tuinstra et al. 1997) was used to develop NILs for the grain density locus in TX9425 from two F_2 populations, TX9425/Franklin and TX9425/Gairdner. TX9425 is characterized by the trait combination of dense, short spikes and short awns (DSS) and both Franklin and Gairdner are characterized by the combination of loose, long spikes and long awns (LLL). A single co-dominant SSR marker HVM33, which was the SSR marker most closely linked to the peak of the QTL controlling these traits in the DH population (Wang et al. 2010), was used to select F_2 individuals heterozygous at the marker locus following the method as described by Ma et al. (2012). Briefly, heterozygous F_2 plants were identified and self-pollinated. Six F_3 plants from each of the heterozygous plants were raised and a single heterozygous plant was again selected and self-pollinated. This process of selecting heterozygous individuals and self-pollination was repeated until the F_8 generation. Two homozygous lines, one with and the other without the allele of TX9425, were then isolated from each of the F_8 heterozygous plants and were treated as a pair of NILs.

The NILs were assessed by two trials, one pot trial in a glasshouse and the other in a field trial conducted at the CSIRO Research Station at Gatton in Queensland (27°34′S, 152°20′E). The pot trial was conducted using six plants, each in a different pot of 2.0 L. Measurements for each of the traits were obtained from the two tallest tillers for each plant and their averages were used for statistical analysis. The field trial was sown in June 2011. Twenty seeds for each of the NILs were grown in a single 1.5 m row with a 25 cm row spacing. Six measurements were obtained for each trait from the six tallest tillers in each row and the average from the six measurements was used for statistical analysis. Spike length (in centimeter or cm) was measured from the base of a spike to the tip of the terminal spikelet (excluding awns). Length of awns (in cm) was measured from the base of awn to the tip of the terminal. Grain density was measured as the average number of rachis internodes per cm.

Development and assessment of a population segregating primarily for the targeted QTL on 3HL

Three F_7 plants (all belonging to the TX9425/Franklin cross) heterozygous at the HVM33 SSR marker locus were used to generate a population segregating primarily for the targeted locus on 3HL. Self-pollinated seeds from the three F_7 plants were used to generate the population, which consisted of a total of 1,028 individuals. Considering the segregation of the targeted chromosomal region, this large mapping population is equivalent to an F_2 population. Thus, the NIL-derived population was named as $NILF_2$. Each of the $NILF_2$ plants was grown in a 2.0 L pot in a glasshouse. DNA from each of the $NILF_2$ plants was

extracted and genotyped using molecular markers. SL, GD and AL were assessed using three of the tallest tillers for each plant. The NIL-derived population was then further assessed in two field trials, one at the Mt Pleasant Laboratories in Tasmania (47°46′S, 147°14′E) (sown in July 2010) and the other at the CSIRO Research Station at Gatton in Queensland (sown in June 2011). A single row plot consisting of about 15 $NILF_3$ plants was used for each of the two field trials. Thus the total number of plants grown between these two field trials was over 30,000. The trial at Mt Pleasant Laboratories used 2.0 m row plots with 0.4 m row spacing . The trial at Gatton used 1.5 m row plots and 0.3 m row spacing. For those lines which did not segregate, six tillers in the middle of each row were selected for measuring SL, GD and AL. For those lines segregating for the traits of interest, six measurements were taken for each of the spike types. The mean values of the six measurements were used for statistical analysis.

Molecular marker analysis and linkage map construction

Methods used for the DNA isolation and marker analysis were as described by Li et al. (2009). PCR reactions for the amplification of SSRs were carried out in a total volume of 12 µl containing 25 ng genomic DNA, 0.2 µM of forward and reverse primer, 3 mM $MgCl_2$, 0.2 mM dNTPs and 0.5 U Taq DNA polymerase. During PCR reactions the marker products were labeled with α-[^{33}P]dCTP (3,000 ci/mmol). Reactions were run on a Gene Amp PCR System 2700 thermocycler (PE Applied Biosystems, Foster City, CA) programmed with the cycling conditions: one cycle of 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at the appropriate annealing temperature (ranging from 50 °C to 56 °C depending on the marker) and 1 min at 72 °C, with a final extension step of 5 min at 72 °C. The amplified products were then mixed with an equal volume of loading dye, denatured at 95 °C for 5 min, and 3.8 µl samples was run on a denaturing 5 % polyacrylamide (20:1) gel at 90 W for 2 h. The gels were subsequently dried using a gel dryer for 30 min at 80 °C and exposed to Kodak X-Omat X-ray film for 4–6 days. A total of 32 SSR, 14 CAPS (cleaved amplified polymorphic sequences), 4 SNP and 4 Indel markers were used to analyze the 12 pairs of NILs. Ten of the CAPS markers were derived from the EST sequences published earlier and the other four were derived from DArT marker sequences generated in this study (Table S1). Polymorphic markers among the NIL pairs were used in analyzing the NIL-derived population. Linkage analysis was carried out using the computer package JoinMap (version 4.0, Van Ooijen 2006).

Data analysis

Statistical analysis were performed using the GenStat for Windows, 13th edition (copyright Lawes Agricultural Trust, Rothamsted Experimental Station, UK) and the SPSS statistics 17.0 for Windows statistical software package (SPSS, Inc., Chicago, IL). For each trial, the following mixed-effects model was used:

$$Yij = \mu + ri + gj + wij$$

where Yij = observation on the jth genotype in the ith replication; μ = general mean; ri = effect due to ith replication; gj = effect due to the jth genotype; wij = error or genotype by replication interaction, where genotype was treated as a fixed effect and that of replicate as random and that of genotypes fixed. The effects of replicate and genotype for each trait were determined using ANOVA. Pearson correlation coefficient was estimated between traits and trials. The Duncan's new multiple range test of One-Way ANOVA analysis (Duncan 1955) was employed to detect possible differences among the means.

Results

Genetics of SL, GD and AL based on the analysis of near isogenic lines

When assessed using SSR marker HMV33, five heterozygous plants were identified from the cross TX9425/Franklin and seven from the cross TX9425/Gairdner. Similar to those individuals with homozygous Franklin or Gairdner alleles, all of the 12 heterozygous individuals were characterized by the trait combination of LLL. However, they produced both DSS and LLL progenies. All of the plants obtained during the generation of the NILs gave the phenotypic combination of either DSS or LLL but no other combination of the three traits was observed. As only six plants were grown for each of the heterozygous individuals identified at each generation, the numbers of progeny were too small to analyze the segregation ratios between LLL and DSS individuals. However, the ratio of LLL to DSS individuals seemed to fit the expected 3:1 segregation ($p < 0.01$) for a single Mendelian factor, with DSS being recessive. The two isolines for each of the 12 pairs of NILs showed the contrasting phenotypic combinations of DSS and LLL (Fig. 1).

Analysis of variance showed that genotypic effects for each of the three traits as well as the trial effects were all highly significant in the trials conducted on the 12 pairs of NILs (Table 1). Differences between the two isolines for each of the three traits were all highly significant for each of the 12 NIL pairs in the field (Fig. 2) as well as in the

glasshouse trial (Table S2). The average GD across the 12 DSS isolines was 5.40 and that for the 12 LLL isolines was 3.52, showing that the allele from TX9425 increased GD by 34.8 % on average across the 12 pairs of NILs. The average SL across the 12 DSS isolines was 6.14 and that across the 12 LLL isolines was 9.26, showing that the TX9425 allele reduced spike length by 33.7 % on average. The average AL across the 12 DSS isolines was 3.29 and that across the 12 LLL isolines was 8.80, showing that the TX9425 allele reduced AL by 62.6 % on average. Highly significant correlations were detected among the three traits across the 12 pairs of NILs are at both the glasshouse and the Gatton field trials (Table S3).

Fig. 1 Different spike morphology of two isolines for one of the NIL pairs obtained in this study: dense, short spike and short awns (DSS) (*left*) and long, loose spike and long awns (LLL) (*right*). The length of the bar is 2 cm

Genetics of SL, GD and AL based on the analysis of a population segregating primarily for the targeted chromosome segment

Of the 1,028 $NILF_2$ plants assessed, 770 gave the LLL phenotype and the other 258 DSS. None of the $NILF_2$ plants showed any other combination of the three traits. The segregation ratio of 770:258 fits the expected 3:1 ratio for a single Mendelian factor ($p < 0.001$) with DSS being recessive.

The 258 $NILF_2$ plants with DSS phenotype produced only DSS progenies at the Gatton trial thus they were classified as homozygous recessive. SL of these $NILF_3$ plants ranged from 5.08 to 7.49 with an average of 6.37, GD ranged from 5.12 to 6.39 with an average of 5.65, and AL ranged from 2.63 to 4.57 with an average of 3.48. Of the 770 $NILF_2$ plants characterized as LLL, 278 produced only LLL plants and thus they were classified as homozygous dominant. SL of these $NILF_3$ plants ranged from 8.38 to 12.88 with an average of 10.05, GD ranged from 2.75 to 3.70 with an average of 3.31, and AL ranged from 7.52 to 11.33 with an average of 9.19. The other 492 $NILF_2$ plants characterized as LLL produced both DSS and LLL progenies thus they were classified as heterozygous. SL of the lines with DSS phenotypic combination derived from these heterozygous $NILF_2$ plants ranged from 4.52 to 7.27 with an average of 6.21, GD ranged from 4.92 to 6.57 with an average of 5.70, and AL ranged from 2.77 to 4.40 with an average of 3.48. SL of the lines with LLL phenotypic combination derived from these heterozygous $NILF_2$ plants ranged from 8.40 to 11.87 with an average of 9.93, GD ranged from 2.84 to 3.78 with an average of 3.38, and AL ranged from 7.53 to 11.65 with an average of 9.04. The distributions and averages of the three traits among the $NILF_3$ lines produced from either the homozygous or heterozygous $NILF_2$ plants were not significantly different (Fig. 3). The ratio of 258:492:278 fits the expected 1:2:1 ($p < 0.01$) ratio for a single Mendelian factor. Similarly, the 258 $NILF_2$ plants characterized by DSS also produced only DSS progenies at the Mt Pleasant trial. Of the 770 $NILF_2$ characterized by LLL, 279 produced LLL progenies only and the other 491 produced both LLL and DSS progenies at the Mt Pleasant trial. The ratio of 258:491:279 again fits the expected 1:2:1 segregation ($p < 0.01$) for a single Mendelian factor. Highly significant correlations were detected among the three traits measured at either the Gatton or the Mt Pleasant trial (Table 2). Across the two field trials, the presence of the TX9425 allele increased GD

Table 1 Variance analysis of awn length, spike length and grain density across the 12 pairs of NILs

Source	Dependent variable	*df*	Mean square	*F*	*P*
Genotype	AL	23	144.2	134.5	< 0.01
	SL	23	35.8	84.6	< 0.01
	GD	23	13.6	105.2	< 0.01
Trial	AL	1	188.3	175.7	< 0.01
	SL	1	28.7	67.8	< 0.01
	GD	1	9.1	70.4	< 0.01
Residual	AL	263	1.1		
	SL	263	0.4		
	GD	263	0.1		

AL awn length, *SL* spike length, *GD* grain density

by 64.6 %, decreased SL by 38.8 %, and reduced AL by 62.7 % on average. Similar to those observed among the $NILF_2$ plants, the over 30,000 $NILF_3$ plants at the two field trials could also be easily placed into either the DSS or LLL group. Other combination of the three traits was not observed from any of the over 30,000 $NILF_3$ plants tested at the two field trials.

Progenies produced by five of the $NILF_2$ plants were different between the two field trials. All of the five $NILF_2$ plants were characterized by the LLL phenotypic combination. Two of the $NILF_2$ produced only LLL plants at the Gatton trial but they produced both LLL and DSS plants at the Mt Pleasant trial. Similarly, three of the $NILF_2$ plants produced only LLL plants at the Mt Pleasant trial but they all produced both LLL and DSS $NILF_3$ plants at the Gatton trial. The difference was most likely due to the limited numbers of plants grown for each of the $NILF_2$ plants. Thus, all of these five $NILF_2$ plants were treated as heterozygous individuals in the mapping analysis (below).

Genetic mapping of the targeted 3HL region using the NIL-derived population

Of the 32 SSR, 14 CAPS, 4 SNP and 4 Indel markers tested (Table S1), seven were polymorphic among the 1,028 $NILF_2$ plants. They formed a linkage group covering a total

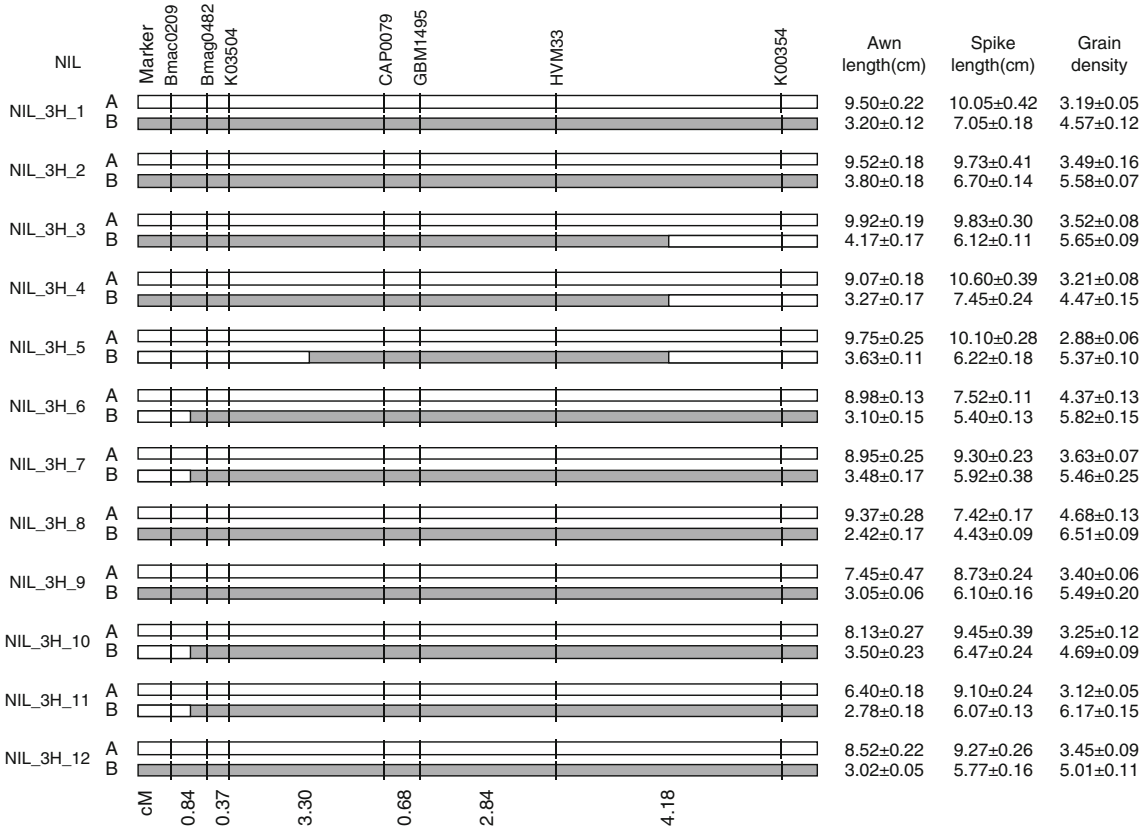

Fig. 2 Genotypes of NILs used for mapping the locus controlling DSS for the targeted chromosomal region on chromosome 3H. Chromosomal regions for the DSS lines with TX9425 alleles are depicted in *dark grey*, and those for the LLL with either Franklin or Gairdner alleles are in *white*. Marker loci and centiMorgan scale are indicated according to their genetic distances estimated from the NIL-derived population. Phenotypic values (mean ± SE) of the 12 pairs of NILs from the Gatton field trial

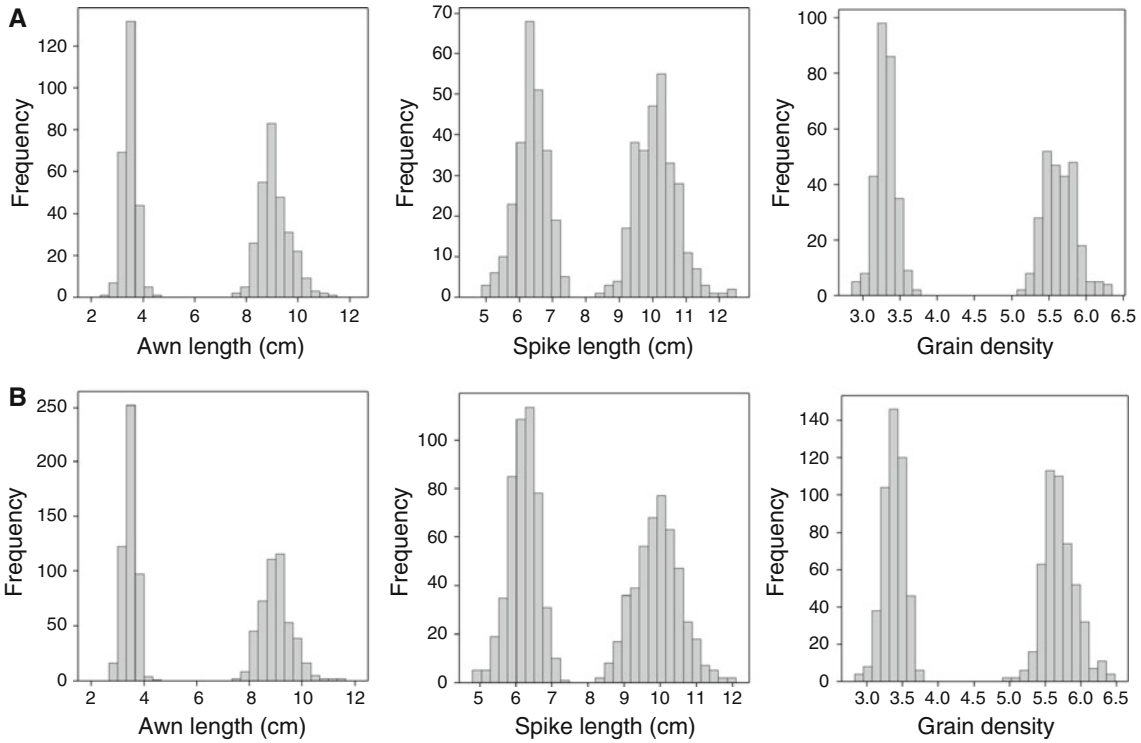

Fig. 3 Distributions of awn length, spike length and grain density of the $NILF_3$ lines derived from (**a**) the 258 homozygous DSS and the 278 homozygous LLL $NILF_2$ plants, and (**b**) the 492 heterozygous $NILF_2$ plants

Table 2 Correlation coefficients between awn length, spike length and grain density obtained from the NIL-derived population

Trial	Trait	Gatton			Mt Pleasant		
		AL	SL	GD	AL	SL	GD
Gatton	AL	1.00					
	SL	0.91**	1.00				
	GD	−0.98**	−0.93**	1.00			
Mt Pleasant	AL	0.94**	0.85**	−0.94**	1.00		
	SL	0.84**	0.77**	−0.83**	0.84**	1.00	
	GD	−0.94**	−0.86**	0.94**	−0.95**	−0.89**	1.00

AL awn length, *SL* spike length, and *GD* grain density

** Correlation significant at $p < 0.01$

distance of 12.70 cM and the locus controlling the three different SM-related traits was mapped between markers GBM1495 and HVM33 (Fig. 4). As both of the flanking markers are known to be on the long arm of chromosome 3H, the locus controlling these traits of spike morphology is on this chromosome arm.

Based on the profiles of the seven polymorphic markers, the 258 homozygous DSS lines could be placed into six groups. The numbers of loci with TX9425 alleles varied from one (6 lines) to all seven (185 lines). However, significant differences were not detected for any of the three traits among the six groups of DSS lines (Fig. 4).

Structured mapping of the targeted 3HL region using NILs

Based on the genetic profiles of the seven polymorphic markers, the 12 DSS isolines could be placed into four different groups: alleles for all of the seven markers were derived from TX9425 for DSS isolines belonging to NIL pairs 1, 2, 8, 9 and 12; alleles for all but marker K00354 were derived from TX9425 for DSS isolines belonging to NIL pairs 3 and 4; alleles for all but marker Bmac0209 were derived from TX9425 for DSS isolines belonging to the NIL pairs 6, 7, 10 and 11; and alleles for three of the

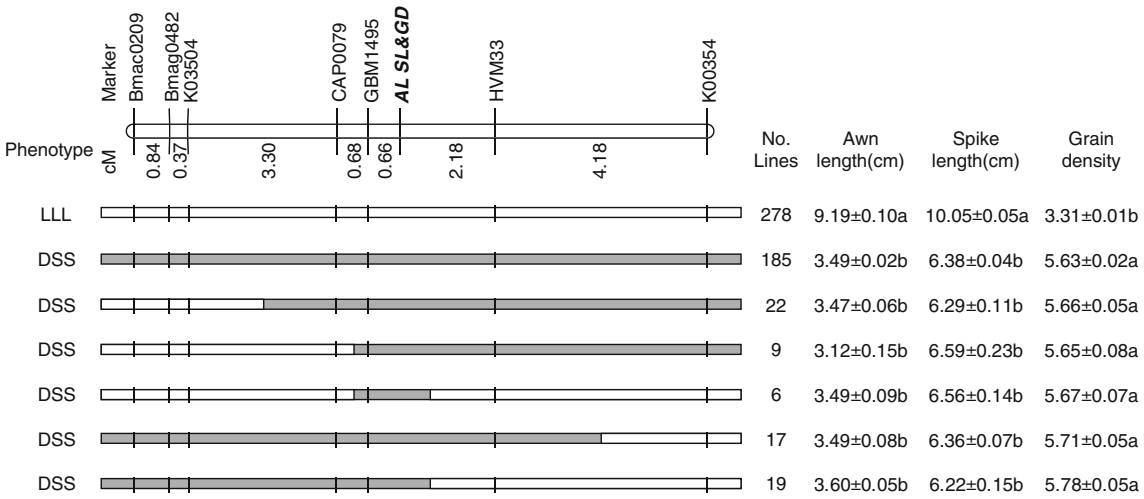

Fig. 4 A linkage map obtained from analyzing the NIL-derived population. Chromosomal regions with TX9425 alleles are depicted in *dark grey*, and those with Franklin alleles are in *white* (*left*). Phenotypic values (mean ± SE) of the homozygous DSS and LLL plants were from the NIL-derived population grown at the Gatton field trial. The different letters denote statistically significant differences at $P < 0.05$ based on the One-Way ANOVA Duncan's new multiple range test

markers, CAP0079, GBM1495 and HVM33 were derived from TX9425 for the DSS isoline belonging to the NIL pairs 5 (Fig. 2). Based on these marker profiles among the 12 pairs of NILs, the locus controlling DSS could be mapped in an interval of 7.62 cM covered by markers CAP0079, GBM1495 and HVM33.

Discussion

Twelve pairs of NILs and a NIL-derived population consisting of 1,028 lines were developed in this study. Data from analyzing these genetic stocks showed that the three traits investigated in this study, including GD, SL and AL, are likely controlled by a single-locus with the phenotype of DSS being recessive. This locus was mapped to a 2.84 cM interval flanked by markers GBM1495 and HVM33.

Different numbers of QTL controlling the three different SM-related traits were detected between the study based on the use of a six-row genotype (Sameri et al. 2006) and a two-row genotype (Wang et al. 2010). However, both of these studies detected a similar chromosomal region on the long arm of chromosome 3H influencing all three traits. As only two combinations of these traits, either DSS or LLL, were observed from all of the materials obtained in this study (including 1,028 $NILF_2$ plants, over 30,000 $NILF_3$ plants, and several hundreds of plants obtained during the generation of the NILs), it is clear that the three SM-related traits from the donor parent TX9425 are likely controlled by a single locus. This is in contrast to the finding that different genes seem to be responsible for these same traits (Zhang 2000; Taketa et al. 2011), and that the locus affecting GD located near the centromere on 7H in a two-row barley genotype is clearly not affecting AL (Shahinnia et al. 2012). It is important to understand why some of these loci control one trait only while others have pleiotropic effects on several traits of spike morphology. Answering this question may require the cloning of the genes responsible from the different sources and comparing their functions. The genetic stocks developed in this study, including the NILs, the NIL-derived population and the markers flanking the targeted region, would be invaluable in such an effort.

In addition to the 3HL locus investigated in this study, a second locus on chromosome 2H was also found to affect GD in TX9425 (Wang et al. 2010). In fact the effect of the latter was even larger than the former, and the presence of both loci increased GD by 44.9 % (Wang et al. 2010). Thus, it was anticipated that the presence of the 3HL locus alone should increase GD by a maximum of half of the amount caused by the presence of both loci. However, data from the NILs and the NIL-derived population showed that the presence of the 3HL locus alone increased GD by 41.7 % which accounted for 92.9 % of the total reduction by both the 2H and 3H loci. Similarly, in addition to the 3HL locus, there was also a second locus with slightly smaller effect affecting SL in TX9425 (Wang et al. 2010). Results from this study showed that, again the single 3HL locus was more effective than what could be anticipated. The 3HL locus alone reduced SL by 36.3 % which accounted for about 75.8 % of the total reduction by the presence of both of the loci together (Wang et al. 2010). It would be of interest to investigate if the presence of the alternative locus alone would also increase GD or SL by a much larger proportion than the contribution it makes when

present together with the 3HL locus or other genes affecting these traits. It is also interesting to note that for the single-locus controlled AL, effects of the 3HL locus estimated in this study based on the NILs or the NIL-derived population were not significantly different from that obtained by using the doubled haploid population (Wang et al. 2010).

NILs have been used effectively to develop markers closely linked to a locus of interest in the current study. NILs can also find a wide range of other applications including the study of phenotypic effects attributable to a particular gene or locus. NILs offer unique advantages in such studies, such as only two isolines are involved in assessing the effect of a particular allele in a particular genetic background and the genetic background determining morphological and phenological characters that commonly influence phenotypic assessments of quantitative traits is essentially fixed (Hoogendoorn et al. 1990; Youssefian et al. 1992; Miedaner and Voss 2008; Liu et al. 2010; Yan et al. 2011). Clearly, the quality of NILs developed by the HIF method depends on the quality of flanking markers. The tightly linked markers developed in this study can be used to generate improved NILs for the 3HL locus with further reduced chromosome segments differentiating each pair of the NILs.

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